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Research Article

pH Fractionation and identification of proteins: Comparing column chromatofocusing versus liquid isoelectric focusing techniques

In proteomic investigations, a number of different separation techniques can be applied to fractionate whole cell proteomes into more manageable fractions for subsequent analysis. In this work, utilizing HPLC and mass spectrometry for protein identification, two different fractionation methods were compared and contrasted to determine the potential of each method for the simple and reproducible fractionation of a bacterial proteome. Column-based chromatofocusing and liquid-based isoelectric focusing both utilized pH gradients to produce similar results in terms of the numbers of proteins successfully identified (402 and 378 proteins) and the consistency of proteins identified from one experiment to the next (<10% change). However, there was limited overlap in the protein sets with <50% of the proteins identified as common between the sets of proteins identified by the different systems. In addition to the numbers of proteins identified and consistency of those identified, the reduced monetary costs of experimentation and increased assay flexibility produced by using isoelectric focusing was considered in order to adopt a system best suited for comparative proteomic projects.

Keywords: Chromatofocusing / *Escherichia coli* / Isoelectric focusing
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1 Introduction

How bacteria, including human pathogens, survive and persist within varied environments and cause disease is dependent on the complement of proteins the bacteria are able to produce at any given time. To understand bacterial mechanisms, we must be able to identify and measure the proteins produced by the organism under clearly defined conditions. Well-defined reverse-phase liquid chromatography-mass spectrometry based techniques commonly known by the acronym MudPIT (Multidimensional protein identification technology) for the identification of large collections of proteins exist and are readily applicable to bacterial research [1]. However, the expected size of bacterial proteomes can be relatively large; a bacteria like *Escherichia coli* is expected to be capable of expressing greater than 4000 different proteins [2]. For this reason, it is in some conditions preferable to select a portion of the entire proteome from which to identify and measure the quantity of proteins contained within the fraction. There are several different well described techniques for sub-dividing a bacterial proteome for investigation. Some techniques preferentially isolate a sub-cellular class of proteins from a sample, such as membrane proteins or cytoplasmic proteins [3]. Other techniques isolate the entire proteome but then fractionate it into more manageable units with fewer numbers of total proteins in the subsequent fractions [4–8]. The most common technique used to fractionate a proteome is 2-D gel electrophoresis, which utilizes isoelectric focusing based on the isoelectric point (pI) of the individual proteins as the first separation step [9–12]. Isoelectric focusing is often conducted using immobilized pH gradients on gel strips [13, 14]. However, the separation of proteins based on pI can also be accomplished using column-based (CB) chromatofocusing (CF) [15] and liquid-based (LB) isoelectric focusing (IEF) [16–27]. An advantage of using CB-CF or LB-IEF is that the proteins are separated and eluted in liquid state, making downstream techniques such as liquid chromatography-based

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Abbreviations: **CB**, column based; **CF**, chromatofocusing; **LB**, liquid based; **LC-MS**, liquid chromatography mass spectrometry; **PLGS**, ProteinLynx global server; **UPLC**, ultra pressure liquid chromatographer

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mass spectrometry (LC-MS) easier to perform and less time consuming for multiple samples [28, 29]. We compared and contrasted CB-CF and LB-IEF systems for use in conjunction with a downstream quadrupole time-of-flight mass spectrometer (Q-TOF MS) for the fractionation and identification of whole proteome samples. The immediate goal of the presented research was to identify a fractionation technique that worked well with time-of-flight mass spectrometry allowing for the identification of a large number of fractionated proteins in a reproducible manner while remaining sufficiently cost effective to allow for the practical investigation of multiple protein samples. The ultimate goal of this investigation is the development of a reliable and cost-effective technique for managing whole proteomic comparisons of microbial samples.

