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Optimization of mass spectrometric parameters improve the identification performance of capillary zone electrophoresis for single-shot bottom-up proteomics analysis

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Abstract

The effects of MS1 injection time, MS2 injection time, dynamic exclusion time, intensity threshold, and isolation width were investigated on the numbers of peptide and protein identifications for single-shot bottom-up proteomics analysis using CZE-MS/MS analysis of a *Xenopus laevis* tryptic digest. An electrokinetically pumped nanospray interface was used to couple a linear-polyacrylamide coated capillary to a Q Exactive HF mass spectrometer. A sensitive method that used a 1.4 Th isolation width, 60,000 MS2 resolution, 110 ms MS2 injection time, and a top 7 fragmentation produced the largest number of identifications when the CZE loading amount was less than 100 ng. A programmable autogain control method (pAGC) that used a 1.4 Th isolation width, 15,000 MS2 resolution, 110 ms MS2 injection time, and top 10 fragmentation produced the largest number of identifications for CZE loading amounts greater than 100 ng; 7218 unique peptides and 1653 protein groups were identified from 200 ng by using the pAGC method. The effect of mass spectrometer conditions on the performance of UPLC-MS/MS was also investigated. A fast method that used a 1.4 Th isolation width, 30,000 MS2 resolution, 45 ms MS2 injection time, and top 12 fragmentation produced the largest number of identifications for 200 ng UPLC loading amount (6025 unique peptides and 1501 protein groups). This is the first report where the identification number for CZE surpasses that of the UPLC at the 200 ng loading level. However, more peptides (11476) and protein groups (2378) were identified by using UPLC-MS/MS when the sample loading amount was increased to 2 µg with the fast method. To exploit the fast scan speed of the Q-Exactive HF mass spectrometer, higher sample loading amounts are required for single-shot bottom-up proteomics analysis using CZE-MS/MS.

Keywords

capillary zone electrophoresis; bottom-up proteomics; optimized performance; ultraperformance liquid chromatography; *Xenopus laevis*

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Conflict of interest

The authors declare no competing financial interest.

Appendix A

Supplementary data related to this article can be found on the journal's web page.

1. Introduction

Capillary zone electrophoresis-tandem mass spectrometry (CZE-MS/MS) is attracting increasing attention for proteomics analysis due to its high sensitivity,^{1–3} fast separation speed,⁴ small volume sample consumption, orthogonality to reversed-phase liquid chromatography (RPLC), and performance with mass-limited samples.^{5,6,7} A number of recent papers have focused on improvements in sample preparation,⁸ preconcentration,^{9–11} and fractionation for CZE-based bottom-up proteomics.^{6,12}

Mass spectrometric parameters have been investigated to improve the identification rates for single-shot bottom up proteomics using RPLC-MS/MS.^{13–21} To the best of our knowledge, there has been no published report that studied the effects of the mass spectrometric parameters on the identification performance of CZE-MS/MS method for bottom up proteomics analysis. In this work, we investigated the effects of mass spectrometric parameters on peptides and protein identification rates for single-shot bottom up proteomics analysis by using CZE-MS/MS and compared those results with RPLC-MS/MS, each operating under their optimized conditions. The results demonstrate that the optimized mass spectrometric parameters are not only related to the sample loading amount but also to the separation method.

2. EXPERIMENTAL SECTION

2.1 Reagents and Chemicals.

Formic acid (FA), acetic acid (HOAc), bovine pancreas TPCK-treated trypsin, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-Aldrich (St. Louis, USA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Uncoated fused silica capillary (50 μm i.d. $\times$ 350 μm o.d.) was purchased from Polymicro Technologies (Phoenix, AZ). Water was deionized by a Nano Pure system from Thermo Scientific (Marietta, OH).

2.2 Preparation of *Xenopus laevis* sample.

The method used for the preparation of the *Xenopus laevis* tryptic digest is described in Supporting Information.

