Zhenbin Zhang[1]
Thomas Albanetti[2]
Travis Linkous[2]
Christopher J. Larkin[2]
Ronald Schoner[2]
James B. McGivney IV[2]
Norman J Dovichi[1]

[1]Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, USA
[2]BioPharmaceutical Development, MedImmune LLC, One MedImmune Way, Gaithersburg, MD, USA

Received August 25, 2016
Revised November 1, 2016
Accepted November 3, 2016

Research Article

Comprehensive analysis of host cell impurities in monoclonal antibodies with improved sensitivity by capillary zone electrophoresis mass spectrometry

Four methods were compared for analysis of host-cell protein (HCP) impurities in a recombinant mAb. First, CZE-MS/MS was used to analyze the digest of an HCP sample following extraction of the mAb with proteins A and L affinity columns; 220 protein groups and 976 peptides were identified from the depleted HCP digest. Second, a nanoACQUITY UltraPerformance LCH system was also used to analyze the depleted HCP digest; 34 protein groups and 53 peptides from 50 ng of the depleted HCP digest and 290 protein groups and 1011 peptides were identified from 1 μg of the depleted HCP digest. Third, 185 protein groups and 709 peptides were identified by CZE-MS/MS from the HCP digest without depletion. Fourth, a strong cation exchange SPE was coupled to CZE-ESI-MS/MS using online pH gradient elution for analysis of the HCP digest without depletion. A series of five pH bumps were applied to elute peptides from the strong cation exchange monolith followed by analysis using CZE coupled to a Q Exactive HF mass spectrometer; 230 protein groups and 796 peptides were identified from the HCP digest without depletion.

Keywords:
Cationic solid phase extraction / CZE-MS/MS / Electrokinetically pumped nanoelectrospray / Host cell proteins / pH bumps DOI 10.1002/elps.201600390

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

mAbs are produced by expression in a nonhuman host cell line, such as the Chinese hamster ovary (CHO) cell line [1–4]. The recombinant protein is affinity purified, but low levels of host cell proteins (HCPs) inevitably contaminate the final products. HCPs have potential risks that include immunogenicity, adjuvant activity, and proteolytic activity [5]. Therefore, identification and quantification of HCPs are required to protect patient safety. HCPs are present at low concentrations (1–100 ppm), their concentrations cover a wide dynamic range, and their detection is confounded by the presence of a large excess of mAbs.

Estimates of total HCPs are most commonly performed using an ELISA assay based on an anti-host cell antibody [6]. However, ELISAs are silent on the identity of the HCPs and only estimate the total HCP levels [7]. Alternatively, 2DE can be used to characterize HCPs, but this technology is semi-quantitative, is very labor intensive, and requires additional analytical methods for HCP identification [8].

MS has attracted attention for the identification and quantitation of HCPs in biopharmaceutical samples [9–11]. Typically, the mAb is removed from the sample using proteins A and L affinity columns. The depleted sample is digested using trypsin, peptides are separated using RPLC or CZE, and ESI MS/MS is used for identification. Several reports describe the use of 2D chromatography-ESI-MS/MS for identification of HCPs from depleted samples [7, 12].

Compared to RPLC, CZE has lower loading capacity and poorer concentration detection limits due to its small injection volume. However, recent advances using stacking and the dynamic pH junction have produced dramatic improvements in CZE concentration detection limits [13–17]. CZE has received increasing attention in proteomics analysis in

Correspondence: Dr. Norman J. Dovichi, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, USA
E-mail: ndovichi@nd.edu

Abbreviations: **CHO**, Chinese hamster ovary; **FA**, formic acid; **HCP**, host-cell protein; **LPA**, linear polyacrylamide; **SCX**, strong cation exchange

Colour Online: See the article online to view Figs. 1–6 in colour.

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

recent years due to the development of sensitive and robust CZE-MS interfaces [18–22]. Moreover, CZE has dramatically improved performance compared to RPLC for mass-limited samples, has much lower carry-over between runs, and can provide faster separations [23, 24].

There have been reports of the analysis of HCPs in a recombinant humanized mAb by using CIEF-MS/MS and CZE-ESI-MS/MS to identify HCPs and estimate their absolute amount [25–28]. In most of these studies, the antibody was depleted before analysis to reduce the background signal from the mAb itself. However, protein depletion extends the analysis time, and there is concern that hitchhiker proteins may be lost in the depletion step.

