

Proteomic Study of Leukemia Cell Line (K562/CR3) using Free Flow Electrophoresis (FFE) Coupled with LC/MS

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Introduction

The requirement for prefractionation in proteomic analysis is linked to the challenge of performing such an analysis on complex biological samples and identifying low level components in the presence of numerous abundant housekeeping and structural proteins. The employment of a preliminary fractionation step results in a reduction of complexity in an individual fraction and permits more complete LC/MS analysis. Free flow electrophoresis (FFE), a solution-based preparative isoelectric focusing technique, fractionates and enriches protein fractions according to their charge differences and is orthogonal in selectivity to the popular reversed phase HPLC fractionation step. In this paper, we explore the advantages of a combination of FFE and liquid chromatography/mass spectrometry (LC/MS) to extend the dynamic range of a proteomic analysis of a complex cell lysate K562/CR3.

Methods

Cell line K562/CR3 was expanded to $\sim 2 \times 10^7$ cells. The cells were lysed by explosive decompression and mixed 1:1 with lysis buffer (2M thiourea/7M urea/4% CHAPS/1% dithreitol/2% Pharmalyte lysis buffer). The cell lysate of K562/CR3 were separated into 96 fractions with free flow–isoelectric focusing (FF-IEF) using a prototype instrument, type OCTOPUS (FFEweber GmbH, Germany). The linear pH gradient was from 3.0-11.5. The fractions were subject to SDS-PAGE (NuPAGE 4-12% Bis-Tris gel, Invitrogen). Thirty five fractions from 27 to 61 (pH 4.75 – 9.60) had visible protein bands after silver staining (SilverQuest, Invitrogen). These fractions were reduced, alkylated, and the proteins were precipitated with acetone and then digested with trypsin.

The digested sample was analyzed with LC/MS (LCQ DecaXP, ThermoFinnigan). The LC/MS conditions were as follows: Mobile phases were 0.1% formic acid in water (A) and acetonitrile (B). The flow rate was maintained at 2 μ L/min after flow split. The column was ThermoHypersil C-18 (180 μ m \times 10cm). The temperature of the ion transfer tube was set at 185°C. The spray voltage was set at 3.3kV and the normalized collision energy was set at 35% for MS/MS. Protein identification was obtained through a database search using SEQUEST algorithm.

Preliminary Results

In this study, K562/CR3 cell lysate were fractionated into 96 vials using FFE and the pH was measured for each fraction. The cell lysate, with and without (as a control) FFE prefractionation, were analyzed by SDS-PAGE and LC/MS. Only those fractions that

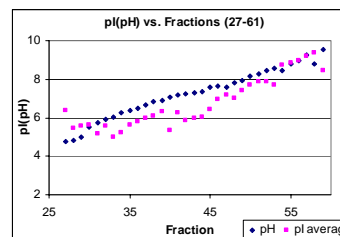
showed bands on SDS-PAGE were chosen for LC/MS analysis. The LC/MS results of 35 FFE fractions, pH from 4.75 to 9.60, showed a significant improvement in protein identifications after FFE prefractionation, both in quantity (Table 1) and quality (Table 2). Using cross correlation value, (Xcorr of greater than 1.5, 2.0, 2.5 for +1, +2, +3 charge states respectively), 736 proteins were identified with more than 2 peptides.

SEQUEST* Xcorr Score	35 FFE Fractions	Cell Lysate
(1.5, 2.0, 2.5)	(736) 2078	(31) 70
(1.8, 2.5, 3.0)	(169) 387	(17) 39
(1.8, 2.5, 3.5)	(155) 319	(17) 36

Table 1. Comparison of protein identity before and after FFE fractionation. (Left)

*The number in bracket denotes proteins with two or more hits.

Figure 1. Comparison of pI average and pH gradient for FFE fractions. (Right)



Top 10 Protein Identification	Sequence Coverage(%)	
	FFE-LC/MS	LC/MS
1 FRUCTOSE-BISPHOSPHATE ALDOLASE A	70.60	23.35
2 ACTIN, CYTOPLASMIC 2	38.40	24.53
3 GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE	60.90	17.61
4 ACTIN, ALPHA SKELETAL MUSCLE	28.91	11.41
5 HEAT SHOCK PROTEIN HSP 90-BETA	43.65	16.02
6 PEPTIDYL-PROLYL CIS-TRANS ISOMERASE A	74.55	8.48
7 HEAT SHOCK 70KDA PROTEIN 1	29.80	3.74
8 TRIOSEPHOSPHATE ISOMERASE	63.05	8.03
9 ALPHA ENOLASE	40.09	11.06
10 KERATIN, TYPE II CYTOSKELETAL 1	40.22	1.86

Theoretical pIs of top ranking proteins (with more than 2 hits) in each fraction were averaged and compared with the pH of that fraction. The gradient of pI average for all fractions is consistent with the pH gradient (Figure 1). In some cases, mismatches between pI of protein and

Table 2. Comparison of sequence coverage for top 10 protein identifications.

pH of the corresponding fraction suggested possible post-translation modifications. The comparison of theoretical and measured pI values can be used to identify false protein assignments based solely on MS data.

By using FFE prefractionation, some important low abundant proteins were identified, such as integrins and semaphorins. Figure 2 shows the MS/MS spectrum of integrin alpha-V.

In this study, FFE coupled with LC/MS, proved its reproducibility and capability of analyzing complex protein samples and identifying proteins with high confidence.

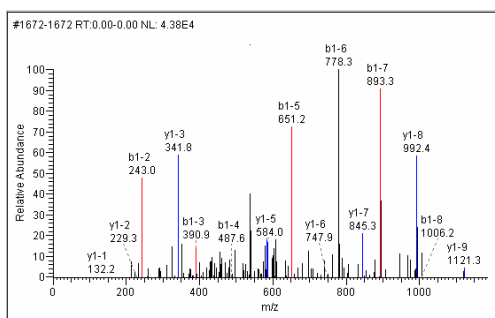


Figure 2. Peptide MS/MS spectrum of integrin alpha-V.

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