First-Dimension Separation With the MicroRotofor™ Cell Prior to SDS-PAGE and LC-MS/MS Analysis

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Introduction

Free-flow isoelectric focusing (IEF) is a gel-free method for separating proteins based on their isoelectric point (pI) in a liquid environment and in the presence of carrier ampholytes. This method has been used with the Rotofor® cell at the preparative scale to fractionate proteins from samples containing several hundred milligrams of protein; see the references listed in Bio-Rad bulletin 3152. The MicroRotofor cell applies the same method to much smaller protein samples without dilution, separating and recovering milligram quantities of protein in a total volume of about 2 ml.

In this study, we modified the traditional two-dimensional gel electrophoresis (2-D PAGE) proteomics workflow by using the MicroRotofor cell for a liquid-based first-dimension separation prior to SDS-PAGE. This approach has been applied using the Rotofor cell (Davidsson 2002, Peirce et al. 2004), and is particularly effective when proteins of interest are insoluble in gel-based IEF media. Here an *E. coli* extract containing a few milligrams of protein was separated on the MicroRotofor cell. This was followed by one-dimensional SDS-PAGE of the ten resulting fractions, in-gel tryptic digestion of selected protein bands, and mass spectrometry for protein identification. The results were compared to published *E. coli* 2-D PAGE data (Swiss-Prot *E. coli* database).

Methods

Bacterial Growth

E. coli XL1-Blue cultures (Stratagene Corp.) were grown overnight at 37°C from single colonies in 12.5 ml Luria broth in 50 ml tubes. Cultures were cooled on ice and then centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was removed, the pellets were washed with ice-cold sterile water, and the centrifugation step was repeated. The supernatant was removed, and the pellets were frozen in liquid nitrogen. Cultures were kept at –80°C until use.

Cell Disruption and Protein Extraction

Pellets obtained from 50 ml *E. coli* cultures were pooled and resuspended in 1.9 ml of 8 M urea, 50 mM DTT. Cells were disrupted by sonication using four 10 sec sonicator pulses separated by 1 min incubations on ice. To clarify the sample, the extract was centrifuged 2 x 15 min at 14,000 x g. The protein content of the supernatant was estimated using Bradford reagent (Bio-Rad protein assay dye reagent concentrate).

Free-Flow IEF Using the MicroRotofor Cell

The MicroRotofor cell was assembled and used according to the protocol outlined in the instruction manual. For fractionation, the sample was adjusted to contain 2.5 mg protein in 4% CHAPS, 8 M urea, 20 mM DTT, 2% Bio-Lyte® ampholytes (pH 3–10). The cell was set to position II (for denaturing conditions), and separation was performed under 1 W constant power. During the run, which was completed within 2 hr 50 min, the voltage increased from 130 V to 450 V. The ten fractions were harvested immediately following separation.

Fraction Analysis

The pH of each fraction was estimated using pH paper. A portion (1/20) of each fraction was then separated by SDS-PAGE using a Mini-PROTEAN® 3 cell and a 10% polyacrylamide gel. Following separation, the gel was fixed for 1 hr in a solution containing 40% ethanol and 10% acetic acid, stained overnight with colloidal Coomassie Blue G-250 stain (Fisher Scientific; Candiano et al. 2004), and then transferred to 0.1% acetic acid. No destaining was required. The gel was imaged with an ImageScanner II flat-bed scanner (GE Healthcare). Gel bands were excised with a scalpel, diced into 1–2 mm^2 pieces, and stored individually at –80°C until tryptic digestion.

Tryptic Digest

Gel pieces were washed twice for 10 min in 100 µl of 25 mM ammonium bicarbonate (ABC), twice in 1:3 acetonitrile (ACN):ABC or until the Coomassie stain was completely removed, and twice in 1:1 ACN:ABC or until the gel pieces became opaque or white. Gel pieces were then dried for 20 min in a SpeedVac vacuum centrifuge concentrator (Thermo Electron Corp.). Cystines were reduced by incubation of the gel pieces in 20 µl of 20 mM DTT in ABC for 45 min at 56°C. Excess liquid was removed, and the gel pieces were overlaid with 30 µl of 10 mg/ml iodoacetamide in ABC and incubated for 1 hr in the dark. Gel pieces were then washed three times with 1:1 ACN:ABC and dried for 20 min in the vacuum concentrator.

The dried gel pieces were rehydrated in ABC containing 12.5 ng/µl sequencing grade trypsin (Sigma-Aldrich), and after rehydration, the gel pieces were covered with 15 µl of ABC and incubated overnight at 37°C. Peptides were then extracted 3 times with 30 µl of 1:1 ACN:5% trifluoroacetic acid (TFA). Supernatants were pooled and concentrated to dryness. The resulting digests were kept at –20°C until analyzed by mass spectrometry.

