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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.

2.2. Stable transfection on HEK 293 cells

HEK 293 cells were maintained in DMEM supplemented with 10% FBS. BACE and BACE2 encoded pcDNA3 vectors were tagged by 1D4 which is a 10 amino-acid C-terminal tag derived from bovine rhodopsin [28]. All these vectors were transformed on JM109 cells and purified using a Plasmid Midi Kit. Transfection was carried out using Lipofectamine2000 based on the manufacturer's protocols. BACE, BACE2 constructs and pcDNA3 plasmid were transfected in HEK 293 cells with a DNA to Lipofectamine2000 ratio of 1 μg/10 μl. Fresh growth medium was replaced 24 h after transfection. Selective medium containing 0.5 mg/ml G418 was added the following day. Medium was continually exchanged every 1–2 days for 1 week for the neomycin to act on the non-transfected cells. Stable transfected cells were maintained in growth medium with 0.5 mg/ml G418.

2.3. Western blot analysis

Cells that stably expressed BACE and BACE2 each contained in a 10 cm diameter dish were detached and briefly centrifuged. Half of the cell pellet was re-suspended in Laemmli sample buffer (Invitrogen, Carlsbad, CA, USA) to get the whole cell lysate. Lysates were separated by a 4–12% SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA) and then transferred to a PVDF membrane (Imobilon-P, Millipore, Billerica, MA, USA) with a semidry transfer system Semi-Phor TE-77 (Hoefer, San Francisco, CA, USA). After transferring, the membrane was blocked with 5% nonfat milk in PBS overnight followed by incubating with the primary antibody anti-1D4 (1:500) for 1 h at room temperature. The membrane was then probed by HRP-conjugated secondary antibody and labeled by Western Lightning Chemiluminescence reagent.

2.4. BACE and BACE2 activity assay

Cells were detached from culture dishes and washed with cold PBS buffer three times. Harvested cells were lysed in 0.3 ml of 0.5% NP-40/0.5 M NaCl/phosphate buffer, pH 7.4, containing protease inhibitor mixture and 0.5 μM PMSF, and incubated for 2 h at room temperature with 1D4 antibody which was bound to Ultralink Immobilized protein G beads (Pierce, Rockford, IL, USA). Samples were washed four times with lysis buffer and twice with incubation buffer (2.5% acetic acid/ammonium acetate, pH 5.5). Immunoprecipitates bound to 1D4-protein G beads were mixed with 100 μl of incubation buffer containing 50 μM synthetic peptides, and incubated at 37 °C for 2 h. Protein G beads were removed by centrifugation and the supernatant was analyzed by LC–MS/MS with an Esquire HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Nano-HPLC separation was performed with an LC packings Ultimate Nano-HPLC system equipped with a Famos micro autosampler and a Swichos micro column switching module (Dionex, Sunnyvale, CA, USA). Reverse phase solvents were (A) 0.1% TFA in water and (B) 0.1% TFA in 95% acetonitrile. Samples were first injected by the autosampler and loaded onto a micro trap column (C18 PepMap, 300 μm × 1 mm, 5 μm, LC packings) at a flow rate of 50 μl/min with solvent A. The loaded sample was continuously washed with solvent A for 3 min to remove salts. Peptides were then eluted at a flow rate of 300 nl/min to an analytical column (C18 PepMap, 75 μm × 150 mm, 3 μm, 100 Å, LC packings) and separated using the following gradient: 0% B for 0–3 min, 20–70% B for 3–45 min, 90% B for 45–55 min, and 0% B for 55–65 min. The eluant from the analytical column was sprayed on-line with a nano-spray emitter to the mass spectrometer. The nano-spray emitter was made in house by etching 20 μm × 360 μm fused silica capillary with HF. The spray potential was set as 1300–1500 V. LC–MS/MS mass spectra were acquired using Hystar software (version 2.3, Bruker Daltonics, Billerica, MA, USA). MS/MS data acquisition was set in automatic mode with active exclusion based on peak intensity and a selection of exclusion peak lists. Data analysis and processing were using Bruker Daltonics DataAnalysis software (version 3.1).

