Technical Note

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One-Step Procedure for Peptide Extraction from In-Gel Digestion Sample for Mass Spectrometric Analysis

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Protein identification from samples resolved by one-dimensional and two-dimensional gel electrophoresis is highly dependent on the recovery of trypsin-digested peptides prior to mass spectrometric analysis. The commonly used two-step protocol for extracting tryptic peptides, involving high-volume organic solvent extraction and cleanup via microscale reversed-phase micropipet tip or microcolumn is not only limited by significant sample loss but is also costly and very labor-intensive. We report here a simple one-step procedure for simultaneous peptide extraction and cleanup by incubating a small piece of C18 Empore Disk (3M) with the in-gel digested solution. We show that the direct Empore Disk-based peptide extraction procedure is convenient, economical, and has higher efficiency as compared with the commonly used two-step protocol for peptide preparation prior to MS analysis.

Mass spectrometry (MS)-based proteomics is a highly sensitive method for protein identification and characterization.[1] Currently, there are two common MS-based proteomic approaches: multidimensional protein identification technology (MudPIT)[2] or shotgun proteomics where peptides from in-solution digestion of the whole proteome are usually analyzed by multidimensional LC−MS/MS, and gel-based proteomics where peptides eluted from one-dimensional or two-dimensional gel electrophoresis (1D GE or 2D GE)[3] are normally analyzed by MALDI-MS or LC−MS/MS. These two complementary approaches both play important roles in the discovery of diagnostic biomarkers[4,5] and therapeutic targets.[6,7]

In the gel-based approach, protein identification is largely dependent on the efficient peptide preparation from the excised 1D GE gel slices or 2D GE gel spots. Here a peptide concentration/purification step to remove MS-interfering contaminants (e.g., salts and detergents) prior to MS analysis is often required not only to increase protein identification score and sequence coverage of abundant proteins but also to enhance the capability of identifying low abundance proteins. The conventional method of peptide preparation typically consists of two steps: first, peptide elution from the gel using repetitive organic solvent extraction after in-gel digestion, followed by removal of organic solvent in a vacuum centrifuge and, second, peptide concentration/purification by binding the peptides to reversed-phase material in microcolumns or microtips such as the commonly used ZipTip (Millipore, Billerica, MA). This procedure is fairly efficient but is accompanied by several shortcomings. First, the use of high organic content to extract peptides from in-gel digested solution requires processes such as vacuum drying and sample transfer, which result in up to 50% sample loss.[8] Second, commercial microscale reversed-phase micropipet tips or microcolumns are expensive and their use is labor-intensive. Third, the binding properties of these tips/columns (e.g., forming primary flow) and the need for relatively large elution volumes have been found to be incompatible for downstream MS analysis.[9] The newly developed C18 microspin column[10] and microtips (e.g., StageTip)[11] could enhance peptide binding and elution but demonstrated little improvement in

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simplifying the tedious procedures and relieving labor-intensive steps.

The ideal peptide extraction and cleanup method should be easy to handle, inexpensive, and with minimal number of steps to reduce sample loss and possible contamination. We describe here a simple one-step procedure, where a small piece of C18 Empore Disk (3M, St. Paul, MN) was corked and added directly into the in-gel digested solution and incubated for 3 h. The peptides in solution bind to the hydrophobic beads that were immobilized homogeneously on a Teflon matrix. Small interfering molecules can be washed off easily by immersing the Empore Disk into a washing buffer, followed by peptide elution via a small-volume of MS-compatible solvent. We show that peptides prepared by this method could be accurately and efficiently identified by MS. Therefore, this peptide extraction and cleanup method is convenient, inexpensive, and highly efficient in peptide preparation from gel bands/spots for MS analysis.

EXPERIMENTAL SECTION

Cell Culture, Protein and Peptide Sample Preparation. BL21 strain of *Escherichia coli* was cultured in LB medium (Pronadisa, Hispanlab, Madrid, Spain) at 37 °C overnight. The cells were harvested by centrifugation at 12000*g* for 10 min and washed twice with phosphate-buffered saline. The cells were resuspended in lysis buffer (25 mM NaCl and 2 mM EDTA in 50 mM Tris, 4 °C) and subjected to intermittent sonication using a Vibra Cell high-intensity ultrasonic processor (Jencon, Leighton Buzzard, Bedfordshire, UK). The unbroken cells and debris were removed by centrifugation at 12000*g* at 4 °C for 10 min. Proteins in the supernatant were precipitated using methanol/chloroform as described[12] and resuspended in 6 M urea/2 M thiourea. Protein concentration was determined using 2-D Quant Kit (GE Healthcare, Milwaukee, WI). Aliquots of the protein sample were then stored at −80 °C until analysis.

Standard protein BSA solutions (Sigma-Aldrich, St. Louis, MO) with protein amount ranging from 1 pmol to 1 fmol were prepared in a suitable volume before loading onto a SDS−PAGE gel. Commercial tryptic-digested BSA peptides (Michrom Bioresources, Auburn, CA) with peptide amount ranging from 5 pmol to 1 fmol were dissolved in 100 mM ammonium bicarbonate/0.1% trifluoroacetic acid (TFA) and stored at −80 °C until analysis.

One- and Two-Dimensional Gel Electrophoresis. For 1D GE separation, protein samples were separated as described[13] using 4−12% (w/v) gradient precast NuPAGE Novex Bis-Tris gels (Invitrogen, Carlsbad, CA) with an XCell SureLock Mini-Cell (Invitrogen). For 2D GE analysis, 200 μg of proteins was diluted with DeStreak Rehydration Solution containing 0.5% ampholytes (GE Healthcare) to a final volume of 420 μL. This protein solution was applied to ReadyStrips (24 cm) to be passively rehydrated at room temperature for 16 h. Proteins were then subjected to isoelectric focusing at 50 V for 3 h; 500 V for 1 h; 1000 V for 1 h before increasing linearly to 8000 V in 1 h, 8000 V for 3 h, and finally rapidly ramping to and maintaining at 10 000 V for a total of 40 000 Vh using an Ettan IPGphor III isoelectric focusing system (GE Healthcare). Subsequently, the ReadyStrips were equilibrated with buffer A (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 30% glycerol, and 2% (w/v) dithiothreitol), followed by buffer B (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 30% glycerol, and 2.5% (w/v) iodoacetamide) before being applied to 10% polyacrylamide gel for the second-dimension separation. The electrophoresis on the gel was carried out at 17 w/gel at 10 °C for ∼5 h in an Ettan DALTsix Electrophoresis System (GE Healthcare). The protein gels were stained using either a Silver-Quest Silver Staining Kit (Invitrogen) or SimplyBlue solution (Invitrogen). The 2D gels were scanned using an Xcise scanner (Proteome Systems Ltd. and Shimadzu-Biotech, Kyoto, Japan), and the spots of interest were cut and transferred into 96-well microplates with the Xcise scanner system for the subsequent tryptic digestion.