Protein Identification by Mass Spectrometry

Peptides from tryptic digests were reconstituted in 10 µl of 0.1% TFA and then separated and analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using an UltiMate Plus HPLC system (Dionex Corp.) and an Esquire HCT mass spectrometer (Bruker Daltonics Inc.). Peptides were trapped on a 10 mm C_{18} trapping column (Dionex Corp.) and separated on a 15 cm, 75 µm inner diameter C_{18}-PepMap column (Dionex Corp.) packed with 3.5 µm particles. A binary LC gradient was formed from 2% ACN, 0.1% formic acid (FA) (solvent A) and 90% ACN, 0.1% FA (solvent B). Sample (1 µl) was loaded onto the trap column, and the C_{18} PepMap column was equilibrated for 10 min in 100% solvent A. Peptides were separated in a 60 min gradient of 0–80% solvent B, followed by a 20 min wash with 100% solvent B (solvents were obtained from Rathburn Chemicals, Ltd).

Mass spectra were acquired by scanning *m/z* 300–1,700 using parameters optimized for *m/z* 900. The trap ion charge control was 150,000, the maximum acquisition time was 100 ms, and three spectra were averaged. Tandem mass spectra were analyzed with DataAnalysis software (Bruker Daltonics Inc.), and the resulting peak lists were searched using Mascot protein identification software (http://www.matrixscience.com) using the *E. coli* database and 2 Da tolerance in MS, 0.4 Da tolerance in MS/MS, up to three missed tryptic cleavages, and singly, doubly, and triply charged ions. Cysteines were assumed to be carbamidomethylated, and methionine oxidiation was considered a variable modification. Only proteins identified by at least two peptides were considered. The Mascot MudPIT scoring system was used since typically several proteins per gel band could be identified.

Results and Discussion

Protein Separation by Free-Flow IEF

A crude *E. coli* extract containing 2.5 mg protein was separated under denaturing conditions within 3 hr by free-flow IEF using the MicroRotofor cell. Using pH paper, a pH gradient of 3–10 was observed between the cathode and the anode (not shown), indicating that ampholytes and proteins migrated according to their pI. Each fraction was analyzed by SDS-PAGE, and Figure 1 shows that most protein bands were observed in one fraction (and none were observed in more than two fractions), indicating that the proteins were well resolved along the pH gradient. Most proteins were located in fractions 3–7, though a few were observed in fraction 10 (Figure 1).

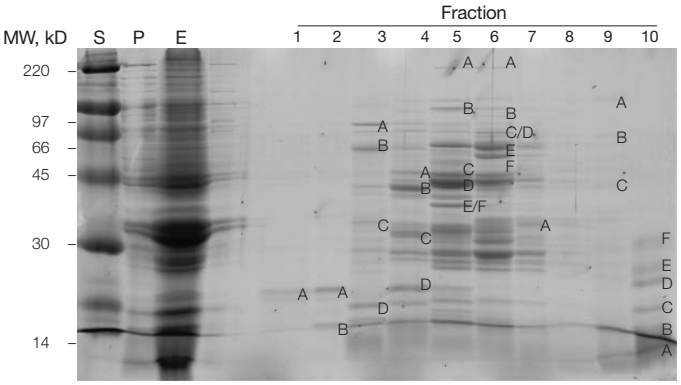

Fig. 1. MicroRotofor cell fractions separated on SDS-PAGE gel. One-twentieth (10 µl) of each fraction (fractions 1–10) was separated by SDS-PAGE and stained with colloidal Coomassie Blue G-250 stain. The protein bands indicated by letters were excised, and 20 were randomly selected for tryptic digest and LC-MS/MS analysis. S, standard; P, pellet; E, crude extract. MW standard, high-range Rainbow molecular weight markers (GE Healthcare).

Protein Identification by LC-MS/MS

To identify proteins and verify their separation according to pI, 20 bands were cut from the SDS-PAGE gel, digested, and identified by LC-MS/MS using Mascot software. Figure 2 shows there was a good correlation between the fraction number from which a protein was isolated and its theoretical pI. The few exceptions might be explained by posttranslational modifications, such as cleavage or phosphorylation.