2 Materials and methods

2.1 Bacterial strains, growth, and whole cell protein extraction

The bacterial strain used in this study designated 43895 is an *E. coli* O157:H7 strain [30]. The bacterial strain was grown and maintained on YESCA plates (per liter of media – 20 g agar, 1 g yeast extract, 10 g casamino acids [Becton Dickinson, Sparks, MD, USA]). *E. coli* strain 43895 was streaked for confluence onto ten YESCA plates each and the plates were incubated at room temperature for 48 h. Each plate was flooded with 5 mL of 50 mM Tris-HCl (pH 8.0) (Invitrogen, Grand Island, NY, USA), and the cells were removed from the plate surface with the use of a plastic cell spreader. Twenty-five milliliters each of the cell suspensions were placed in two separate centrifuge tubes and the cells were pelleted by centrifugation at 5500 × *g* for 10 min. The cell pellets were resuspended in 0.4 mL of 50 mM Tris-HCl (pH 8.0) followed by addition of 1.6 mL of lysis buffer (pH 6.0, 7.5 M urea, 2.5 M thiourea, 12.5% v/v glycerol, 50 mM Tris base, 2.5% m/v *n*-octylglucoside, 6.25 mM TCEP [Tris-carboxyethyl phosphine hydrochloride], 1.25 mM protease inhibitor cocktail [Sigma P2714]) to each of the cell suspensions. Next, the cell suspensions were divided equally into four 2-mL microcentrifuge tubes preloaded with low protein binding 100 micron zirconium beads (OPS Diagnostics, LLC, Lebanon, NJ, USA). The cells in suspension were completely disrupted using the zirconium beads and a mini BeadBeater high-speed bead agitation system (BioSpec Products, Bartlesville, OK, USA). The cells and beads were agitated 3 times for 60 s with 90 s on ice between each repetition. Next, the solution was centrifuged at 21 000 × *g* for 60 min, and the resulting supernatant was collected. The supernatant was stored at −80°C until the day it was to be fractionated by column or MicroRotofor. The total volume of supernatant was divided equally into two parts and PD-10 columns (Amersham Biosciences, Sweden) were then used, following the manufacturer's recommendations, to exchange the total cellular proteins from the lysis mix into a proprietary Start buffer (Beckman-Coulter, CA, USA) for one sample (column separation) and into 1/50th strength Dulbecco's PBS (phosphate buffered saline) for the other sample (liquid separation). The total concentration of proteins present in each of the resulting preparations was measured by means of a BCA protein assay (Pierce, IL, USA).

2.2 Column-based chromatofocusing

The CB-CF separation method was described previously [16] and is as follows: The whole cell protein preparations in the proprietary start buffer (pH 8.5) were diluted to a concentration of 1 mg/mL (5 mL total) with additional start buffer. The CF separation utilized a chromatofocusing column (250 × 2.1 mm i.d.) (Eprogen, Downers Grove, IL, USA) that generated a pH gradient from 8.5 to 4 using the proprietary start and elute buffers (pH 4.0); this was accomplished over 185 min at a flow rate of 0.2 mL/min. Liquid fractions were collected using an automated fraction collector during the focusing processes based on time or change in the pH of the fractions. The pH values of the eluted fractions were measured by an in-line pH meter. Protein separation was monitored by UV absorbance at 280 nm allowing for the identification and collection of fractions containing proteins for use in further analysis. The chromatofocusing system used in these experiments is part of the Proteolab PF2D system (Beckman-Coulter).

2.3 Liquid-based isoelectric focusing

The whole cell preparations (2.25 mg total protein) in 1/50th strength Dulbecco's PBS were separated using a MicroRotofor system (Bio-Rad, CA, USA) following the manufacturer's recommended instructions. The protein preparations with volumes of 2.5 mL each had 125 μL of a 40% 3–10 pH range ampholyte solution (Bio-Lyte, Bio-Rad) added; resulting in a final concentration of 2% ampholytes in the samples. The samples were loaded into the focusing chambers and cooled to a temperature of 10°C for 15 min. Following the cooling step, the samples were electrophoresed under the conditions of 1 W of constant power applied to the chamber with potential limits of 350 V and current limits of 20 mA. Constant power was applied to the chamber until the voltage reached a plateau and then remained stable for 30 min. These conditions resulted in focusing runs of between 2.5 and 3 h, but never exceeded 3 h. After focusing, the ten resulting fractions were collected for further analysis. The pH values of the separated fractions were measured by applying 20 μL from each fraction to wide-range pH indicator strips.

