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A rapid cIEF–ESI–MS/MS method for host cell protein analysis of a recombinant human monoclonal antibody

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ABSTRACT

A rapid and reproducible system that couples capillary isoelectric focusing to a high-resolution mass spectrometer was developed for on-line analysis and identification of protein digests. Magnetic microsphere-based immobilized trypsin was used for protein digestion to reduce the digestion time to 10 min, with a total analysis time of 4 h. A three-protein-mixture (myoglobin, BSA, cytochrome c) with a molarity ratio of 1:10:50 was successfully digested and identified. This system was also used to analyze host cell protein impurities in a recombinant humanized monoclonal antibody product in which the sample was product-depleted using affinity capture on protein A/protein L columns prior to analysis. A database search identified 37 host cell proteins with peptide and protein identity probability greater than 0.9.

1. Introduction

Recombinant proteins are becoming increasingly important as therapeutics. Recombinant proteins are produced by expressing a desired human gene in a host cell. After culture, the target protein is purified through multiple chromatographic steps to high levels of purity. However, trace amounts of proteins produced by the host cell line can be present in the final product. Because host cell proteins (HCPs) can potentially impact product quality and safety, it is important to quantitate and characterize the HCPs present in biotherapeutic products to mitigate these risks [1,2].

The amount of contaminating HCP present in a biotherapeutic is typically measured using either an SDS-PAGE based visualization method or an immunoassay [2,3]. Both of these methods are suitable for measuring HCP levels but neither of the methods is able to identify the actual HCPs present in the test sample. Other characterization methods for HCPs, such as two-dimensional gel electrophoresis followed by gel extraction and identification by mass spectrometry, tend to be labor-intensive and cumbersome, which limits their routine use [3–5].

Capillary isoelectric focusing (cIEF) is a powerful enrichment and separation technique for proteins and peptides. Compared to conventional capillary electrophoresis, the focusing properties of cIEF allow use of much larger injection volumes, which facilitates analysis of dilute analyte [6–8]. cIEF has been coupled with a number of detection techniques, including UV absorbance [9,10], laser-induced fluorescence [11–14] and mass spectrometry (MS) [15–19]. While laser-induced fluorescence produces outstanding detection limits with cIEF, the limited information content of the technique frustrates attempts to identify components.

Mass spectrometry provides an information-rich signal that is ideally suited for peptide identification. Electrospray ionization (ESI) dominates applications of capillary electrophoresis for peptide analysis. However, on-line coupling of cIEF with ESI–MS for biological samples, especially protein digests, can be challenging. First, sheath flow CE–MS interfaces tend to dilute the sample, which makes identification of low concentration peptides difficult. Second, the ampholytes used in cIEF both suppress peptide ionization and compete with peptides during tandem mass spectrometry analysis [20].

The need for carrier ampholytes has been eliminated from isoelectric focusing as reported for a cIEF–ESI-–MS method of a relatively high concentration complex peptide mixture and periplasmic protein digest from *Escherichia. coli* [21,22]. Although high sample concentrations were successfully used to perform

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autofocusing in this cIEF–ESI–MS method, poor generation of a pH gradient for low concentration samples and trace species was still a significant problem.

In this report, we describe a cIEF–ESI–MS/MS system that was able to detect HCPs in a product-depleted recombinant monoclonal antibody.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma Aldrich Co. (St. Louis, MO, USA) unless otherwise stated. LPA-coated fused-silica capillaries (50 μm i.d., 150 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Pharmalytes (3–10) were purchased from GE Healthcare (Piscataway, NJ, USA). Acetonitrile (ACN) and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Carboxyl functionalized magnetic microspheres (BioMag®Plus carboxyl, mean diameter ~1.5 μm) were purchased from Bangs Laboratories, Inc. (Fishers, IN, USA). Water was deionized by a Nano Pure system from Thermo Scientific (Marietta, OH, USA). Recombinant human IgG was prepared by MedImmune. NAb protein A and protein L spin columns were purchased from Thermo Scientific. All mass spectrometric experiments were performed using an LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Preparation of magnetic microsphere based immobilized trypsin

The procedure for activation of carboxyl functionalized magnetic microspheres and trypsin immobilization has been described elsewhere [23,24].

