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Electroblotting through a tryptic membrane for LC-MS/MS analysis of proteins separated in electrophoretic gels

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Digestion of proteins separated *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) remains a popular method for protein identification using mass-spectrometry based proteomics. Although robust and routine, the in-gel digestion procedure is laborious and time-consuming. Electroblotting to a capture membrane prior to digestion reduces preparation steps but requires on-membrane digestion that yields fewer peptides than in-gel digestion. This paper develops direct electroblotting through a trypsin-containing membrane to a capture membrane to simplify extraction and digestion of proteins separated by SDS-PAGE. Subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) identifies the extracted peptides. Analysis of peptides from different capture membrane pieces shows that electrodigestion does not greatly disturb the spatial resolution of a standard protein mixture separated by SDS-PAGE. Electrodigestion of an *Escherichia coli* (*E. coli*) cell lysate requires four hours of total sample preparation and results in only 13% fewer protein identifications than in-gel digestion, which can take 24 h. Compared to simple electroblotting and protein digestion on a poly(vinylidene difluoride) (PVDF) capture membrane, adding a trypsin membrane to the electroblot increases the number of protein identifications by 22%. Additionally, electrodigestion experiments using capture membranes coated with polyelectrolyte layers identifies a higher fraction of small proteolytic peptides than capture on PVDF or in-gel digestion.

Introduction

This paper examines the use of electroblotting through trypsin-containing membranes to identify proteins separated in SDS-PAGE. Remarkably, gel electrophoresis is still the most common biochemical technique for separating proteins for applications such as identification of biomarkers and protein-antibody interactions.[1-7] However, identifying the proteins in specific gel bands typically requires additional steps such as western blotting or in-gel digestion with subsequent LC-MS/MS analysis. Western blotting is a relatively low throughput application because it requires an antibody for each protein of interest.

In-gel digestion and LC-MS/MS can readily identify proteins in electrophoretic gels, but common in-gel digestion protocols require multiple labor-intensive steps that take up to 24 h. These steps include excision of specific gel regions, extensive staining and destaining, protein reduction and alkylation, in-gel digestion, extraction of peptides into solution, and desalting prior to LC-MS/MS.[8-12]

Electroblotting provides an alternative strategy for extracting the proteins from electrophoretic gels. [13-15] In this technique, an applied voltage moves negatively charged, SDS-coated proteins from a gel toward a porous PVDF or nitrocellulose capture membrane (**Fig. 1A**). Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry can detect intact protein on the capture substrate,[13, 15-16] but enzymatic on-membrane digestion and peptide extraction enable identification of a wider range of proteins using LC-MS/MS. Nevertheless, digestion on capture substrates and extraction of proteins and peptides from PVDF and nitrocellulose may prove incomplete and require special detergents.[13, 16-22]

To simplify protein identification after electroblotting, this work explores electrodigestion (**Fig. 1B, C**). This technique employs an enzyme-containing membrane between the electrophoretic gel and the capture membrane in the electroblotting apparatus. Proteins migrate from the gel and are digested as they pass through the enzymatic membrane. The resulting peptides adsorb on the capture membrane. Compared to electroblotting combined with on-membrane digestion, electrodigestion eliminates the separate digestion

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step. Additionally, electrodigestion may yield more peptides than on-membrane digestion after electroblotting.[15-22]

We aim to increase the number of protein identifications in electrodigestion-based methods by replacing MALDI-TOF-MS