2.4 Protein sample dialysis and digestion

All protein fractions collected from either CB-CF or LB-IEF were dialyzed against 50 mM ammonium carbonate to remove any chemicals that might interfere with trypsin digestion of the proteins or mass spectrometry of the resulting

peptides. Dialysis was performed using individual 3500 relative molecular mass cut-off mini-dialysis cassettes (Slide-A-Lyzer, ThermoFisher Scientific, Waltham, MA, USA) containing 200 μL of one of the collected fractions. Dialysis proceeded for 24 h with the buffer switched for fresh buffer after the first 2 h of dialysis. After dialysis, 100 μL of each protein fraction was digested with trypsin. First, the samples were reduced using 1 μL of 0.5 M DTT dissolved in ammonium carbonate and incubated at 57°C for 20 min. The samples were subsequently alkylated using 2.7 μL of 0.55 M iodoacetamide and incubated at room temperature for 20 min in the dark. Next, 1 μL of 1% m/v surfactant (ProteasMAX, Promega, Madison, WI, USA) was added to the protein samples and digested with the addition of 1.8 μL of 1 μg/μL Trypsin (TrypsinGold, Promega) at 37°C for 12 h. The samples were acidified using 4 μL 10% v/v formic acid and stored at −20°C until assayed by mass spectrometry.

2.5 Peptides separation and mass spectrometry

Tryptic peptides from the corresponding sample were injected into a Nano-Acquity ultra pressure liquid chromatographer (UPLC) (Waters Co. Milford, MA, USA). The UPLC was connected online to a quadrupole-time-of-flight analyzer (Q-TOF) Synapt G1 mass spectrometer (Waters Co.). Triplicate analysis of each fraction were performed by the injection of 2–3 μL of the sample into a Symmetry C18 trapping column (300 μm × 1 cm) (Waters Co.) using a 5-μL injection loop with a flow of 10 μL/min and with a solvent composition of water/acetonitrile (97:3, 0.1% v/v formic acid) for peptide trapping and washing. After 3 min, the trapping column was switched to connect on line with a BEH C18, 1.7 μm column (100 μm × 10 cm) (Waters Co.) with a solvent flow set at 450 nL/min and with the following linear gradient for peptides separation: water/acetonitrile (97:3) (0.1% v/v formic acid), 1 min, to a final composition over 60 min of water/acetonitrile (40:60) (0.1% v/v formic acid) 5 min. At the end of the gradient, the column was washed with water/acetonitrile (15:85) (0.1% v/v formic acid) for 5 min and returned to the initial condition with an equilibration time of 25 min.

The Q-TOF mass spectrometer was operated in the MS^E acquisition mode with a NanoLock-Spray probe, with the collision energy set at 6 V (low energy, for intact peptide detection) and ramped from 15 to 40 V (high energy, for peptide fragmentation) at an interval of 1 s. The reference mass-lock electrospray was pumped at 350 nL/min with an Acquity Auxiliary pump (Waters, Co) with a solution containing 300 fmol of Glu-fibrinopeptide (*m/z* 785.8426) (water/acetonitrile (75:25) 0.1% v/v formic acid), and sample for 1 s at intervals of 30 s to calibrate the TOF analyzer. Prior to the injection of each set of samples, the TOF analyzer calibration was checked for mass accuracy within 3 ppm with the fragmented ions (30 V collision energy) of the Glu-fibrinopeptide.

2.6 Data analysis and protein identification

MS^E raw data were submitted for data analysis and protein identification using $IDENTITY^E$ algorithm with the search engine ProteinLynx Global Server (PLGS) version 2.4 (Waters Co.). Files were processed to generate charge state reduced, deisotoped, precursor mass list, and associated products ions. *E. coli* O157 database was used by the search engine and downloaded from www.uniprot.org. Data search parameter include 0.01 Da for precursor ions and 0.03 Da for product ions, fixed modification of cysteine with iodoacetamide, and oxidation of methionine, and formation of pyroglutamine from N-terminal glutamine as variable modifications, allowing one trypsin miscleavage. PLGS results were submitted to Scaffold (Proteome Software Inc., Portland, OR, USA) for further statistic and comparative analysis with the restrictions of a minimum of 95% confidence for protein and peptide identification, and one peptide sequence as minimum requirement for protein identification.

3 Results

The pH fractions produced by the LB-IEF system using carrier ampholytes with a 3–10 pH range were compared against the 10 fractions collected from the CB-CF system within the dynamic fractionation range of the system, pH 8.5 to 4, that were shown to contain proteins based on UV (280 nm) absorbance. The pH ranges or values for each of the fractions in both of the separations for the different techniques are listed in Table 1. The pH values from the CB-CF are reported as a range starting with the pH value of the eluting sample at the time collection began for the fraction and ending with the pH value when the fraction collector switched to the next collection tube. Alternately, a single pH value was recorded for the LB-IEF focusing since it was determined by measuring the overall pH value of the individual fraction chambers after the separation was complete. The pH values for the fractions derived from the CB-CF are more precise and consistent

