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Multisegment Injections Improve Peptide Identification Rates in Capillary Zone Electrophoresis-based Bottom-up Proteomics

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Abstract

While capillary zone electrophoresis (CZE) provides dramatically improved numbers of peptide identifications compared with reversed-phase chromatography for bottom-up proteomics of mass limited samples, CZE inevitably produces lower numbers of peptide identifications than RPLC for larger samples. One reason for this poorer performance is the dead time between injection of samples and subsequent appearance of the fastest moving component. This dead time is typically 25% of the separation window in CZE, but is only 5% of the separation window in gradient elution RPLC. This dead time can be eliminated in CZE by use of a multisegment injection mode where a series of samples is analyzed by injecting each sample while the preceding sample is still being separated. In this paper, we demonstrate that capillary zone electrophoresis employing sequential injections can produce a doubling in peptide identification rate with no degradation in separation efficiency.

Keywords

capillary zone electrophoresis; bottom-up proteomics; multisegment injection

1. Introduction

Capillary zone electrophoresis (CZE) was introduced to the general analytical community by Lukacs and Jorgenson in two seminal publications that appeared in 1981 and 1983 (1,2). These pioneering papers present impressive demonstrations of separation of important classes of biological molecules, including the human urine metabolome, tryptic digest of a protein, and top-down analysis of human blood serum. However, detection was limited to optical methods 35 years ago, and the systems were unable to positively identify components.

Fenn and colleagues' development of electrospray ionization (ESI) as a tool to interface liquid chromatography with mass spectrometry provided a very important tool for identification of analyte (3–4). Olivares and colleagues very quickly followed Fenn's early publications, and developed an ESI-CZE interface with mass spectrometry (CZE-ESI-MS) (5).

The community rapidly adopted CZE-ESI-MS for analysis of a wide range of standard proteins and peptides (6). Tong and colleagues appear to be the first to apply CZE-ESI-MS to the bottom-up analysis of a complex sample, the tryptic digest of the *S. cerevisiae*

ribosomal complex (7). A total of 136 peptides were identified using a multistep elution-solid phase extraction technique with an LTQ mass spectrometer as detector. Tong's pioneering work has been followed by a number of publications applying CZE-ESI-MS to the bottom-up analysis of increasingly complex prokaryotic and eukaryotic proteomes (8–18).

As a result of these studies, it is clear that CZE offers several advantages compared to RPLC for this analysis: CZE produces many more peptide and protein identifications from mass limited samples (17), a simple model has been developed for the migration time of peptides to increase confidence of identifications (19), and on-column digestion has been developed to simplify sample manipulations (20–21).

As a major disadvantage of CZE compared with RPLC for bottom-up proteomic analysis, CZE consistently produces fewer peptide and protein identifications per unit time than RPLC for samples greater than a few hundred nanograms in mass. In a comparison of CZE and RPLC for the single-shot analysis of the HeLa proteome, CZE produced 10,000 peptide identifications compared with 40,000 using RPLC, in both cases using a 90-minute separation (18). This smaller number of identifications in CZE is a result, in part, of the dead time before the fastest migrating components reach the detector. In the HeLa proteome study, the separation window for CZE was roughly 20 to 90 minutes, and few peptides were identified in the first 20 minutes of the separation. In contrast, gradient elution RPLC produced a separation window from 5 to 90 minutes, providing 25% more opportunity for peptide detection.

A solution to this issue of a long dead time between injection and the appearance of peaks in CZE was introduced by Larmann and Jorgenson in 1993 as part of an RPLC-CZE interface (22), and redeveloped in a modified form by Kuehnbaum and colleagues in 2013 for analysis of metabolites (23). In these methods, a series of injections is performed at time periods greater than the separation window but less than the total analysis time. This technology has been applied this method to the analysis of metabolites in human plasma (24–26), and demonstrated efficient generation of calibration curves, standard addition analysis, and serial dilutions (23).

In this manuscript, we employ the segmented injection method to increase the peptide identification rate in CZE. In our protocol, a series of samples are analyzed where the injections are timed so that each injection is performed before the preceding sample has completely migrated from the capillary. Through careful timing, the dead time at the start of an electropherogram is occupied by analyte from the preceding electropherogram. We demonstrate a doubling in the peptide identification rate through this method.

