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Single-shot proteomics using capillary zone electrophoresis-electrospray ionization-tandem mass spectrometry produces more than 1,250 *E. coli* peptide identifications in a 50-minute separation

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Abstract

Capillary zone electrophoresis (CZE)-electrospray ionization-tandem mass spectrometry (ESI-MS/MS) was optimized and applied for analysis of 1–100 ng *E. coli* protein digests in a single run (single-shot analysis). The system employed an electrokinetically-pumped nanospray interface, a coated capillary, and stacking conditions for sample injection. More than 1,250 peptides were identified by optimized single-shot CZE-ESI-MS/MS with 100 ng digest loaded and 50 min analysis time. When 10 ng and 1 ng digests were loaded, about 1,000 and 600 peptides were identified in a single-shot analysis, respectively. Compared with single-shot ultra-performance liquid chromatography (UPLC)-ESI-MS/MS, CZE-ESI-MS/MS produced fewer peptide IDs (1,377 ± 128 vs. 1,875 ± 32) for large sample loading amounts (100 ng) with the same mass spectrometer time (50 min). However, when the loaded digest was mass limited (1 ng), CZE-ESI-MS/MS generated many more peptide identifications than UPLC-ESI-MS/MS (627 ± 38 vs. 342 ± 113). In addition, CZE-ESI-MS/MS and UPLC-ESI -MS/MS provided complementary peptide level identifications. These results suggest that CZE-ESI-MS/MS may be useful for large-scale, comprehensive, and confident proteomics analysis.

Capillary electrophoresis (CE) provides rapid and efficient separations of biological molecules [1–3]. CE-electrospray ionization-tandem mass spectrometry (CE-ESI-MS/MS) is an interesting tool for protein analysis [4]. However, the large-scale application of the CE-ESI-MS/MS for protein and peptide analysis has been hampered by much poorer performance than ultrapressure liquid chromatography (UPLC)-ESI-MS/MS.

A number of studies have used CE-ESI-MS/MS for bottom-up based proteomics analysis [5–16]. One set of papers employed a sheathless electrospray interface with a porous capillary tip as the nanospray emitter [5–8]. Faserl *et al.* evaluated the suitability of the interface for peptide analysis [7]. They applied the interface for CE-ESI-MS/MS analysis of Arg-C-digested rat testis linker histones, identifying 135 peptides and eight non-histone H1 proteins from 6 ng of sample. They also studied the same sample using nano-liquid chromatography (nLC)-ESI-MS/MS. The UPLC system identified 129 peptides and 23 non-histone HI proteins. Wang *et al.* developed online solid phase microextraction preconcentration, fractionation, and transient isotachophoresis CE–ESI-MS/MS procedure with the porous tip interface for a moderately complex protein mixture analysis [8]. They did comparative proteomic analysis of CE and nLC for series of sample loading amounts,

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and found complementary identifications for 100 ng of *Pyrococcus furiosus* tryptic digest. CE identified three times more proteins from a 5 ng digest than nLC.

This group developed and applied an electrokinetically pumped sheath-flow electrospray interface [9–15]. Li *et al.* used the interface for capillary zone electrophoresis (CZE)-ESI-MS/MS analysis of the tryptic digest of a sample of intermediate protein complexity, the secreted protein fraction of *Mycobacterium marinum* [11]. Eleven fractions were generated by reversed-phase LC (RPLC) and each fraction was analyzed by the CZE-ESI-MS/MS system. In total, 334 peptides corresponding to 140 proteins were identified in 165 min of mass spectrometer time from ~1 ng of sample. Roughly 40 peptides were detected in each 15-min long CZE separation. With comparable instrument time and 250 ng sample loading, 388 peptides corresponding to 134 proteins were identified by nLC-ESI-MS/MS. The CZE-MS and nLC-MS peptide and protein IDs were complementary; CZE-ESI-MS/MS tended to identify more basic and hydrophilic peptides with low molecular masses. In addition, the CZE-ESI-MS/MS system with the electrokinetically pumped sheath-flow interface generated low amole level peptide detection limit with an LTQ-Orbitrap Velos as the detector [10, 12], and high zeptomole level peptide detection limit when coupled to a triple-quadrupole mass spectrometer using multiple reaction monitoring [13]. Finally, capillary isoelectric focusing-ESI-MS/MS was used with the interface for the analysis of host-cell proteins in a recombinant monoclonal antibody [14]. Thirty-seven proteins were identified in the 30-minute long separation. An improved cIEF-ESI-MS/MS system employed amino acids as ampholytes; the low m/s of amino acids minimized interference by the ampholytes in peptide analysis [15]. The amino acid based cIEF-ESI-MS/MS, system identified 112 protein groups and 303 unique peptides in triplicate runs of a RAW 264.7 cell homogenate protein digest.