Table 1. The pH values of the various fractions produced by CB-CF and LB-IEF

Fraction number	CB-CF fractionation 1	CB-CF fractionation 2	LB-IEF fractionation 1	LB-IEF fractionation 2
1	7.4–7.1	7.3–7.0	1	1
2	7.1–6.8	7.0–6.7	2	1
3	6.8–6.5	6.7–6.4	3	2
4	6.5–6.2	6.4–6.1	4	4
5	6.2–5.9	6.1–5.8	5	5
6	5.9–5.6	5.8–5.5	6	6
7	5.6–5.3	5.5–5.2	7	7
8	5.3–5.0	5.2–4.9	8	9
9	5.0–4.7	4.9–4.6	9	12
10	4.7–4.4	4.6–4.3	13	13

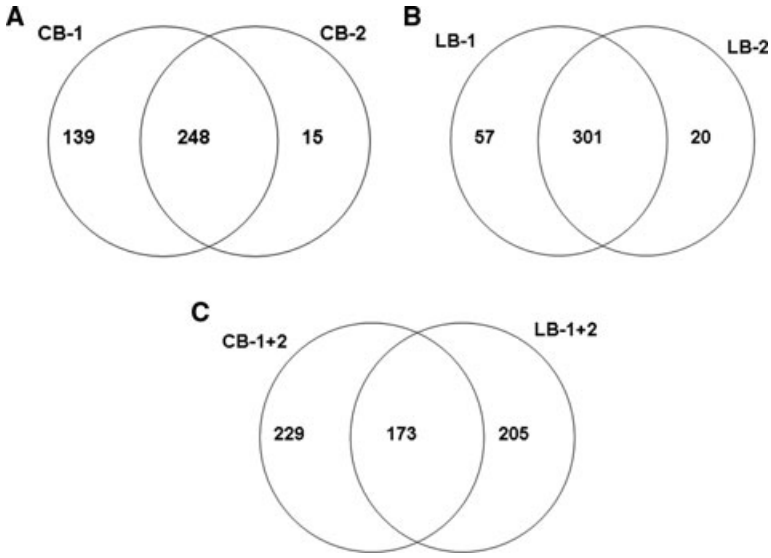

Figure 1. Venn diagrams comparing the numbers of proteins separated and identified by each technique through two different fractionations. A comparison of fractionation 1 using the CB-CF method (CB-1) and fractionation 2 using the CB-CF method (CB-2) (A). A comparison of fractionation 1 using the LB-IEF method (LB-1) and fractionation 2 using the LB-IEF method (LB-2) (B). A comparison of fractionations 1 and 2 using the CB-CF method (CB-1+2) and fractionation 1 and 2 using the LB-IEF method (LB-1+2) (C).

from one separation to the next compared to the LB-IEF (Table 1). However, the overall pH range available for fractionation was much smaller in the CB-CF versus the LB-IEF.

The overall effects of the two different techniques on the numbers and consistency of protein identification are presented in Fig. 1. The CB-CF separations demonstrated good consistency with regards to the protein set identified ($\geq$ 95% confidence) in the second separation CB-2 as compared to the first separation CB-1 (Fig. 1A). There were only 15 different proteins identified in the second fractionation CB-2 that were not also identified in CB-1. However, there were 139 proteins that were originally identified in CB-1 that were not also identified from CB-2. The 402 proteins identified by this method represent roughly 10% of the 4000+ open reading frames (ORFs) identified in *E. coli* O157:H7 strains. This number of proteins, though appearing low, is consistent with other fractionation and mass spectrometry-based identification techniques for this bacteria.

The LB-IEF fractionation demonstrated good consistency in the protein set identified from the second complete LB-IEF separation LB-2 compared to the set from the first LB-IEF separation LB-1 (Fig. 1B), which is similar to the results seen with the CB fractionation. Again, there was a relatively small number of proteins (20 proteins) identified in LB-2 that were not also previously identified from the fractions separated by LB-1. Again, there were a larger number of proteins (57 proteins) that were identified in LB-1 that were not also identified in LB-2. There was little difference from the CB-CF fractionation method in regards to the total numbers of proteins identified by the LB fractionation method, which resulted in the identification of a total of 378 proteins using the LB-IEF method.