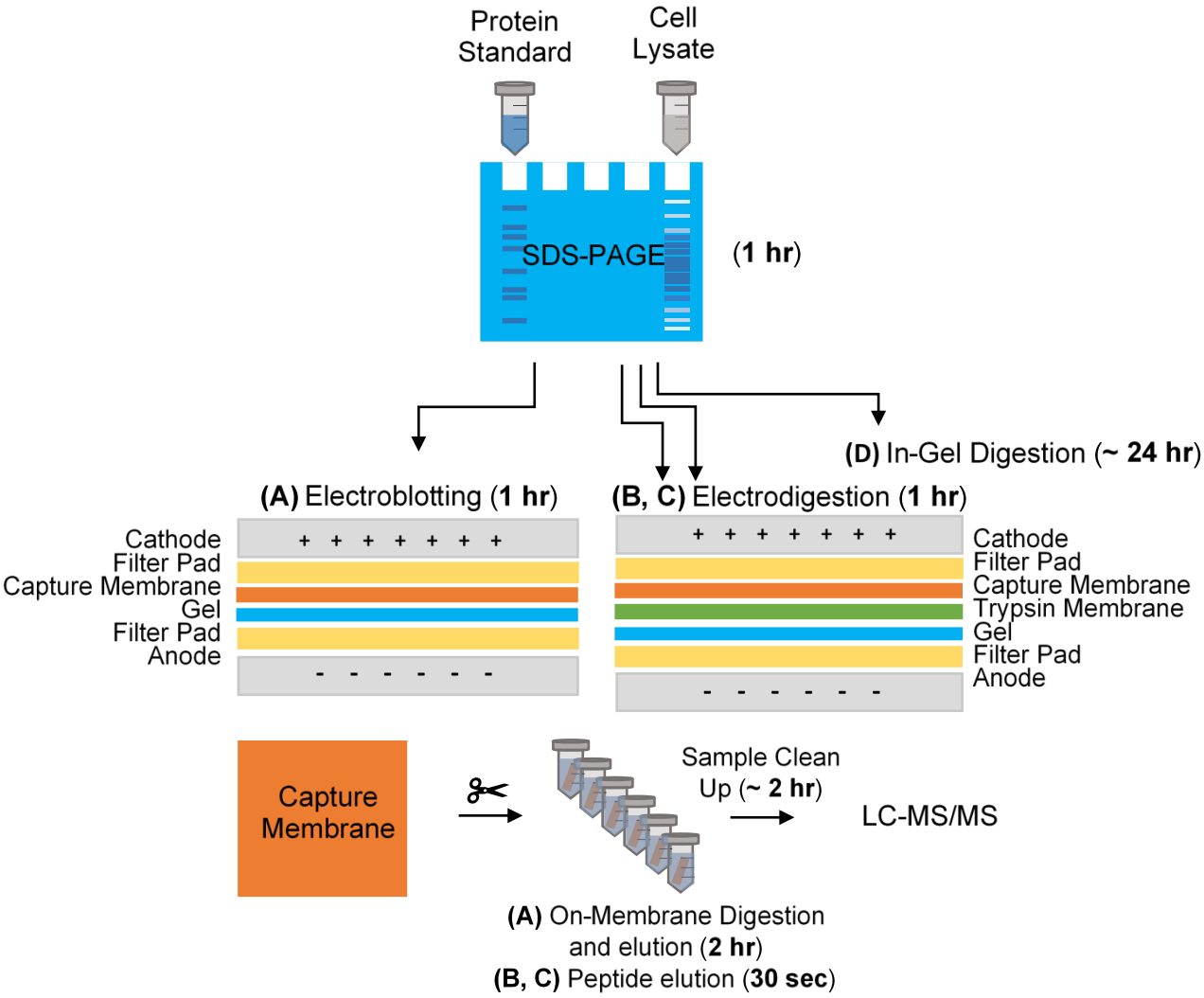

Fig. 1 Four methods for analysis of proteins separated using gel electrophoresis. **(A)** Electroblotting onto PVDF before on-membrane digestion and extraction. **(B)** Digestion during electroblotting, capture on polyelectrolyte-modified nylon, and peptide elution. **(C)** Digestion during electroblotting, capture on PVDF, and peptide elution. **(D)** In-gel digestion using a multistep procedure (not shown).

Bienvenut and coworkers aimed to improve MALDI mass spectrometry imaging using a technique they called the molecular scanner.[13] This approach combines enzymatic digestion with electroblotting of tissue samples or 2D gels and creates a tryptic peptide spatial pattern on the capture membrane. MALDI-MS imaging can reveal the peptide locations and identities.[23-29] To compare their method with in-gel and on-membrane digestion, Bienvenut and coworkers electrodigested nine standard proteins that span a broad range of molecular weights. Using the MALDI-TOF scanning approach, they identified only six of the nine proteins.[13]

with LC-MS/MS and by using more efficient trypsin and capture membranes. LC-MS/MS is better suited than MALDI for identification of a large number of peptides.[29-32] Moreover, we use proteolytic membranes with a high trypsin loading. Bienvenut and coworkers employed trypsin membranes with 0.90 ± 0.20 µg of active trypsin/cm^2. Because their enzyme capacity was low, they stacked two enzymatic membranes into their electroblotting apparatus and employed a complicated square wave alternating voltage program that took a total of 12–18 h.[13] By comparison, our membranes contain 120 to 390 µg of trypsin/cm^2, depending on the immobilization method.[33-35] With this increase in enzyme concentration, digestion can occur in residence times as short as milliseconds.[35-36] For

proteins with mobilities of $2x10^{-4}$ $cm^2/(V·s)$, [37-38] an electric field of 8 V/cm, and a membrane thickness of 120 µm, the residence time in the trypsin membrane during electrodigestion will be a few seconds.

We also examine replacement of the traditional PVDF capture membrane with porous nylon modified with polyelectrolytes to improve capture and elution of peptides. Layer-by-layer absorption of polyelectrolytes within membrane pores produces charged surfaces that may bind charged peptides more effectively than PVDF. Moreover, if peptides adsorb throughout the multilayer film, the coating will increase the number of binding sites. The polyelectrolyte film could also partially block pores and prevent small peptides from passing through the membrane during blotting.

This paper compares protein identification using in-gel digestion, on-membrane (PVDF) digestion after electroblotting, and two electrodigestions: first using a nylon capture membrane coated with a poly(acrylic acid) (PAA)/polyethyleneimine (PEI) film and second using a traditional PVDF membrane. In these comparisons we use the four workflows depicted in **Fig. 1** to analyze a standard protein mixture as well as a whole-cell lysate. Although the four techniques yield similar numbers of protein identifications, electrodigestion takes less than four hours whereas in-gel digestion requires more manipulation and can take twenty-four hours. Electrodigestion employs common western blot apparatuses and provides a convenient alternative to time-consuming in-gel procedures.

Experimental

Reagents

Nylon membranes (LoProdyne LP, pore size 1.2 µm, 110 µm thickness) were acquired from Pall Corporation (Port Washington, New York). Poly(sodium 4-styrenesulfonate) (Mw ~ 70,000) (PSS), sodium chloride, trypsin (from porcine pancreas type IX-S, lypholized powder), hydrochloric acid, poly(acrylic acid) solution (Mw ~ 100,000), branched polyethylenimine (Mw ~ 25,000) , iodacetamide, acetonitrile, ammonium bicarbonate (ABC), and sodium dodecyl sulphate (BioReagent, suitable for electrophoresis) were purchased from Sigma Aldrich (St. Louis, Missouri). Sequencing grade modified trypsin was obtained from Promega (Madison, Wisconsin), and Mini-Protean 4-20% TGX precast gels were purchased from Bio Rad (Hercules, California). HiPPR Detergent Removal columns (0.1 mL), dithiothreitol (DTT, molecular biology grade), unstained protein molecular weight marker, extra thick western blotting filter paper, and Pierce C-18 Spin Columns, were acquired from Thermo Scientific (Waltham, Massachusetts). Methanol, glycine, and ultra-pure Tris were purchased from VWR (Radnor, Pennsylvania), and low fluorescence PVDF (0.45 µm) was obtained from Azure Biosystems (Dublin, California). Zwittergent 3-16 detergent was purchased from EMD Millipore (Burlington, Massachusetts).

Immobilization of Trypsin

Immobilization of trypsin was performed as previously described.[34] A 2.5 x 3.5 cm piece of nylon was cleaned with UV-ozone for 10 min and inserted into a home-made aluminium holder attached to a peristaltic pump. 100 mL of water was passed through the membrane, and then 100 mL of 20 mM PSS in 0.5 M NaCl adjusted to pH 2.3 was circulated through the system for 20 min at 2 mL/min. 100 mL of water was then passed through the membrane before circulating 100 mL of a 1 mg/mL trypsin solution through the membrane at 2 mL/min for 1 hour. Lastly, 100 mL of 1 mM HCl was passed through the membrane, which was then dried under nitrogen and stored until use.