2.5. Strong anion exchange (SAX)-HPLC separation

Cell lysates were collected as described above and total protein concentration was detected by Bradford assay. Analysis of cell lysates was performed using a SAX HPLC column (4.6 mm × 100 mm, Source 15Q, Amersham, Piscataway, NJ, USA) attached to the DIONEX Auto-Ion HPLC instrument with a 2 ml sample injection loop. UV absorbance signals (280 nm) were collected by 757 Absorbance Detector (Applied Biosystems, Foster City, CA, USA) and were translated to digital signals using a LabJack U12 (LabJack, Lakewood, CO, USA). Buffer A (20 mM Tris buffer, pH 7.0) and buffer B (1 M NaCl, 20 mM Tris buffer, pH 7.0) were used as mobile phases for all samples. Fractions were collected using SF-2120 fraction collector (Advantic MFS Inc., CA, USA).

For reproducibility experiments, three 1 mg aliquots of HEK 293 cell lysate were used. In each experiment, the sample was diluted to a total injection volume of 1.5 ml and then loaded to the column. A linearly increased step-gradient from 100% buffer A to 100% buffer B at a flow rate of 1.0 ml/min was used. After injection, the column was equilibrated in buffer A for 5 min. Fraction 1 was collected from 2 to 5 min during equilibration time. Then fractions 2–11 were collected by increasing elution buffer B concentration from 10%, 20% to 100% with 10% increase in each step. Future studies will employ non-linear step gradients to maximize separation resolution. Each fraction corresponded to one elution condition was collected in a 3-min interval. The total elution time was 35 min. 1 ml of each fraction was used for TCA precipitation and resuspended in 20 μl sample buffer. For reproducibility comparison, three replicate fractions were loaded onto a 4–12% SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA) in neighboring lanes to allow rapid visual comparison.

For BSA spiking experiments, 10 μg and 20 μg BSA were spiked into 1 mg pcDNA3-transfected HEK 293 cell lysate separately. For ovalbumin spiking experiments, 1 μg, 5 μg, 10 μg, and 20 μg ovalbumin were spiked into 1 mg pcDNA3-transfected HEK 293 cell lysate separately. The SAX HPLC (4.6 mm × 100 mm, Poros QE, PerSeptive Biosystems, Foster City, CA, USA) was used at the flow rate of 2.0 ml/min. Each sample was diluted to a total injection volume of 1.5 ml. A linearly increased step-gradient from 100% buffer A to 100% buffer B at a flow rate of 2.0 ml/min was used. Fractions were collected as described before. Each fraction corresponded to one elution condition and was collected in 6 ml total volume. One milliliter of each fraction was used for TCA precipitation and re-suspended in 20 μl sample buffer. Corresponding fractions of individual samples were loaded on neighboring lanes on 4–12% SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA).

For BACE, BACE2 overexpressing cells and pcDNA3-transfected cells comparison experiments, 2 mg of each cell lysate was used. Each sample was diluted to a total injection volume of 1.5 ml. After injection, the column was equilibrated in buffer A for 5 min. A linearly increased step-gradient described as before was used and the flow rate was set at 2.0 ml/min. Fractions were collected as described before. Each fraction corresponded to one elution condition and was collected in 6 ml total volume. One milliliter of each fraction was used for TCA precipitation and re-suspended in 20 μl sample buffer. Corresponding fractions of individual samples were loaded on neighboring lanes on 8–16% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA).

2.6. SDS-PAGE analysis

SDS-PAGE was carried out by the method of Laemmli under denatured conditions in different gradient polyacrylamide gels; each sample (after TCA precipitation) was dissolved in 20 μl sample buffer followed by heating 10 min at 70 °C. Gels were run at a constant voltage of 200 V. After completion of electrophoresis, the gels were stained with Coomassie G-250 stain buffer. Bio-Rad precision plus protein standards were used as standard molecular weight markers. After staining and destaining, gels were scanned by a laser scanning densitometer (ImageQuant Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) and analyzed by ImageQuaNT software.