2.3. Sample preparation

Bovine serum albumin (BSA, 0.5 mg/mL) dissolved in 100 mM ammonium bicarbonate (pH 8.0) was denatured at 90 °C for 10 min, followed by the standard reduction and alkylation processes with dithiothreitol (DTT, ≥99.5%), iodoacetamide (IAA, ≥99%). Then, the digestion was performed by incubating the proteins for 12 h at 37 °C with trypsin at a trypsin:protein ratio of 1:30 (w/w).

A three-protein-mixture including BSA (1 mg/mL, 14 μM), cytochrome c (0.017 mg/mL, 1.4 μM), and myoglobin (0.0049 mg/mL, 280 nM) dissolved in 100 mM ammonium bicarbonate (pH 8.0) was denatured at 90 °C for 10 min, followed by the standard reduction and alkylation processes with DTT and IAA. Then, the sample was mixed with equal volume of water. For immobilized trypsin digestion, 30 μL of protein solution was incubated with 200 μg trypsin immobilized beads at 37 °C for 10 min. The digests were diluted 1:1 with water and stored at −20 °C for use.

The recombinant human IgG was depleted from test samples using protein A and protein L spin columns. Briefly, protein A spin-columns were equilibrated with 1× phosphate buffered saline (PBS; pH 7.2). The IgG sample was diluted with PBS and added to 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.

The flow through fraction from the Protein A/L columns (0.045 mg/mL, 500 μL) was dried in an Eppendorf concentrator. The dried sample was dissolved in 100 μL 100 mM NH_4HCO_3 with 1 M urea and denatured at 90 °C for 10 min, followed by standard reduction and alkylation process with DTT and IAA. Then, digestion was performed by incubating 50 μL denatured proteins with 400 μg immobilized trypsin magnetic beads for 10 min at 37 °C. The digests were stored at −20 °C for use.

2.4. cIEF–ESI–MS/MS

The instrument is based on a capillary electrophoresis system that we have described earlier [25]. A commercial linear polyacrylamide coated capillary (50 μm i.d., 50 cm long, Polymicro) was used for the cIEF separation. The anode end of the capillary was placed in formic acid (0.1%, pH 2.5), and the cathode end was placed in 0.3% ammonium hydroxide (pH 11). The capillary was filled with sample prepared in a 0.4% Pharmalyte (3–10) solution by purging the solution through the capillary at 2 psi for 3 min. Focusing voltage was applied at 360 V/cm for 10 min. After focusing, the cathode end of the capillary was inserted into the emitter of the electrospray interface [26] and chemical mobilization was performed with the sheath flow buffer (50% methanol, 0.05% formic acid). The electric field was kept at 330 V/cm during mobilization.

2.5. Data acquisition and processing

Full MS scans were acquired in the Orbitrap mass analyzer over the 395–1900 m/z range with resolution 60,000 (at 400 m/z). The 12 most intense peaks with charge state ≥2 were selected for sequencing and fragmented in the ion trap with normalized collision energy of 35%, activation $q=0.25$, activation time of 10 μs, and one microscan. Peaks selected for fragmentation two or more times within a 45 s window were excluded from selection for an additional 45 s.

For standard protein samples, database searching of the raw files was performed in Proteome Discoverer 1.2 with the SEQUEST search engine against ipi.bovin.v3.68.fasta (for BSA and cytochrome c), equine.fasta (for myoglobin). Peptides identified with confidence value as "high" were considered as positive identification.

For the HCP sample, the raw files were first transferred to mgf files. Database searching of mgf files was performed with the MASCOT search engine against SwissProt Rodent. Trans-Proteomic Pipeline (TPP) version 4.4 was used to filter the database search results with both peptide probability and protein probability higher than 0.9.

3. Results and discussion

3.1. Effect of sheath flow on chemical mobilization

In this system, the sheath flow liquid serves as both the chemical mobilization buffer for cIEF and the entrainment buffer for electrospray ionization. Three kinds of sheath flow buffers were investigated: 50% methanol and 0.05% acetic acid; 50% methanol and 0.05% formic acid; 50% methanol and 0.1% formic acid. Fig. 1 presents the current profiles. Curve *a* is from cIEF chemical mobilization using 0.05% acetic acid in the sheath flow. Curves *b* and *c* employed 0.05% formic acid and 0.1% formic acid, respectively. Current was low and did not change significantly during chemical mobilization with 50% methanol and 0.05% acetic acid.