2.3 Preparation of linear polyacrylamide coated capillary.

The linear polyacrylamide (LPA) coated capillary was prepared by using a surface-confined aqueous reversible addition-fragmentation chain transfer (SCRAFT) polymerization method.²²

2.4 CZE-ESI-MS/MS analysis.

A PrinCE Next 840 Series autosampler (from PrinCE Technologies) was used for sample injection and CZE separation. The sample was injected by pressure. Separation voltage was applied at the injection end of the capillary with the autosampler. An electrokinetically pumped nanospray interface was used to couple the CZE separation capillary to a Q Exactive HF mass spectrometer (Thermo Scientific). The electrospray emitter was made

from a borosilicate glass capillary (1.0 mm o.d. $\times$ 0.75 mm i.d., 10 cm long) pulled with a Sutter instrument P-1000 flaming/brown micropipette puller; the size of the emitter opening was 15-20 μ m. The electrospray sheath electrolyte was 10% (v/v) methanol with 0.5%FA. The background electrolyte was 1 M HOAc in water. The 50 μ m i.d. $\times$ 350 μ m o.d. $\times$ 99 cm LPA coated capillary was used as the separation capillary. The *Xenopus laevis* proteins digest dissolved in 30 mM NH_4HCO_3 was used as the standard sample. 1.6 kV was applied to the sheath flow reservoir for electrospray. The mass spectrometer's operating parameters are described below.

2.5 UPLC-ESI-MS/MS analysis.

An ACQUITY UPLC M-Class system (Waters, Milford, MA, USA) with an ACQUITY UPLC M-Class Peptide BEH C18 column (Waters, 100 μ m $\times$ 100 mm, 1.7 μ m, 300 Å) was coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) for peptide separation and identification. Mobile phase A (0.1%FA in water) and mobile phase B (0.1% FA in ACN) were used for gradient separation. Peptides were automatically loaded onto a commercial C18 reversed phase column and flushed with 2% mobile phase B for 10 min at a flow rate of 0.9 μ L/min, then followed by the gradient: 10–12 min, 2–8% B; 12–70 min, 5–30% B; 70–74 min, 20–80% B; 74–79 min, 80% B; 79–80 min, 80–2% B; 80–90 min, 2% B. The eluted peptides from the C18 column were pumped through a capillary tip for electrospray.

2.6 Mass Spectrometer Operating Parameters.

A Q Exactive HF mass spectrometer (Thermo Scientific) was used in this work. The mass spectrometer was programmed in data-dependent mode. The S-lens RF level was set at 60, and heated capillary at 300 °C. Full scan resolution was set to 60,000 at m/z 200. Full scan target was 3.00E+06. Mass range was set to m/z 350-1,800. Target value for fragment scans was set at 1.00E+06. A fixed first mass of 100 was used. Normalized collision energy was set at 28. Other parameters are optimized in this work.

2.7 Database Searching.

Database searching of the raw files was performed in both Proteome Discoverer 1.4 (Thermo) with MASCOT 2.5 and Maxquant 1.5.8.3. The *Xenopus laevis* database (version 9.1) was downloaded from Xenbase (<http://www.xenbase.org/>, RRID:SCR_003280). Database searching for the reversed database was also performed to evaluate the false discovery rate. 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 0.05 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 was used to filter the peptide identification, and the corresponding false discovery rate on peptide level was less than 1%. On the protein level, protein grouping was enabled.

3. RESULTS AND DISCUSSION

We investigated the effect of a number of mass spectrometer operating parameters on peptide and protein identifications in the CZE-MS/MS analysis of the *Xenopus* embryo proteome.