2.7. In-gel tryptic digestion

The gel bands for protein identifications were cut and destained in 50% methanol/5% acetic acid solution overnight, followed by treatment with DTT for reduction of disulfide bonds and iodoacetamide for alkylation [32]. After equilibration in 100 mM NH_4HCO_3 solution, the gel bands were dehydrated in acetonitrile. The remaining solvent was evaporated by using CentriVap (Labconco, Kansas City, MO, USA). Trypsin solution was added with the concentration of 20 ng/μl and allowed to react with the gels overnight at 37 °C. Peptide extraction solutions were concentrated and desalted using C18 ziptip (Millipore, Billerica, MA, USA). Solution A (0.1% TFA in deionized water) was used for washing and solution B (0.1% TFA and 50% acetonitrile) was used for elution.

2.8. Mass spectrometry and protein identification

One microliter of concentrated peptide solution was mixed with 1 μl matrix solution (α-CHCA saturated in solution of 0.1% TFA and 50% acetonitrile) and then was spotted on a 7 × 7 sample target. An OmniFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) was used for analysis. The spectrum was externally calibrated by angiotensin I, ACTH clip (1–17), and ACTH clip (18–39), and analyzed by Bruker FlexAnalysis software. MASCOT was used for peptide mass mapping by searching NCBInr database. The database search was performed based on following parameters: taxonomy (Homo Sapiens), enzyme (trypsin), missing cleavage (1), mass tolerance (0.1–0.3 Da), modification (carbamidomethylation for cysteine and oxidation for methionine).

In-gel digests of gel bands of interest of BSA spiking sample were also analyzed by ESI-FTICR-MS/MS experiment. A Bruker Daltonics Apex-Q 7T FTICR-MS was used to acquire the mass spectral data using XMASS as the data acquisition software program. The voltage on the capillary of ESI was set between 2050 V and 2350 V. A syringe pump used to introduce the solutions was set between 15 μl/h and 25 μl/h. The ions enter the instrument through a glass capillary and then pass through a hexapole followed by a quadrupole then a second hexapole. The quadrupole was used to select the specific *m*/*z* ions of interest to be fragmented. These ions were accumulated and were activated by CAD in the second set of hexapoles (collision cell energy was set to 30 eV) between 1 s and 2 s to acquire MS/MS data. The ions were then sent to the ICR cell using electrostatic lenses. The FTICR data were processed using ICR-2LS software package [33].

3. Results and discussion

3.1. Overexpression of BACE and BACE2 in cells and BACE activity assay

C-terminal tagged BACE and BACE2 were subcloned into pcDNA3 separately and were stably transfected into HEK 293 cells. HEK 293 cell lines were employed here since BACE was discovered in HEK 293 expression library analysis [27]. Thus, BACE likely fulfills some natural functional roles in HEK 293 cells. Additionally, HEK 293 cells were used previously to identify BACE and to analyze its proteolytic function by many other reported studies [27,28,30,31]. After transfection, all cells were grown in the culture media

containing 0.5 mg/ml G418. Untransfected HEK 293 cells and cells transfected with pcDNA3 vector were prepared as control for cells stably expressing BACE and BACE2. Expression of BACE and BACE2 was identified by Western blot analysis (Fig. 1) using the anti-1D4 antibody for C-terminal 10 amino acid tag [28]. The C-terminal labeling enables the detection of the exogenous protein and consequently facilitates the detection of differences in its amount in different cells by one Western blot analysis. For example, only one band corresponding to a molecular mass of about 70 kDa was detected in BACE overexpressing cells and was not found in all other cells. Furthermore, only one band corresponding to a molecular mass of about 50 kDa was detected in BACE2 overexpressing cells and was not found in all other cells. Both of them were close to their expected masses [28]. These data illustrate stable overexpression of BACE and BACE2 in these newly developed cell lines.