To date, the largest number of identifications in a single CE analysis (single-shot analysis) is on the order of 50 peptides [7, 8, 11]. This mediocre performance might be due to several reasons. First, the loading capacity of CE is usually limited, especially for CZE, which will limit peptide identification. Second, the separation performance may degrade when a large amount of peptide is loaded onto the capillary. Third, relatively fast CZE separations have been used, which limit the number of tandem mass spectra that can be generated during the separation. Fourth, the mass spectrometer and its parameter settings may not have been optimized. Finally, no highly complex proteomic sample has yet been analyzed by CZE-ESI-MS/MS, and the limited complexity of samples studied to date will limit the number of possible identifications.

To improve the peptide identifications of single-shot CE-ESI-MS/MS analysis, we coupled the electrokinetically pumped sheath-flow electrospray interface with an improved CZE-ESI-MS/MS system for analysis of 1–100 ng *E. coli* digest. The CZE system employed a coated capillary, which reduced electroosmotic flow and increased the separation time, which allowed for generation of a large number of tandem spectra. The sample buffer was manipulated to facilitate stacking, which allowed injection of a large amount of sample. High-energy collisional dissociation [17] was applied for peptide fragmentation in an LTQ-Orbitrap Velos, due to its better performance for bottom-up proteomics analysis compared with collisionally induced dissociation [18, 19].

Materials and Methods

Materials

Bovine pancreas TPCK-treated trypsin, urea, ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and formic acid (FA) were purchased from Fisher

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Scientific (Pittsburgh, PA, USA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, IE, USA). Water was deionized by a Nano Pure system from Thermo Scientific (Marietta, OH, USA). Linear polyacrylamide (LPA)-coated fused capillary (50 μm i.d./150 μm o.d.) was purchased from Polymicro Technologies (Phoenix, AZ, USA). Complete, mini protease inhibitor cocktail (provided in EASYpacks) was purchased from Roche (Indianapolis, IN, USA).

Preparation of the E. coli sample

Solid lysogeny broth (LB) was used to make the agar plates for *E. coli* culture. Solid LB was prepared by dissolving 3 g NaCl, 3 g tryptone, 1.5 g yeast extract, and 6 g of agar in 300 mL of deionized water. Liquid LB medium (without agar) was also prepared for *E. coli* culture by mixing 10 g NaCl, 10 g tryptone, and 5 g of yeast extract in 1 L of deionized water. All media, plates, and other utensils and flasks were appropriately autoclaved before use. Frozen cultures of *E. coli* (Dh5-Alpha) were thawed and plated on the prepared agar plates. After incubated at 37°C for 24 h, single colonies were isolated and grown in tubes with 4 mL liquid LB medium and incubated in a shaker at 37°C overnight. When the tubes' contents turned opaque, the liquid medium with *E. coli* was transferred into new flasks, and shaken overnight at 37°C.

The liquid LB medium with *E. coli* was centrifuged, and the resulting *E. coli* pellets were washed with PBS for three times. Then, the *E. coli* pellets were suspended in 8 M urea and 100 mM Tris-HCl (pH 8.0) buffer supplemented with protease inhibitor, and sonicated for 15 min on ice for cell lysis. The lysate was centrifuged at 18,000 x g for 15 minutes, and the supernatant was collected. The protein concentration was measured by the BCA method. An aliquot of protein (900 μg) was precipitated by cold acetone overnight at −20°C. After centrifugation, the pellet was washed again with cold acetone. The resulting protein pellet was dried at room temperature.

The dried *E. coli* proteins (900 μg) were dissolved in 300 μL of 100 mM NH_4HCO_3 (pH 8.5) with 8 M urea, and denatured at 37 °C for 60 min followed by reduction with DTT (8 mM) at 60 °C for 1 h and alkylation with IAA (20 mM) at room temperature for 30 min in dark. Then 1.2 mL of 100 mM NH_4HCO_3 (pH 8.5) was added to reduce the concentration of urea to less than 2 M. Finally, an aliquot of 120 μg treated proteins was digested by incubation with trypsin at a trypsin/protein ratio of 1/30 (w/w) for 4 h at 37 °C. The digests were acidified with FA (0.5% final concentration) 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). The dried protein digests were dissolved in 0.1% FA or in 50% (v/v) ACN with 0.05% FA for CZE-ESI-MS/MS analysis, and in 0.1% FA for UPLC-ESI-MS/MS analysis.

CZE-ESI-MS/MS

The CZE-ESI-MS/MS system was similar to that reported previously [9–12]. High voltage was supplied by two Spellman CZE 1000R power supplies. The LPA-coated separation capillary (60 cm, 50 μm i.d./150 μm o.d.) was coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) with an electrokinetically pumped sheath-flow electrospray interface [9]. The borosilicate glass emitter was pulled in a Sutter pipette puller to a ~6 μm ID tip. Voltage programming was controlled by LabView software. For separation, 250 V/cm was applied for 100 ng and 300 V/cm for 1 ng and 10 ng *E. coli* digests analysis. Electrospray was powered by 1.5 kV. Sample injection was performed by pressure, and the injection volume was about 24 nL for 100 ng and 10 nL for both the 1 ng and 10 ng *E. coli* digest analyses.

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Full MS scans were acquired in the Orbitrap mass analyzer over the m/z 395–1800 range with resolution 30,000 (at 400 m/z). Ten most intense peaks with charge state 2 were fragmented in the higher-energy collisional dissociation collision cell, and analyzed by the Orbitrap mass analyzer with resolution 7,500. One microscan was used. Normalized collision energy was set as 40%. For MS and MS/MS spectra acquisition, maximum ion inject time was set as 500 ms and 250 ms, respectively. The precursor isolation width was 2 Da. The target value for MS and MS/MS was set as 1.00E+06 and 5.00E+04, respectively. Dynamic exclusion was applied for the experiments. Peaks selected for fragmentation more than once within 25 s were excluded from selection for 25 s.

UPLC-ESI-MS/MS

A nanoACQUITY UPLC system with a UPLC BEH 130 C18 column (Waters, 100 μm i.d. × 100 mm length, 1.7 μm particles) was coupled to a LTQ Orbitrap Velos instrument via a pulled capillary tip as the electrospray tip for peptide separation and identification. The RPLC gradient (A: 0.1% (v/v) FA in water; B: 0.1% (v/v) FA in ACN) was as follows: 0–8 min, 2% B; 8–10 min, 2–10% B; 10–30 min, 10–30% B; 3–32 min, 30–85% B; 32–40 min, 85% B; 40–40.5 min, 85–2% B; 40.5–50 min, 2% B. The flow rate was 1.2 μL/min. The sample injection volume was 1 μL. The electrospray voltage was 1.5 kV and the MS parameters were the same as that used for CZE-ESI-MS/MS analysis.

Data analysis

Database searching of the raw files was performed in Proteome Discoverer 1.3 with MASCOT 2.2.4 against the SwissProt database with taxonomy as *Escherichia coli* (13,477 sequences). Database searching of the reversed database was also performed in order to evaluate the false discovery rate (FDR) [20, 21]. The database searching parameters included full tryptic digestion and allowed up to two missed cleavages, precursor mass tolerance 10 ppm, and fragment mass tolerance 0.05 Da. Carbamidomethylation (C) was set as fixed modifications. Oxidation (M) and deamidated (NQ) were set as variable modifications.

On peptide level, peptide confidence value as high was used to filter the peptide identification, and the corresponding FDR on peptide level was less than 1%. On protein level, protein grouping was enabled.

Results and discussion

Optimization of the CZE-ESI-MS/MS for E. coli protein digests analysis

To improve the number of identifications from single-shot CZE-ESI-MS/MS, we first varied the sample buffer composition. Two sample buffers were evaluated with a 60 cm LPA coated separation capillary: 0.1% (v/v) FA and 0.05% (v/v) FA with 50% (v/v) ACN, S-Fig. 1 in supporting material I. The loading amount of *E. coli* digest was 60 ng, and injection volume was about 20 nL. For the digests dissolved in 0.1% (v/v) FA, the separation capacity and peptide identifications were poorer than that produced with the separation buffer containing 0.05% (v/v) FA with 50% (v/v) ACN. For digests dissolved in 0.05% (v/v) FA with 50% (v/v) ACN, the digests were stacked at the beginning of the separation due to the lower conductivity of the buffer compared with the 0.1% (v/v) FA separation buffer [22, 23]. This stacking reduced the effect of the large injection volume on the separation performance, resulting in higher separation capacity, more peptide identifications (1,132 vs. 815), and higher base peak intensity (8.85E7 vs. 3.90E7). Furthermore, 80% of the peptides identified using the 0.1% FA buffer (656) were found in the identification using 0.05% FA + 50% ACN.

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We also evaluated the effect of capillary length on the peptide identifications using 60 cm and 40 cm LPA-coated capillaries. The 60 cm separation capillary generated more peptide IDs than 40 cm capillary (1,520 vs. 1,184) with the same sample loading amounts (100 ng *E. coli* digests), likely due to the longer separation time, which allowed generation of ~50% more tandem mass spectra. Therefore, 0.05% (v/v) FA containing 50% (v/v) ACN as sample buffer and 60 cm LPA-coated separation capillary were used for subsequent experiments.

Comparison of single-shot CZE-ESI-MS/MS and UPLC-ESI-MS/MS for 1–100 ng E. coli digests

The same *E. coli* digest loading amounts (1–100 ng) and same mass spectrometer time (50 min) were applied for both CZE-MS/MS and UPLC-MS/MS, Fig. 1 The number of identified peptide and protein group IDs for different sample loading amounts with both methods are listed in Table 1. For a relatively large sample loading amount (100 ng), single-shot CZE-ESI-MS/MS generated 1,377 ± 128 peptides and 312 ± 29 protein groups (mean ± standard deviation of the distribution), which was roughly 25% poorer than single-shot UPLC-ESI-MS/MS. When the sample loading amounts were decreased to 10 ng, a comparable number of peptide IDs was generated from CZE-MS/MS and UPLC-MS/MS (997 ± 54 vs. 1,108 ± 104). Interestingly, when the loaded digest was mass limited (1 ng), CZE-MS/MS generated the same number of protein groups and 83% more peptide IDs compared with UPLC-MS/MS. The observation that CE-MS/MS produces more protein and peptide IDs is consistent with earlier results [8, 12]. In addition, CZE-ESI-MS/MS is reasonably reproducible for analysis of complex protein digests in terms of identified number of peptides and protein groups (relative standard deviation less than 10% for triplicate runs). However, the peptide intensity from UPLC-ESI-MS/MS is dramatically lower than that from 10 ng and 100 ng samples. Weak peptide signal leads to more random peptide identification, which makes the identification reproducibility of UPLC-ESI-MS/MS for 1 ng sample analysis poorer than for 10 ng and 100 ng samples.

To further characterize the analysis of 1 ng *E. coli* digests, we extracted peaks of five peptides identified by both CZE-MS/MS and UPLC-MS/MS, and obtained their peak intensity information, S-Table 1. Unexpectedly, the peptide intensity from CZE-MS/MS was about one order of magnitude higher than that from UPLC-MS/MS. In addition, CZE-MS/MS generated many more tandem spectra (2,029 ± 200 vs. 837 ± 139), peptide spectral matches (PSMs) (879 ± 41 vs. 398 ± 59), and peptide IDs (627 ± 38 vs. 342 ± 113). It is worth mentioning that the relative standard deviations in peptide intensity generated by triplicate CZE-MS/MS and UPLC-MS/MS were comparable (S-Table 1), which suggests that the CZE-ESI-MS/MS might be useful for quantitative proteomics analysis.

We also determined the overlap of peptides and protein groups from triplicate CZE-MS/MS and UPLC-MS/MS analysis of 1–100 ng *E. coli* digests, Fig.2. CZE-MS/MS and UPLC-MS/MS were complementary at the peptide level for 100 ng and 10 ng *E. coli* digests analysis, and to some extent complementarities also existed on protein group level, which agreed with previous reports [11]. The complementarity on peptide level was useful for improvement of protein sequence coverage, resulting in more confident identification. As an example, triplicate UPLC-ESI-MS/MS generated sequence coverage of 78% for DNA-directed RNA polymerase subunit omega with 100 ng loaded per run, and combination of CZE-MS/MS and UPLC-MS/MS produced 92% sequence coverage for the protein. As another example, triplicate UPLC-ESI-MS/MS only produced 4.6% sequence coverage for FKBP-type peptidyl-prolyl cis-trans isomerase slyD with 100 ng loading amount per run. Addition of the CZE-MS/MS results increased its sequence coverage to 49%.

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We analyzed the physical features including molecular weight, m/z, pI values and GRAVY values of identified peptides in CZE-ESI-MS/MS and UPLC-ESI-MS/MS for 10 ng sample loading amount (S-Fig. 2 in supporting material I).

Like our earlier results, CE-ESI-MS/MS tends to identify more basic peptides, and also tends to identify more hydrophilic peptides compared with UPLC-ESI-MS/MS.[11] These peptides are poorly retained on the reversed-phase chromatography stationary phase. Unlike our earlier study, there was no significant trend for the identification of small peptides. This result might be due to two reasons. First, the sample injection methods used in the two studies are different. Our earlier work employed electrokinetic injection, which will tend to bias against slow-moving and larger peptides. In contrast, this paper employed pressure injection, which will provide an unbiased sampling of peptides with respect to their size-to-charge ratio. Second, no pre-fractionation of peptides was performed before CZE-ESI-MS/MS analysis in this work, which makes the trend not as evident as previous reports. According to the mass and m/z distributions of peptides (S-figure 2A, B), single shot CZE-ESI-MS/MS identified more very large peptides (MW>3000 Da) than LC-MS/MS, which might be due to difficult elution of these peptides from the UPLC column.

Conclusions

Single-shot CZE-ESI-MS/MS generates more than 1,250 peptide IDs from complex protein digests. Slightly fewer peptide IDs were identified compared with single-shot UPLC-ESI-MS/MS for 100 ng digests. In contrast, CZE-ESI-MS/MS produced more peptide IDs than UPLC-ESI-MS/MS for 1 ng digest. In addition, CZE-ESI-MS/MS and UPLC-ESI-MS/MS were complementary for peptide identifications. These results indicated that the CZE-ESI-MS/MS may be an important alternative for large-scale, more comprehensive, and confident proteomics analysis.