Despite the fact that the CB and LB separation methods resulted in similar numbers of proteins identified (Fig. 1A and B), the protein sets identified from each of the fractionation systems differ from one another greatly. Of the total 402 proteins identified from the CB-CF fractions and the 378 proteins identified from the LB-IEF fractions, these two protein sets only have 173 proteins in common (Fig. 1C). This number represents less than 50% of the total proteins identified in the fractions separated by either system.

In addition to comparing the overall consistency of protein identification from one fractionation to the next when utilizing either of the systems, a comparison of the consistency of the proteins identified by the individual pH fraction from one separation to the next was made (Fig. 2). The CB-CF system was less consistent in the proteins sets produced between individual experiments for fractions ranging from pH 7.4 to 5.8 when compared to the more acidic fractions ranging from pH 5.9 to 4.3 (Fig. 2A). The LB-IEF method did not demonstrate any overall superior consistency compared to the CF method for protein identification when comparing individual pH fractions from one experiment to the next (Fig. 2B). Fractions 2, 3, and 9 showed marked reductions in the consistency of the proteins identified in one LB-IEF fractionation compared to the next fractionation. Better levels of consistency were seen in fractions with acidic pH values (fractions 1, 4, and 5), and fractions with neutral or basic pH values (fractions 6, 7, 8, and 10).

It should also be noted that proteins separated by the CB or LB methods did not always segregate into single fractions; rather, individual proteins occasionally distributed across multiple fractions. Figure 3 illustrates how the CF or IEF techniques are able to focus the protein samples more sharply resulting in individual proteins being segregated to a limited number of fractions. The data from both of the CB-CF experiments demonstrate relatively sharp protein focusing with greater than 80% of the total proteins appearing in 3 or less fractions (Fig. 3A). The two LB-IEF experiments produced protein focusing results with a slight reduction in sharpness compared to the CF focusing with 63% of the proteins focused by the IEF method appearing in 3 or less fractions after separation (Fig. 3B).

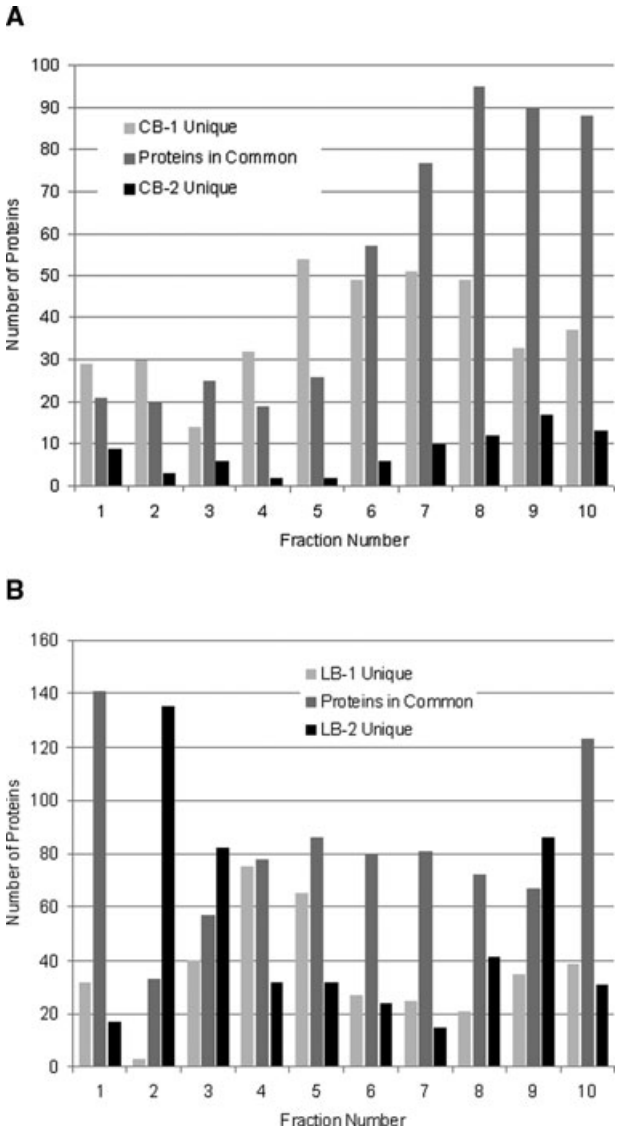

Figure 2. Fraction by fraction comparison of the consistency of the protein sets identified in a specific fraction from one separation to the next for CB-CF fractions (A) and for LB-IEF fractions (B).