Conventional Peptide Extraction and Cleanup from In-Gel Digestion. Protein gel bands or spots were in-gel digested as described[14] with some modifications. Briefly, after in-gel digestion using trypsin, the digested solution was transferred into a clean 0.6-mL tube. Fifty microliters of 50% acetonitrile (ACN)/5% formic acid (FA) was added to the gel pieces and sonicated for 30 min. This extraction procedure was repeated three times, and a total of ∼150 μL of extracts was collected. All extracts were pooled and concentrated to less than 10 μL using an SPD 2010 SpeedVac system (Thermo Electron, Waltham, MA). Thereafter, the sample was diluted with 0.1% FA in HPLC water to 100 μL for direct LC−MS/MS analysis or reconstituted with TFA to a final concentration of 0.1% and subjected to sample cleanup steps using C18 ZipTips (Millipore) prior to MALDI-TOF/TOF-MS analysis. The C18 ZipTips were conditioned with 100% ACN and then equilibrated three times with 0.1% TFA. The peptides were bound to the ZipTip pipet tip by aspirating and dispensing the sample for at least 15 cycles, washed with 0.1% TFA, and eluted by 20 μL of elution buffer (75% ACN, 0.1% TFA).

Empore Disk Peptide Extraction and Cleanup from In-Solution and In-Gel Digestion. To quantitatively evaluate the efficiency of the Empore Disk in-solution extraction method, two different sizes of Empore Disk pieces were cut with the tip of a 200-μL pipet (0.5 mm in diameter) and a 1-mL pipet (0.75 mm in diameter). The cut-out Empore Disks were first wetted with 100% ACN and conditioned with 100% methanol, followed by direct immersion into the solutions with 1 pmol, 100 fmol, and 10 fmol of commercial digested BSA peptides, respectively. After incubation for 3 h at room temperature, the Empore Disks were washed with 200 μL of 0.1% of TFA. The bound peptides were incubated in 15 μL of elution buffer (75% ACN, 0.1% TFA) for 30 min. The eluted peptides were used for the subsequent MALDI-TOF/TOF-MS or LC−MS/MS analysis. For the MALDI-TOF/TOF-MS analysis, only 2 μL of elution buffer was used.

For peptide extraction after in-gel digestion, the trypsin activity was stopped by addition of TFA to a final concentration of 0.1%. The Empore Disks were then immersed into the digestion solution and incubated for 3 h to extract peptides from the gel. The subsequent procedure was the same as that of in-solution extraction.

MALDI-TOF/TOF-MS Analysis. The peptide sample was mixed with an equal amount of matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% ACN and

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spotted onto a 384-well stainless steel MALDI target plate (Applied Biosystems, Foster City, CA). An ABI 4800 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems) was used to analyze the spotted samples. Both MS and MS/MS spectra were recorded in the combination mode and submitted for database searching. Typically, 1800 shots were accumulated for each MS spectrum while 4500 shots were accumulated for each MS/MS spectrum. For protein ID, combined MS and MS/MS data were submitted via GPS Explorer (version 3.6, Applied Biosystems) to Mascot server (version 2.0, Matrix Science) for database searching. Swissprot database (version 54.4, including 287 050 entries, and 105 598 259 residues) was utilized for the search. Cysteine carbamidomethylation and methionine oxidation were selected as fixed and variable modifications, respectively, and two missing cleavages were allowed. Precursor ion tolerances were set to <0.15 Da, and MS/MS fragment ion error tolerances were set to <0.3 Da. Proteins were accepted as true positives if they had significant scores of $p < 0.05$.

LC−MS/MS Analysis. The extracted *E. coli* peptide samples were vacuum-centrifuged to ~1−10 μL and then reconstituted to 100 μL with 0.1% FA in HPLC water. The samples were then injected by an autosampler and online desalted in a Zorbax peptide trap (Agilent, Palo Alto, CA). The peptide separation was carried out with a home-packed nanobored C18 column (75 μm i.d × 15 cm, 5-μm particles) directly into a picofrit nanospray tip (New Objective, Wubrun, MA), operating at a flow rate of ~200 nL/min after a splitter with a 53-min gradient. Solvent A was 0.1% FA/99.9% H_2O; Solvent B was 0.1% FA/99.9% ACN. The gradient included a 35-min linear gradient from 5 to 35% ACN/0.1% FA, 10-min gradient from 35 to 60% ACN/0.1% FA, and 3-min gradient from 60 to 80% ACN/0.1% FA and 5 min of 80% ACN/0.1% FA. The C18 column was conditioned with 5% ACN/0.1% FA for 10 min between each run. The LTQ-Orbitrap (Thermo Fisher Scientific, Inc., Bremen, Germany) was run in a data-dependent mode by performing MS/MS scans (in LTQ) for the eight of the most intense peaks from each MS scan (in Orbitrap). The machine was operated at a nanospray voltage of 1.8 kV, without sheath gas and auxiliary gas flow, ion-transfer tube temperature of 180 °C, collision gas pressure of 0.85 mTorr, and normalized collision energy at 35% for MS/MS. Ion selection threshold was set to 500 counts for initiating MS/MS while activation q was set to 0.25 and activation time to 30 ms. The MS scan range was 350−2000 m/z. Dynamic exclusion was activated with an exclusion duration of 30 s. The MS/MS data were submitted to Sequest protein sequence database search engine for peptide/protein identification. For Sequest protein ID, the raw data was batch searched with Bioworks Browser (version 3.3, Thermo Finnigan). In order to estimate the false positive rate (FPR), the search was performed with "target" (forward database) and "decoy' (reverse database) as described.[15] The FPR was controlled below 1.5% for Sequest identification. The database was NCBI *E. coli* database (version 4, including 54 969 entries). Carbamidomethyl at cysteine residue was set as static modification; while oxidation at methionine and phosphorylation at serine, threonine, or tyrosine residues were set as variable modifications. A maximum of four post-translational modifications were allowed for each peptide. Peptide tolerance was 100 ppm, and fragment ions tolerance was 1 amu. Full enzymatic cleavage by trypsin was selected, with a maximum of two missed cleavages. Matched peptides were prefiltered by requiring XCorr cutoffs of 1.5, 2.5, and 3.5 for +1, +2, and +3 charged peptides, respectively, as well as a DeltaCN cutoff of 0.08 and a peptide probability cutoff of 0.05. Peptide identification was confirmed with the probability score calculated by Bioworks Browser. Peptide quantitation was based on the area of the extracted ion chromatogram (XIC) of precursor ion calculated by Bioworks Browser 3.3, with a mass tolerance of 0.05 Da, and five smoothing points.

