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Technique review

Solution phase isoelectric fractionation in the multi-compartment electrolyser: A divide and conquer strategy for the analysis of complex proteomes

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Abstract

Sample complexity frequently interferes with the analysis of low-abundance proteins by two-dimensional gel electrophoresis (2DGE). Ideally, high abundance proteins should be removed, allowing low-abundance proteins to be applied at much higher concentrations than is possible with the unfractionated sample. One approach is to partition the sample in a manner that segregates the bulk of extraneous proteins from the protein(s) of interest. Solution phase isoelectric focusing in the multi-compartment electrolyser generates fractions of discrete isoelectric point (pI) intervals allowing isolated narrow segments of a proteome to be analysed individually by 2DGE. It is particularly useful for the isolation of low-abundance proteins of extremely basic or acidic pI.

Keywords: *immobilised pH gradient, multi-compartment electrolyser, proteomics, solution phase isoelectric focusing, two-dimensional gel electrophoresis*

INTRODUCTION

One strategy for analysing complex proteomes involves a 'divide and conquer' approach in which the sample is fractionated to generate less complex protein subpopulations for analysis. Fractionation based on protein charge can be accomplished by several methods, including ion exchange chromatography,[1] free flow electrophoresis[2,3] or solution phase isoelectric focusing (IEF).[4,5] Preparative solution phase IEF in the multi-compartment electrolyser (MCE) produces fractions of narrow isoelectric point (pI) unit intervals allowing isolated narrow segments of a proteome to be analysed individually. Moreover, fractions obtained with the MCE can be concentrated several times, providing a good opportunity to visualise low abundance proteins. This is of key importance, since many low-abundance proteins may be indices of nascent pathologies, potential drug targets or regulated by therapeutic regime.

Of the 4,377 genes transcribed in *Escherichia coli*, it is estimated that only 4,290 are actually translated into proteins. This number of proteins is increased to an undetermined number following post-translational modifications; however, fewer than 3,000 of these proteins have been isolated by two-dimensional gel electrophoresis (2DGE). This is largely due to the broad concentration range over which proteins are expressed, spanning at least six orders of magnitude, such that very low-abundance proteins

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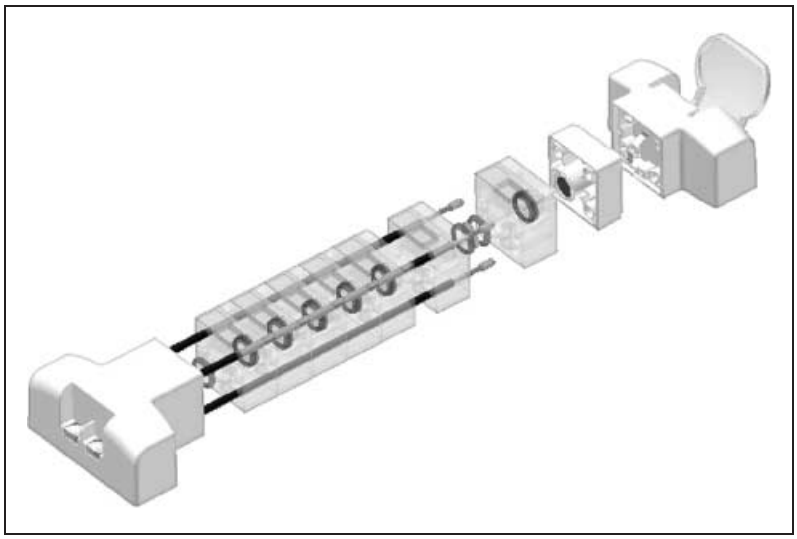

Figure 1: The multi-compartment electrolyser (MCE). The seven-chamber MCE can partition a sample into five pI intervals using six different isoelectric membranes

are not detected against the 'background' of medium- to high-abundance proteins. Preparative solution phase IEF of *E. coli* lysate in the MCE is capable of generating fractions as narrow as 0.5 and 1.0 pI intervals across the pH 3–11 range (Figure 1). Concentration and individual analyses of these fractions on corresponding narrow-range immobilised pH gradients (IPGs) detected nearly three times as many proteins as were detected in the unfractionated bacterial lysate.