To demonstrate that BACE and BACE2 in transfected cells are active, lysates of cells transfected with plasmids

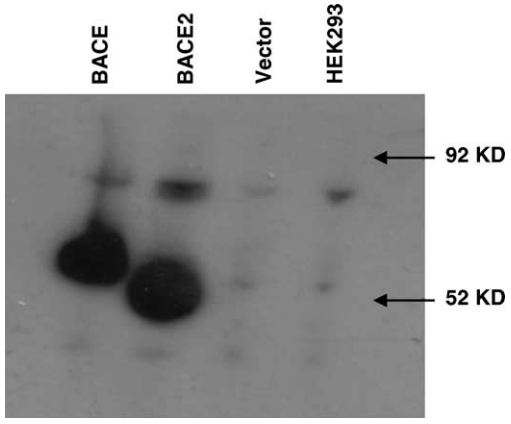

Fig. 1. Western blot analysis of BACE overexpression and BACE2 overexpression in HEK 293 cells vs. controls of vector-transfected HEK 293 cells and untransfected HEK 293 cells.

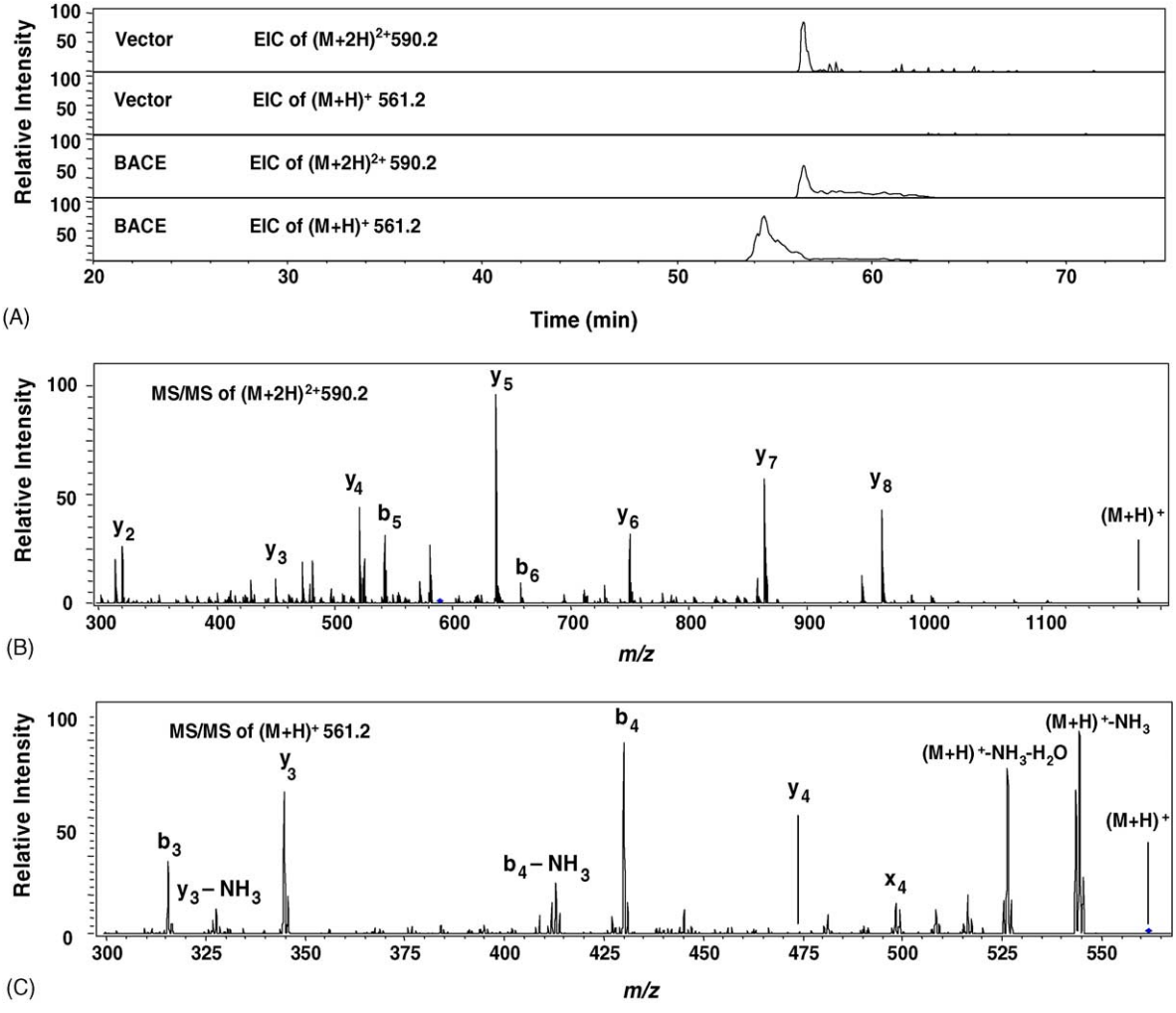

Fig. 2. LC–MS/MS analysis of enzyme assay reaction mixture. (A) Extracted ion chromatogram (EIC) of full length peptide ($(M + 2H)^{2+}$ at 590.2) and N-terminal product ($(M + H)^{+}$ at *m*/*z* 561.2) for vector control and BACE, respectively; (B) MS/MS mass spectrum of full length peptide and (C) MS/MS spectrum of N-terminal product.

encoding tagged BACE, BACE2, or vector alone were incubated with 1D4-protein G beads. Beads were washed extensively and incubated with peptide SEVNLDAEFR which is a well-known BACE substrate. The elution was then analyzed by LC–MS/MS. As shown in Fig. 2, peptides incubated with 1D4-protein G beads from vector only cell lysates exhibited a $(M+2H)^{2+}$ peak at *m*/*z* 590.2, corresponding to the full-length peptide (Fig. 2B). The same peptide incubated with beads from BACE overexpressing cell lysates has produced a $(M+H)^{+}$ peak at *m*/*z* 561.2 as well as a $(M+2H)^{2+}$ peak at *m*/*z* 590.0. The peak at *m*/*z* 561.2 agrees well with the mass of the peptide, DAEFR, the expected C-terminal fragment ($(M+H)^{+}=561.612$) resultant from BACE hydrolysis of the incubated substrate. Fig. 2C is the MS/MS spectrum of peak 561.2 which indicates that the sequence of this peak is DAEFR. No peak at *m*/*z* 561.2 was found when the peptide was incubated with 1D4-protein G beads from vector only cell lysates. The same fragment peak was also obtained from peptide incubated with BACE2 (data not shown here) as expected since both proteases have been shown to cleave this substrate [31].