To evaluate the detection of HCPs without protein A/protein L depletion using CZE-ESI-MS/MS, we spiked 12 standard proteins at five concentration levels into a recombinant mAb sample. The mixtures were digested with trypsin without depletion and analyzed using both CZE-MS/MS and RPLC-MS/MS. Both methods could detect peptides from spiked impurity proteins at the 100 ppm level [28].

In this paper, we evaluate bottom-up proteomic analysis of both depleted and nondepleted HCP digests in a series of four experiments. In the first two experiments, CZE and ultraperformance liquid chromatography (UPLC) were used for analysis of depleted HCP digests. In the third experiment, CZE was used for analysis of nondepleted HCP digests. In the fourth experiment, we investigate CZE coupled with a strong cation exchange (SCX) SPE system for analysis of nondepleted HCP digests.

This SCX monolith allows concentration of dilute tryptic digests [15, 16]. The digests are loaded onto the monolith in an acidic electrolyte. Components can be eluted using a basic solution, which deposits the sample in the separation capillary in a plug of the basic solution. The separation is performed with an acidic background electrolyte, which results in concentration of analyte through formation of a dynamic pH junction. In this paper, we employ a series of elution buffers of increasing pH. These pH bumps result in successive elution of peptides from the SCX monolith with very low carry-over.

2 Materials and methods

2.1 Reagents and chemicals

Bovine pancreas tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin, urea, ammonium bicarbonate (NH_4HCO_3), DTT, iodoacetamide, and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). ACN and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Water was deionized by a Nano Pure system from Thermo Scientific (Marietta, OH, USA). Fused capillaries and linear polyacrylamide (LPA) coated capillaries were purchased from Polymicro Technologies (Phoenix, AZ).

2.2 Cell culture

The CHOK1SV host cell line was derived from the CHOK1 cell line (European Collection of Cell Cultures accession number 85051005) by Lonza Biologics in 1990. CHO K1SV host cells (Lonza Biologics, Slough, Berkshire, UK), Clone 1 of CHO K1SV stably expressing mAb A, were used in this experiment for material generation. All cell lines were cultured in serum-free maintenance media with the CHO K1SV host cells supplemented with 6 mM glutamine. A shake flask fed-batch overgrowth assay was set up with a working volume of 350 mL at 0.6 million viable cells in a serum-free growth media. Samples (1.5 mL) were taken for monitoring cell growth, metabolites, pH, and osmolarity during the duration of the fed-batch overgrowth. The cultures were fed a glucose and nutrient feed throughout the duration of the culture. One hundred twenty-five milliliters samples were taken for HPLC titer measurements on day 5 and then centrifuged at 2000 RPM for 10 min, and the supernatants were filtered with 0.2 mm Nalgene filters and stored at 4°C.

2.3 mAb depletion

Two experiments employed a depleted mAb. The supernatant sample was depleted of mAb using proteins A and L chromatography. Protein A spin columns were equilibrated with 1× phosphate buffered saline (PBS; pH 7.2). The mAb supernatant sample was diluted with PBS and loaded onto a column. Following a 10-min incubation at room temperature with gentle mixing by inversion, the flow-through was collected and immediately added to an equilibrated protein L spin column. The protein L column was incubated and mixed as described above. The protein L flow through fraction was collected and stored at −80°C until used.

2.4 Protein precipitation

Protein concentration was measured by a bicinchoninic acid assay. Two hundred micrograms samples were taken from CHO supernatant and precipitated with cold acetone overnight at −20°C. After centrifugation, the pellets were washed again with cold acetone. The resulting protein pellets were dried at room temperature.

2.5 Protein digestion

Protein pellets were dissolved in 152 μL of 100 mM NH_4HCO_3 (pH 8.5) with 8 M urea and denatured at 37°C for 1 h followed by reduction with 16 μL of 100 mM DTT at 37°C for 1 h and alkylation with 32 μL of 100 mM iodoacetamide at room temperature for 40 min in the dark. Then, 700 μL of 100 mM NH_4HCO_3 (pH 8.5) was added to 100 μL of the sample to reduce the concentration of urea to less than 1 M. The proteins were digested with trypsin at a trypsin/protein ratio

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

of 1/25 w/w for 17 h at 37°C. Digests were then acidified with 10% TFA to terminate the reaction. The tryptic digests were desalted with tC18 SepPak columns (Waters, Milford, MA, USA), followed by lyophilization with a vacuum concentrator (Thermo Fisher Scientific, Marietta, OH, USA).