MATERIALS

The IsoelectrIQ[2] IEF MCE instrument and ImagepIQ image analysis software were from Proteome Systems Limited (North Ryde, NSW, Australia). The Barocycler NEP-2017 and PULSE Tubes were from Pressure BioSciences (West Bridgewater, MA, USA). The Xcise robotic system was from Shimadzu Corporation (Columbia, MD, USA). The Axima CFR Plus mass spectrometer was from Kratos Analytical (Manchester, UK). The B-193 conductivity meter was from Horiba (Kyoto, Japan), distributed by Davis Innotech (Baltimore, MD, USA).

The MCE kit for Low Solubilising Proteins (sample solubilization solution, tributylphosphine, acrylamide, chamber solution, electrode solution and isoelectric membranes), IPGs (pH 3–10, 3–5, 4–7, 5–8 and 8–11), sodium dodecyl sulphate (SDS) equilibration buffer, polyacrylamide GelChips and ProteomIQ Blue Gel Stain were from Proteome Systems Incorporated (Woburn, MA, USA). Lyophilised *E. coli* K-12 cells and protease inhibitor cocktail for bacterial and yeast cells were from Sigma Chemical Company (St Louis, MO, USA). ULTRA-15 and ULTRAFREE-0.5 centrifugal filtration devices were from Millipore Corporation (Billerica, MA, USA). Bradford reagent and AG 501-X8 ion-exchange resin were from Biorad (Hercules, CA, USA).

SAMPLE PREPARATION

Sixty milligrams of lyophilised *E. coli* K12 cells were reconstituted in 9 mL of Sample Solubilisation Solution from the MCE kit. This consisted of 7M urea, 2M thiourea and 1 per cent 3-(4-heptyl)phenyl 3-hydroxypropyl dimethylammonio propane sulfonate (C7BzO).[6] To the cell suspension, 5 mM tributylphosphine, 5 mM ethylene diamine tetra-acetic acid and protease inhibitor cocktail were added.[7] The suspension was divided into six PULSE Tubes™ and subjected to ten cycles at 20°C in the Barocycler pressure cycler.[8] Each cycle consisted of 20 seconds at 35,000 PSI followed by 20 seconds at 0 PSI. The resulting lysates were pooled and centrifuged at 12,000 RCF to remove cellular debris. The supernatant was alkylated for two hours following the addition of 40 mM Tris and 10 mM acrylamide. The alkylation reaction was terminated by ultrafiltration[9] using an ULTRA-15 centrifugal filtration device with 10 kDa nominal molecular weight limit (NMWL). Protein concentration was estimated using the Bradford reagent and the ultrafiltered sample was diluted to approximately 1.2 mg/ml with 7M urea, 2M thiourea and 1 per cent C7BzO that had been ion-exchanged with AG 501-X8 resin. Sample conductivity was measured and was lower than 200 μS/cm.

Table 1: Number of proteins detected in unfractionated and fractionated cell lysates

Sample	Fold concentration	Number of spots detected
Unfractionated cell lysate	1×	925 ± 10
MCE pH 3–5 fraction	10×	410 ± 5
MCE pH 5–6.5 fraction	3×	1,048 ± 9
MCE pH 6.5–8 fraction	2×	619 ± 13
MCE pH 8–11 fraction	10×	240 ± 11
Total of MCE fractions	-	2,317 ± 38

MCE, multi-compartment electrolyser

MCE ELECTROPHORESIS

Six milligrams of *E. coli* protein were fractionated in a six-chamber MCE partitioned by membranes of pH 3, 5, 6.5, 8 and 11. This created chambers of pH 3–5, 5–6.5, 6.5–8 and 8–11 intervals. Five millilitres of cell lysate (1.2 mg/ml) were added to the pH 5–6.5 chamber. The two distal chambers contained 5 ml of electrolyte solution from the MCE kit consisting of 7M urea and 2M thiourea. The remaining chambers were filled with 5 ml of chamber solution consisting of 7M urea, 2M thiourea and 1 per cent C7BzO. Carrier ampholytes were not included in any of the MCE chamber solutions. Other MCE fractionations used isoelectric membranes of 0.5 and 1.0 pH intervals.