Immobilization of Polyelectrolyte layers

Immobilization of polyelectrolytes was performed as previously described.[33] A 2.5 x 3.5 cm piece of nylon was cleaned with UV-ozone for 10 min and inserted into a home-made aluminium holder attached to a peristaltic pump. 100 mL of water was passed through the membrane, and then 100 mL of 10 mM PAA in 0.5 M NaCl adjusted to pH 2.3 was circulated through the system for 20 min at 2 mL/min. 100 mL of water was passed through the membrane before circulating 100 mL of a 2 mg/mL PEI solution (pH 6) through the system at 2 mL/min for 20 min. Lastly, 100 mL of water was passed through the membrane, which was then dried under nitrogen and stored until use.

Cell Lysis

E. coli (MG1655) cells were grown while shaking for 6 hours at 37 °C, in LB at a 1:400 dilution from an overnight culture at 200 RPM. Cells were then pelleted by centrifugation at 6,000 x g for 10 min, washed in cold phosphate-buffered saline and lysed by 100 µm Zr bead beater (Biospec) 3x passes, 30s/pass in 600 µL of a solution containing 50 mM TRIS (pH 8), 100 mM NaCl, 25 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.05% Triton x-100. Extracted protein was then clarified by centrifugation at 12,000 g for 12 minutes. Protein concentration was determined via micro BCA (Pierce) according to the manufacturer's instructions.

SDS-PAGE

One-dimensional SDS–PAGE was performed using standard methods on the Bio-Rad Mini-Protean system, with 4-20% TGX precast 8.6 x 6.7 cm polyacrylamide minigels.[39] For separation of a standard protein mixture, 10 µL of a mixture of six native proteins (0.1-0.2 µg of each protein) in reducing buffer was loaded onto the gel after being denatured at 100 °C for 10 min. Similarly, in electrophoresis of lysate proteins, 30 µL (100 µg of total protein) of *E. coli* cell lysate was loaded after being denatured at 100 °C and diluted 1:1 in reducing buffer. The gels were then run at 140 V for 45 min.

Electroblotting and On-Membrane (PVDF) Digestion

After SDS-PAGE, gels were soaked in transfer buffer (0.1 wt% SDS, 20 vol% MeOH, 2.5 mM Tris, and 19.2 mM Glycine) for 15 minutes. The PVDF capture membrane and filter pads were also soaked for 15 minutes in transfer buffer. The electroblot was then assembled with a filter pad, gel, PVDF capture membrane, and another filter pad **Fig. 1**). Samples were blotted with the Thermo Scientific™ Owl™ HEP Series Semidry Electroblotting System at 10 V for 45 min. After blotting, the PVDF capture membrane was removed, and using the ladder on the membrane as a guide, the area from 2 kDa to 250 kDa was cut into six pieces. To elute and digest proteins, each piece was placed into a microcentrifuge tube and soaked in 100 µL of digestion buffer (10 ng/100 µL trypsin, 0.3 wt% zwittergent 3-16, 10 vol% acetonitrile (ACN), 100 mM ABC) for 2 h at 37°C.[37-38]

Electrodigestion

Electrodigestion employed the same procedure as electroblotting except that a trypsin membrane was placed between the gel and the capture membrane (**Fig. 1B, C**). (The trypsin membrane was soaked in transfer buffer for 15 min prior to cell assembly.) After electrodigestion and sectioning of the capture membrane, each piece of the membrane was placed into a microcentrifuge tube and vortexed for 30 seconds in elution buffer (0.3 wt% zwittergent 3-16, 100 mM ABC) to collect peptides. In one case, a trypsin membrane used in a previous electrodigestion of the standard protein mixture was placed between an empty, or "blank", gel piece and a PVDF capture membrane. Electrodigestion of the blank gel was done as described for the samples.

In-gel Digestion

Gels were stained with Coomassie Brilliant Blue (CBB) R250 and destained by shaking in 10% acetic acid in 40% methanol for thirty minutes followed by rinsing in water and shaking for 30 min in a solution containing 7% acetic acid, 1% glycerol, and 5% MeOH in water . The area from 2 kDa to 250 kDa (in a single lane) was then cut into six pieces of approximately equal length. The gel pieces were dehydrated in 2:1 ACN:50 mM aqueous ABC and rehydrated in 50 mM ABC in incremental 5-minute steps until CBB was removed. After immersion in 50 mM dithiothreitol for 20 minutes at 60 °C proteins in the gel pieces were alkylated using 20 mM iodoacetamide for 12 min in the dark at room temperature. The gel pieces were then rehydrated with 50 mM ABC and dehydrated with 2:1 ACN:50 mM ABC in 5-minute steps until any residual CBB was removed. The pieces were then dried down by speed vac and immersed in 20 µl of 20 µg/mL trypsin in 50 mM ABC. These samples were then placed on ice for one hour to allow trypsin to rehydrate into the gel. Proteins in the gel were then digested at 37 ℃ overnight. After digestion, the supernatant was collected in a new microcentrifuge tube. The gel pieces were then rinsed with 75 µL of 30% ACN, 0.1% formic acid (FA) for 5 minutes and the rinse was collected and combined with the previous supernatant. To quench digestion, 15 µL of 5% aqueous FA was added to the aliquots. Samples were then dried down by speed vac and desalted as described below.