2.6 Direct CZE-ESI-MS/MS analysis

Single-shot CZE was used to analyze both depleted and non-depleted mAb digests. The CZE-ESI-MS/MS system consists of two high-voltage power supplies (Spellman CZE 1000R) and an electrokinetically pumped electrospray interface to couple the CZE separation capillary to a mass spectrometer [23]. The electrospray emitter was made from borosilicate glass capillary (1.0 mm od × 0.75 mm id, 10 cm long) pulled with a Sutter instrument P-1000 flaming/brown micropipette puller to produce a 10–15 μm emitter. The electrospray sheath flow buffer was 10% v/v methanol/water with 0.5% FA.

A Polymicro (Phoenix AZ USA) LPA-coated capillary (50 μm id × 150 μm od × 85 cm length) was used for the CZE separation. The background electrolyte was 1 M acetic acid (HAc) in water. Approximately 25 nL of a 2 mg/mL digest solution (50 ng) was injected by pressure. Voltage of 19.5 kV was applied at the injection end of the capillary for separation and 1.5 kV on the sheath flow reservoir for electrospray. The electrophoretic current was typically ~6 μA. The sample flow rate was roughly 30 nL/min, which is 3% of the UPLC flow rate. Voltage programming was controlled by LabView software.

2.7 SCX-SPE-CZE-ESI-MS/MS analysis with online pH gradient elution

An SCX-CZE system was used to analyte a nondepleted mAb digest. The SCX SPE-CE-ESI-MS/MS system was similar to a previous report [16]. A 50-μm id and 150-μm od fused silica capillary was filled with a mixture of acetic acid, PEG, urea, tetramethyl orthosilicate, vinyltrimethoxysilane (0.6 mL), 3-sulfopropyl methacrylate potassium salt, and AIBN for formation of the monolith. The resulting sulfonate SCX hybrid column was then flushed with methanol and water to remove the porogen and other residual components. Finally, the sulfonate SCX hybrid monolithic column was cut to a length of 1 cm. The sulfonate SCX hybrid monolith was connected to the LPA-coated capillary by a tubing sleeve with 180 μm id and a polyether ether ketone (PEEK) union. The SCX column was first conditioned by flushing with 1 M HAc for 10 min. After ~100 nL of the sample was loaded onto the SCX-SPE column by pressure, the system was flushed with 1 M HAc to remove unretained components.

Online pH gradient elution was performed by sequential pressure injecting plugs of five elution buffers: 30 mM ammonium formate, pH 3; 30 mM ammonium formate, pH 4; 30 mM ammonium acetate, pH 5; 30 mM ammonium bicarbonate, pH 7; and 30 mM ammonium bicarbonate, pH 8. Each solution was injected for ~40 s at ~30 PSI pressure for an estimated volume of 100 nL. After injecting each elution buffer, the background electrolyte was changed back to 1 M HAc, and normal CZE-ESI-MS/MS analysis was carried out under the same condition as direct CZE-ESI-MS/MS. The analysis required 5 h.

2.8 UPLC-ESI-MS/MS analysis

A nanoACQUITY UltraPerformance LCH system (Waters, Milford, MA, USA) with a UPLC BEH 130 C18 column (Waters, 100 μm × 100 mm, 1.7 μm) was coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) for analysis of a depleted mAb digest. Buffers A (0.1% FA in water) and B (0.1% FA in ACN) were used as mobile phases for gradient separation. Peptides were loaded onto a commercial C18 RP column (Waters, 100 μm × 100 mm, 1.7 μm particle, BEH130C18, column temperature 40°C) and flushed with 2% buffer B for 10 min at a flow rate of 1 μL/min, then followed by a gradient: 10−11 min, 2−8% B; 11−71 min, 8−30% B; 71−72 min, 30−80% B; 72−77 min, 80% B; 77−78 min, 80−2% B; and 78−90 min, 2% B. The eluted peptides from the C18 column were pumped through a capillary tip for electrospray with the electrospray voltage of 1.5 kV, and the MS parameters were the same as that used for CZE-ESI-MS/MS analysis.