3.2. Method development

A schematic representation of this method is illustrated in Fig. 3. In the initial implementation, cell lysates are fractionated off a strong anion exchange (SAX) column and fractions are then run on a 1D gel for relative quantitation. This approach allows a lane-by-lane comparison of samples for relatively easy identification of changes in protein abundance, either visually or with computer analysis. Since much larger total protein amounts can be loaded on the SAX column as compared with IPG strips, much greater amounts of protein are available on the gel for improved quantitation and protein identification.

For this study, we used the cell lysis method described above to collect the protein sample from HEK 293 untreated cells. Fig. 4A illustrates the UV chromatograms during SAX fractionation of this cell lysate. Also shown in this figure are the elution buffer concentration steps employed for fractionation. These data indicate the qualitative nature of the UV chromatogram, since most fractions that showed strong UV peaks also showed a lot of bands on corresponding lanes in gels. However, in some cases, for example in fraction 7, very little protein was detected on the gel or by Bradford assays (data not shown), indicating UV absorbance in the fraction was dominated by non-protein species.

After SAX fractionation, samples were concentrated and desalted by TCA precipitation. During TCA precipitation, protein loss might have occurred due to the acetone wash. However, previous studies [34] reported the effects of protein precipitation methods on sample preparation. Their results with 2D gel electrophoresis showed all spots detected before TCA precipitation were also detected after TCA