Sample Clean up, Mass Spectrometry, and Data Processing

Following separation and digestion, detergent was removed using HiPPR Detergent Removal Spin Columns, and digests were desalted using 0.1 mL Pierce PePClean C18 Spin Columns following the manufacturer's instructions. The desalted peptide mixture was analysed by nano-ultrahigh performance liquid chromatography (n-UHPLC). Samples (2.5 µL) were injected onto a 100 mm × 75 µm C18-BEH column (Waters, Billerica, MA) and separated over a 60 min gradient from 5 to 35% B on a nano-Acquity system (Waters) flowing at 900 nL/min. Solution A was 0.1% FA in H_2O, and solution B was 0.1% FA in ACN. MS/MS was performed on a Q-Exactive Hybrid Quadrupole–Orbitrap instrument (Thermo, San Jose, CA) running a Top-12 data-dependent method, where a single mass spectrum at a resolution of 70, 000 was acquired, and the top 12 precursors were selected for fragmentation. Raw LC-MS/MS files were processed by MaxQuant version 1.6.7.0.[42-43] MS/MS spectra were searched against the *E. coli* (Strain K12) proteome (4,391 proteins),[44] or in the case of the molecular weight standard, a combined list of the standard protein sequences along with common contaminants. MaxQuant analysis parameters included a precursor mass tolerance of 30 ppm for the initial search, a precursor mass tolerance of 6 ppm for the main search, and an FTMS MS/MS match tolerance of 30 ppm. We set trypsin as the specific enzyme. For analysis of in-gel digestion experiments, variable modifications included N-terminal acetylation (Acetyl Protein N-term), methionine oxidation (M), deamidation (NQ), Gln → pyro-Glu, and Glu → pyro-Glu, while the fixed modification was carbamidomethyl on cysteine. For analysis of electroblotting and electrodigestion experiments variable modifications were set as described above, but carbamidomethyl on cysteine was not selected as a fixed modification. The minimal peptide length was set to seven amino acids, the maximum peptide mass was 4600 Da, and the maximum number of missed cleavages was three. Label free quantitation (LFQ) was turned on for calculation of LFQ intensities. For protein quantification, modifications included oxidation (M), acetyl (protein N-term) and deamidation (NQ), and the "discard unmodified counterpart peptides" was unchecked. The false discovery rate was 1%.

Results and Discussion

This study compares protein identification using the four protocols in **Fig. 1** to extract and digest proteins from electrophoretic gels prior to LC-MS/MS analysis. As a first step, we analyse a mixture of standard proteins and compare the number of identified peptides and protein sequence coverages in each method. To compare the four methods with a more complex protein mixture, we analyse an *E. coli* cell lysate. These studies examine the type of proteins and peptides identified and the number of shared protein and peptide identifications to help us understand differences in digestion and

capture efficiencies among the methods. The results also allow us to examine whether spatial resolution is preserved during electrodigestion.

Analysis of a Standard Protein Mixture

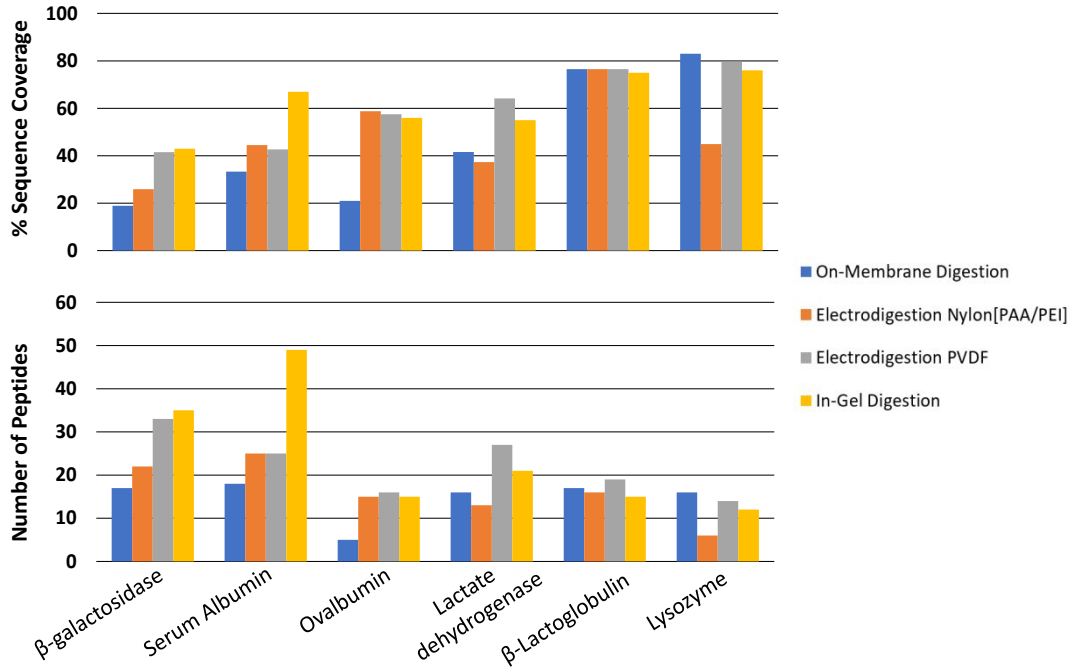

Fig. 2 Number of peptides and % sequence coverage in the analysis of a standard protein mixture using electrophoresis and the four different protocols in **Fig. 1**. The number of peptides includes all peptides found from any piece of capture membrane or gel in either of the two replicates without redundancy.

As an initial test of the different extraction/digestion methods, we separated 6 commercial proteins using SDS-PAGE and subsequently extracted and digested these proteins using the four different protocols in **Fig. 1**. **Fig. 2** summarizes the numbers of identified peptides and % sequence coverages found in analyses of all gel or capture pieces across two experimental replicates. The four methods produce multiple peptides for each protein, and sequence coverages are always >18%. All of the techniques lead to similar sequence coverages and numbers of peptides.

In each method the strongest signal for a given protein appears in the extract from the gel piece corresponding to the protein's molecular weight**. Fig. 3** shows the label free quantitation (LFQ) intensities detected for each protein in every membrane piece for