Statistical Analysis. All of the experiments were repeated at least three times unless stated otherwise. Results were averaged from the same set of experiments. Standard deviations were calculated, shown as error bars. Paired Student's t-test was used for the comparison of protein scores, sequence coverages, and peptide numbers between the common proteins identified by both Empore Disk-based and conventional ZipTip-based methods from 1D or 2D GE in-gel samples. $p < 0.05$ was taken as significant.

RESULTS AND DISCUSSION

Direct Empore Disk-Based Peptide Extraction Is Compatible With MALDI-TOF/TOF-MS Analysis. The efficiency of the peptide extraction and concentration method with Empore Disk from solution was evaluated using known amounts of commercial digested BSA peptides in 100 mM ammonium bicarbonate buffer. Twenty-three peptides were identified with a high degree of confidence by MALDI-TOF/TOF-MS analysis from 10 fmol of BSA digest, with 38% sequence coverage. When 100 fmol of BSA tryptic peptides was used, 30 peptides were identified and the sequence coverage reached 50%. Interestingly, higher sample amount did not always lead to better sequence coverage of the protein. The reason might be the precursor ions of high-abundance peptides suppressed the signals from low-abundance peptides (Supporting Information (SI) Table S-1).

We next tested the efficiency of the procedure for in-gel digested samples. BSA solution aliquotes of various amounts ranging from 1 pmol to 1 fmol were separated by SDS−PAGE and silver-stained. All bands from different lanes were excised for in-gel digestion. The peptides were extracted with Empore Disk as described in the Experimental Section. BSA was consistently identified from the bands with sample concentrations of 1 pmol and 500 fmol by the MALDI-TOF/TOF-MS (SI Table S-1). Occasionally, BSA could be identified from the 10-fmol gel bands (data not shown). The results indicated that the one-step Empore Disk procedure was an effective method for peptides extraction method from both solution and gel matrix for MALDI-TOF/TOF-MS analysis.

Peptide Extraction Efficiency from Solution. ZipTip is widely employed for peptide extraction and cleanup from both in-solution and in-gel digested proteins. It is fairly efficient, but falls short as an ideal tool because of its high price, significant sample loss in multistep sample preparation, time-consuming, and the need to change buffers before LC−MS/MS analysis. The one-step peptide extraction approach based on Empore Disk as described above could be a viable alternative method in that it is low cost, less laborious, and compatible with subsequent MALDI-TOF/TOF-MS and LC−MS/MS analyses. To explore this possibility, we compared the peptide extraction efficiency of Empore Disk with the well-established conventional ZipTip method using

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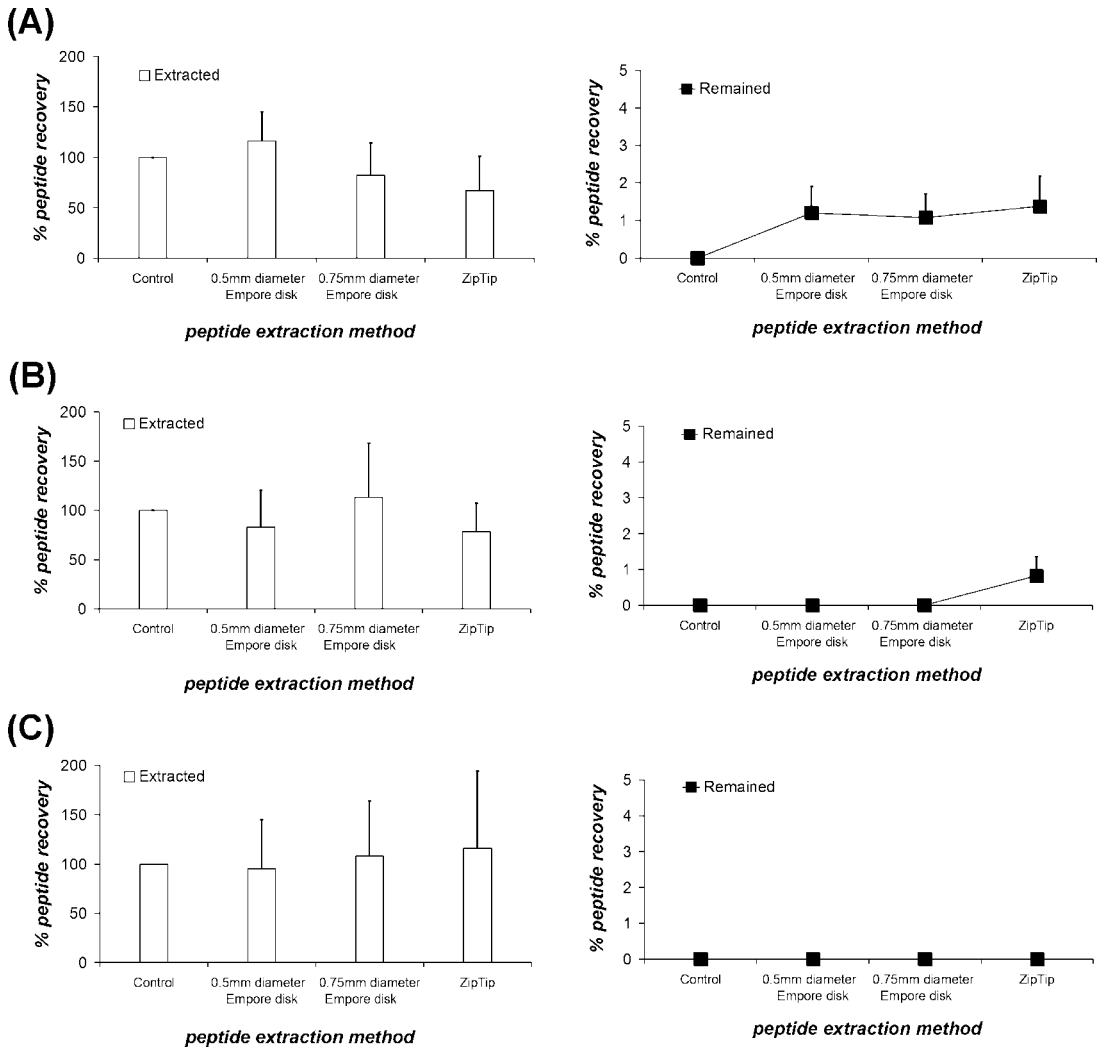

