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Chromatographic prefractionation prior to two-dimensional electrophoresis and mass spectrometry identifies: Application to the complex proteome analysis in rat liver

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Abstract

In this paper, we describe an approach for fractionating complex protein samples from rat liver prior to two-dimensional gel electrophoresis (2-DE) using reversed-phase high-performance liquid chromatography (RP-HPLC). The whole lysate of liver tissue was prefractionated by RP-HPLC with an optimal multi-stage linear gradient elution. Successive fractions were analyzed using 2-DE and selected spots were identified by MALDI-TOF-TOF mass spectrometry. The reproducibility of this prefractionation technology allows pooling of several consecutive runs of the same sample, resulting in a highly enrichment of low abundance proteins. Computer-assisted calculation showed that the total spot number of samples prefractionated by RP-HPLC was nearly five times as many as that of unfractionated sample. The choice of Coomassie Blue staining rather than silver staining indicated that RP-HPLC prefractionation can provide strong enrichment effect which enabled us to visualize additional and less abundance proteins. Chromatographic enrichment was also demonstrated by the peptide mass fingerprint data, which gave mass spectra with increased number of peptide detected and improved signal intensity.

Keywords: Reversed-phase high-performance liquid chromatography; Two-dimensional gel electrophoresis; Proteomics; MALDI-TOF-TOF

1. Introduction

The traditional two-dimensional gel electrophoresis (2-DE) technique was introduced a quarter of a century ago [1–3] and is still a very widely used method for broad proteomic separations [4–7]. It is an orthogonal separation technique in which proteins are separated by two different physicochemical principles. After in-gel digestions, the digested peptides were identified by MALDI or ESI spectrometry. The most significant disadvantage of the 2-DE technique lies in the difficulty to detect low abundance proteins in the presence of high abundance proteins. In proteomic research, many disease-associated proteins or drug targets are low abundance proteins, which exist with amounts of femtomole or less and therefore are difficult to be identified with 2-D gel. In order to visualize these proteins in the gels, it is necessary to increase the relative amounts of low abundance proteins in the sample. Merely increasing the amount of protein loaded to the 2-D gel is often insufficient, because high abundance proteins will dominate the gels and can hide low abundance proteins. Moreover, with high protein loadings, resolution is lost and, therefore, it is difficult to distinguish the closely spaced protein spots. Considerable effort is being devoted to the development of prefractionation methods as means of enriching the content of low abundance proteins in samples for 2-DE. The basic idea of prefractionation is to segregate sample proteins

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into distinguishable fractions containing limited numbers of proteins.

Several approaches are widely used to enrich the low abundance proteins: chromatographic, electrophoretic and centrifugal subcellular fractionation. An important chromatographic approach is affinity chromatography (AC) [8–12] that can be used to reduce the complexity of the peptide by selecting the specific target protein or peptide. With the development of quantitative analysis of complex protein mixture, the immunoaffinity chromatography, immobilized metal-affinity chromatography (IMAC) [11] and isotope-coded affinity tags (ICAT) techniques [12] are widely used as prefractionation method. However, these techniques only enrich a few kinds of proteins with strong specificity and these approaches are not appropriate for global analysis. Reversed-phase chromatography [13,14] and anion-exchange chromatography [15] are also used. Preparative liquid electrophoretic methodologies [16–20] greatly depend on instrumental setup. Several typical instrumental setups are reported: rotationally stabilized focusing apparatus (Rotofor) [16], continuous free-flow IEF [17] and multifunctional electrokinetic membrane apparatus (Gradflow) [18,19]. One main disadvantage of this strategy is that the fractions collected from above equipments contain high amount of ampholytes, which will obstruct the downstream separation and analysis. Subcellular fractionation of organelles [21] is a traditional approach that based on the differences in size or density of these organelles. But the small amount of tissues or cell limits the application of this method.

Liver cancer is one of the most fatal diseases worldwide, especially in Asia. The mortality rate has been raised up to the second among malignancies in China. Up till now, there is no an effective remedy to cure malignant cancer of liver except liver transplant. However, liver transplant is an operation with highly difficulty and high cost, due to the immunological rejection of the host to the donor liver. Therefore, it is very significant to study liver proteome. The establishment of technology platform of the liver proteome represents an important first step. The traditional 2-DE technique has been insufficient and prefractionation techniques prior to 2-DE were employed more and more. In this work, RP-HPLC was selected to prefractionate proteins, due to its advantages, such as orthogonal separation mechanism of hydrophobicity, high resolution, good reproducibility and purification effect. Moreover, there has no report about whether the prefractionation technique of RP-HPLC is suitable to be applied into human liver tissue protein. Rodents, such as rat, are widely utilized as experimental animals for biological and toxicity studies because this specie is homologous greatly with human being. Therefore, in this study, we used rat as animal model to assess the feasibility of RP-HPLC prefractionation for liver tissue proteins. A multi-stage linear gradient elution was employed and optimized. The experimental results have proved that RP-HPLC prefractionation technique can be applied into liver proteomics study. The prefractionation technique and experimental conditions have been used in human liver proteomics project (HLPP) and the large-scale identification of human liver protein has begun and is still in progress.

2. Materials and methods

2.1. Materials

Acetonitrile (ACN) and trifluoroacetic acid (TFA) (HPLC grade) from Merck (Darmstadt, Germany), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), phynylmethylsulfonyl fluoride (PMSF), sequencing grade trypsin, dithiothreitol (DTT), α-cyano-4-hydroxycinnamic acid (CHCA) and urea were obtained from Sigma (St. Louis, MO). All chemical regents are of analytical grade. Deionized water is produced by Millipore system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Normal rat liver was obtained from Liver Cancer Institute in Zhongshan Hospital, Fudan University. The liver tissue was cut into small pieces and washed with glacial NaCl solution (0.9%) to remove blood and some possible contaminants. Then, the tissue debris were rapidly mixed with lysis buffer, containing 8 M urea, 2% (w/v) CHAPS, 50 mM DTT and 1 mM PMSF. Tissue sample was homogenized in ice bath. The resulting homogenate was swirled for 20 min and centrifuged for 10 min at 15,000 × *g*. The supernatant was collected. Protein concentration of sample was measured by the Bradford assay [22], using bovine serum albumin (BSA) as standard.

2.3. RP-HPLC

Liquid chromatography separation was performed with a LC-2010A system and fractions were collected using FRC-10A fraction collector combined with a SCL-10A controller (Shimadzu Corp., Japan). Integrated Shimadzu LC-2010A system consisted of a quaternary low pressure gradient pump, autosampler, on-line degasser, block heating-type column oven and UV–vis detector with a D_2 lamp. Shimadzu Class-VP station was used to acquire and process data. The separation procedure was fulfilled in 25 °C. Protein sample was loaded onto the C18 reversed-phase column (250 mm × 4.6 mm, 5 μm, 300 Å, Hypersil, eliteHPLC, China) preceded by a 20 mm × 4.6 mm (5 μm, 300 Å, Hypersil, eliteHPLC) guard column. The buffer solutions used were 0.1% TFA in water (buffer A), 100% acetonitrile–0.1% TFA (buffer B) (all v/v). A linear gradient elution had the following profile after injection: 10 min of 100% buffer A, 20 min linear gradient from 0% to 30% buffer B, then 80 min linear gradient from 30% to 90% buffer B, 5 min of 90% buffer B and 2 min back to 0% buffer B. Three hundred micrograms of total proteins was loaded onto the column in one run. The flow rate is 0.8 mL/min, considering the column