2.9 Mass spectrometers operating parameters

An LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was used for initial experiments comparing CZE and RPLC for analysis of the depleted HCP digest. The ion transfer tube temperature was held at 300°C. The S-Lens RF level was 50.00. The mass spectrometer was programed in data-dependent mode. A top 20 method was used. Full MS scans were acquired with the Orbitrap mass analyzer over m/z 380–1800 range with resolution of 60 000 (m/z 200) and the number of microscans set to 1. The target value was 1.00×10^6, and the maximum injection time was 250 ms. For MS/MS scans, the 20 most intense peaks with charge state ≥2 were sequentially isolated and further fragmented in the CID mode following one full MS scan. The normalized collision energy was set at 30.

In the online pH gradient elution experiment, a Q Exactive HF instrument (Thermo Scientific) was used. The mass spectrometer was programed in data-dependent mode. A top 20 method was used. The S-lens RF level was set at 50, and heated capillary at 300°C. Full scan resolution was set to 60 000 at m/z 200. Full scan target was 3.00×10^6 with a maximum fill time of 30 ms. Mass range was set to 375–1500. Target value for fragment scans was set at 1.00×10^5, and intensity threshold was kept at 1.00×10^5. Isolation width was set at 1.4 Th. A fixed first mass of 100 was used. Normalized collision energy was set at 28.

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

2.10 Database searching

Database searching of the raw files was performed in Proteome Discoverer 1.4. MASCOT 2.2.4 was used for database searching against UniProt database with taxonomy as *Cricetulus griseus* (Chinese hamster; www.uniprot.org; Proteome ID UP000001075). The corresponding reversed database was used to evaluate the false discovery rates. The database searching parameters included full tryptic digestion and allowed up to two missed cleavages, the precursor mass tolerance was set at 10 ppm, and fragment mass tolerance was 0.5 Da for the data obtained on the LTQ-Orbitrap Velos mass spectrometer and 0.05 Da for the data obtained on the Q Exactive HF instrument. Carbamidomethylation (C) was set as fixed modifications for the sample treated by alkylation. Oxidation (M) and deamidated (NQ) were set as variable modifications. On the peptide level, peptide confidence value as high was used to filter the peptide identification, and the corresponding false discovery rates at the peptide level were less than 1%. On the protein level, protein grouping was enabled.

3 Results

3.1 Comparison of UPLC and CZE analysis of depleted protein digest

Fifty nanograms of the depleted HCP digest was analyzed by CZE-MS/MS and UPLC-MS/MS (Fig. 1). Only 34 protein groups and 53 peptides were identified using UPLC. In contrast, 220 protein groups and 976 peptides were identified using CZE. The peptide identification information is listed in the Supporting Information.

When the loading amount was increased to 1 μg for UPLC, 290 protein groups and 1011 peptides were identified (Supporting Information Fig. S1). These results are similar to the results obtained using CZE analysis of 50 ng of depleted HCP digest.

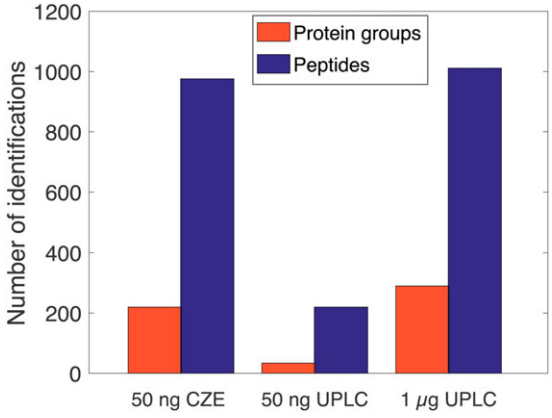


Figure 1. Effect of the separation methods for the depleted host cell protein digest at the amounts listed. For CZE, 50 μm id × 150 μm od × 85 cm long LPA-coated capillary; 1 M HAc separation buffer; 19.5 kV separation voltage; 1.5 kV spray voltage; and LTQ-Orbitrap Velos mass spectrometer.