3.1 Effect of MS1 injection time.

Figure 1 presents the total ion electropherogram for the injection of 100 ng of *Xenopus laevis* embryo tryptic digest. The first components migrate at roughly 20 minutes and the last components migrate at 100 minutes. The figure also presents the MS1 injection time for the separation of this sample. The fill time minimizes at ~2 ms for the portion of the electropherogram with the highest base-peak intensity, and reaches a maximum value of ~14 ms for the lowest intensity portion of the electropherogram. The inverse relationship between total ion current and fill time is expected; the greater the total ion current, the less time is required to fill the trap. We then varied the maximum injection time from 10 to 50 ms for the analysis of the *Xenopus* digest using CZE-MS/MS, Figure S1. The number of protein identifications maximized with a 15 ms MS1 injection time, which produced 5% more protein identifications than a 10 ms injection time, and a 15 ms injection time was used in the following studies.

3.2 Effect of dynamic exclusion time.

Dynamic exclusion times were varied from 20 to 60 s, Figure 2. More MS/MS spectra and peptide spectral matches (PSM) were obtained at the shorter dynamic exclusion time. However, larger numbers of MS/MS spectra do not always lead to more identifications. When the dynamic exclusion time was set at 20 s, 5476 peptides and 1259 protein groups were identified. When the dynamic exclusion time was increased to 30 s, the number of identified peptides and protein groups increased to 5590 and 1349, respectively, but decreased when the dynamic exclusion time was further increased to 60 s. A single factor *ANOVA* analysis confirmed that dynamic exclusion time significantly influenced the number of identified protein groups ($p=0.0049$) and peptides ($p=0.0016$). The dynamic exclusion time was set at 30 s for subsequent experiments.

The optimal dynamic exclusion time will depend on the peak width of the identified peptides. We analyzed the distribution of the retention length of the identified peptides based on the data from MaxQuant software. As shown in Figure S2, the peak width of the identified peptides was mainly between 20 s and 40 s, with a median of ~27 s, which is close to the optimal exclusion time.

3.3 Effect of intensity threshold.

Three different intensity thresholds were investigated: 5E4, 1E5, and 2E5, Figure 3. As expected, more MS/MS spectra were obtained at the lowest intensity threshold. However, the number of PSM, peptide IDs, and protein IDs maximized at the intermediate intensity threshold. A single factor *ANOVA* analysis confirmed that the intensity threshold influenced the number of identified protein groups ($p=0.0088$) and peptides ($p=0.041$).

3.4 Effect of isolation width.

Three isolation widths were studied: 0.4, 1.4, and 2.4 Th, Figure 4. Fewer MS/MS spectra were acquired at the 0.4 Th isolation width because the narrow isolation width produces decreased ion transmission. A similar trend was found for the number of PSM. However, a larger isolation width can lead to the co-migration and cofragment of peptides with similar mass, and we observed a decrease in the number of peptide and protein identifications at the 2.5 Th isolation window. A single factor *ANOVA* analysis also demonstrated the isolation width significantly influenced the number of identified protein groups ($p=8.7E-4$) and peptides ($p=3.2E-5$). The optimum isolation window of 1.4 Th was used for the following experiments.

3.5 Acquisition method comparison.

Based on the above results and previously reports,^{18–21} four acquisition methods were developed, Table 1. A pAGC method was based on a 32 ms transient but allowed for up to 110 ms MS2 fill times, making use of the automatic gain control (AGC) feature.¹⁹ The different acquisition methods were evaluated as a function of loading amount of *Xenopus* protein digest for the CZE-MS/MS analysis, Figure 5. The experiments were performed as technical duplicates. The faster method produced the most MS/MS spectra for the different sample loading amounts, and the number of the MS/MS spectra decreased in the order of fast, pAGC, and sensitive methods. However, the larger number of MS/MS spectra didn't always lead to more PSM and identifications. When the sample loading amount is lower than 100 ng, the sensitive method generated more PSM than other methods, and the number of PSM decreased in the order of pAGC, fast, and faster methods. The same trend was also found for the number of identifications.