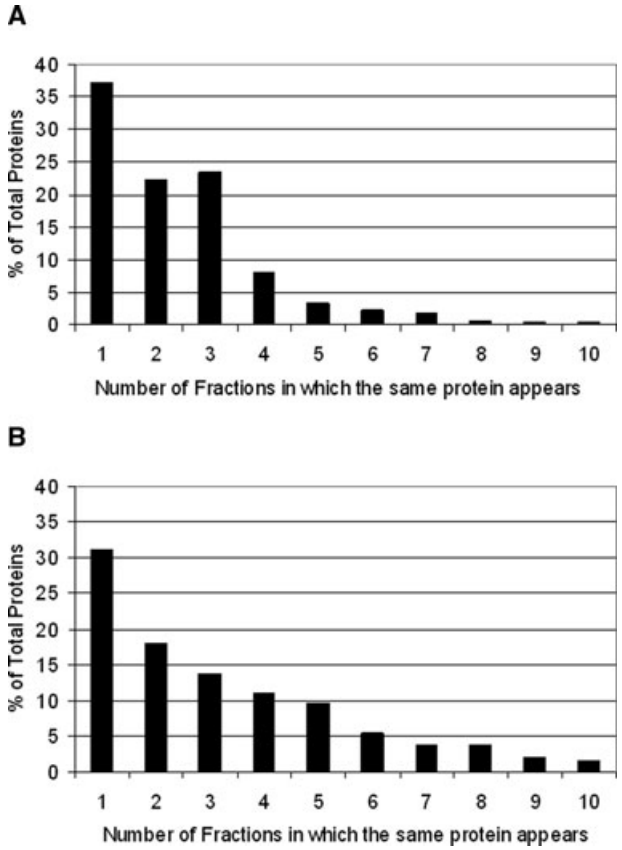

Figure 3. The number of individual fractions in which the proteins were identified presented as the percent of the total proteins identified from the CB separation (A) and from the LB separation (B).

4 Discussion

Two fractionation techniques were compared to determine their individual effects on protein identification through mass spectrometric analysis. The two techniques demonstrated similar profiles in terms of numbers of proteins identified and to a lesser degree the consistency of those proteins identified (Fig. 1A and B). However, the fractions selected for analysis in the results section only included fractions from the two different techniques produced within pH gradients, which therefore resulted in a separation of the proteomes based on the pI values of the individual proteins. For the CB-CF fractionation, this means that only fractions from pH 8.5 to 4.0 which were shown to contain proteins using a UV detector were analyzed. These were only 10 of the 42 total fractions collected and spanned a pH range from 7.4 to 4.3. For the LB-IEF system, a 3–10 ampholyte solution was used to produce a larger pH gradient (pH 1–13) that encompassed all 10 fractions of the separation. Since the IEF system's pH range encompasses the pH range of the CF system, one might expect that the set of proteins identified from the fractions of the IEF system would encompass the set of proteins identified from the CF fractions. However, this is not the result as there are proteins from the sets identified from the two techniques that are unique to one or the other technique alone (Fig. 1C). An alternate analysis was also performed that only used the IEF fractions spanning the pH range from 4 to 8, similar to the range for the CF system. This alternative analysis did little to increase the number of identified proteins common to both techniques. When the fractions were limited to those from pH 4 to 8, it resulted in 39 fewer proteins being identified by the IEF method and 23 fewer proteins in common with the CF fractions (data not shown). It is important to note that because of the different loading capacities of the two methods, the CB system is able to load a protein quantity over two-fold larger than the protein quantity for the LB method (5 mg versus 2.25 mg). Therefore, it is possible that the proteins

uniquely identified from the CF fractions represent low concentration proteins that, because of the diminished sample quantities loaded for IEF fractionation, were not present in sufficient quantities after IEF fractionation to be identified.

While the numbers of proteins identified by both fractionation systems is only roughly 10% of the total proteins that the target bacteria is believed to make, these numbers are similar to the numbers of proteins identified from 2-D gel electrophoresis separation techniques [31–34]. However, all of these methods fall well short of proteomic experiments using nonfractionated MudPIT approaches in terms of the numbers of proteins identified [35–37]. In order to increase the numbers of identifiable proteins in each fraction, it may be necessary to increase the concentrations of proteins being loaded into the different systems. Unfortunately, the amount of protein loaded into the CB-CF system was the maximum recommended amount (5 mg); any further increases in protein concentration often results in clogging of the CF column and loss of data. With the LB-IEF system, there exists some flexibility to increase the samples' protein concentrations in future experiments; this hopefully will increase the numbers of proteins identified from the fractions separated by this technique.