Electrophoresis was performed at 18°C with Peltier cooling in the computer-controlled IsoelectrIQ^2 multi-channel IEF instrument. The contents of each chamber were continuously stirred using magnetic stir bars during solution-phase IEF. Current was limited to 0.8 mA, permitting a natural voltage ramp from 100–1,500 V over 2–3 hours, depending on the initial conductivity of sample and chamber solutions. IEF was continued until a value of 10 kVh was reached. The fractions were aspirated from each chamber and the pH and conductivity were measured. Prior to IPG analysis, the fractions were concentrated and exchanged into 7M urea, 2M thiourea, and 4 per cent 3-[(3-cholamidopropyl)dimethylammonium]-1-propane sulphonate (CHAPS) by ultrafiltration in ULTRA-15 or ULTRAFREE-0.5 centrifugal filtration devices.

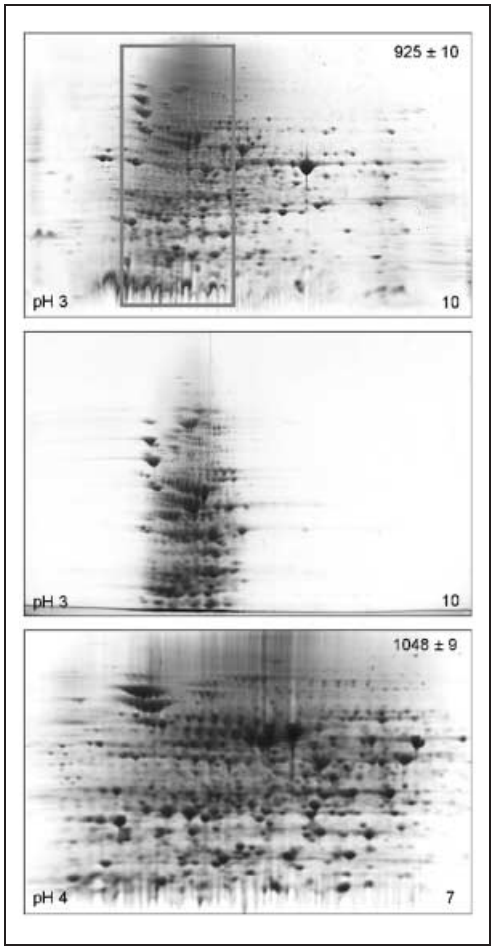

Figure 2: Unfractionated *Escherichia coli* lysate (top gel) revealed 925 ± 10 proteins. The multi-compartment electrolyser isolated a pH 5–6.5 fraction that was not contaminated by proteins outside this pH interval, as confirmed on an immobilised pH gradient (IPG) pH 3–10 (middle gel). The corresponding interval is indicated in the unfractionated lysate (rectangle, top gel). When the pH 5–6.5 fraction was concentrated 3× and separated on an IPG pH 4–7 (bottom gel), 1,048 ± 9 protein spots were detected in duplicate gels. The number in the lower corner of each gel indicates pH range and direction of the first dimension IPG

2DGE

Following concentration, 200 µl of each fraction was used to rehydrate duplicate IPG strips. Rehydration was allowed to proceed for 4–6 hours. IPGs were focused in the IsoelectrIQ^2 at 18°C using a non-

linear ramp from 0.1–10 kV for ten hours, followed by 10 kV until 100 kVh were attained. Immediately following IEF, the IPG strips were equilibrated twice for ten minutes in SDS equilibration buffer. Unless otherwise indicated, IPG strips were positioned on second dimension 6–15 per cent polyacrylamide GelChips and SDS polyacrylamide gel electrophoresis was performed at 20°C for two hours. GelChips were stained with ProteomIQ Blue Gel Stain. Spot counts were obtained using ImagepIQ 1.0 image analysis software.

PROTEIN MASS FINGERPRINTING (PMF)

Protein spots were excised, trypsin digested and spotted on matrix-assisted laser desorption/ionisation plates using the Xcise robotic system. PMF was performed on the Axima CFR Plus mass spectrometer.