Figure 1. Percentage of peptide recovered from and remained in solution containing 1 pmol (A), 100 fmol (B), and 10 fmol (C) of BSA by 0.5-mm-diameter Empore Disk, 0.75-mm-diameter Empore Disk, and ZipTip. The percentages were calculated as average of top 5 detected peptides. Area of XIC of peptide (extracted or remained)/area of XIC of peptide (direct injection of the same amount of peptide) × 100%. The data were averaged from three experiments.

identical BSA tryptic digests. The 0.5-mm-diameter disks and 0.75-mm-diameter disks were excised from Empore Disk using 200-μL and 1-mL plastic pipet tips, respectively. The disks were then incubated with the same volume of BSA digest solution of varying concentrations. LTQ-Orbitrap was used for semiquantitative analysis of the samples. After the peptide extraction, the remaining or the flow-through solution was also analyzed. Control experiments were performed as direct injection of the same amount of BSA tryptic peptides. Analysis of the samples was done in a sequential order starting with samples of the lowest to highest concentration to minimize the carryover effect. The raw data were submitted to Sequest or Mascot for protein sequence database searching. The extracted ion chromatograms of the identified peptides were integrated by Bioworks Browser. The areas of the remainder and extracted peptides were normalized with the control.

Figure 1 shows the percentage of peptide recovery based on five of the detected peptides that generated the strongest ion signals. The recovery was normalized against the control. At 10 fmol of BSA tryptic peptides, the peptide recoveries by both Empore Disk and ZipTip were nearly 100%, and this was confirmed by the undetectable peptide levels remaining in the extracted solution (Figure 1B and C). When the amount of BSA tryptic peptides was increased to 1 pmol, a small amount of peptides (~1−2%) remained in the solution after extraction (Figure 1A). These data suggest that the peptide binding capacity of the 0.5- and 0.75-mm-diameter Empore Disk and ZipTip are comparable.

Comparison of Conventional and Empore Disk-Based In-Gel Extraction for MALDI-TOF/TOF-MS Analysis. The conventional in-gel digestion followed by peptide extraction using ZipTip is laborious and usually results in significant sample loss. To evaluate if the one-step Empore Disk-based technique could be an alternative method of extracting peptide from gel, we first compared the yield and efficiency of peptide extraction by the two methods with a large (50 μg) and a small (1 μg) amount of *E. coli* cell lysate. The 50 and 1 μg of *E. coli* cell lysates were both separated by 1D GE in duplicate. One of the duplicated samples was subjected to the Empore Disk method, while the other was used for the conventional peptide extraction. After separation, the former (50 μg) gels were stained with Coomassie

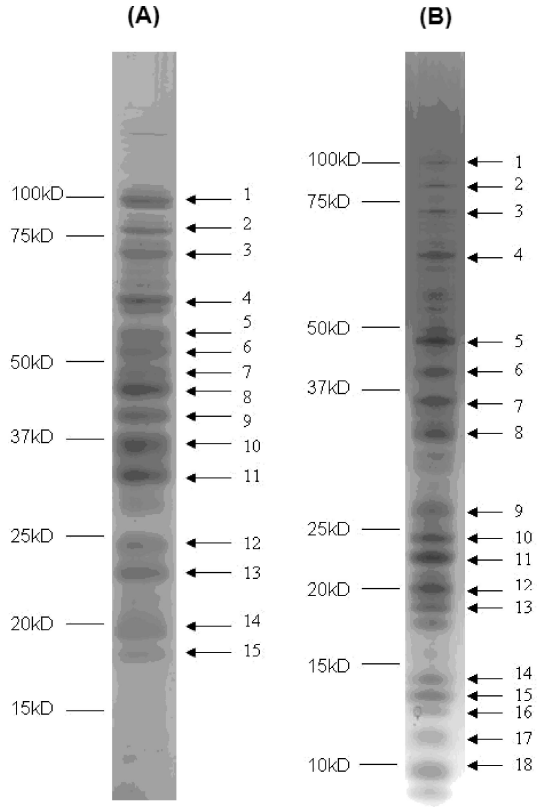

Figure 2. 1D GE separation of *E. coli* cell lysate. (A) The 50 μg of *E. coli* cell lysate was separated by 4−12% (w/v) gradient precast gel and was stained by Coomassie Blue. Fifteen bands were cut and subjected to both conventional in-gel extraction method followed by ZipTip desalting and Empore Disk extraction method. (B) One microgram of *E. coli* cell lysate was separated by 4−12% (w/v) gradient precast gel and was stained by Silver. Eighteen bands were cut and subjected to both conventional in-gel extraction method followed by ZipTip desalting and Empore Disk extraction method.

blue and the latter (1 μg) with silver. Protein bands were excised and subject to in-gel trypsin digestion. The tryptic peptides were then extracted using either the conventional ZipTip- or Empore Disk-based methods.

For the large (50 μg) lysate samples, 15 bands from each of the duplicate gels were selected for the in-gel digestion followed by peptide extraction and cleanup using either the conventional ZipTip- or Empore Disk-based methods, respectively (Figure 2A). In the conventional protocol, peptides were extracted with 50% ACN/5% FA solution. The sample was dried and reconstituted with 10 μL of 5% ACN/0.1% TFA prior to desalting by ZipTip. In the Empore Disk-based procedure, peptide extraction and desalting were achieved simultaneously in an aqueous solution, as peptides continuously diffused out from the gel and bound to the hydrophobic beads in Empore Disk. Subsequent MALDI-TOF/TOF-MS analysis of the two samples prepared using the ZipTip or Empore Disk approaches identified a total of 31 proteins. Each approach identified 25 proteins (Table S-2, SI). Six proteins were unique to each approach, and 19 proteins were identified by both approaches. For identification of these sharing proteins, the Empore Disk approach showed a higher Mowse score than the ZipTip approach (Table S-2, SI).

For the small (1 μg) lysate samples, 18 gel bands were selected for peptide extraction by either ZipTip- or Empore Disk-based methods followed by MALDI-TOF/TOF-MS analysis (Figure 2B). We identified a total of 26 distinct proteins from the 18 selected bands. Of these, 26 proteins were identified from 12 protein bands using the Empore Disk approach while only 5 proteins were identified from 4 protein bands by the ZipTip approach. Average Mowse scores for the five common proteins in Empore Disk data set were 90% higher than that in ZipTip (Table 1). The above experiments were repeated another three times, and the results consistently showed that the Empore Disk-based method enabled identifying more proteins with higher Mowse scores from the set of excised gel bands than ZipTip.

The efficiency of the two methods in extracting peptides from protein spots in 2D GE stained by silver was also compared. 2D GE separation of *E. coli* extracts was performed in duplicate, the gels were stained with silver, and a set of 33 protein spots was excised from each of the duplicate protein gels (Figure S-1, SI). Peptide extraction was carried out by either one of the two methods. A total of 35 proteins from 33 spots were identified. Of these, 30 (86%) were identified in samples extracted using Empore Disk method, whereas only 17 (49%) proteins were identified from 27 spots using the conventional ZipTip method (Table S-3, SI). For the 12 common proteins identified by both ZipTip and Empore Disk, the latter showed significantly higher Mowse score ($p < 0.05$) as evaluated by paired Student's *t*-test. Proteins identified by Empore Disk had 43% higher Mowse scores on average, although sequence coverage of the two methods was comparable.

The MALDI-TOF/TOF-MS analysis of peptides extracted from both tryptic digest of 1D GE and 2D GE showed that the Empore Disk method was more sensitive for low-abundance silver-stained gel bands/spots. However, the MALDI signal quality usually depends on multiple parameters such as the "sweet spots" problem where the analyte is not evenly distributed over the sample spot.[16] Although the results were not quantitative, the general observation was that both methods were equally good for the high-abundance proteins as there was no significant difference for the protein ID in Coomassie blue-stained gel bands. However, for silver-stained gel spots/bands or low-abundance proteins, more proteins were identified in Empore Disk extracts than in ZipTip extracts, suggesting that the Empore Disk peptide extract was of better quality.

Peptide Extraction Efficiency from Tryptic Digested 2D GE Spot. We employed semiquantitative LC−MS/MS to investigate the peptide extraction efficiency of the two methods for samples separated with 2D GE. Duplicated 2D GE of *E. coli* cell lysate were run, and 24 gel spots (Figure S-2, SI) with 1.4-mm diameter were excised with an Xcise gel cutter (ProteomeSystems) from each gel into a 96-well microtiter plate. The gel spots were subsequently digested with trypsin by the Xcise robotics. One set of samples was extracted manually with ZipTip, while the other set was prepared with the 0.5-mm-diameter Empore Disk method. After elution from the ZipTip and Empore Disk, the samples were injected into LTQ-Orbitrap as pairs of the same spots for semiquantitative analysis. The raw data file was submitted to Sequest for protein ID, and the area of the identified peptides was integrated by setting the mass range to 0.05 Da for extracted ion chromatogram integration.

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Table 1. Proteins Identified from Silver-Stained 1D GE Separation of *E. coli* Cell Lysate by Conventional (ZipTip Desalting) and Empore Disk-Based Method, Respectively

protein information			ZipTip		Empore Disk	
band no.	accession no.	protein description	protein score	Seq Cov (%)	protein score	Seq Cov (%)
2	P0A6M9	elongation factor G (EF-G)	59	32	118	35
4	P0A6F6	60-kDa chaperonin (protein Cpn60) (groEL protein)	180	32	183	19
5	P0A6N2	elongation factor Tu (EF-Tu) (P-43)	232	50	448	52
6	P0A799	phosphoglycerate kinase (EC 2.7.2.3)			61	33
7	P02931	outer membrane protein F precursor (Porin ompF) (outer membrane protein 1A)	86	19	164	10
	P0A9B3	glyceraldehyde-3-phosphate dehydrogenase A (EC 1.2.1.12) (GAPDH-A)			71	16
	P0ABK5	cysteine synthase A (EC 2.5.1.47) (*O*-acetylserine sulfhydrylase A) (*O*-acetylserine (Thiol)	61	40	266	56
	P0A868	transaldolase A (EC 2.2.1.2)			55	44
8	P61890	malate dehydrogenase (EC 1.1.1.37)			123	24
	Q8FLD1	transaldolase B (EC 2.2.1.2)			79	35
	P0A868	transaldolase A (EC 2.2.1.2)			65	24
	P0A6P2	elongation factor Ts (EF-Ts)			93	39
	P39325	ABC transporter periplasmic-binding protein ytfQ precursor			58	15
	P37902	glutamate/aspartate periplasmic-binding protein precursor			77	25
	P16700	thiosulfate-binding protein precursor			60	17
9	P0AEU1	histidine-binding periplasmic protein precursor (HBP)			103	40
	P62708	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (EC 5.4.2.1) (phosphoglyceromuta			70	34
10	P0AEQ4	glutamine-binding periplasmic protein precursor (GlnBP)			262	46
	P30859	arginine-binding periplasmic protein 1 precursor			89	45
11	P0A806	ribosome recycling factor (ribosome-releasing factor) (RRF)			113	40
	P0AFH8	osmotically inducible protein Y precursor			184	27
	P0ADV7	protein yrbC precursor			63	21
	P00448	superoxide dismutase [Mn] (EC 1.15.1.1) (MnSOD)			78	28
12	P0AGD4	superoxide dismutase [Fe] (EC 1.15.1.1)			108	40
13	P69783	glucose-specific phosphotransferase enzyme IIA component (EC 2.7.1.-)			66	10
	P23869	peptidyl-prolyl cis−trans isomerase B (EC 5.2.1.8) (PPIase B) (rotamase B)			62	23
14	P76290	protein yecO			61	46

From the 24 excised gel spots, 17 distinct proteins were identified from 16 gel spots using the ZipTip method, while 29 proteins were identified from 20 gel spots using the Empore Disk method. Among these identified proteins, 14 proteins were identified by both methods. Proteins identified by Empore Disk extraction showed significantly higher scores ($p < 0.05$) and higher peptide numbers ($p < 0.01$) than those using ZipTip as evaluated by paired Student's *t*-test. On average, proteins identified by Empore Disk have 58% higher scores and 57% higher peptide numbers than those identified by ZipTip. The most significant peptides of each of the 14 proteins were selected for quantitative comparison of peptide peak areas. The result showed that peptides identified by Empore Disk extraction had higher intensities than those identified by ZipTip extraction. The ratio of the XIC areas and the standard deviations were calculated and shown in Table 2. Interestingly, both methods detected some unique proteins, 15 unique proteins were detected by the Empore Disk method, while only 3 proteins were unique to the conventional method.

In general, peptides extracted from gel spots using the Empore Disk method yielded more protein IDs with higher scores than those extracted by the ZipTip method. However, we did not find any significant difference for peptides extracted from solution digests of BSA. This indicates that the binding efficiency of both Empore Disk and ZipTip are approximately the same. Thus, the observed difference was likely due to the higher peptide extraction efficiency from the gel matrix by the Empore Disk incubation method.

To determine why Empore Disk has a higher peptide extraction efficiency from gel matrix, we calculated the area of extracted ion chromatogram of each peptide from the identified gel spot and the ratios of the area between Empore Disk and ZipTip methods were determined. As some of the gel spots contained more than one protein, and some proteins have more than 40 tryptic peptides, we did not include all the peptides in the area ratio calculation, including only those peptides producing relatively strong ion signals. All outlying peptides were not included in the area ratio calculation. Figure 3 shows a typical pair of XIC of peptides used for the extraction efficiency calculation. Figure 3A is the XIC of the MAPPQISAEVLK peptide extracted by the Empore Disk, while Figure 3B is the XIC of the peptide extracted by ZipTip. The ratio of XIC for this peptide is 10.1 (Empore Disk: ZipTip).

The GRAVY values of each identified peptide were calculated. We found that the area ratio was dependent only slightly on the hydrophobicity of the peptides as shown in Figure 4B. Although both the Empore Disk and tryptic-digested gel spots were incubated in a low percentage of organic solvent (5% ACN) for peptide extraction, this method still slightly favors hydrophobic peptides.

One possible explanation for the more efficient peptide extraction by the Empore Disk is that, unlike proteins, most peptides are readily soluble in aqueous solution. As peptides diffuse out of the gel matrix, the more hydrophobic peptides tend to bind strongly to the Empore Disk. This effectively reduces the

Table 2. Proteins Identified from 2D GE Separation of *E. coli* Cell Lysate by Conventional (ZipTip desalting) and Empore Disk-Based Method Followed by LC–MS/MS

protein information			ZipTip			Empore Disk			quantitation			
spot no.	protein gi number	protein description	peptides	probability	score	peptides	probability	score	unique peptide detected	peptide number used for quantitation	ratio (Empore Disk/ZipTip)	SD
1	16128008	chaperone Hsp70, cochaperone with DnaJ	153	2.22×10^{-15}	220.30	263	1.11×10^{-15}	320.38	34	20	13.066	2.709
1	16128878	30S ribosomal subunit protein S1	14	5.96×10^{-10}	60.20	104	1.00×10^{-30}	180.31	18	12	12.791	2.739
2	16131968	Cpn60 chaperonin GroEL, large subunit of GroESL	139	5.48×10^{-12}	120.25	241	3.33×10^{-15}	210.35	21	7	56.221	4.495
2	16128421	peptidyl-prolyl cis/trans isomerase (trigger factor)				2	6.83×10^{-7}	10.14	1			
3	90111690	aspartate ammonia-lyase	29	1.30×10^{-7}	80.21	54	1.58×10^{-12}	110.29	11			
4	90111690	aspartate ammonia-lyase				76	4.14×10^{-13}	120.28	12			
5	16128088	GTP-binding tubulin-like cell division protein	127	3.72×10^{-11}	140.28	124	2.69×10^{-12}	170.31	18	9	6.287	0.571
6	16128147	glutamate-1-semialdehyde aminotransferase (aminomutase)	62	4.62×10^{-10}	70.24	64	8.87×10^{-12}	80.27	9	4	7.573	0.734
7	16130533	heat shock protein				25	1.42×10^{-12}	30.28	3			
8	16130826	fructose-bisphosphate aldolase, class II	46	9.44×10^{-13}	30.25	88	9.21×10^{-14}	70.26	7	4	251.052	136.659
9	16130815	ribose 5-phosphate isomerase, constitutive	27	1.34×10^{-11}	30.24	24	1.36×10^{-11}	30.23	4	4	8.44	2.485
9	16131119	stringent starvation protein A				4	1.09×10^{-6}	10.16	4			
10	16128723	phosphoglyceromutase 1	29	2.35×10^{-10}	40.20	44	3.49×10^{-10}	80.19	8	5	17.938	9.866
11	16131680	uridine phosphorylase	12	5.13×10^{-5}	10.18	7	3.05×10^{-6}	20.18	2	2	52.035	25.82
13	16130909	alcohol dehydrogenase, NAD(P)-dependent	3	1.85×10^{-6}	10.14				1			
14	16130029	galactitol-1-phosphate dehydrogenase, Zn-dependent and NAD(P)-binding	36	1.14×10^{-7}	40.18	20	1.30×10^{-8}	40.19	5	5	10.154	13.348
14	16131091	predicted transporter subunit: ATP-binding component of ABC superfamily				1	4.59×10^{-9}	10.20	1			
15	16130231	acetate kinase A and propionate kinase 2	6	1.75×10^{-5}	10.16				1			
16	16129204	oligopeptide transporter subunit	1	7.33×10^{-7}	10.17	11	1.89×10^{-14}	20.28	*2*	1	16.031	
17	16131639	transcription termination factor				1	1.71×10^{-4}	10.19	1			
17	16130419	predicted peptidase				2	6.16×10^{-4}	10.14	1			
18	16131262	tryptophanyl-tRNA synthetase	7	4.36×10^{-8}	10.17	11	2.49×10^{-7}	30.18	*4*	1	1.112	
18	16129733	glyceraldehyde-3-phosphate dehydrogenase A				10	6.64×10^{-10}	30.18	3			
19	16131968	Cpn60 chaperonin GroEL, large subunit of GroESL				1	1.31×10^{-9}	10.20	1			
19	16132049	predicted sugar transporter subunit: periplasmic-binding component of ABC superfamily				4	5.45×10^{-6}	10.16	1			
22	90111448	uracil phosphoribosyltransferase	2	5.91×10^{-8}	10.22	18	2.09×10^{-12}	20.24	*2*	1	9.295	
22	16128453	adenine phosphoribosyltransferase				4	7.48×10^{-6}	20.22	2			
23	16128126	pantothenate synthetase				6	3.30×10^{-8}	30.25	3			
23	16129982	predicted subunit with GalU				3	6.37×10^{-6}	10.17	1			
24	16128126	pantothenate synthetase	1	5.29×10^{-7}	10.16				1			
24	16128704	succinyl-CoA synthetase, NAD(P)-binding, alpha subunit				7	1.55×10^{-6}	40.18	3			
24	16131968	Cpn60 chaperonin GroEL, large subunit of GroESL				1	6.02×10^{-5}	10.18	1			

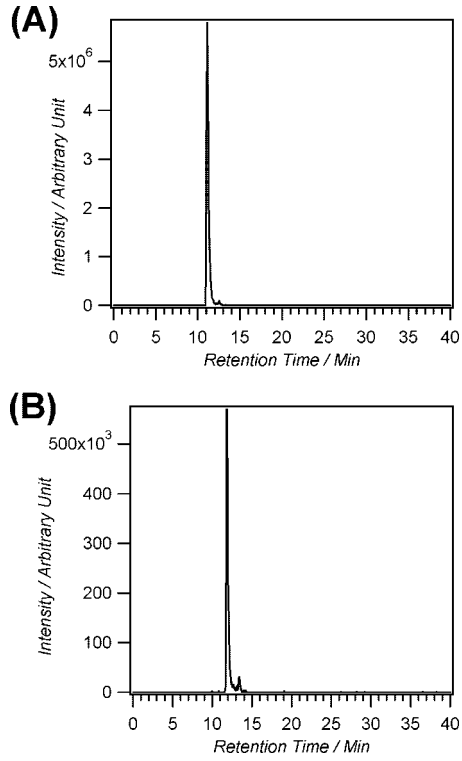

Figure 3. Extract ion chromatogram of peptide MAPPQISAEVLK at 476.603 Da ± 20 ppm by Empore Disk and ZipTip. Gel spot 1 was identified to be gi 16128008 ref NP_414555.1, chaperone Hsp70, cochaperone with DnaJ of *E. coli*. XIC of peptide MAPPQISAEVLK by Empore Disk (A) and ZipTip (B) are shown. Integrated chromatograph areas of Empore Disk and ZipTip are 1.74×10^6 and 1.72×10^5, respectively. Ratio of XIC of Empore Disk to ZipTip is 10.1.

hydrophobic peptide concentration in the solution and enhances diffusion of peptide from gel matrix to solution.

Although the extraction efficiency was not highly dependent on peptide hydrophobicity (Figure 4B), we found it was highly correlated to peptide length and peptide mass. Figure 4A shows that the XIC area ratios are highly dependent on peptide length up to 18 amino acids. When the peptide size exceeded 18 amino acids, the extraction efficiency reached its plateau. As large peptides diffuse slower than the small peptides from the gel matrix to solution, this result might suggest that the peptide extraction efficiency was limited by its diffusion out of gel matrix or that large peptides were more susceptible to sample loss in the ZipTip handling procedure. Moreover, the incubating solvent affects the pore size of the gel matrix; the gel pore can be dehydrated and shrunk by organic solvent. In our experiment, the peptide extraction by the Empore disk method was performed in 95% H_2O/5% ACN/0.1% TFA, while the conventional solvent extraction method used 50% H_2O/50% ACN/0.1% TFA. The high organic solvent used in the conventional method would reduce the gel pore size and affect the extraction of large peptides. Taken together, by simultaneously incubating the gel spots with Empore Disk for 3 h in 95% water, we ensured minimal sample handling steps, sufficient time, and optimal conditions for continuous streaming of peptides from the gel matrix to the Empore Disk.

