INTEGRATED PAPER

Evaluation of Solution Phase Isoelectrofocusing as Part of Proteomics Strategies

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(Received August 19, 2004; Accepted January 5, 2005)

Solution isoelectrofocusing has been evaluated for prefractionation of nuclear proteins from human cancer MCF-7 cells. Two separation devices were exercised using commercial polyacrylamide membranes, which carry embedded immobiline mixtures to establish pH boundaries between chambers. Protein recovery was quantified and 2-D gel electrophoresis was used to evaluate resolution. Solution isoelectrofocusing provides satisfactory separations and decreases the complexity of the protein mixture, however rates of recovery need to be improved.

1. Introduction

It is widely agreed that the current bottleneck in the proteomics pipeline is sample preparation, and particularly protein fractionation not peptide preparation or mass spectrometric analysis. Fractionation or separation of proteins before they are cleaved to peptides offers many advantages as a proteomics strategy. The characteristics of proteins that govern their separation are more diverse than those of peptides; thus higher resolution can be achieved. Subsequent peptide mixtures are simpler, allowing more peptides to be analyzed from each protein, and thus providing higher protein sequence coverage. Protein identifications are more reliable and reproducible when fractionation is carried out at the protein level.

Among the most commonly used methods for protein separation or fractionation are two dimensional gel electrophoresis and reverse phase high pressure liquid chromatography. A third technique, the application of isoelectrofocusing to separate proteins in solution has also been advocated by several prominent proteomics laboratories.^{1)–4)} This separation, based on the isoelectric points of the proteins, is envisioned as complementary and orthogonal methodology to reverse phase HPLC or gel electrophoresis. In this technique a mixture of proteins is sorted under the influence of an electrical field into a series of chambers, which are bounded by membranes that maintain different pH values.

Thus far, however, the method has been challenging for the non-specialist to implement. In previous (unpublished) work in this laboratory, the membranes that separate the cells have proven fragile, and protein overlap between the cells has been extensive and irreproducible. The evaluation reported here has been carried out with a new generation of robust membranes derivatized to maintain appropriate pH values. The membranes were used successfully in two com-

mercial apparatus. Separation and reproducibility were evaluated by 2D gel electrophoresis, and the validity for proteomics application was confirmed by excising spots from the gel and identifying the protein by micro-sequencing and bioinformatics.

2. Experimental

2.1 Materials and reagents

All reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 Isolation of nuclei and preparation of nuclear proteins

The Nuclei Pure Prep nuclei isolation kit (Sigma, product No. NUC-201) was used to isolate and purify MCF-7 nuclei according to user instructions with slight modifications. Cultured MCF-7 cells were harvested at 95% confluence. The cultures were treated with trypsin and centrifuged at 500 *g*, then washed twice with PBS. The cell pellets were weighed and added to hypotonic lysis buffer in a ratio of 1 g cells to 10 mL buffer with 0.5% Triton X-100. Then they were vortexed for 10 s and were set on ice for 5 min. The cell lysate was stained with 0.4% trypan blue solutions (Sigma) to monitor if the cells were completely lysed. When no intact MCF-7 cells could be observed under a light microscope, then the cell lysate was centrifuged through a sucrose cushion at 30,000 *g* for 45 min to purify the nuclei. The nuclei pellets were washed twice with nuclei storage buffer and spun down at 500 *g*. A little portion of the nuclei was observed under a fluorescence microscope, stained with propidium iodide. The nuclei were weighed and nuclear protein extraction solutions were added in a ratio of 4 mL solution every 1 g of nuclei. The nuclei pellets were resuspended in the NaCl buffers described earlier and vortexed vigorously for 15 s every 10 minutes, for a total of 40 minutes on ice. Then the suspension was centrifuged at 16,000 *g* for 10 min. The supernatant fraction was immediately transferred to new pre-chilled tubes and snap-frozen in aliquots with liquid nitrogen and stored at -80°C .

2.3 Sample fractionation by solution isoelectrofocusing using Amika device

A five-chamber separation device and an electrophoresis tank (Amika Corp., Columbia, MD) were used in

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Table 1. Protein Recoveries in Isoelectrofocusing Experiments

Device	Initial loading	Fraction pH 3–5	Fraction pH 5–8	Fraction pH 8–11	Sample recovery
Amika	1.6 mg/1.5 mL	304 μ g	302 μ g	254 μ g	54%
	2.0 mg/1.5 mL	540 μ g	620 μ g	14 μ g	59%
	2.0 mg/1.5 mL	197 μ g	635 μ g	362 μ g	60%
MCE	8.0 mg/5 mL	429 μ g	400 μ g	126 μ g	12%

Fig. 1. 2-D Gel array of the entire nuclear protein mixture, pH 3 to 10.