The pAGC method generated more PSM for a 200 ng sample loading amount. The number of PSM decreased in the order of sensitive, fast, and faster methods, and the number of peptide and protein identifications decreased in the order of fast, sensitive, and faster methods.

We also analyzed the distribution of the cycle time (The total time - full scan including the tandem MS scans) and the retention length as defined by Maxquant for the peptides under each experimental conditions, Figures S3 and S4. The cycle time under different experimental conditions was typically between 0.19 s and 0.25 s, with a median of 0.22 s. The peak width of the identified peptides under different experimental conditions was typically between 23 s and 33 s, with a median of ~27 s, Figure S5, which is similar to the results we obtained when optimizing the dynamic exclusion time.

3.6 Effect of MS/MS injection time.

The pAGC method consistently produced more peptide and protein identifications than the faster method, irrespective of sample loading amount. The main difference between these methods is the MS2 injection time. To study the effect of MS2 injection time, electropherograms were generated under an acquisition method with different MS/MS injection times, Figure 6. The number of MS/MS spectra decreased monotonically with an increase of MS2 injection time. However, the number of PSM, peptide IDs, and protein IDs

all maximized with an injection time of ~100 ms and decreased at longer MS2 injection times. This result suggests that MS2 must be optimized to ensure good quality MS/MS spectra when the sample loading amount is limited (100 ng in this case). The decrease in performance for longer MS2 injection times is a result of the long duty cycle, which resulted in poor precursor fragmentation.

A top-20 method was used wherein the 20 most intense precursor ions from each parent ion spectrum were fragmented. The effect of the MS2 injection time on the total number of identified unique peptides for the 20 MS/MS scans was analyzed, Figure 7. As expected, the numbers of peptide identifications from the later MS/MS scans increased with MS2 injection time. For example, when the MS2 injection time was 15 ms, only 19 peptides were identified from the seventh MS/MS scan, 9 peptides from the eighth MS/MS scan, and no peptides were identified after the eighth MS/MS scan. This result shows that twelve of the MS/MS scan are useless due to the low intensities of the MS/MS spectra. When the MS2 injection time was increased to 45 ms, the numbers of unique peptides identified from the seventh and eighth MS/MS scan events increased to 149 and 92, respectively, and two unique peptides could be identified from the fourteenth MS/MS scan. No peptides were identified for later MS/MS scans.

When the MS2 injection time was increased to 75 ms and longer, the numbers of peptides identified from the first and the fourth MS/MS scan events decreased. The maximum number of peptides identified in the earlier MS/MS scans was usually reached for shorter MS2 injection times, and the increased identifications from the later MS/MS scans were at the expense of the decreased identifications from the earlier MS/MS scans, presumably because of the effect of dynamic exclusion. Therefore, it is necessary to optimize MS2 injection time and the corresponding loop count (top N) to maximize the identification performance of CZE-MS/MS method for shotgun proteomics analysis.

3.7 Comparison with UPLC-ESI-MS/MS.

The different acquisition methods were also tested against different loading amounts of *Xenopus* protein digest using a 90 min UPLC separation, Figure 8. Similar to CZE-MS/MS, the faster method always results in the highest number of MS/MS spectra. For sample loading amounts less than 100 ng, more peptides were identified using the sensitive method. Similar numbers of peptides were identified from a 200 ng sample using the sensitive (6032 peptides), the fast (6025 peptides) and the pAGC methods (5993 peptides). In contrast, a different trend was obtained in CZE-MS/MS, where the pAGC method gave the largest number of identifications (7218 peptides). Clearly, the optimized mass spectrometric parameters are dependent on the separation method.

The fast method outperforms the other three methods using UPLC-MS/MS for sample loading amounts higher than 200 ng. When the sample loading amount is higher than 1 μ g, more peptides were identified by using the faster method (8980 peptides) than the sensitive method (8688 peptides). The faster method never provided the best identification number, even when the sample loading amount was increased to 2 μ g. This result differs from an early report, where a faster method provided the best identification number for 1 μ g of HeLa

digest.¹⁹ However, a similar trend was obtained for the pAGC method, which provided the second best number of identifications, irrespective of the sample loading amounts.