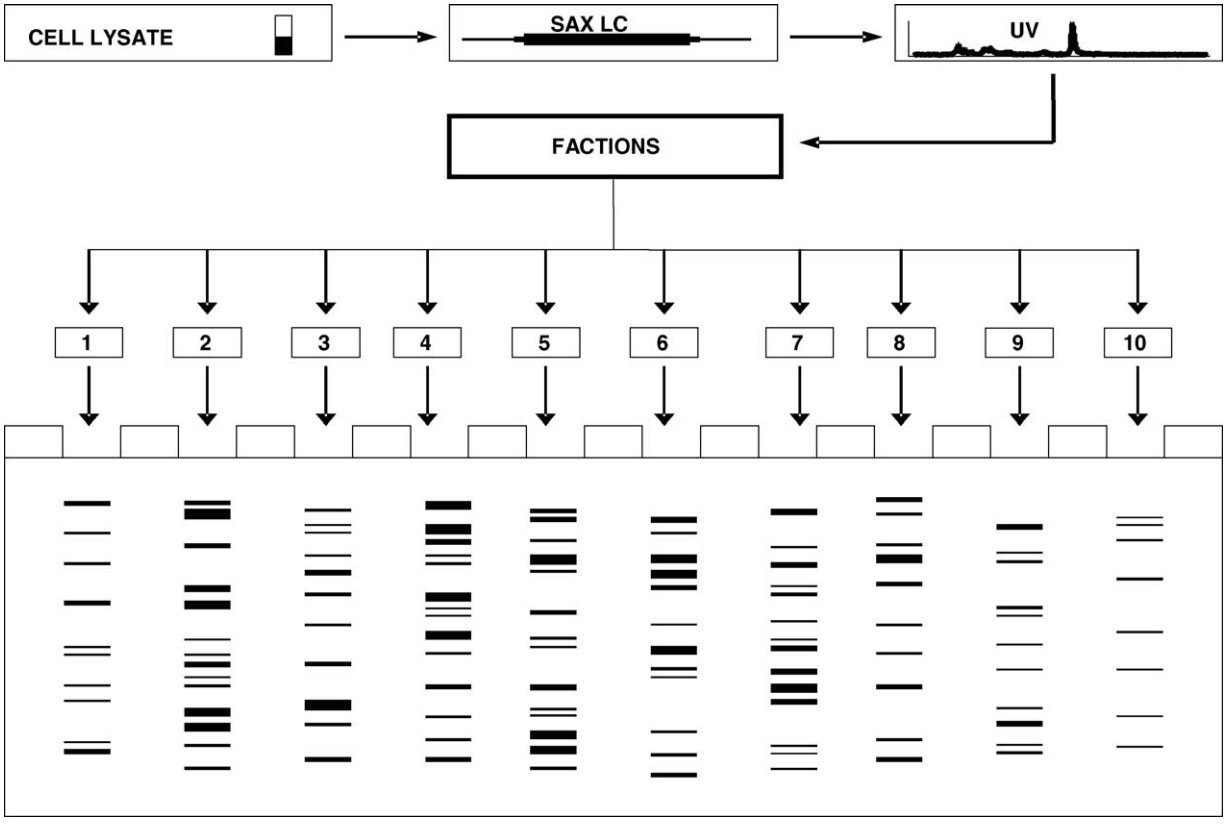

Fig. 3. Schematic diagram illustrating combined liquid chromatography gel electrophoresis separation of proteins.