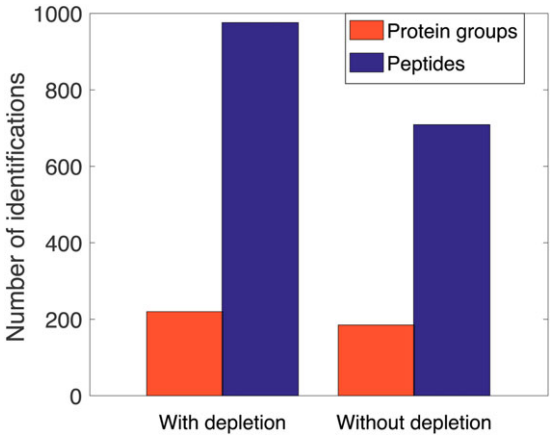


Figure 2. Effect of depletion. CZE experimental conditions are same as Fig. 1.

3.2 Effect of mAb depletion

Host cell protein digests with and without depletion were analyzed by CZE (Fig. 2). Two hundred twenty protein groups and 976 peptides were identified from 50 ng of the depleted HCP digest, and 185 protein groups and 709 peptides were identified from 50 ng of the sample without depletion. The peptide identification information is listed in the Supporting Information. The high abundance peptides produced by digestion of the mAb suppressed the identification of the low abundance host cell proteins.

The overlaps in peptide and protein identifications between the sample with depletion and without depletion are shown in Fig. 3. Thirty-nine percent of the peptides identified from the depleted sample were not identified in the sample without depletion. Sixteen percent of the peptides were identified in the sample without depletion but not in the depleted sample; presumably, these peptides derive from high abundance proteins that were removed during the depletion process.

The p*I* distributions of all identified peptides from the sample with and without depletion are shown in Fig. 4, and the p*I* distribution of unique peptides in the depleted sample and sample prepared without depletion are shown in Fig. 5. Most of the peptides identified from the samples have p*I* values less than 7.

3.3 Online pH gradient fractionation of HCP digests that are not mAb depleted

The results shown in Fig. 5 suggest that fractionation based on p*I* may improve HCP identification in samples that are not depleted. We employed online pH gradient fractionation based on an SCX monolith that was prepared at the proximal

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

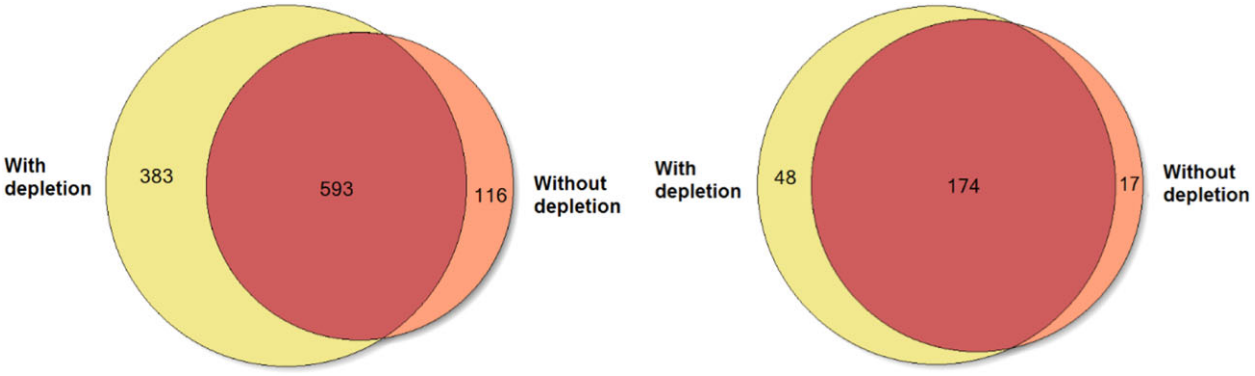


Figure 3. Overlaps between the host cell protein digest with depletion and without depletion. Peptides are shown on the left and proteins on the right.