In contrast, the current increased during chemical mobilization with 50% methanol with 0.05% formic acid and 50% methanol with 0.1% formic acid. The current increased more quickly with 0.1% formic acid than with 0.05% formic acid; a higher concentration of formic acid speeds mobilization and results in a narrow separation window. This short separation window is not always

desirable because it limits the number of tandem mass spectra that can be accumulated during a separation. To broaden the separation window and increase the peptide number and protein sequence coverage, 50% methanol with 0.05% formic acid was used as sheath flow in the following experiments.

3.2. Reproducibility of the cIEF–ESI–MS/MS system

A BSA digest (0.1 mg/mL) was used to evaluate the reproducibility of the cIEF–ESI–MS/MS system. Peptides (25 ± 4) were identified with high confidence producing a sequence coverage of $44 \pm 6\%$ in a triplicate analysis. The peptide intensity in the three runs was also analyzed. Table 1 presents the peak intensity of five peptides extracted from spectra obtained in the triplicate runs. The relative standard deviation in the peak intensity ranged from 3% to 8%.

3.3. Analysis of a three-protein-mixture

To evaluate the performance of the cIEF–ESI–MS/MS system, a three-protein-mixture was prepared with a 50-fold range in concentration; the mixture contained 7 μM BSA (0.5 mg/mL), 0.7 μM cytochrome c (0.0095 mg/mL), and 140 nM myoglobin (0.0025 mg/mL). The mixture was digested with trypsin immobilized on magnetic particles at 37 °C for 10 min. The protein digest was diluted 1:1 with water, mixed with ampholytes, and analyzed by cIEF–ESI–MS/MS. Table 2 presents the number of identified peptides and the sequence coverage of the three proteins. The analysis consistently identified at least one peptide from the dilute myoglobin sample in the presence of a 50-fold excess of BSA.

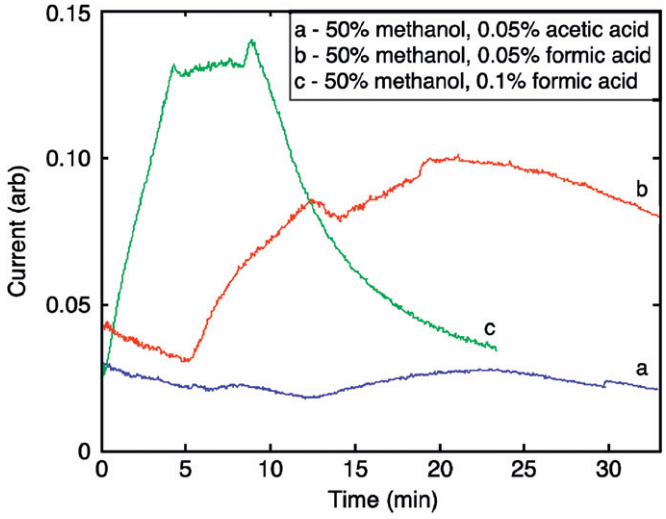

Fig. 1. Current profiles for the mobilization during capillary isoelectric focusing with different sheath flow buffers: (a) 50% methanol and 0.05% acetic acid; (b) 50% methanol and 0.05% formic acid; and (c) 50% methanol and 0.1% formic acid.

The injection volume employed in this experiment is equal to the capillary volume. The amount of myoglobin taken for the analysis is 70 fmol (1.3 ng).

3.4. Analysis of HCP sample

A recombinant antibody sample was product-depleted using standard protein A and protein L affinity columns. The sample required product depletion since the concentration of the antibody was in vast excess, compared to the concentration of contaminating HCPs. The depleted sample was digested for 10 min using immobilized trypsin and then analyzed by the cIEF–ESI–MS/MS system; 53 peptides were identified by tandem mass spectrometry. There is a monotonic but nonlinear relationship between the observed migration time and the predicted isoelectric point for these peptides (see Fig. 2). A plot of pI vs. 1/(Migration time—offset) is linear ($r=0.97$) for an offset of 11 min. This offset reflects the time necessary for the ampholytes to begin to exit the capillary. The inverse relationship reflects the positive chemical mobilization.

