Fractionation by Liquid-Based Isoelectric Focusing With the MicroRotofor™ Cell: Improving the Resolution of Low-Abundance Proteins on 2-D Gels

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Introduction

The effective study of low-abundance proteins by 2-D electrophoresis often requires a fractionation step. Sample fractionation lowers the overall complexity of a sample and enriches low-abundance proteins relative to the original sample. Following fractionation, proteins that may have been undetectable are often present at sufficient quantities for downstream analysis, such as by 2-D electrophoresis or mass spectrometry. To be effective, a fractionation method must be reproducible and provide high yields of protein. We investigate the reproducibility and effectiveness of fractionation with the MicroRotofor cell, which employs liquid-based isoelectric focusing (IEF) to separate protein samples by pI.

Methods

Mouse liver (1 g tissue) was extracted using the ReadyPrep™ total protein extraction kit, and protein concentration was determined with the *RC DC*™ protein assay. The sample was treated with the ReadyPrep™ reduction/alkylation kit, and then diluted to 0.6 mg/ml protein in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 mM TBP, 0.001% Bromophenol Blue, 2% Bio-Lyte® 3/10 ampholyte). For fractionation, 2.7 ml diluted sample was loaded into the MicroRotofor cell and separated using 1 W (constant) for 2.5 hr. The pH, volume (calculated by weight/density, 1.1 g/ml), and protein concentration (measured with the *RC DC* protein assay) were measured for each of the ten fractions collected, and fractionations were performed in triplicate. Prior to 2-D electrophoresis, fractions were treated with the ReadyPrep 2-D cleanup kit and protein pellets were resuspended in IEF buffer (pH range matching that of the IPG strip used).

First-dimension separations were performed using 11 cm ReadyStrip™ IPG strips, pH 3–10 or pH 4.7–5.9, and a PROTEAN® IEF cell. Second-dimension SDS-PAGE was performed on 8–16% Criterion™ Tris-HCl precast gels using a Criterion Dodeca™ cell. Gels were stained with Flamingo™ fluorescent gel stain and imaged with a Molecular Imager FX™ Pro Plus system. Gel images were analyzed with PDQuest™ 2-D analysis software.

Results

Reproducibility

Analysis of the pH gradients and recovery of sample volumes and protein quantity across the three replicate fractionations demonstrated that the MicroRotofor cell generates reproducible separation conditions (Figure 1).

Recovery

Volume: Regulated vacuum harvesting by the MicroRotofor cell allowed recovery of 86–89% of the original sample volume (Table 1). Fraction volumes of 215–247 µl (mean = 229 µl) were recovered.

Protein: Protein recovery is influenced by a number of factors, including sample type, protein load, and buffer conditions. Using mouse liver samples, we observed that, on average, 77% of the initial amount of protein loaded was recovered following fractionation (Table 1).

Table 1. Recovery of volume and protein. Total volume and protein recovered are shown as a percentage of the total loaded for separation.

Fractionation Run	Volume Recovery	Protein Recovery
1	89%	74%
2	89%	80%
3	86%	78%

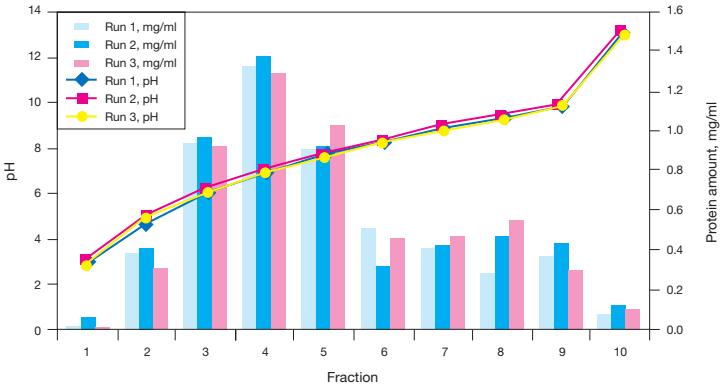

Fig. 1. Reproducibility of the pH gradient and protein distribution generated by the MicroRotofor cell. Plotted in the line graph are the pH values of each of the ten fractions generated in three separations of the same mouse liver sample. Plotted in the bar graph are the amounts of total protein found in each fraction.

2-D Gel Electrophoresis

Figure 2 shows representative 2-D gels of unfractionated sample and of fractions 2–4 from the MicroRotofor separation. These samples were separated on broad-range (pH 3–10) IPG strips, and the spot patterns and relative protein abundance between fractionated and unfractionated material were compared. These gels show that the MicroRotofor fractions have clearly delineated pH boundaries, indicating effective separation of the mouse liver protein sample by IEF.

Fraction 3 from all three fractionation runs was also separated by 2-D electrophoresis using micro-range (pH 4.7–5.9) IPG strips. PDQuest analysis of the three resuting gels revealed a correlation coefficient of 0.7 (Figure 3).

To demonstrate the level of enrichment that is attainable using the MicroRotofor cell, 40 µg fractionated sample was separated by 2-D electrophoresis using micro-range (pH 4.7–5.9) IPG strips, and compared to separations of even higher protein loads of unfractionated sample (Figure 4). Increasing the load of unfractionated sample impaired resolution without improving detection of low-abundance proteins.