RESULTS AND DISCUSSION

Preparative solution-phase IEF in the MCE produced fractions of narrow pI unit intervals for 2DGE analysis. When the MCE fractions were concentrated and focused on narrow-range IPGs, nearly three times as many proteins were detected in the corresponding 2D gels (Table 1), including many low-abundance proteins not seen in the unfractionated lysate. Figure 2 illustrates that, whereas 925±10 spots were detected in the unfractionated lysate, 1,048±9 spots were detected in the pH 5–6.5 fraction alone. Figures 3, 4 and 5 show concentrated pH 3–5, 6.5–8 and 8-11 fractions, respectively, as analysed on narrow-range IPGs and by 2DGE. The total number of spots detected by 2DGE of the MCE fractions exceeded 2,300. Some overlap of proteins was observed, but this appears to be limited to proteins of pI corresponding closely to the pH of the delimiting membrane (Figure 6 and Table 2). From

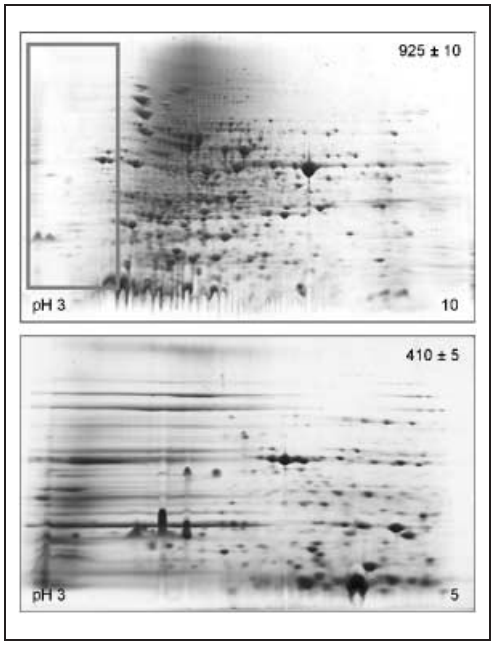

Figure 3: The multi-compartment electrolyser pH 3–5 fraction concentrated 10× and separated on an immobilised pH gradient pH 3–5 (bottom gel). 410 ± 5 protein spots were detected in duplicate gels. The corresponding interval is indicated in the unfractionated lysate (rectangle, top gel)

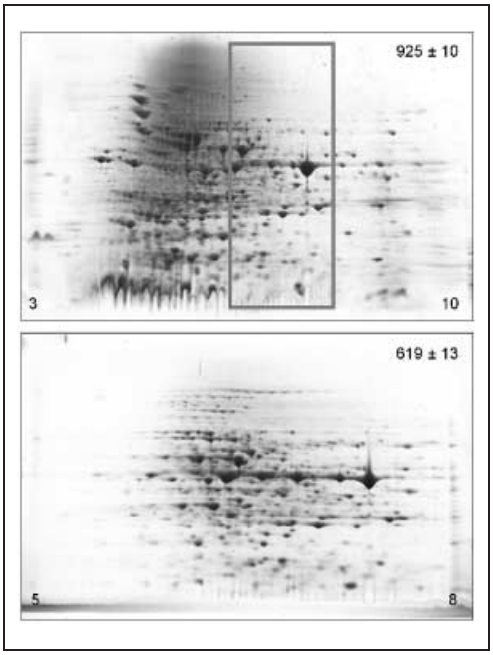

Figure 4: The multi-compartment electrolyser pH 6.5–8 fraction concentrated 2× and separated on an immobilised pH gradient pH 5–8 (bottom gel). 619 ± 13 protein spots were detected in duplicate gels. The corresponding interval is indicated in the unfractionated lysate (rectangle, top gel)