Comparison of the Free-Flow IEF Method and Conventional 2-D PAGE

The major bands in the SDS-PAGE gel correlated to spots on the 2-D gel electrophoretic separations of *E. coli* proteins in the Swiss-Prot ECOLI database (http://ca.expasy.org/cgi-bin/map2/def?ECOLI). For example, band 3B corresponded to spot 2D-000KMU (identified as 60 kD chaperonin, groEL protein), 3D to 2D-000LMU (phosphotransferase system enzyme II (EC 2.7.1.69), glucose-specific, factor III), 4B to 2D-000KZ9 (phosphoglycerate kinase), 5C to 2D-000KV0 (enolase), 5D to 2D-000KWF (elongation factor Tu), 6E to oligopeptide-binding protein (spot 2D-000KNL) and inosine-5'-monophosphate dehydrogenase (EC 1.1.1.205, spot 2D-000KOE), and 7A to 2D-000L4W glyceraldehyde-3-phosphate dehydrogenase). In most bands, more than one protein could be identified, such as chaperone dnaK (spot 2D-000KIM) and 30S ribosomal protein S1 (2D-000KIV) in band 3A, which were partially resolved by 2-D PAGE.

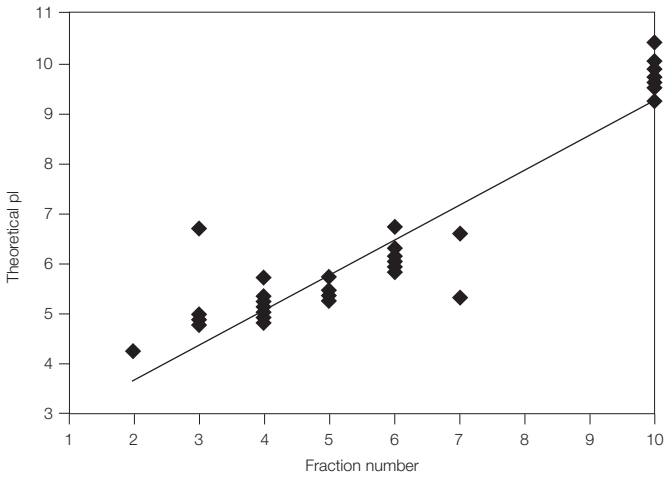

Fig. 2. LC-MS/MS analysis of protein bands from MicroRotofor cell fractions. Twenty excised protein bands from the gel shown in Figure 1 were randomly selected for tryptic digest and LC-MS/MS analysis. A positive correlation exists between the fraction number (thus, the pH of the fractions) and the theoretical pI of proteins identified by Mascot searches.

Each of the ten MicroRotofor fractions produced 10–50 protein bands when separated by SDS-PAGE, and 2–5 proteins were identified from each band. We estimate that 500 or more proteins could theoretically be identified using this approach. This value might be increased by using longer SDS-PAGE gels, providing a convenient alternative to large format 2-D PAGE gels. Narrowing the pH range used or refractionation could also further increase separation, thereby maximizing the number of proteins that can be identified. Alternatively, a MudPIT approach could be used to identify proteins in MicroRotofor fractions, thereby eliminating the need for further protein separation.

Conclusions

Use of the MicroRotofor cell for first-dimension separation prior to SDS-PAGE offers several advantages over traditional 2-D PAGE. First, samples can be run in native or denaturing conditions. Second, the method is particularly useful for separation of proteins that are insoluble in gel-based IEF media. Since proteins can be loaded into the MicroRotofor cell in a urea/thiourea/CHAPS buffer without dilution or precipitation, it may be possible to use this method to fractionate membrane proteins. Third, concentration of crude protein extracts is not necessary before separation with the MicroRotofor cell, so there is no sample quenching due to protein loss during precipitation/resolubilization procedures. Fourth, the experimental setup for the MicroRotofor cell/SDS-PAGE workflow is simpler than the traditional 2-D PAGE approach, and the consumables are less expensive. Finally, with the MicroRotofor cell, unlike with some other chromatographic techniques, prefractionated samples can be directly analyzed on mini gels without including any concentration steps, thus speeding and facilitating the prefractionation workflow prior to LC-MS/MS analysis.

References

Bio-Rad Laboratories, The Rotofor cell in the public domain: selected references demonstrating the versatility of the Rotofor system, Bio-Rad bulletin 3152 (2004)

Candiano G et al., Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis, Electrophoresis 25, 1327–1333 (2004)

Davidsson P, Combination of 2-D gel and liquid-phase electrophoretic separations as proteomic tools in neuroscience, Bio-Rad bulletin 2859 (2002)

Peirce MJ et al., Expression profiling of lymphocyte plasma membrane proteins, Mol Cell Proteomics 3, 56–65 (2004)

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