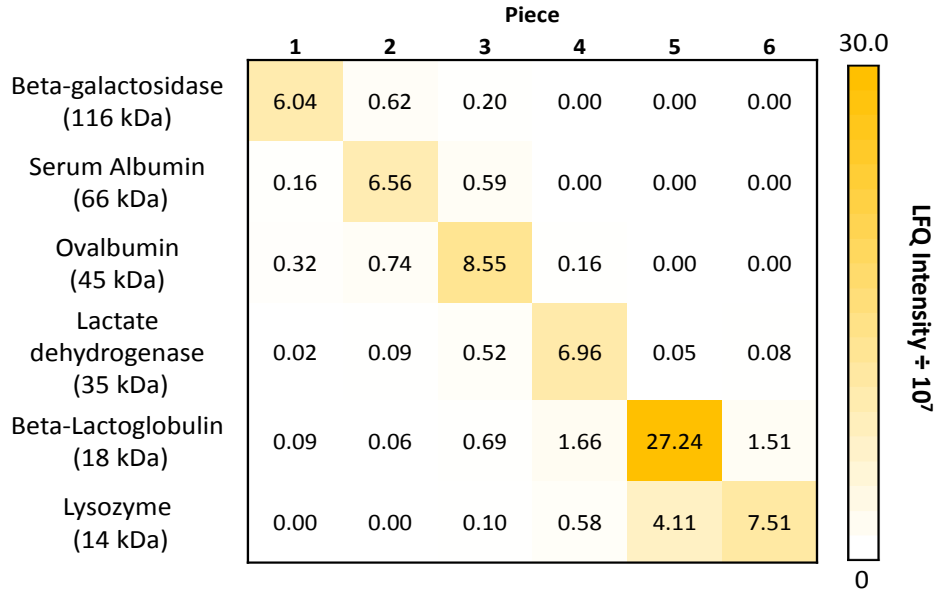

Fig. 3 Average LFQ intensities (arbitrary units ÷ 10^7) of six standard proteins in sequential pieces of a nylon[PAA/PEI] capture membrane. The x-axis shows pieces arranged in decreasing order of expected molecular weights. Intensities are the average of two experimental replicates.

Table 1. Number of distinct protein identifications, distinct peptide identifications, MS/MS spectra, percent of spectra identified (ID%), and total number of protein and peptide identifications per replicate from analyses of *E. Coli* cell lysate using each of the four methods. The number of distinct proteins and distinct peptides includes all proteins and peptides found in either of the two replicates without redundancy.

Method	Distinct Proteins	Distinct Peptides	MS/MS Spectra	ID%	Protein ID's		Peptide ID's	
					Replicate 1	Replicate 2	Replicate 1	Replicate 2
On-Membrane Digestion	1,229	10,077	81,549	42.8	1,053	1,153	7,058	8,469
Electrodigestion Nylon [PAA/PEI]	1,469	10,464	125,076	33.2	1,381	1,401	7,932	8,488
Electrodigestion PVDF	1,498	10,196	119,155	31.2	1,400	1,405	8,224	7,807
In-Gel Digestion	1,711	15,065	154,973	36.6	1,508	1,665	11,281	13,102

electrodigestion using a nylon[PAA/PEI] capture membrane. Although detection of multiple proteins in a single piece occurs, the intensity of the targeted protein is generally an order of magnitude higher than the other protein intensities in that piece. This result shows that proteins and peptides do not rapidly diffuse laterally during electrodigestion and can be excised at positions suggested by a standard molecular weight ladder. Results similar to those in Figure 3 also occur with electrodigestion using a PVDF capture membrane, in-gel digestion, and electroblotting with on-membrane digestion (see **Figs. S1-S3**).

We also examined the possibility of reusing trypsin membranes in subsequent electrodigestions. Our biggest concern with reusing the trypsin membrane is protein or peptide carry over from previous experiments. To analyse possible carry over, a trypsin membrane previously used in an electrodigestion of the standard protein mixture was placed between an empty, or "blank", gel piece and a PVDF capture membrane. Peptide elution and LC-MS/MS identified around half the number of peptides found in the first experiment (data not shown). Thus, we do not recommend reusing the membrane.

Identified Proteins in *E. Coli* cell lysate

For a more extensive comparison, we analysed an *E. coli* cell lysate using each method in experimental duplicate. **Table 1** summarizes the protein and peptide identifications made across the four methods. In-gel digestion yields the highest number of protein identifications (1,711), both electrodigestion methods identify essentially equivalent numbers of proteins (1,469 and 1,498, respectively), and on-membrane digestion yields 1,229 protein identifications. Electrodigestion requires much less time than in-gel digestion and identifies only ~13% fewer proteins. The numbers of identified proteins are similar to other methods that employ GELFrEE or gel electrophoresis separations prior to digestion and analysis. For example, Sharma et al. identified 1,190 proteins in an *E. coli* cell lysate after GELFrEE protein fractionation followed by filter-aided sample preparation (FASP) for clean-up and digestion.

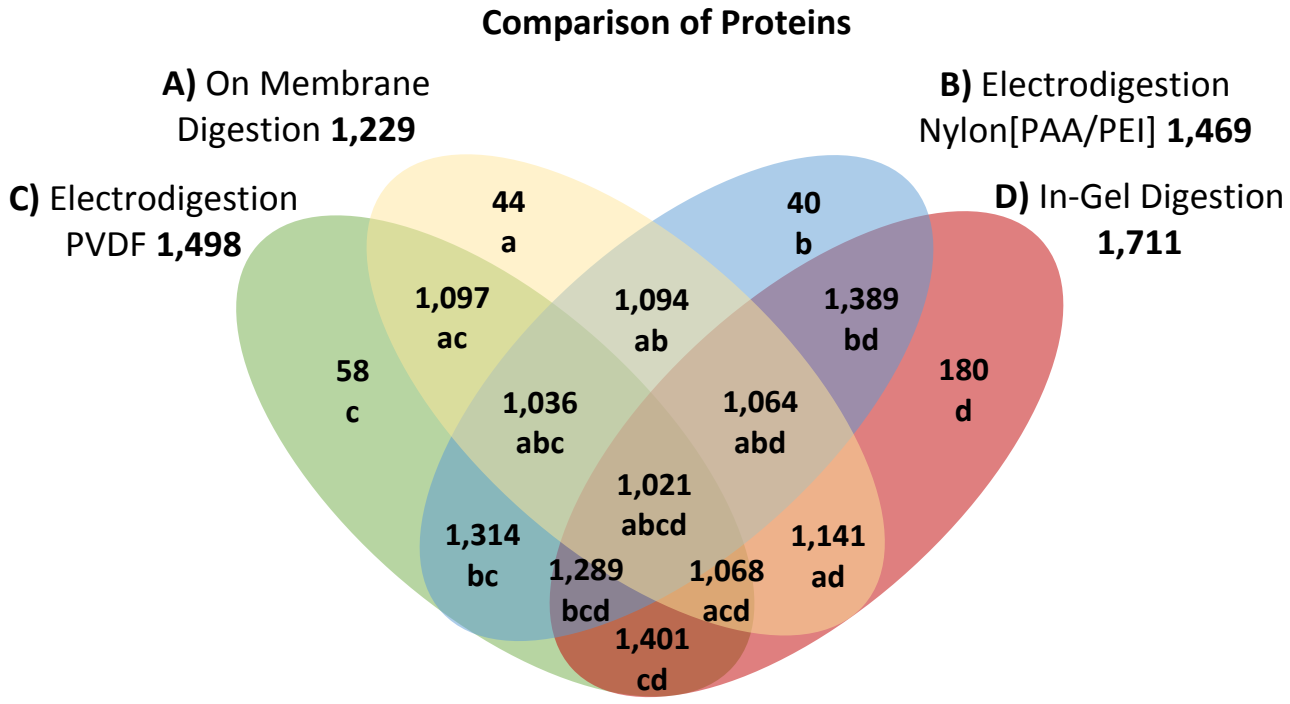

Fig. 4 Comparison of shared protein identifications in the analysis of an *E. Coli* cell lysate using the four protocols shown in **Fig. 1**.

Antberg et al. identified 688 proteins after SDS-PAGE and in-gel digestion of 100 µg of a human mantle lymphoma cell lysate.[45-48]

The four different protocols lead to a high overlap among the identified proteins. As **Fig. 4** shows, for the two electrodigestion methods (B and C), shared protein identifications (Venn Diagram region bc) account for approximately 88% of the total number of proteins identified in either method. The similarities in these two procedures, which differ only in the capture membrane, likely lead to this result. Similarly, around 89% of proteins identified in the on-membrane (A) digestion are shared with either electrodigestion method (regions ac and ab). The strong correspondence of protein identifications in these three methods suggests that the addition of a trypsin membrane does not greatly alter electrotransfer. Although in-gel digestion leads to the most protein identifications, it only identifies 180 proteins (region d in **Fig. 4**) that are not observed in the other methods. Thus, electroblotting and electrodigestion could serve as alternatives to the time-consuming in-gel procedure.

To achieve large numbers of protein identifications, we loaded large amounts of *E. coli* cell lysate onto our gel. This results in relatively poor separation of proteins (see **Fig. S4**). Nevertheless, we wanted to see if specific molecular weights appeared in the appropriate pieces of gel or capture membranes. **Fig. 5** shows LFQ intensities and molecular weights of *E. coli* cell lysate proteins identified in each piece of gel or membrane. The average molecular weights of

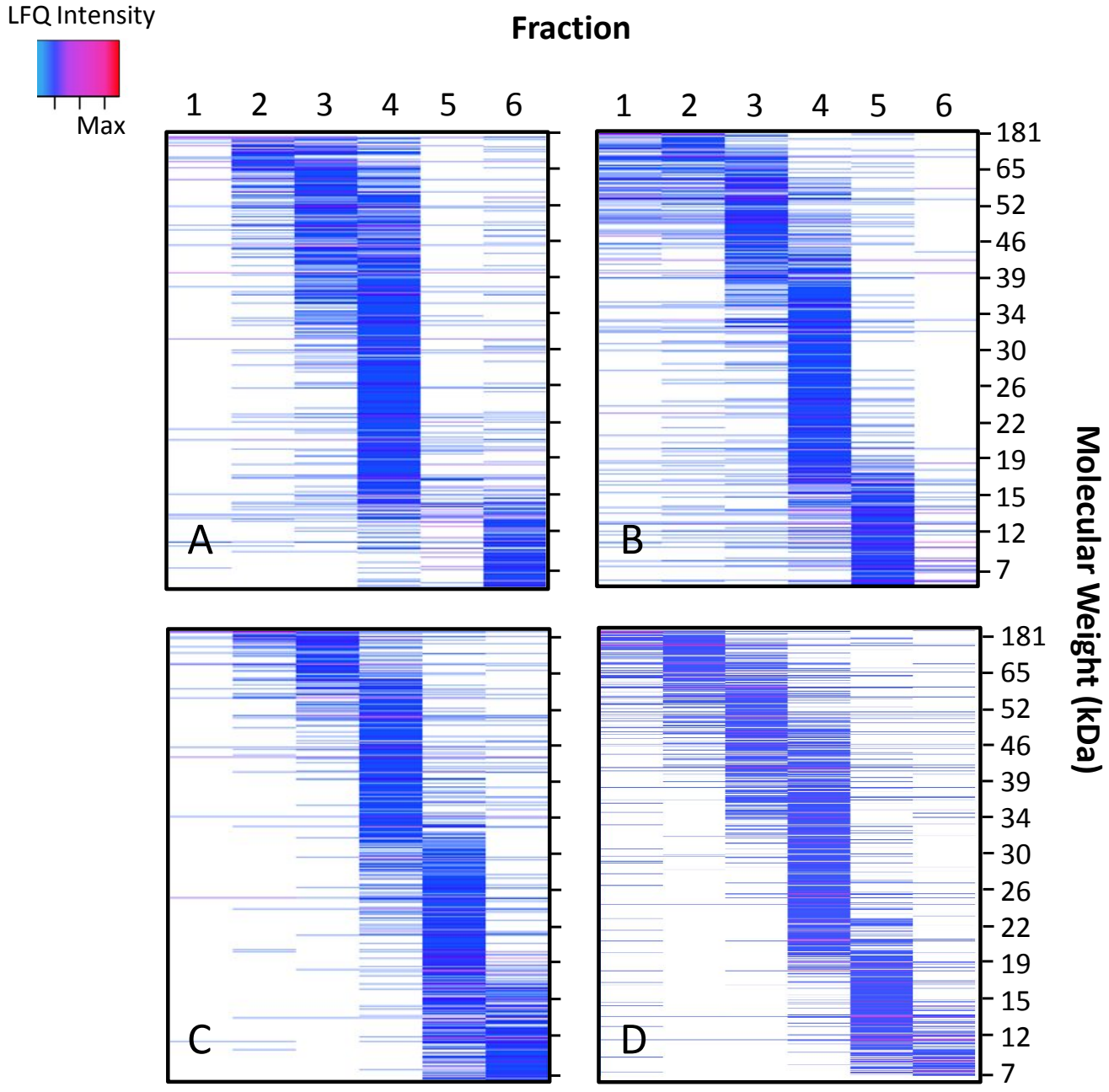

Fig. 5 Average Protein LFQ intensity per piece of gel or capture membrane plotted by molecular weight for **(A)** On-Membrane Digestion **(B)** Electrodigestion with a nylon[PAA/PEI] capture membrane, **(C)** Electrodigestion with a PVDF capture membrane, and **(D)** In-Gel Digestion.

identified proteins decrease in pieces excised further down the gel. However, proteins of higher-than expected and lower-than expected molecular weight appear, albeit at lower abundance, throughout the gel. The similar trends in **Fig. 5** for in-gel, electroblotting, and electrodigestion suggests that the SDS-PAGE separation controls the distribution of proteins in all the techniques.

All methods predominately identified proteins with molecular weights below 60kDa, and there was no obvious difference among the distribution of molecular weights for proteins identified with different techniques. The molecular weight distribution is likely not a result of transfer efficiency from the gel, but instead reflects the naturally occurring distribution of protein molecular weights in *E. coli* cell lysate (see **Fig. S5**).[49] Additionally, protein identifications unique to in-gel digestion show a similar molecular weight distribution (see **Fig. S6**). Of these unique proteins, only 11% produced a label free quantitation (LFQ) intensity in both replicates. This indicates that the higher number of unique proteins identified after in-gel digestion is likely a result of identifying low-abundance species rather than a bias toward a certain protein characteristic. In electroblotting or electrodigestion, protein or peptide losses due to adsorption on trypsin or capture membranes may decrease signals from low-abundance proteins.

The experiments reported above used unmodified trypsin (from porcine pancreas type IX-S, lyophilized powder) because previous studies employed unmodified trypsin to produce nylon enzymatic membranes.[33-34] Thus, to fairly compare the different methods, we used unmodified trypsin in each experiment. However, we did perform in-gel digestion with sequencing grade trypsin to see if this substantially increases the number of protein identifications. The sequencing grade trypsin in-gel digestion gave 1,800 protein identifications. This value is 5% more than the number of proteins identified using the unmodified trypsin, so use of unmodified trypsin should allow a fair comparison.

Identified Peptides in *E. Coli* Cell Lysate

We compared the peptides identified in each method (see **Fig. 6**) to examine digestion and capture efficiencies. Interestingly, the two different capture membranes used during electrodigestion do not adsorb highly orthogonal sets of peptides. Shared peptide identifications (region bc) in the two electrodigestion protocols are approximately 72% of the total peptides identified in either method (B or C). In contrast, around 55% of peptides identified in either electrodigestion method (B or C) are shared with on-membrane digestion (regions ab or ac). Proteolysis of proteins bound to a membrane apparently produces different peptides than electrodigestion. In-gel digestion gives the largest number of peptides and includes around 85% of peptides identified in either electrodigestion (B or C).

The difference in the number of peptides identified using in-gel or electrodigestion likely does not stem from incomplete electrodigestion. **Fig. 7** compares the digestion efficiency of the four analysis protocols in terms of the number of identified missed cleavages. The percentage of peptides with missed cleavages is about the same for electrodigestion and in-gel digestion. On membrane digestion shows the largest percentage of peptides with one or more missed cleavages. Digestion of proteins bound to a

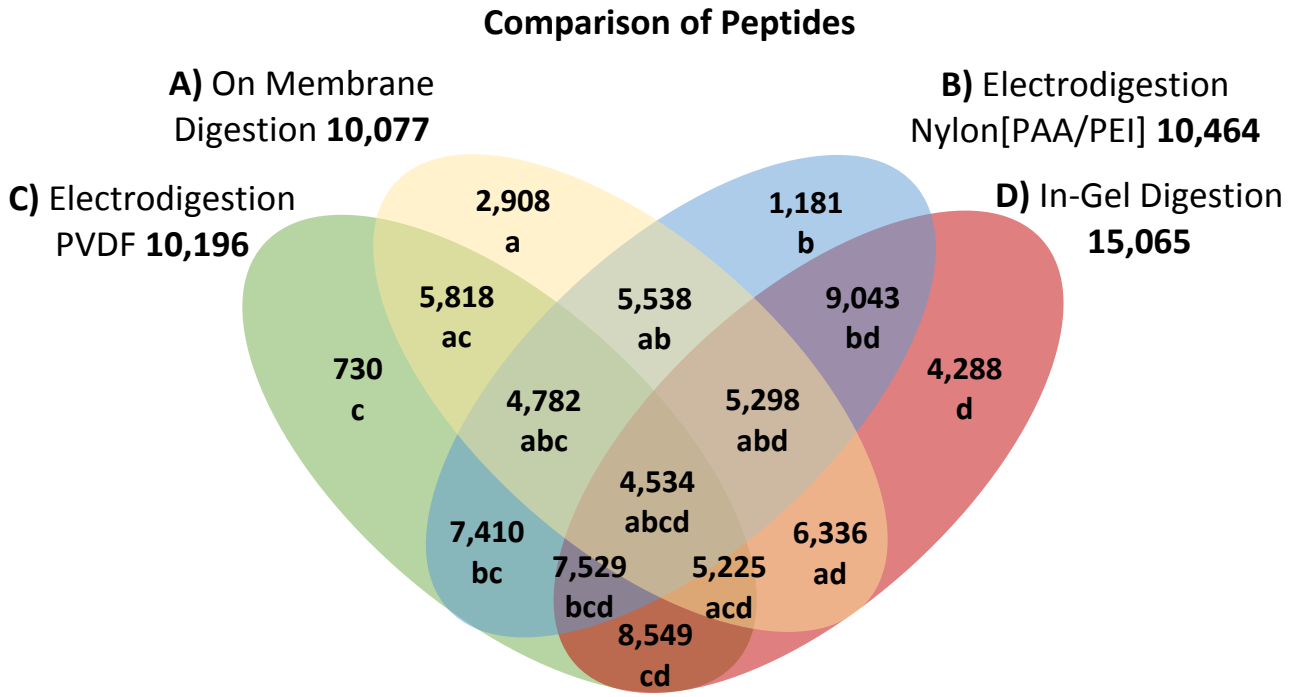

Fig. 6 Comparison of shared peptide identifications in the analysis of an *E. Coli* cell lysate using the four protocols shown in **Fig. 1**.