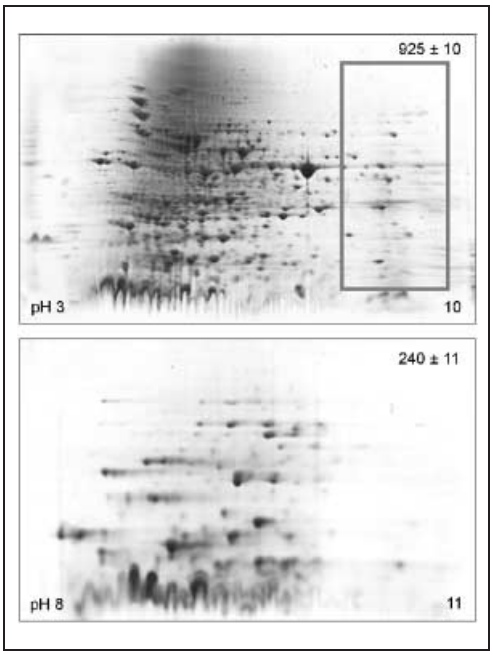

Figure 5: The multi-compartment electrolyser pH 8–11 fraction concentrated 10× and separated on an immobilised pH gradient pH 8–11 (bottom gel). 240 ± 11 protein spots were detected in duplicate gels. Second-dimension polyacrylamide gel electrophoresis was performed on 11 per cent polyacrylamide gels. The corresponding interval is indicated in the unfractionated lysate (rectangle, top gel)

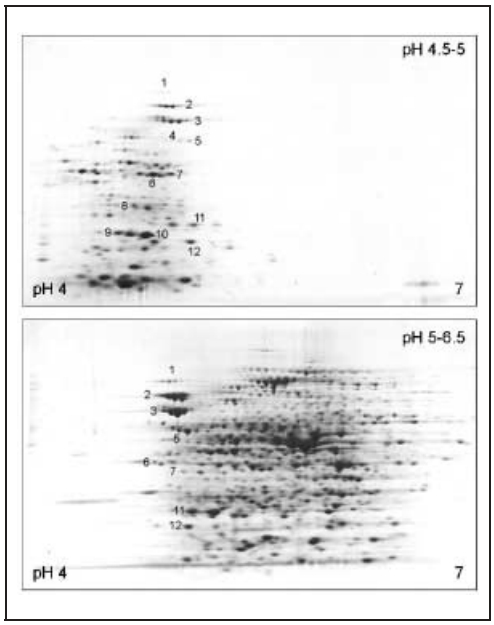

Figure 6: Twelve spots excised from two-demensional gels of multi-compartment electrolyser (MCE) of 0.5 and 1.5 pH intervals. MCE fractions pH 4.5–5 and 5–6.5 were separated on immobilised pH gradients pH 4–7. Ten of the 12 spots were identified by protein mass fingerprinting, with statistical significance. These proteins are identified in Table 2. Eight of the 12 spots were isolated in both fractions. The theoretical pIs of these proteins ranged from 4.75–4.90; corresponding closely to the pH of the delimiting pH 5 membrane.

Table 2: Proteins numbered in Figure 6 identified by protein mass fingerprinting

Spot number	Protein	MW (kDa)	pI
1	Unknown protein, 2D PAGE	87.9	4.87
2	Hsp70	69	4.83
3	Hsp60	57	4.79
4	ATP synthase, beta-subunit	50.3	4.9
5	ATP synthase, beta-subunit	50.3	4.9
6	Phosphoporin, outer membrane pore protein E	36.9	4.8
7	ADP-L-glycero-D-mannoheptose 6-epimerase	34.8	4.8
8	Thiazole moiety	26	4.83
9	–	–	–
10	Glucose-specific component IIA	17.5	4.79
11	–	–	–
12	Thiol peroxidase	17.8	4.75

2D PAGE = two-dimensional polyacrylamide gel electrophoresis
MW = molecular weight

these data, the resolution of the MCE is estimated to be 0.1–0.2 pI units.

CONCLUSION

MCE fractionation of *E. coli* lysates, followed by concentration and individual analysis of the resulting fractions on corresponding narrow-range IPGs, facilitated the detection of nearly three times as many proteins as were detected in the unfractionated bacterial lysate. MCE fractionation would appear to be conducive to direct analysis by alternative methods such as liquid chromatography-mass spectrometry.

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