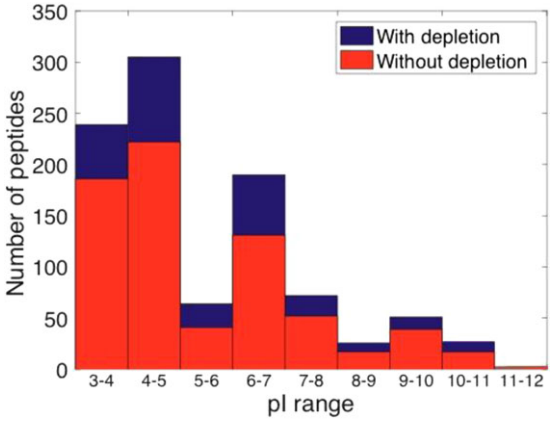


Figure 4. p*I* distribution of all identified peptides in the mAb depleted sample.

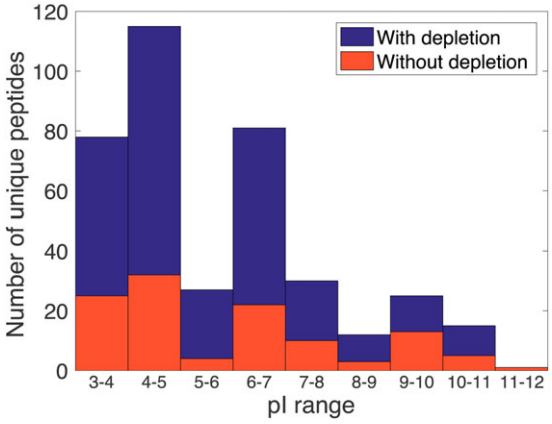


Figure 5. p*I* distribution of peptides found only in the depleted HCP digest (blue) and only in the HCP digest prepared without depletion (orange). p*I* was calculated using MATLAB's isoelectric function.

tip of the separation capillary and used for SPE of peptides [15,16]. Before each experiment, a 50 ng aliquot of HCP digest without depletion was loaded onto the SCX SPE monolith. The monolith was then flushed with 1 M HAc, followed by five successive elutions using buffers at pH 3, 4, 5, 7, and 8. After each injection of the elution buffer, the separation electrolyte was changed back to 1 M HAc for CZE-ESI-MS/MS analysis. An Orbitrap Q-Exactive HF mass spectrometer was used in this experiment.

Two hundred thirty protein groups and 796 peptides were identified from the HCP digest that was not depleted. The peptide identifications from each elution step are listed in the Supporting Information. Overlaps between the successive eluting steps are shown in Fig. 6. In accordance with our previous work, overlaps between the adjacent eluting steps were very small [16]. In this case, the complexity of the sample was decreased in each pH gradient elution step, which improved the detection of low abundance peptides.

We also analyzed the overlaps between the peptides identified in each elution step and the peptides identified from the single-shot analysis of the depleted HCP digest. 69% of peptides identified from the first elution step with the elution buffer of pH 3 were also identified from the depleted HCP digest. When the pH value of the elution buffer was increased to 4 and 5, 54 and 49% of the identified peptides were also identified from the sample with depletion, respectively. When the pH value of the elution buffer was increased to 8, 42% of the identified peptides were also identified in the depleted HCP digest. It seems that the overlaps decreased with an increase of the pH values of the elution buffers. This result is consistent with the p*I* values of the peptides in Figs. 4 and 5. Most of the HCP peptides could be eluted with buffers with pH lower than 7.

4 Discussion

We report four experiments in this manuscript. The first two experiments compare CZE and UPLC for analysis of host cell proteins in a sample that has been depleted of mAb using protein A/protein L columns. CZE generates many more protein and peptide identifications for 50-ng loading amounts. A 1-μg loading amount using UPLC produced a