the experiments. The device includes five Teflon dialysis chambers (500 μ L-volume each) connected in tandem plus two terminal Teflon caps (Amika Corp.). The adjacent separation chambers were divided by membranes with the desired pH values (ProteomeSystems, Woburn, MA), and two 3.5-kDa dialysis membranes (MFPI, Seguin, TX) were put at each end of the terminal chambers. Two O-rings (12 mm i.d., Scientific Instrument Services, Ringoes, NJ) were used to seal each membrane between two chambers. A sample of MCF-7 nuclear protein (1.6 mg) was solubilized in 1.5 mL of IPG rehydration buffer and loaded into the three separation chambers. The two terminal chambers were filled with electrode buffers, 7 mM phosphoric acid (anode) and 20 mM lysine/20 mM arginine (cathode). The assembled device was put into the electrophoresis tank and the two parts of the tank were filled with anode and cathode electrode buffers, respectively. A model 1,000/500 power supply (BioRad, Hercules, CA) was used for isoelectrofocusing. Typically, 150 V was used for 1 h (initial $\sim$ 0.7 mA, final $\sim$ 0.4 mA), 250 V for 1 h (initial $\sim$ 1.4 mA, final $\sim$ 0.2 mA), and then 1,000 V for overnight (final 0.17 mA). After fractionation, solutions ($\sim$ 500 μ L each) were removed from the three chambers and the surfaces of the membranes and the inside walls of the chambers were washed with 200 μ L of rehydration buffer. The rinses were combined with the sample fractions. The membranes were removed and soaked in 250 μ L rehydration buffer for 1 h to extract proteins and combined with the appropriate sample fractions. In order to evaluate the separation efficiency, 25% of each fraction was concentrated with a centrifugal filter (5 kDa, Millipore, MA) and separated on pH 3–10 2D-PAGE.

2.4 Sample fractionation by multi-compartment electrolyzer (MCE)

Proteome Systems IsoelectrIQTM MCE was used in these experiments and all the materials were from Proteome systems (Woburn, MA). Five chambers (5 mL each) were assembled according to the instructions and an MCP-7 nuclear extract (8 mg) was dissolved in the MCE Sample Solubilizing Solution and loaded into the central chamber. Two chambers at each end were filled with MCE Electrode Solution and other chambers were filled with MCE Chamber Solution. A two-step isoelectrofocusing program was used, 100–1,500 V slow ramp for 8 h, and then 1,500 V for 8 h. After separation, each fraction was removed and the membranes and inside walls of the chambers were rinsed with chamber buffer. The membranes were extracted with 500 μ L rehydration buffer for 1 h. The rinses and extracts were combined with the corresponding chamber fractions. Each fraction was concentrated with centrifugal filter (5 kDa, Millipore, MA) and separated on pH 3–10 2D-PAGE.

2.5 Two-dimensional electrophoresis

For the isoelectric focusing step, linear 11 cm IPG strips pH 3–10 (Bio-Rad, CA) were rehydrated in sample solution for 12 h, and IEF was performed in a Protean IEF Cell (Bio-Rad). The second-dimensional gels were Tris-HCl 8–16% IPG well gels (BioRad). Staining was with Coomassie Stain (Bio-Rad).

2.6 Determination of protein concentration

Protein concentration and recoveries of each device were determined by Bradford Protein Assay (BioRad) and a Beckman DC 530 UV/Vis spectrophotometer was used.

2.7 In-gel tryptic digestion and mass spectrometry analysis

Fig. 2. Two dimensional gel arrays of protein fractions separated by isoelectrofocusing on the Amika device. (A) Fraction pH 3-5; (B) fraction pH 5-8; (C) fraction pH 8-11.

Fig. 3. Two dimensional gel arrays of protein fractions separated by isoelectrofocusing on the MCE device. (A) Fraction pH 3-5; (B) fraction pH 5-8; (C) fraction pH 8-11.