It is worthwhile pointing out the rapid improvement in performance of CZE-ESI-MS/MS for bottom-up proteomics. The state of the art peptide and protein IDs for many years was from Yate's laboratory [24], which identified 136 peptides and 66 proteins from a yeast ribosome digest in eleven 30-min long CZE separations of fractions generated using solid-phase extraction. This group reported 344 peptides and 140 proteins from the secretome of *M. marinum* in eleven 15-min long CZE separations of fractions generated using HPLC separation. In this paper, we report over 1,250 peptide and 300 protein identifications in a 50-min long single shot CZE separation. The performance of CZE-ESI-MS/MS will undoubtedly improve as sample preparation, separation conditions, electrospray conditions, and mass spectrometer operating parameters are further optimized. If so, CZE will be a powerful competitor to UPLC for bottom-up proteomic studies of complex samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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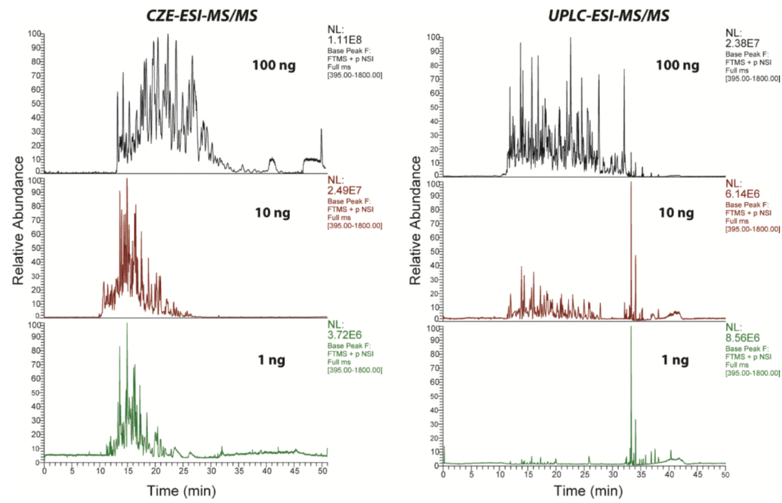

Figure 1.
Base peak electropherograms/chromatograms of 1–100 ng *E. coli* digests analyzed by CZE-ESI-MS/MS and UPLC-ESI-MS/MS.

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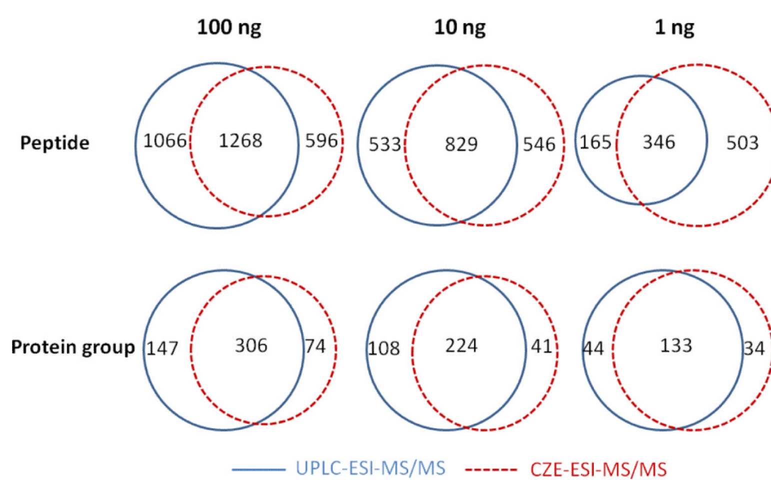

Figure 2.
Overlap of peptide and protein groups from triplicate CZE-ESI-MS/MS and UPLC-ESI-MS/MS analysis of 1–100 ng *E. coli* digests.

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Table 1

Identification results of single-shot CZE-ESI-MS/MS and UPLC-ESI-MS/MS for 1–100 ng *E. coli* protein digests analysis.

***E. coli* digests loading amount (ng)**	**Single shot CZE-ESI-MS/MS (triplicate runs)**		**Single shot UPLC-ESI-MS/MS (triplicate runs)**	
	Protein groups	**Peptides**	**Protein groups**	**Peptides**
100	312 ± 29*	1,377 ± 128	395 ± 3	1,875 ± 32
10	212 ± 19	997 ± 54	280 ± 9	1,108 ± 104
1	142 ± 10	627 ± 38	140 ± 25	342 ± 113

*Uncertainties are standard deviation of the mean.