After database searching with MASCOT and filtering with TransProteomic Pipeline (TPP, v 4.4), 37 HCP proteins were identified with

Table 2
Identification of three-protein-mixture by cIEF–ESI–MS/MS.

	BSA (0.25 μg, 3.5 pmol)		**Cytochrome c (4.8 ng, 350 fmol)**		**Myoglobin (1.3 ng, 70 fmol)**	
	Number of peptides	Sequence coverage (%)	Number of peptides	Sequence coverage (%)	Number of peptides	Sequence coverage (%)
Run 1	36	63.0	2	17.1	2	20.3
Run 2	38	61.6	3	31.4	1	20.3
Run 3	32	61.1	3	27.6	1	9.8

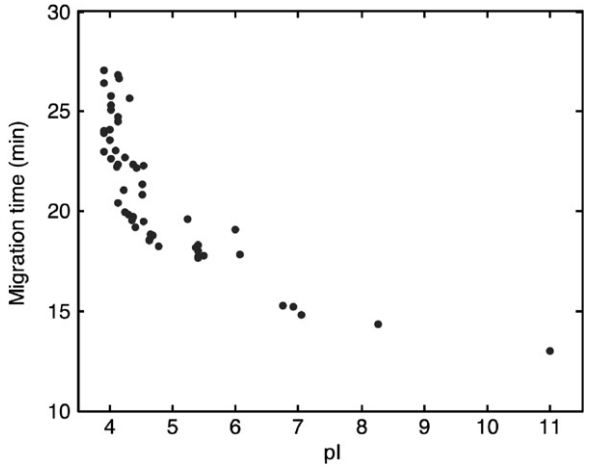

Fig. 2. Calculated pI value vs. observed migration time of identified peptides from the HCP sample. The pI values of peptides were calculated with TPP, and the migration time was obtained from the extracted peptide spectrum.

Table 1
Extracted peptide intensity in triplicate analysis of BSA digests.

Peptide m/z	**653.3617**	**751.8099**	**820.4724**	**788.8879**	**847.7277**
Sequence	HLVDEPQNLIK	EYEATLEEccAK	KVPQVSTPTLVEVSR	LKPDPNTLcDEFK	QEPERNEcFLSHKDDSPDLPK
Charge	2	2	2	2	3
Run 1	5.69×10^8	1.36×10^8	1.92×10^8	1.11×10^8	4.59×10^7
Run 2	4.99×10^8	1.32×10^8	2.12×10^8	1.16×10^8	4.63×10^7
Run 3	5.02×10^8	1.17×10^8	1.82×10^8	1.19×10^8	4.92×10^7

sequence coverage ranging from 1.5% to 60.4% (mean=11.7%) with peptide and protein probability higher than 0.9 after triplicate cIEF–ESI–MS/MS runs of the same sample. The total analysis time including sample preparation, digestion, and cIEF–ESI–MS/MS analysis is about 4 h.

A cIEF–MS system for protein and peptide analysis has been previously described Kuroda et al. [26] demonstrated application of on-line cIEF–MS for absolute quantification of standard peptides and proteins using an ampholyte concentration of 1%. Good linearity ($R^2=0.998$) between angiotensin II concentration and peak area was obtained. However, they employed a co-axial sheath flow interface and a high sheath flow rate (2 μL/min), which led to sample dilution. The detection limit of their system for a standard peptide, analyzed in the absence of other peptides, was 220 nM (0.22 μM). By comparison, a detection limit of 70 nM was found for a myoglobin peptide using the system described in the report. Moreover, the myoglobin peptide was found in the presence of higher concentrations of BSA and cytochrome c peptides, suggesting that the detection limit of this method for a peptide standard would most likely be even better.

The rapid and sensitive method for HCP analysis presented in this report provides an alternative to conventional two-dimensional gel electrophoresis for analysis of HCPs in recombinant therapeutics, and may prove to be a valuable tool for HCP detection applications.

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