Spots of interested were excised from gels. The gel particles were washed with water-acetonitrile 1:1 (v/v) twice. After all liquid were removed, acetonitrile was added to cover the gel. Then the gel pieces were rehydrated in 0.1 M NH_4HCO_3 for 5 min, and an equal volume of acetonitrile was added and incubated for 15 min. All the liquid was removed and the gel pieces were dried in a vacuum centrifuge. The gel pieces were incubated with 10 mM dithiothreitol–0.1 M NH_4HCO_3 45 min at 56°C and 55 mM iodoacetamide–0.1 M NH_4HCO_3 for 30 min in the dark, respectively. After removing iodoacetamide solution, the gel particles were washed with 0.1 M NH_4HCO_3 –acetonitrile and dried in a vacuum centrifuge. The gel particles were rehydrated in a 50 mM NH_4HCO_3 solution of 12.5 ng/ μL sequencing grade modified trypsin (Promega, Madison, WI) for 45 min at 4°C. Then the enzyme supernatant solution was replaced by 50 mM NH_4HCO_3 buffer and the gel pieces were incubated at 37°C overnight. Peptides were extracted from the gel with 25 mM NH_4HCO_3 and acetonitrile, followed by 5% formic acid and acetonitrile (1:1, v/v). All the extracts from same gel spot were pooled and dried in a Speed Vac. The peptides were redissolved with 10 μL 0.1% aqueous TFA and desalted with ZipTip C_{18} pipette tips (Millipore, Bellerica). An AXIMA-CFR MALDI-TOF (Kratos, Chestnut Ridge, NY) was used to determine peptide mass maps. The instrument was operated in reflectron mode, analyzing positive ions. The laser power was set at 45–50 arbitrary units. Mascot search programs were used for protein identification.

3. Results and Discussion

An important consideration was the yield of separated proteins. Table 1 summarizes protein recovery from each of the chambers and the overall recovery. In two experiments in the Amika apparatus, overall recoveries were 54% and 59%, while recovery from the larger apparatus was lower. It appears that significant amounts of protein were adsorbed into the isoelectric membranes, or precipitated out and were not recovered. The MCE device was designed to fractionate proteins on the 100 mg scale, and our small sample may not be optimal.

The effectiveness of the separation of nuclear proteins from human breast cancer cells was investigated by subjecting the protein fraction recovered from each chamber to classical 2-D gel electrophoresis with Coomassie staining. Figure 1 presents the gel array for the entire nuclear protein fraction, separated on a gel with a pH range for the first dimension separation of 3–10. One hundred and sixty of these spots have been excised, and tryptic peptides have been micro-sequenced to identify 120 different proteins.⁵⁾ In Fig. 2, the 2-D gel arrays are presented for the material recovered in one experiment from the three chambers in the Amika isoelectrofocusing device, and Fig. 3 contains 2-D gel arrays of protein mixtures recovered from the three chambers of the MCH device.

When the set of gels from either of the isoelectrofocusing experiments is compared to the gel in Fig. 1, where the whole nuclear protein fraction is arrayed, it can be seen that significant fractionation has occurred. This is particularly dramatic in the gels in Fig. 3, from the MCH device. It should be noted that all the frac-

Table 2. Proteins Identified from Spots Excised in This Proteomic Strategy

Spot No.	Protein ID	pI	MW	Accession No.	Sequence coverage
1	Calreticulin	4.29	48,141 Da	P27797	24%
2	Prohibitin	5.57	29,804 Da	P35232	37%
3	Actin Beta	5.15	42,408 Da	P60709	22%
4	Stress-70 protein	5.87	73,625 Da	P38646	27%
5	HSP 60	5.70	61,016 Da	P10809	34%
6	Cytokeratin 19	5.04	44,079 Da	P08727	62%
7	hnRNP A1	9.27	38,715 Da	P09651	41%
8	hnRNP A2/B1	8.97	37,430 Da	P22626	35%

Fig. 4. Peptide mass map of the material in spot 1 in Fig. 2A.

Fig. 5. Peptide mass map of the material in spot 5 in Fig. 2C.

tions from both experiments contain proteins that accumulate at the lowest and highest pI values on the gels. This is not yet understood. It also appears that protein is most poorly recovered from the most basic chamber. Nonetheless, the successful fractionation by both devices qualifies isoelectrofocusing as a potential method for analysis of membrane proteins from these human breast cancer cells.

As the final step in demonstrating that isoelectrofocusing can be part of a proteomics strategy, several spots were excised from the gels shown in Fig. 2, and analyzed by peptide mass mapping. Protein identifications are summarized in Table 2, along with their Mascot search scores. Peptide maps (MALDI mass spectra) for the first and last entries are shown in Figs. 4 and 5. In these spectra masses are indicated for the peaks used in the database searches. The peak at m/z 842 in both spectra is a trypsin autolysis peptide.

4. Conclusions

This evaluation of isoelectrofocusing of the human nuclear protein fraction allows us to conclude that isoelectrofocusing can make valuable contributions to

the fractionation of protein mixtures, and can be utilized in strategies designed to identify proteins by mass spectrometry. The new membranes that enabled this study proved sturdy and durable. The fractionation achieved was satisfactory in both of the apparatus tested. Based on our experience, however, methods must be developed to improve protein recovery from the electrophoresis chambers.

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Keywords: Proteomics, Isoelectrofocusing, Nuclear proteins, Multi-compartment electrolyzer, MALDI-TOF