The consistency of the CB-CF fractionation method was reasonable when considering the proteins uniquely identified (15) in CB-2 as a percentage (4%) of the total number of proteins identified (402) in both CB experiments (CB-1 + CB-2) (Fig. 1A). However, this level of consistency was not present when considering the uniquely identified proteins (139) from CB-1 as a percentage (35%) of the total proteins identified in both CB experiments (CB-1 + CB-2). The consistency results for the LB-IEF fractionation method were roughly similar to those seen in the CB method. Again, the consistency was reasonably good when considering the proteins uniquely identified (20) in LB-2 as a percentage (5%) of the total proteins identified (378) in both experiments (LB-1 + LB-2). Similar to the CB method, the level of consistency was again decreased when considering the uniquely identified proteins (57) in LB-1 as a percentage (15%) of the total proteins from both LB experiments (LB-1 + LB-2). Neither of the separation methods investigated presented a strong advantage over the other with regards to the experimental consistency, which is highly desirable in any proteomic investigation in order to achieve statistical confidence from multiple experimental replicates [38]. However, when the consistency of the protein sets identified from individual fractions within either of the separation techniques are compared from one experimental separation to the next, interesting observations appear with regards to the two techniques (Fig. 2). The CB-CF method produced little variability in the pH ranges of the individual fractions collected from one separation to the next. However, the consistency of the protein sets identified in a specific fraction from one experimental separation to the next was superior for proteins identified within fractions derived from the more acidic ranges compared to those from the more neutral ranges (Fig. 2A). However, no explanations for this apparent enhanced consistency in proteins identified within acidic fractions of the CB method are immediately apparent. This observation does present possible challenges to future experiments when comparing the amounts of proteins present in neutral or basic fractions from CB separations. In comparison to the CB method, the LB method demonstrated greater variability in the pH values of the corresponding fractions from one separation to the next (Table 1). Perhaps not surprisingly, the fractions with variation between the pH values from one separation to the next also showed greater variability in the protein sets identified within those fractions from each separation (Fig. 2B). Fractions 2, 3, and 9 varied with regards to their pH values from one separation to the next and also produced the lowest percent values of all 10 fractions for proteins present in both of the corresponding fraction 17%, 32%, and 36%, respectively. This result demonstrates the importance in developing identical pH gradients for the LB-IEF system from one separation to the next in order to ensure that a consistent set of proteins is segregated into specific fractions for further analysis. Additionally, the less variability in the pH values of the individual fractions resulting from CF compared to the IEF may also explain the slightly sharper focusing of the protein samples separated by CF compared to IEF (Fig. 3).

Finally, when we consider the relative costs for using either of these two technologies, some important differences emerge. The CB method relies heavily on relatively expensive consumable items, namely the CF columns and the gradient forming buffers. The chromatofocusing columns are expected to be only capable of performing five focusing runs before requiring replacement at a significant cost, though research efforts have been made to lessen this shortcoming [39]. The LB-IEF method also depends on certain consumable items but the costs of these are only a small fraction of the cost of replacing consumable items for the CB-CF method. Since a proteomic study will need to include multiple biological replicates as well as technical replicates in order to achieve statistical significance, it seems unlikely that such a study could be completed using one column and buffer set [38]. Therefore the consistency of pH values for fractions produced from CB-CF (Table 1) would probably be negatively affected when it became necessary to switch to a new column mid-experiment.

Additionally, the CB-CF method is limited to fractionations over the range of 8.5 to 4.0 while the LB method is capable of producing pH gradients of differing sizes utilizing ampholyte sets with different pH values including 10/3, 5/3, 6/4, 7/5, 8/6, 9/7, 10/8, and 8/5.

5 Concluding remarks

The two focusing methods were shown to produce similar results in terms of the numbers of proteins identified and, to a lesser extent, the consistency of the protein sets identified from one experiment to the next. The CB-CF method

demonstrated a slight advantage in the consistent reproduction of the pH values of the fractions produced and the sharpness of the protein focusing. However, the LB-IEF method was more flexible with regards to separation ranges, was quicker to run, and had a lower cost for running and maintaining the system. Therefore, the LB-IEF method appears preferable for performing proteome fractionations based on pH gradients.

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