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

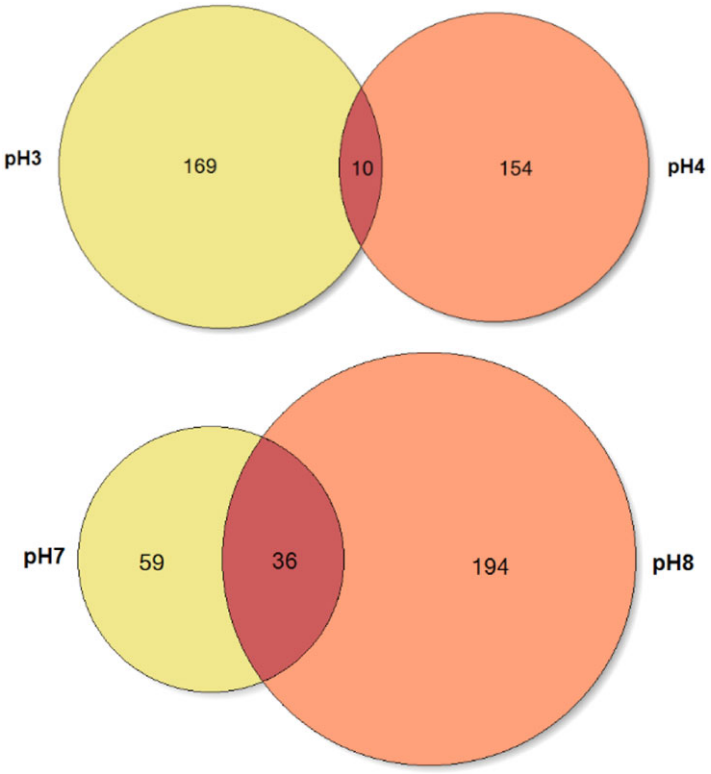


Figure 6. Overlaps of peptide identifications between the adjacent eluting steps during pH gradient elution.

similar number of peptide and protein identifications as the 50 ng loading on CZE.

The third experiment compared the effect of mAb depletion on host cell protein identifications using CZE. We observed ~16% more protein groups and ~28% more peptide identifications from the depleted sample. This difference reflects the large background signal generated by the peptides produced from the very high abundance mAb. Twenty-four proteins were present in the nondepleted sample and were absent from the depleted sample, and are identified in the Supporting Information in the *Proteins not depleted direct CZE* tab. We used isobaric tags for relative and absolute quantitation (iTRAQ) chemistry to quantify protein expression changes in the CHO secretome during culture [27]. Most of the 24 proteins that were in the nondepleted direct CZE experiments but not identified in the depleted sample were also absent from our protein quantification study. That study employed protein A/protein L treatment to remove the mAb. It is likely that most of the 24 proteins identified in this study are indeed hitchhiker proteins that are removed along with the antibody by the protein A/protein L treatment.

The fourth experiment focused exclusively on nondepleted samples. This experiment employed a strong cation exchange monolith to concentrate peptides at the proximal end of the separation capillary. Plugs of increasing pH were used to elute fractions for subsequent separation. By using a basic elution buffer, we formed a pH junction at the start of electrophoresis, which focused peptides after elution. The pH bumps employed in this study (pH 3, 4, 5, 7, 8) generated efficient fractionation with very little overlap of peptide identifications. This approach generated ~10% more peptide and protein identifications than the single-shot analysis used in the third experiment.

There was a remarkable lack of concordance between the peptide identifications using single-shot and pH gradient fractionation analysis of the nondepleted sample. Over half of the peptides identified in one experiment were absent from the other experiment. It appears that the two techniques are complementary. Combination of the two approaches yields 1202 peptide identifications.

We thank Dr. William Boggess in the Notre Dame Mass Spectrometry and Proteomics Facility for his help with this project. This work was sponsored by MedImmune.

The authors have declared no conflict of interest.

5 References

[1] Zhang, Q., Goetze, A. M., Cui, H., Wylie, J., Trimble, S., Hewig, A., Flynn, G. C., *mAbs* 2014, *6*, 659–670.

[2] Baik, J. Y., Joo, E. J., Kim, Y. H., Lee, G. M., *J. Biotechnol.* 2008, *133*, 461–468.

[3] Lim, Y., Wong, N. S. C., Lee, Y. Y., Ku, S. C. Y., Wong, D. C. F., Yap, M. G. S., *Biotechnol. Appl. Biochem.* 2010, *55*, 175–189.

[4] Mohan, C., Kim, Y.-G., Koo, J., Lee, G. M., *Biotechnol. J.* 2008, *3*, 624–630.

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[5] Gao, S. X., Zhang, Y., Stansberry-Perkins, K., Buko, A., Bai, S., Nguyen, V., Brader, M. L., *Biotechnol. Bioeng.* 2011, *108*, 977–982.

[6] Savino, E., Hu, B., Sellers, J., Sobjak, A., Majewski, N., Fenton, S., *BioProcess Intl.* 2011, *9*, 38–47.