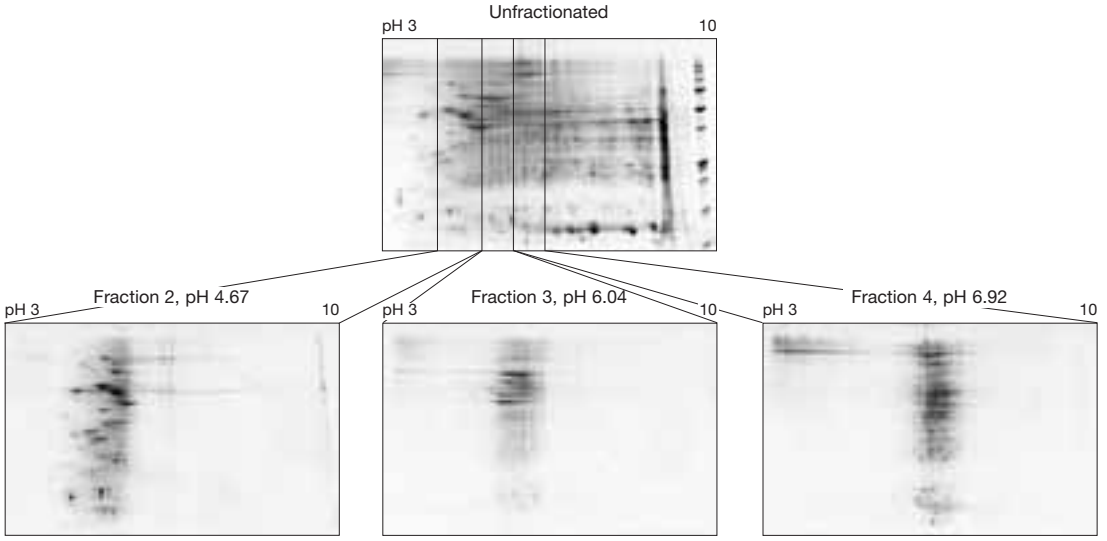

Fig. 2. Clean separation by pI. 2-D separations of unfractionated and fractionated (fractions 2–4) mouse liver samples are shown. First-dimension IEF was performed on broad-range (pH 3–10) IPG strips using 120 µg total protein for analysis of the unfractionated sample, and 20 µg total protein for analysis of fractions. Note the clean pH boundaries of the fractions and the enrichment of proteins within the pH regions they cover.

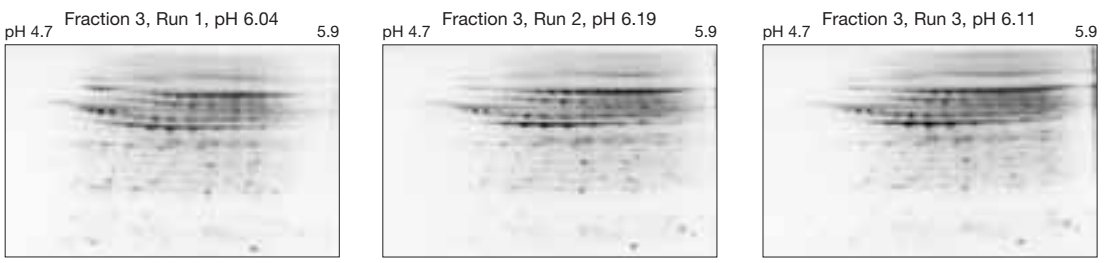

Fig. 3. Reproducibility of fractionation. 2-D separations of fraction 3 from three different MicroRotofor runs are shown. First-dimension IEF was performed on micro-range (pH 4.7–5.9) IPG strips. The correlation coefficient of the three gels was 0.7.

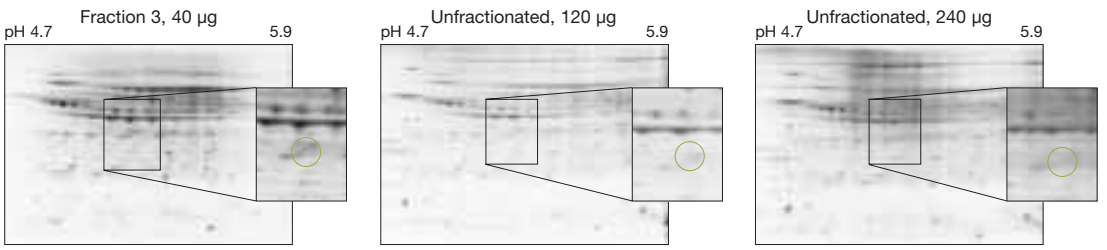

Fig. 4. Resolution of low-abundance proteins following fractionation on a MicroRotofor cell. 2-D separations of unfractionated and fractionated mouse liver samples are shown. First-dimension IEF was performed on micro-range (pH 4.7–5.9) IPG strips to demonstrate the level of enrichment attainable upon fractionation with the MicroRotofor cell. Shown are separations of 40 µg total protein of a MicroRotofor fraction (fraction 3), and 120 µg and 240 µg total protein from an unfractionated mouse liver sample. The same area of each gel is highlighted to show the clear enrichment of proteins in the fractionated sample.

Conclusions

The MicroRotofor cell delivers reproducible pH gradients and sample recovery for the effective fractionation of protein samples and enrichment of low-abundance proteins.