2. Materials and Methods

2.1 Materials and reagents

LC-MS grade water and methanol were purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Acetic acid, formic acid (FA), and hydrofluoric acid (HF) were purchased from Fisher Scientific (Pittsburgh, USA). HCl, NaOH, 3-(trimethoxysilyl) propyl

methacrylate, acrylamide, ammonium persulfate, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, USA). A Nano Pure system from Thermo Scientific (Waltham, MA, USA) was used to generate deionized water. Uncoated fused silica capillary (50 μm i.d./360 μm o.d.) was purchased from Polymicro Technologies (Phoenix, USA). Emitters were prepared from borosilicate glass capillary (1.0 mm o.d., 0.75 mm i.d. and 10 cm length) from Sutter Instrument Company (Novato, USA).

2.2 Capillary coating

A thermally-initiated free-radical polymerization method was used to coat the interior of the capillary with a layer of linear polyacrylamide (27). Briefly, the fused silica capillary (50 μm i.d./360 μm o.d.) was flushed with 1 M HCl for 30 min, water for 10 min, 1 M NaOH for 30 min, water for 10 min, and methanol for 30 min. The capillary was dried with a flow of nitrogen at room temperature for 4 hours. Then, 50 % (v/v) 3-(trimethoxysilyl) propyl methacrylate in methanol was flushed through the capillary for 10 min. With both ends sealed, the filled capillary was left at room temperature for 24 h. The capillary was rinsed with methanol for 20 min and dried under nitrogen.

To prepare the coating, 40 mg of acrylamide was dissolved in 1 mL water, and 2 μL of 5% (w/v) ammonium persulfate (APS) was added to 500 μL of the acrylamide solution. The mixture was vortexed for 30 s and degassed for 5 min with nitrogen. The solution was introduced into the 1.2 m of pretreated capillary by vacuum. With both ends sealed, the capillary was put into 50 $^{\circ}\text{C}$ water bath for 30 min. The polymer that was not bonded to the capillary wall was flushed away by water, and the capillary was stored at room temperature.

2.3 Etching of distal end of the separation capillary

The distal tip of the LPA coated capillary was etched with HF to reduce the outer diameter to about 60 μm (18). The length of the etched segment was about 2 mm. After etching, the capillary was trimmed to a length of 60 cm.

2.4 CZE-ESI-MS/MS analysis

A PrinCE 800 series autosampler was used to inject samples into the separation capillary, and our third generation electrokinetically pumped sheath flow interface was used to couple CE to an LTQ XL (Thermo Scientific) mass spectrometer (28). The electrospray emitter was made from a borosilicate glass capillary (1.0 mm o.d., 0.75 mm i.d., and 10 cm length) pulled with a Sutter instrument P-1000 flaming/brown micropipet puller. The size of the pulled end of the emitter was $\sim 15 \mu\text{m}$.

The autosampler applied 200 V/cm across the LPA-coated capillary (50- μm ID, 60-cm length) for separation, and 1.8 kV was applied with a Bertan high voltage power supply for electrospray. The etched end of the separation capillary was threaded into the spray emitter. The background electrolyte was 5% (v/v) acetic acid, and the sheath buffer was 0.5% (v/v) FA and 10% (v/v) methanol. The sample was injected into the separation capillary by nitrogen pressure (10 psi for 4 s). The sample was a yeast digest prepared at 1 mg/mL in 10 mM ammonium bicarbonate. No spacer plug was injected between runs; a new sample was injected at the times shown below.

An LTQ-XL mass spectrometer (Thermo Fisher Scientific) was used in data dependent acquisition mode. Full MS scans were acquired over the 350–1500 m/z range. The 10 most intense peaks were selected for fragmentation in the ion trap with normalized collision energy of 35%, activation $q = 0.25$, activation time of 30 ms, and one microscan. Peaks selected for fragmentation more than once within 45 s were excluded from selection for 60 s.

2.5 Data analysis

Database searching of the raw files was performed in Proteome Discoverer 1.4 (Thermo) with MASCOT 2.5. The raw files were searched against the SwissProt protein database with taxonomy of *Saccharomyces Cerevisiae* (baker's yeast). The datafiles were divided into three portions corresponding to the three injections, and each portion was searched independently to enable detection of common peptides present in the three injections. The database searching parameters included full tryptic digestion and allowed up to two missed cleavages, the precursor mass tolerance was set at 2 Da, and fragment mass tolerance was 1 Da. Carbamidomethylation (C) was set as a fixed modification. Oxidation (M) and deamidated (NQ) were set as variable modifications. On the peptide level, peptide confidence value as high as possible was used to filter the peptide identification, and the corresponding false discovery rate on peptide level was less than 1%.