[7] Doneanu, C., Xenopoulos, A., Fadgen, K., Murphy, J., Skilton, S. J., Prentice, H., Stapels, M., Chen, W., *mAbs* 2012, *4*, 24–44.

[8] Hayduk, E. J., Choe, L. H., Lee, K. H., *Electrophoresis* 2004, *25*, 2545–2556.

[9] Gupta, P., Lee, K. H., *Trends Biotechnol.* 2007, *25*, 324–330.

[10] Baycin-Hizal, D., Tabb, D. L., Chaerkady, R., Chen, L., Lewis, N. E., Nagarajan, H., Sarkaria, V., Kumar, A., Wolozny, D., Colao, J., Jacobson, E., Tian, Y., O'Meally, R. N., Krag, S. S., Cole, R. N., Palsson, B. O., Zhang, H., Betenbaugh, M., *J. Proteome Res.* 2012, *11*, 5265–5276.

[11] Xu, X., Nagarajan, H., Lewis, N. E., Pan, S., Cai, Z., Liu, X., Chen, W., Xie, M., Wang, W., Hammond, S., Andersen, M. R., Neff, N., Passarelli, B., Koh, W., Fan, H. C., Wang, J., Gui, Y., Lee, K. H., Betenbaugh, M. J., Quake, S. R., Famili, I., Palsson, B. O., Wang, J., *Nat. Biotech.* 2011, *29*, 735–741.

[12] Donato, P., Cacciola, F., Sommella, E., Fanali, C., Dugo, L., Dachà, M., Campiglia, P., Novellino, E., Dugo, P., Mondello, L., *Anal. Chem.* 2011, *83*, 2485–2491.

[13] Foret, F., Szökő, E., Karger, B. L., *Electrophoresis* 1993, *14*, 417–428.

[14] Simonet, B. M., Ríos, A., Valcárcel, M., *Trends Anal. Chem.* 2003, *22*, 605–614.

[15] Zhang, Z., Sun, L., Zhu, G., Yan, X., Dovichi, N. J., *Talanta* 2015, *138*, 117–122.

[16] Zhang, Z., Yan, X., Sun, L., Zhu, G., Dovichi, N. J., *Anal. Chem.* 2015, *87*, 4572–4577.

[17] Osbourn, D. M., Weiss, D. J., Lunte, C. E., *Electrophoresis* 2000, *21*, 2768–2779.

[18] Olivares, J. A., Nguyen, N. T., Yonker, C. R., Smith, R. D., *Anal. Chem.* 1987, *59*, 1230–1232.

[19] Smith, R. D., Barinaga, C. J., Udseth, H. R., *Anal. Chem.* 1988, *60*, 1948–1952.

[20] Whitt, J. T., Moini, M., *Anal. Chem.* 2003, *75*, 2188–2191.

[21] Wojcik, R., Dada, O. O., Sadilek, M., Dovichi, N. J., *Rapid Commun. Mass Spectrom.* 2010, *24*, 2554–2560.

[22] Ramautar, R., Heemskerk, A. A. M., Hensbergen, P. J., Deelder, A. M., Busnel, J.-M., Mayboroda, O. A., *J. Proteomics* 2012, *75*, 3814–3828.

[23] Jorgenson, J., Lukacs, K., *Science* 1983, *222*, 266–272.

[24] Wahl, J. H., Goodlett, D. R., Udseth, H. R., Smith, R. D., *Electrophoresis* 1993, *14*, 448–457.

[25] Zhu, G., Sun, L., Wojcik, R., Kernaghan, D., McGivney, J. B., IV, Dovichi, N. J., *Talanta* 2012, *98*, 253–256.

[26] Zhu, G., Sun, L., Linkous, T., Kernaghan, D., McGivney, J. B., Dovichi, N. J., *Electrophoresis* 2014, *35*, 1448–1452.

[27] Zhu, G., Sun, L., Albanetti, T., Linkous, T., Larkin, C., Schoner, R., McGivney, J. B., Dovichi, N. J., *Biotechnol. Bioeng.* 2016, *113*, 2140–2148.

[28] Zhu, G., Sun, L., Heidbrink-Thompson, J., Kuntumalla, S., Lin, H. Y., Larkin, C. J., McGivney, J. B., Dovichi, N. J., *Electrophoresis* 2016, *37*, 616–622.

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim