Fractionation by Liquid-Phase Isoelectric Focusing in the MicroRotofor™ Cell: Improved Detection of Low-Abundance Proteins

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Introduction

The effective study of low-abundance proteins often requires a fractionation step to reduce overall sample complexity and to elevate the concentration of low-abundance proteins relative to the original sample. Formerly undetectable proteins may be enriched to levels that allow downstream analysis by 2-D gel electrophoresis/mass spectrometry (2-D/MS) and liquid chromatography/mass spectrometry (LC/MS), the two methods most commonly used in proteomics for the separation and identification of proteins. Reduction in sample complexity also minimizes signal suppression effects that may occur in MS analysis of complex samples (Wang et al. 2003).

Isoelectric focusing (IEF), an electrophoretic technique used as the first-dimension separation in a traditional 2-D gel electrophoresis workflow, is also applied as a fractionation technique upstream of both 2-D/MS (Folkesson-Hansson et al. 2004, Puchades et al. 2003, 2005) and LC/MS (Harper et al. 2004) workflows. For 2-D/MS, sample fractionation by IEF can result in a more effective analysis by removing the proteins that are outside the pH range of the selected immobilized pH gradient (IPG) strip. This limits protein precipitation and smearing, which are often the consequences of higher protein loads, and enables the enrichment of proteins in the pI range of interest.

The MicroRotofor cell performs IEF entirely in free solution (liquid-phase IEF). Based on the Rotofor® technology used for decades for liquid-phase IEF of large sample volumes, the MicroRotofor cell was designed for efficient and reproducible IEF of samples with limited availability.

Here, the effectiveness, yield, and reproducibility of fractionation with the MicroRotofor cell were examined. Fractions were analyzed by 2-D electrophoresis using pH 3–10 IPG strips and compared to the unfractionated sample to demonstrate fractionation and protein enrichment. Selected fractions from these replicate runs were separated by 2-D electrophoresis using micro-range IPG strips. The 2-D separations were compared to equivalent separations performed on the unfractionated sample. Analysis of the gels showed improved resolution and representation of low-abundance proteins following fractionation with the MicroRotofor cell.

Methods

Protein from mouse liver tissue (1 g) was extracted using the ReadyPrep™ total protein extraction kit. Total protein concentration was determined with the *RC DC*™ protein assay, and the sample was reduced and alkylated with the ReadyPrep reduction-alkylation kit. For fractionation, the reduced and alkylated sample was diluted to a concentration of 0.6 mg/ml protein in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributylphosphine, 0.001% Bromophenol Blue, 2% w/v Bio-Lyte® 3/10 ampholytes), and a 2.7 ml sample was loaded into the focusing chamber of the MicroRotofor cell. The sample was focused at 1 W (constant) for 2.5 hr, and fractionations were performed in triplicate. The pH, volume (calculated by weight/density, 1.1 g/ml), and protein concentration were measured by the *RC DC* protein assay for each of the ten fractions collected. Prior to analysis by 2-D electrophoresis, the fractions were treated with the ReadyPrep 2-D cleanup kit and resuspended in IEF buffer, containing 0.2% (w/v) ampholytes matching the pH of the IPG strip to be 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 separations were performed using 8–16% Criterion™ Tris-HCl precast gels and a Criterion Dodeca™ cell. Gels were fixed in 40% ethanol, 10% acetic acid, and stained with Flamingo™ fluorescent gel stain. Image acquisition was performed on a Molecular Imager FX™ Pro Plus system, and image analysis was performed with PDQuest™ 2-D analysis software.

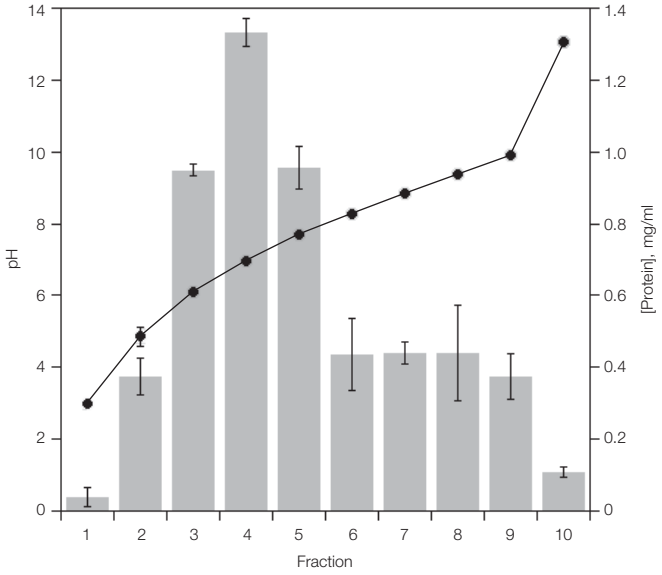

Fig. 1. Reproducibility of the pH gradient and protein distribution generated by the MicroRotofor cell. The line graph shows the mean pH values for each of the ten fractions generated after three separations of the same mouse liver sample. The bar graph shows the mean protein concentration found in each fraction after each of the three separations. Error bars indicate standard deviation.

Results

Reproducibility of Fractionation With the MicroRotofor Cell

The three replicate mouse liver fractionations performed in the MicroRotofor cell generated reproducible pH, volume, and protein quantity profiles, as well as reproducible recovery of sample volume and protein.

pH and protein profiles — The pH gradient generated across the ten fractions showed an average shift of only 0.15 pH units between runs, and the protein concentration of each fraction from the three fractionation runs was also reproducible (Figure 1), indicating reproducible separation and harvesting of mouse liver samples with the MicroRotofor cell.

Sample volume recovery — Regulated vacuum harvesting allowed recovery of 86–89% of the original volume loaded in the focusing chamber (Table 1). The volumes of fractions 2–9 from the three replicate runs ranged from 0.215 to 0.247 ml, differing from the run average fraction volume by ≤6% (Table 2).

Protein recovery — Protein quantitation indicated an average recovery of 77% of the initial protein amount (Table 1). Some of the protein loss may be accounted for by incomplete recovery of sample volume; no precipitate was observed in any of the fractions.

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

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

Table 2. Reproducibility of harvesting. Volumes of fractions 2–9* were calculated from the weight of the solution divided by its density, 1.1 g/ml, and variation was calculated relative to the average fraction volume for each run. The maximum observed variation of 6% is equivalent to 14 µl. The low variability (<6%) between fractions indicates that the focusing chamber geometry and harvesting system are efficient and reliable.

	Variation in Fraction Volume		
Fraction	Run 1	Run 2	Run 3
2	–1%	–1%	–2%
3	–5%	–3%	1%
4	4%	6%	1%
5	–2%	–5%	5%
6	–3%	–4%	–3%
7	5%	4%	–1%
8	5%	5%	5%
9	–2%	–2%	–5%
Average volume	0.227 ml	0.233 ml	0.227 ml

* Fractions 1 and 10 were not used because their volumes were larger than those of fractions 2–9, due to the internal geometry of the focusing chamber.

Analysis of Fractionated Samples by 2-D Electrophoresis

Fractionation efficiency — All ten fractions from the first separation were screened using linear pH 3–10 IPG strips to demonstrate the efficacy of fractionation with the MicroRotofor cell. The 2-D gels of the ten fractions show a clearly delineated separation of the mouse liver protein sample (Figure 2).

Reproducibility of 2-D analysis — Fraction 3 was selected to assess the reproducibility of 2-D electrophoresis of fractionated samples using micro-range pH 4.7–5.9 IPG strips, and the 2-D patterns for these replicate runs were practically indistinguishable (Figure 3). PDQuest software analysis of these gels (Table 3) generated an average correlation coefficient of 0.7, indicating a high degree of reproducibility.

Enrichment of Low-Abundance Proteins

To demonstrate the level of enrichment that is attainable upon fractionation with the MicroRotofor cell, a 40 µg fractionated sample was separated by 2-D electrophoresis using micro-range pH 4.7–5.9 IPG strips, and the resulting 2-D gels were compared to separations of higher protein loads (120 and 240 µg) of unfractionated sample (Figure 4). The enlarged portions of the gels in Figure 4 show that the low-abundance proteins in the 40 µg fractionated sample had identical migration patterns and much higher intensities than the same proteins in the 120 µg and 240 µg unfractionated samples. Whereas increasing the load of unfractionated sample impaired resolution without improving detection of low-abundance proteins, fractionation resulted in the clear enrichment of low-abundance proteins.

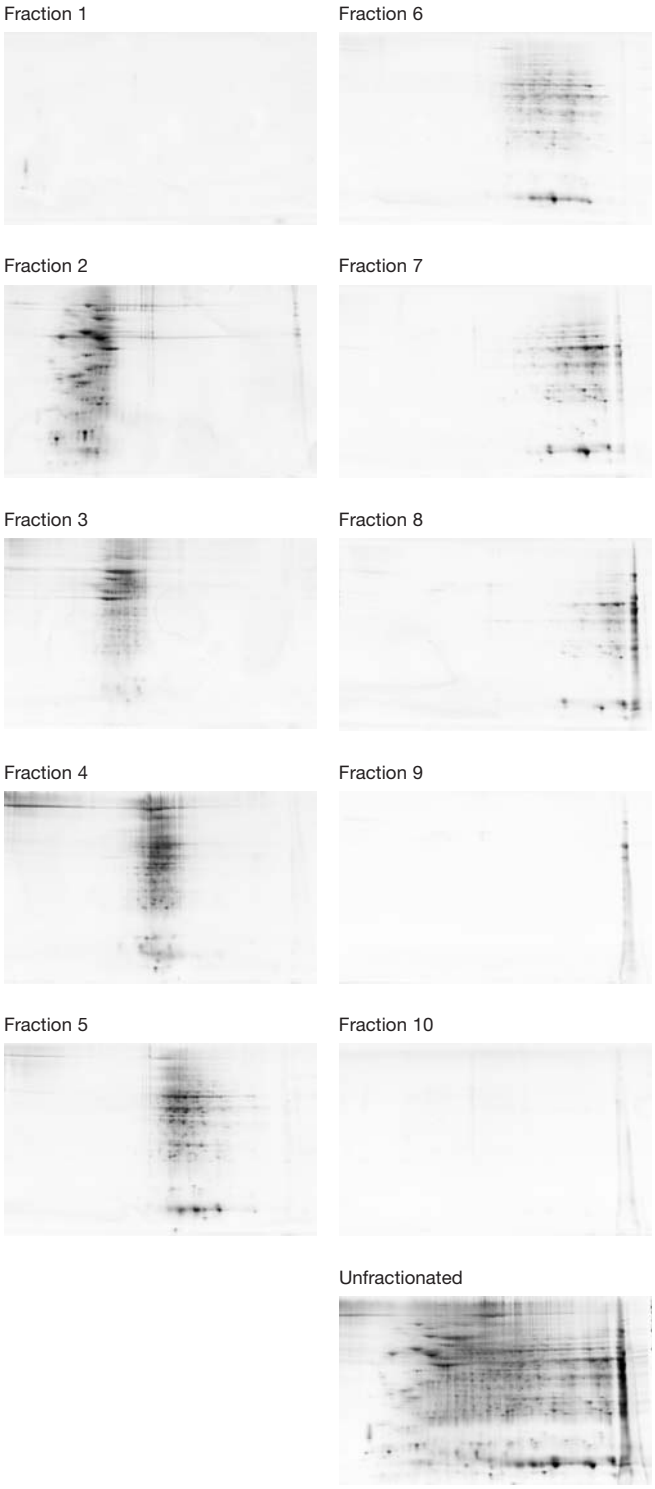

Fig. 2. Clean separation by pI. 2-D separations of fractionated and unfractionated mouse liver samples are shown. First-dimension IEF was performed using broad-range pH 3–10 IPG strips, with 120 µg total protein for analysis of the unfractionated sample and 20 µg total protein for analysis of the fractions.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

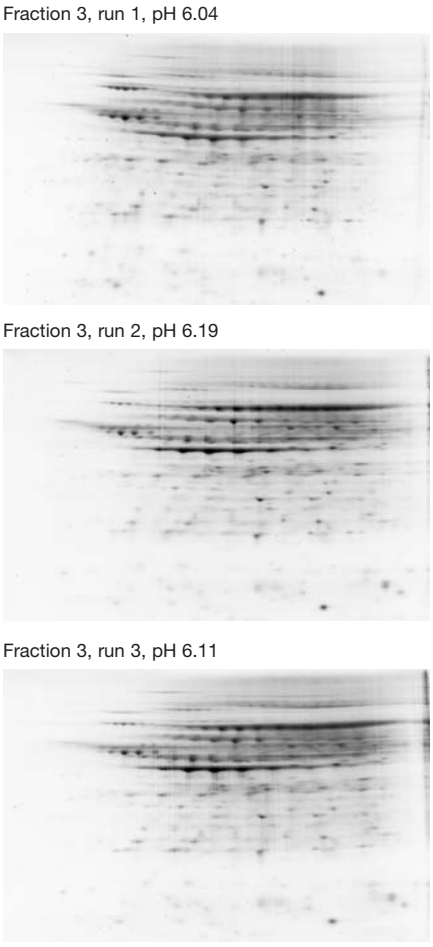

Fig. 3. Reproducibility of fractionation. 2-D separations of fraction 3 from three different MicroRotofor runs are shown. First-dimension IEF was performed using micro-range pH 4.7–5.9 IPG strips.