4. CONCLUSIONS

The effects of the mass spectrometric parameters on the peptide and protein identification numbers for shotgun proteomics analysis by using CZE-MS/MS were investigated in detail. A longer injection time for MS/MS was necessary to increase the number of identified peptides and proteins when the sample loading amount is less than 200 ng for CZE-MS/MS analysis. 7218 unique peptides and 1653 protein groups were identified from 200 ng of the *Xenopus* digest by using the pAGC method.

We compared this CZE with UPLC-MS/MS, where the fast method gave the largest numbers of identifications (6025 unique peptides and 1501 protein groups for 200 ng loading). However, more peptides (11476) and protein groups (2378) could be identified by using UPLC-MS/MS when the sample loading amount was increased to 2 µg with the fast method.

In this work, the faster method never gave the best identification number for the sample loading range from 5 ng to 200 ng for CZE-MS/MS and from 50 ng to 2 µg for UPLC-MS/MS. A recent study showed that the short UPLC gradients could reach deep proteome coverage with faster method and the high peptide loads.²³ A second recent paper also concluded that protein and peptide identifications maximized for injection time > 100 ms.²⁴ Obviously, to take advantage of the fast scan speed of the Q-Exactive HF mass spectrometer, higher sample loading amounts are required. Unfortunately, higher sample loading amounts compromise the CZE separation performance. Therefore, it is necessary to optimize the CZE separation conditions to allow higher sample loading amounts without degradation of its separation performance. The use of the pH-junction for sample loading may provide an opportunity to increase loading without degrading separation efficiency.²⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Total ion electropherogram (blue, left) and MS1 injection time (orange, right) for CZE-MS/MS analysis of the tryptic digest of *Xenopus leavis* embryo. Experimental conditions: Q Exactive HF mass spectrometer; 60 000 resolution; top 7; 50 μm i.d. $\times$ 350 μm o.d. $\times$ 99 cm long LPA coated capillary; 1 M HOAc background electrolyte; 21.6 kV separation voltage; and 1.6 kV spray voltage.

Figure 2. The effects of the dynamic exclusion time on the numbers of tandem spectra (MS/MS), peptide spectral matches (PSM), and peptide and protein identifications for shotgun proteomics analysis by CZE-MS/MS. Experimental conditions are the same as Figure 1.

Figure 3.

The effect of the intensity threshold on the numbers of tandem spectra (MS/MS), peptide spectral matches (PSM), and peptide and protein identifications for shotgun proteomics analysis by CZE-MS/MS. Experimental conditions are the same as Figure 1.

Figure 4.

Effect of the isolation width on the numbers of tandem spectra (MS/MS), peptide spectral matches (PSM), and peptide and protein identifications for shotgun proteomics analysis by CZE-MS/MS. Experimental conditions are the same as Figure 1.

Figure 5. Acquisition method comparison under different sample loading amount for CZE-MS/MS. Other experimental conditions are the same as Figure 1.

Figure 6.

Effect of the MS2 injection time for CZE-MS/MS. Experimental conditions: 15 000 MS2 resolution; top 20. Other experimental conditions are the same as Figure 1.

Figure 7.

Comparison of the numbers of unique peptides identified in each MS/MS scan with different MS/MS injection time using a top-twenty method. Experimental conditions are the same as Figure 6.

Figure 8. Acquisition method comparison under different sample loading amount by UPLC-MS/MS.

Table 1.

Parameters for the acquisition methods.

Method	MS2 Resolution	MS2 fill time (ms)	Top N
<i>Faster</i>	15,000	15	20
<i>Fast</i>	30,000	45	12
<i>Sensitive</i>	60,000	110	7
<i>pAGC</i>	15,000	110	10