Electropherograms were converted into mzXML files and were imported into Matlab for plotting. Selected ion electropherograms were extracted with a 0.5 Da window centered at the selected m/z values.

3. Results and Discussion

Figure 1 presents three sequential injections of a tryptic digest of a yeast sample with varying times between injections. In the bottom trace, samples were injected after the preceding separation was complete. This situation represents a typical experimental condition used for bottom-up proteomic analysis. In the top trace, samples are injected at much shorter time intervals, which results in a negligible dead time between runs.

Figure 2 presents the corresponding peptide identification rate through the separation, where the number of peptides identified per minute tracks the complexity of the electropherogram. The figure clearly shows the inefficient use of the separation time under typical electrophoresis conditions, where large periods of the separation contain no identifications. By shortening the time between injections, a dramatic increase in the use of the mass spectrometer is observed. The shortest time between injections produces a doubling in the average number of identifications per minute. However, there is also a 20% decrease in the total number of identifications at the shortest injection period due to the limited response time of the mass spectrometer. Supporting information provides peptide m/z values and migration times for these separations.

Hydrodynamic injection was employed to load samples, and the resulting parabolic flow profile will induce a component of band broadening. To measure any degradation of peak width due to successive injections, we generated selected ion electropherograms for features dispersed across the electropherogram. A nonlinear least squares algorithm was used to fit a

Gaussian function to the selected ion electropherograms. The number of theoretical plates for the first injection ranged from 15,000 to 150,000, with a median plate count of 64,000. No increase in peak width was observed for successive injections. The mean ratio of peak width for the first and third injections in the top electropherogram of figure 2 was 0.96 ± 0.07 (standard error of mean, $n=9$). Sources of band broadening other than successive injections dominate peak width in these experiments.

This improvement in peptide identification rate comes with penalties. Late migrating peptides from one separation may be overtaken by fast moving peptides from the following injection, scrambling the migration order of peptides. However, this situation is unimportant when performing bottom-up analysis of fractions from the same proteome because it is not necessary to determine the fraction from which a peptide was identified. If it is necessary to identify peptides with a particular injection, a high-accuracy migration time model can be used to estimate the injection time for the peptides (19). We observe fewer total numbers of identifications for shorter times between injections. Low intensity, late migrating peaks will be buried below the higher intensity peptides near the center of the following injection; those low intensity peptides may not generate sufficient intensity to generate a fragmentation spectrum using the top-10 method employed with this mass spectrometer. This 15% decrease in the number of peptide identifications is more than compensated by the doubling of peptide identification rate.

In addition, multisegment injection is not compatible with the large sample-volume concentrator reported in ref 20. Injection of large samples will displace analyte from the capillary, ruining the benefits of multisegment injection. On the other hand, the use of preconcentration followed by a series of pH bumps may be interesting when combined with multisegment injection (21). The use of relatively small volume buffers to generate the pH bumps would not displace an excessive amount of analyte from the distal tip of the capillary, and would provide a simple method of combining on-column fractionation of a sample with efficient separation.

Finally, these experiments were performed using a quite old ion trap mass spectrometer, which has limited response time and mass resolution. It is clear that multisegment injection will produce more interesting results when employed with a more modern mass spectrometer. Nevertheless, these data demonstrate that segmented injection has the potential to at least double the identification rate in CZE-MS analysis of complex proteomes, which should enable CZE to approach the peptide identification rate produced by HPLC, irrespective of sample amount.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Jorgenson JW, Lukacs KD. *Anal Chem.* 1981; 53:1298–1302.
2. Jorgenson JW, Lucacs KD. *Science.* 1983; 222:266–272. [PubMed: 6623076]
3. Yamashita M, Fenn JB. *J Phys Chem.* 1984; 88:4451–4459.
4. Whitehouse CM, Dryer RN, Yamashita M, Fenn JB. *Anal Chem.* 1985; 57:675–679. [PubMed: 2581476]
5. Olivares JA, Nguyen NT, Yonker CR, Smith RD. *Anal Chem.* 1987; 59:1230–1232.
6. Simpson DC, Smith RD. *Electrophoresis.* 2005; 26:1291–1305. [PubMed: 15765477]
7. Tong W, Link A, Eng JK, Yates JR 3rd. *Anal Chem.* 1999; 71:2270–2278. [PubMed: 10405597]
8. Faserl K, Sarg B, Kremser L, Lindner H. *Anal Chem.* 2011; 83:7297–7305. [PubMed: 21848273]
9. Busnel JM, Schoenmaker B, Ramautar R, Carrasco-Pancorbo A, Ratnayake C, Feitelson JS, Chapman JD, Deelder AM, Mayboroda OA. *Anal Chem.* 2010; 82:9476–9483. [PubMed: 21028888]
10. Wang Y, Fonslow BR, Wong CCL, Nakorchevsky A, Yates JR 3rd. *Anal Chem.* 2012; 84:8505–8513. [PubMed: 23004022]
11. Wojcik R, Li Y, MacCoss MJ, Dovichi NJ. *Talanta.* 2012; 88:324–329. [PubMed: 22265506]
12. Li Y, Champion MM, Sun L, Champion PAD, Wojcik R, Dovichi NJ. *Anal Chem.* 2012; 84:1617–1622. [PubMed: 22182061]
13. Ramautar R, Heemskerk AAM, Hensbergen PJ, Deelder AM, Busnel J, Mayboroda OA. *J Proteomics.* 2012; 75:3814–3828wojsik. [PubMed: 22609513]
14. Sun L, Zhu G, Li Y, Wojcik, Yang P, Dovichi NJ. *Proteomics.* 2012; 12:3013–3019. [PubMed: 22888077]
15. Li Y, Wojcik R, Dovichi NJ, Champion MM. *Anal Chem.* 2012; 84:84 6116–1622.
16. Zhu G, Sun L, Yan X, Dovichi NJ. *Anal Chem.* 2013; 85:2569–2573. [PubMed: 23394296]
17. Sun L, Zhu G, Zhao Y, Yan X, Mou S, Dovichi NJ. *Angew Chem.* 2013; 52:13661–13664. [PubMed: 24173663]
18. Sun L, Hebert AS, Yan XJ, Zhao YM, Westphall MS, Rush MJP, Zhu GJ, Champion MM, Coon JJ, Dovichi NJ. *Angew Chem.* 2014; 53:13931–13933. [PubMed: 25346227]
19. Krokhn OV, Anderson G, Spicer V, Sun L, Dovichi NJ. *Anal Chem.* 2017; 89:2000–2008. [PubMed: 28208305]
20. Zhang Z, Sun L, Zhu G, Yan X, Dovichi NJ. *Talanta.* 2015; 138:117–122. [PubMed: 25863379]
21. Zhang Z, Yan X, Sun L, Zhu G, Dovichi NJ. *Anal Chem.* 2015; 87:4572. [PubMed: 25822566]
22. Larmann JP, Lemmo AV, Moore AW, Jorgenson JW. *Electrophoresis.* 1993; 14:439–447. [PubMed: 8354227]
23. Kuehnbaum NL, Kormendi A, Britz-McKibbin P. *Anal Chem.* 2013; 85:10664–10669. [PubMed: 24195601]
24. Kuehnbaum NL, Gillen JB, Kormendi A, Lam KP, DiBattista A, Gibala MJ, Britz-McKibbin P. *Electrophoresis.* 2015; 36:2226–2236.
25. Kuehnbaum NL, Gillen JB, Gibala MJ, Britz-McKibbin P. *Sci Report.* 2014; 4:6166.
26. Yamamoto M, Ly R, Gill B, Zhu Y, Moran-Mirabal J, Britz-McKibbin P. *Anal Chem.* 2016; 88:10710–10719. [PubMed: 27677202]
27. Zhu G, Sun L, Dovichi NJ. *Talanta.* 2016; 146:839–843. [PubMed: 26695337]
28. Sun L, Zhu G, Mou S, Dovichi NJ. *J Proteome Res.* 2015; 14:2312–2321. [PubMed: 25786131]

Appendix A. Supplementary data

Peptide m/z and migration time are listed in a set of Excel spreadsheets. This material is available on the journal's web page.

Figure 1.

Three sequential injections under different time periods between injections. Time between injections are 32.5 min (top), 47 min (2nd), 66.5 min (3rd), and 90 min (bottom). NL – normalization level.

Figure 2. Peptide identification rates for the separations of figure 1. Average number of IDs calculated by dividing the total number of IDs by the total separation time.