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A hybrid LC–Gel-MS method for proteomics research and its application to protease functional pathway mapping

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Abstract

Two-dimensional (2D) gel electrophoresis is the most common protein separation method in proteomics research. It can provide high resolution and high sensitivity. However, 2D gel methods have several limitations, such as labor-intensive procedures, poor reproducibility, and limited dynamic range of detection. In fact, many investigators have returned to couple the one-dimensional (1D) SDS-PAGE with mass spectrometry for protein identification. The limitation of this approach is the increased protein complexity in each one-dimensional gel band. To overcome this problem and provide reproducible quantitative information, we describe here a 2D method for protein mixture separation using a combination of high performance liquid chromatography (HPLC) and 1D SDS-PAGE. The study shows that the step-gradient fractionation method we have applied provides excellent reproducibility. In addition, high mass accuracy of LC–FTICR-MS can allow more confident protein identifications by high resolution and ultra-high mass measurement accuracy. This approach was applied to *comparative proteomics* since protein abundance level changes can be easily visualized with side-by-side vertical comparison in one gel. Furthermore, separation of multi-samples in the same gel significantly reduces run-to-run variation, as is shown with differential image gel electrophoresis (DIGE). Finally, this approach readily incorporates immunological methods to normalize relative abundances of multiple samples within a single gel. This paper presents the results of our developments and our initial application of this strategy for mapping protease function of beta amyloid cleaving enzyme (BACE) in biological systems.

Keywords: Multidimensional protein profiling; SAX; SDS-PAGE; BACE; Mass spectrometry

1. Introduction

The proteome contains information critical to an improved comprehension of life, biological organisms and achieving the paradigm shift now known as "systems biology" [1,2]. Proteomics research technologies are rapidly changing our understanding of complex and dynamic biological systems by providing information relevant to functionally-associated changes in protein abundances, protein–protein interactions, and posttranslational modifications [3–7]. Current proteomics research demands highly efficient analytical systems that are high resolution, high sensitivity, high throughput and robust. The overall challenge of proteomics research is the assignment of function to the large fraction of genes whose functions are currently not known [8,9]. Although several facets of proteomics research currently exist are related to the assignment of functional elements, the primary goals of such research can be classified into two categories including the visualization of changes that occur at the protein level in biological systems and the identification of protein species involved in these changes [7,9,10]. As such, protein separation and mass spectrometry

Abbreviations: AD, Alzheimer's disease; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; SAX, strong anion exchange; FTICR-MS, Fourier transform ion cyclotron resonance mass spectroscopy; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenyl methyl sulfonyl fluoride; MW, molecular weight; DIGE, differential image gel electrophoresis

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technologies have become integral components in much of what is today's proteomics research. However, each of these components places constraints upon the other and proteomics research is only facilitated with integration of the two.

Gel electrophoresis has for some time been the method of choice of biologists and biochemists when faced with the task of protein separation. Gels represent relatively simple technology that can be used for stable, spatial separation of proteins that can then be visualized with a number of stains and dyes [11,12]. The development of immobilized pH gradient (IPG) strips has made 2D gel electrophoresis a reproducible and viable technique that can allow stable separation of thousands of proteins in a single gel image [6,13–16]. This capability has significantly impacted the newly emerging field of proteomics, since it allows high resolution, relatively robust separation of protein mixtures [13]. However, due to the limitation presented by the amount of protein that can be loaded for the IEF separation stage, the identification of low abundance proteins is more difficult [3,17]. For example, for normal analytical 2D gels used for comparative analysis, as with difference image gel electrophoresis (DIGE) analysis [18,19], the recommended protein loading amount is limited to several hundred micrograms of total protein. Additionally, this much protein must be loaded in a restricted volume, placing additional constraints on the applications of 2D gels. These factors place significant limitations on the ability to accurately visualize and quantitate proteins, as well as on the subsequent protein identification strategies. To some extent, these limitations result in proteomics research only being applicable to more abundant proteins, and significantly limit the dynamic range of current proteomics capabilities [16,20–22].

The approach for efficient protein separation presented here involves coupling liquid chromatography as the first dimension, as opposed to isoelectric focusing, followed by additional stages of chromatography and/or SDS-PAGE [21,23,24]. This strategy provides a powerful, flexible and reproducible method to more rapidly separate the large number of proteins present in the proteomes of mammalian cells. The combination of separation techniques exploits the high loading capacity and excellent reproducibility of liquid chromatographic separations [21,25], combined with the attractive features of gel electrophoresis that allow stable spatial separation of proteins that can then independently be visualized for relative quantitation and identification. It has been reported [26] that significant enrichment was obtained for individual proteins by using MonoQ anion exchange chromatography before 1D or 2D PAGE. The feasibility to detect low abundance proteins by combining HPLC and 1D SDS-PAGE has also been demonstrated by Nawarak et al. [17]. This approach also provides the opportunity to gather sample separation information during the first dimension of analysis that can then be used to determine which samples, if any, are subjected to additional stages of liquid chromatography prior to gel analysis. Finally, the last dimension gel analysis also exploits the attractive feature of DIGE that allows multiple samples to be run in the same gel, minimizing difficulties that arise from gel-to-gel comparisons. Because the new approach presented here can allow proteome comparisons for larger numbers of samples, the present methodology will be highly applicable to time-resolved or multi-point proteomics analyses where the protein expression profiles from samples are collected at a multitude of time points, dosages, or other parameters are to be compared.

We demonstrate this new approach with proteomics analysis for improved characterization of the Alzheimer's disease-relevant protease, beta amyloid cleaving enzyme or BACE [27]. BACE and a closely related enzyme BACE2 are two newly identified proteases which are involved in proteolysis of amyloid precursor protein (APP) [28–31]. BACE is a recognized enzyme that initiates generation of beta amyloid peptides, the major components of amyloid plaques found in postmortem brains of Alzheimer's disease patients. Characterization of BACE and BACE2 activity and involvement in Aβ generation in cells is critical to improved understanding of these proteases. In this paper, we present the initial application of our novel profiling system to the study of overexpression of BACE and BACE2 in HEK 293 cells. The initial results showed that this new separation strategy provides more reproducible visualization of protein abundance level changes and offers much greater flexibility for comparative analysis of biological systems.

2. Experimental

2.1. Chemicals and reagents

The HEK 293 cell line used for these studies was obtained from Clontech (Palo Alto, CA, USA). Antibiotics G418 from GibcoBrl, Lipofectamine2000, and pcDNA3 vector were purchased from Invitrogen (Carlsbad, CA, USA). BACE and BACE2 overexpressing pcDNA3 plasmids were kindly provided by Dr. Michael Farzan, Harvard Medical School. Plasmid Midi Kit was purchased from QIAGEN (Valencia, CA, USA). Competent cells JM109, reporter gene β-gal, porcine trypsin, and 5× cell lysis buffers were from Promega (Madison, WI, USA). Antibody anti-1D4 was purchased from Chemicon (Temecula, CA, USA). DMEM, iodoacetamide, DTT, PMSF, and protein inhibitor mixture were purchased from Sigma–Aldrich (St. Louis, MI, USA). FBS was from HyClone (Logcen, UT, USA). Goat anti-mouse HRP-conjugated secondary antibody, protein assay reagent, and gel staining buffer were from Bio-Rad (Hercules, CA, USA). Western Lightning Chemiluminescence reagent was from Perkin-Elmer (Wellesley, MA, USA). Precast SDS-PAGE gels were either from Bio-Rad or from Invitrogen as indicated below. Sample buffer, running buffer, and gel electrophoresis instruments were from these two companies correspondingly.