Table 3. PDQuest software analysis of gels shown in Figure 3. The gels shown in Figure 3 were analyzed with PDQuest 2-D analysis software to identify the total number of spots, number of matching spots, and coefficient of variation (CV) for each run. Values obtained for the gel from run 1 was used as the basis for comparison.

Run	# Spots	# Matched	CV
1	314	—	1.0
2	302	293	0.68
3	324	313	0.70

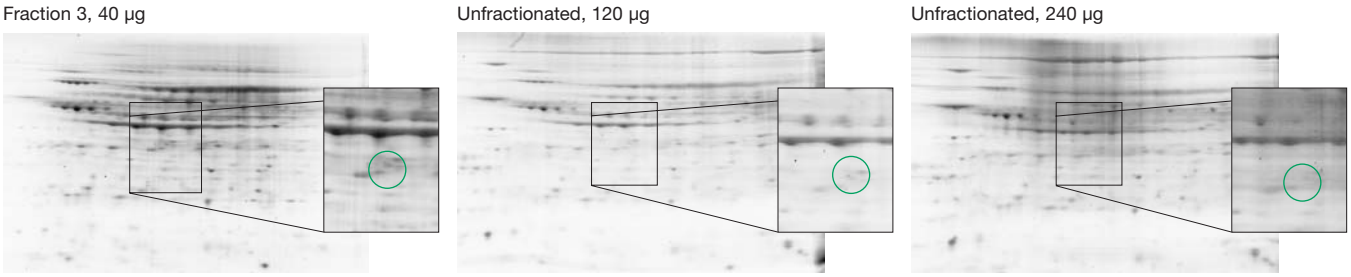

Fig. 4. Resolution of low-abundance proteins following fractionation in a MicroRotofor cell. 2-D separations of fractionated and unfractionated mouse liver samples are shown. First-dimension IEF was performed using micro-range pH 4.7–5.9 IPG strips to demonstrate the level of enrichment attainable upon fractionation with the MicroRotofor cell.

Discussion

Proteomic studies employing 2-D electrophoresis often aim to maximize the number of distinguishable individual protein species. Using narrow- or micro-range IPG strips for the first-dimension IEF separation increases the resolution of the technique; however, simply increasing the total protein load in order to bring the low-abundance proteins within the detection threshold has the undesirable side effect of incomplete and inconsistent protein intake into the IPG strip, and less effective focusing, as evidenced by smearing in the 2-D pattern (Berkelman et al. 2004).

Here, fractionation by liquid-phase IEF has been used successfully to decrease sample complexity, enhance the resolution and representation of low-abundance proteins, and improve the overall effectiveness of 2-D gel electrophoresis. Liquid-phase IEF is an effective fractionation technique: protein loss through isoelectric precipitation is minimized by the use of highly chaotropic solutions and relatively high concentrations of carrier ampholytes, and sample proteins are not exposed to gels or other separation matrices, which can result in protein loss through adsorption.

The MicroRotofor cell simplifies liquid-phase IEF and reduces sample volume requirements. The rocking motion of the separation chamber prevents protein precipitation and settling, and the temperature control option results in reproducible separations with minimal protein modification. The carefully engineered focusing chamber and harvesting system generate reproducible fractionation with high protein recovery.

Conclusions

- Fractionations performed in the MicroRotofor cell are reproducible in terms of run-to-run fraction pH, fraction volume, and protein yield
- Fractionation with the MicroRotofor cell allows for more effective 2-D gel separations using narrow- and micro-range IPG strips by increasing the effective sample load, which minimizes the horizontal streaking seen with unfractionated sample. Fractionation preserves the overall relative abundance and position of protein spots in 2-D gels with respect to the unfractionated sample
- Protein fractionation in the MicroRotofor cell improves the 2-D resolution of low-abundance proteins that are not clearly detectable in the unfractionated sample regardless of sample load. Increased sample loads of unfractionated sample simply leads to increased streaking, which reduces resolution and obscures low-abundance proteins
- The MicroRotofor cell fulfills the requirements for an effective fractionation system and can handle samples in a volume and mass range appropriate for analysis by 2-D electrophoresis

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