Comparison of Conventional and Empore Disk-Based In-Gel Extraction of Whole Proteome for LC−MS/MS Analysis. To determine if the whole proteome extraction via Empore Disk could be as efficient as the conventional method, 30 μg of *E. coli* proteins (per lane) was separated by 1D GE. Each of the two lanes was excised equally into 23 slices and was extracted by Empore Disk and the conventional method respectively for LC−MS/MS analysis. The raw data were submitted to Sequest protein database search engine through Bioworks Browser. Peptide number identified in the Empore Disk-based extraction was statistically equivalent to that identified by the conventional method, with a ratio (Empore Disk: conventional method) of 1.00 ± 0.12 ($n = 3$) (data not shown).

This suggested that there were residual peptides remaining in the gels after extraction by the conventional method. This can be confirmed by performing another round of Empore Disk peptide extraction on the gels. Interestingly, we identified an additional 207 ± 29 ($n = 4$) peptides from the gel that have been

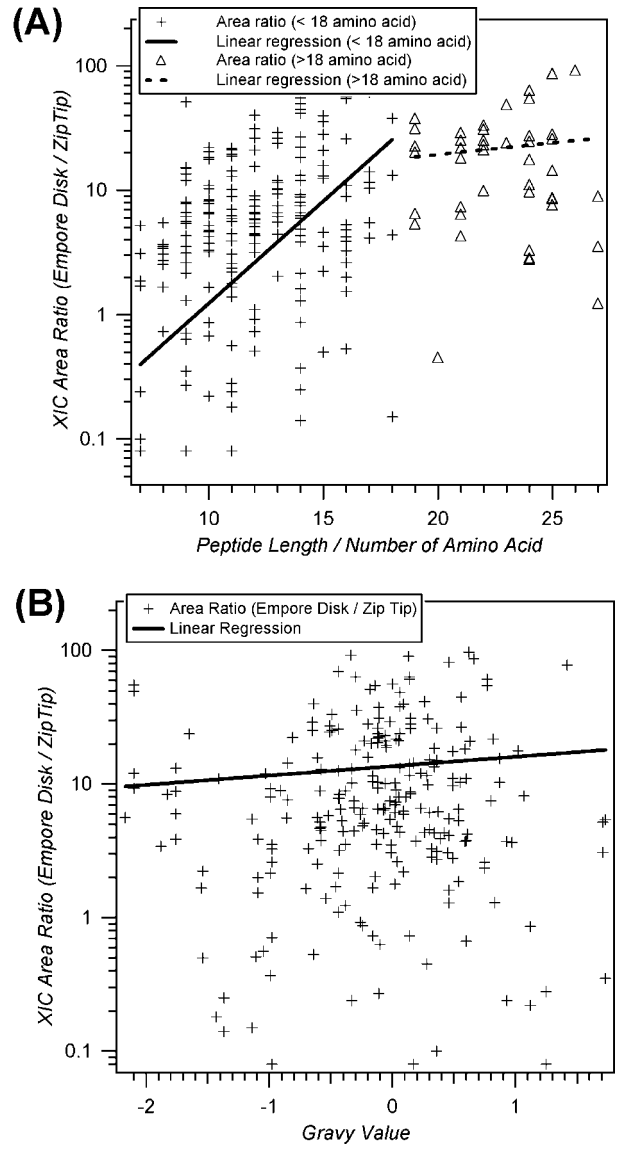

Figure 4. Relative peptide extraction efficiency of 0.5-mm Empore Disk vs ZipTip, peptides were from 24 2D GE gel spots of *E. coli* cell lysate. (A) shows that the relative peptide extraction efficiency is highly dependent on the peptide size/length. A small peptide could be easily extracted by both methods, but a large peptide that diffused slowly favored the continuous extraction by incubation of gel spot with Empore Disk in the in-gel tryptic digestion mixture. (B) shows that the relative peptide extraction efficiency is only slightly dependent on GRAVY value/hydrophobicity of the peptides.

extracted by conventional method. This indicated that the conventional method did not completely extract all peptides from gels.

The relative peptide extraction efficiency of the two methods was semiquantitatively analyzed by integrating the XIC areas of identified peptides with Bioworks Browser. As the total number of used unique peptides with $p < 0.05$ was 3202, the average XIC area ratio of the Empore Disk to the solvent extraction was calculated for each peptide length. Figure S-3 (SI) shows that the relative peptide extraction efficiency is dependent on the peptide length. As the ZipTip was not used in the sample preparation, the result suggested that the Empore Disk was a more efficient method in large peptide extraction from gel matrix than the conventional organic solvent.

CONCLUSIONS

We show that the Empore Disk approach is a simple and highly sensitive one-step method suitable for peptide extraction and cleanup prior to the MS analysis, resulting in substantial cost- and time-saving. By incubating a small piece of Empore Disk with the in-gel digested solution, we speculate that the hydrophobic C18 beads with high peptide affinity and binding capacity in Empore Disk would continuously extract peptides from the solution as soon as the peptides diffused out of the gel matrix. The bound peptides in the disks can be easily eluted out using a small amount of elution buffer for subsequent MS analysis. The whole procedure requires few hands-on steps: cutting the Empore Disk, dropping it into the in-gel digestion solution, washing it, and peptide elution. Furthermore, certain steps in the conventional method, such as repetitive peptide extraction via organic solvent that results in shrinking the gel pores and limiting peptide diffusion, vacuum drying, pipetting, and transferring of the sample between vials can be avoided. Immobilization of the C18 hydrophobic beads onto Teflon matrix enables simultaneous concentration and desalting of the peptides and simplifies the process of peptide elution,[17] thus eliminating the need for an extra desalting step and increasing the efficiency of peptide elution.[18] In addition, Empore Disk extraction can be easily adapted to accommodate different amounts of analytes by cutting disks of different sizes to achieve appropriate peptide binding capacity, as well as being applied in high-throughput experiments.

Despite the simplicity of the Empore Disk method, protein detection sensitivity and accuracy were not compromised. The sensitivity was most evident in extraction of low-abundance peptides from silver-stained gels. This simple one-step Empore Disk method was not only a cost-, labor-, and time-saving protocol, it also exhibited higher peptide extraction efficiency and quality for downstream MS analysis.

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