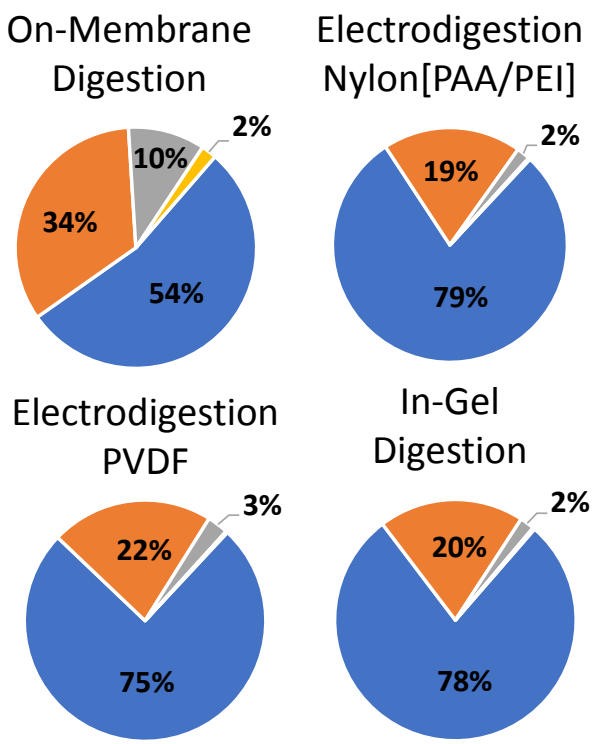

Fig. 7 Percentages of identified peptides with 0, 1, 2, and 3 missed cleavages after electrodigestion, in-gel digestion, and on-membrane digestion of *E. Coli* cell lysate.

membrane is likely less efficient than proteolysis in a gel or in the pores of a trypsin-containing membrane.

Additionally, the methods showed no obvious difference in the types of peptides and proteins identified in terms of peptide isoelectric point and hydrophobicity, or protein molecular mass. All methods produced a distribution of peptides that favoured a pI of 3-5 or 6-7 (**Fig. S7**). Moreover, based on GRAVY scores, around 60% of the peptides are hydrophilic (**Fig. S8**). Peptides uniquely identified after in-gel digestion also follow these trends and do not show bias toward any particular isoelectric point (see **Fig. S9**), peptide length (see **Fig. S10**), or number of missed cleavages (see **Fig. S11**). Additionally, 55% of the peptides uniquely identified after in-gel digestion are also hydrophilic (GRAVY score < 0).

Reproducibility

We analysed the reproducibility of the methods by comparing the number of shared protein identifications per replicate (see **Fig. S12**). All methods identified over 80% of proteins in both replicates. Additionally, as a semiquantitative comparison of reproducibility we plotted the LFQ intensities of identified proteins and peptides in replicate 1 versus those in replicate 2 (see **Figs. S13** and **S14**). If the analyses were identical the plots would give a straight line with a slope of one. Figure **S13** shows that the protein intensities in each method approximately follow this trend. The most reproducible method (electrodigestion with PAA/PEI nylon capture membranes) had an R^2 value of 0.9477. Apart from on-membrane digestion, which produced an R^2 value of 0.7947, the other methods have R^2 values of 0.9-0.95. We also compared the ratio of protein and peptide LFQ intensities in each replicate. The average percent difference in these LFQ values was 28 ± 24% and 52 ± 43% for proteins and peptides respectively, when using electrodigestion with a PAA/PEI nylon capture membrane. This result is similar to in-gel digestion which produced an average percent difference of 38 ± 35% and 49 ± 43% for proteins and peptides respectively (see **Table S1**). These numbers do not include proteins and peptides that did not show an LFQ intensity in both replicates.

Limitations of Electroblotting and Electrodigestion

The ~13% and 29% decrease in protein identifications (compared to in-gel digestion) after electrodigestion and electroblotting, respectively, may stem from several factors. One concern with electroblotting and electrodigestion is the possible passage of proteins and peptides through the capture membrane. Allowing longer blotting times for migration of larger proteins risks the loss of small proteins and peptides that have already reached the capture membrane and may pass through. In **Fig. S15**, electrodigestion with nylon[PAA/PEI] shows the highest percentage of identified small peptides (7-11 amino acids), suggesting that coating the capture membranes with polyelectrolyte layers may help to avoid passage of peptides through the membrane. Another concern with electrodigestion is inefficient capture of positively charged peptides. Although, 0.1% SDS is in the transfer buffer, for small hydrophilic, positively charged peptides, SDS adsorption may not lead to negatively charged peptides. However, compared to the other methods, we do not see a disproportionately small number of basic peptides in analysis of electrodigested proteins. In the case of electroblotting with on-membrane digestion, ineffective elution of large hydrophobic proteins from the PVDF membrane may lead to lower numbers of identified proteins.

Conclusions

Electrodigestion is a relatively simple technique for extracting and digesting proteins from an electrophoretic gel. This procedure could be useful for identifying protein bands in SDS-PAGE and in the future might serve in combination with MALDI-MS imaging of gels or tissue. Compared to in-gel digestion, electroblotting, or GELFrEE techniques, electrodigestion allows identification of nearly the same number of proteins. In only 4 hours of total sample preparation, electrodigestion of an *E. coli* cell lysate results in around 1,500 protein identifications, whereas in-gel digestion leads to identification of around 1,700 proteins after nearly 24 hours of sample manipulation. Moreover, coating electrodigestion capture membranes with polyelectrolyte layers may help to avoid passage of small peptides through the membrane. Addition of a trypsin membrane to the electroblotting system eliminates a separate digestion step and may also increase protein identifications compared to on-membrane digestion because peptide elution is less difficult than proteolysis of proteins bound to PVDF. However, on-membrane digestion of an *E. coli* cell lysate still enables identification of around 1,200 proteins after only 6 hours of sample

preparation and could serve as a time-saving alternative to in-gel digestion. Additionally, electrodigestion and electroblotting use western blotting apparatuses that are common in many labs.

Conflicts of interest

There are no conflicts to declare.

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