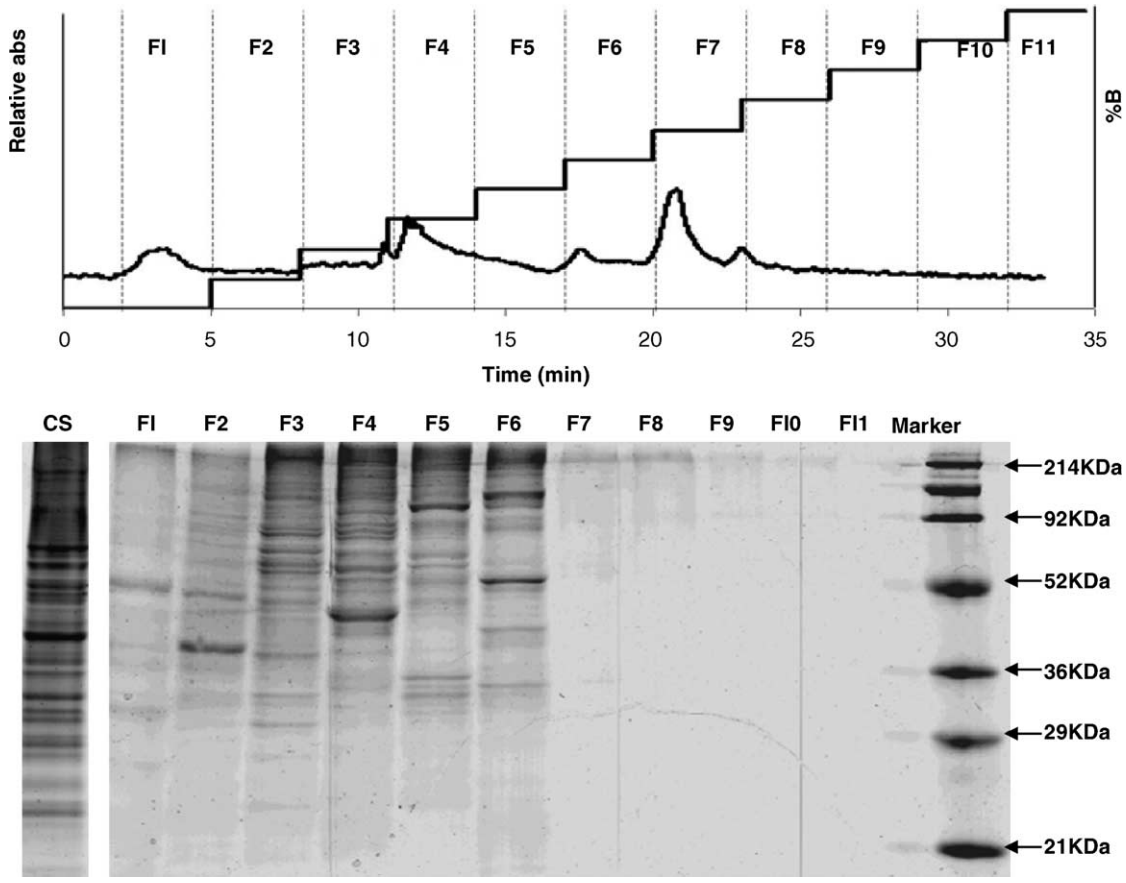

Fig. 4. SAX HPLC separation and gel separation of fractions. (A) UV chromatogram. The step-gradient elution profile is shown here. F1 to F11 represent the fractions collected at 3 min intervals corresponding to different elution condition, and CS represents unfractionated cell lysate. (B) 8–16% SDS-PAGE gel (Bio-Rad) image of individual fractions.

precipitation, including low MW IgG light chain proteins (MW about 20 kDa).

Fig. 4B illustrates corresponding gel separation for the fractions resultant from SAX fractionation shown in Fig. 4A. Most proteins eluted from fractions 1 to 6. After comparing with the gel image of unfractionated sample, we found that the sample complexity of fractions was reduced after the SAX fractionation. Also, different fractions have significantly different band patterns, which indicate substantially different proteins are present in each fraction. These data showed that SAX fractionation can clearly divide the proteome into several less complicated protein mixtures.

3.3. Reproducibility study

We also used untransfected HEK 293 cell lysates to demonstrate the reproducibility of this new approach. After protein concentration measurement, 3 mg of total protein was used and diluted with buffer A into 4.5 ml total volume. Three 1.5 ml aliquots were injected separately to test the reproducibility of this new method. After each run, the column was washed with a large amount of buffer B and then equilibrated by buffer A. The analysis of blank injections verified that no signals were resultant from carryover between each sample.

Fig. 5A illustrates the UV absorption chromatogram acquired with triplicate analysis of the HEK 293 cell lysate described above. We evaluated the reproducibility of retention times based on the major peaks marked in Fig. 5A. Calculated reproducibility of retention times is listed in Table 1, and the average R.S.D. value of 1.3% is obtained. For stepwise gradient LC separations, there is a standard deviation of the time of individual steps formation (instrumental parameter) [35]. This constant deviation can result in a small deviation of peak elution time related to the beginning of a

Table 1
Reproducibility of the retention time based on the major peaks in Fig. 5A

Peak no.	Retention time (min)					
	First	Second	Third	Average	S.D.	R.S.D. (%)
1	3.02	3.023	3.02	3.02	0.0096	0.32
2	10.65	10.27	9.85	10.06	0.4010	3.99
3	11.4	11.17	10.88	11.02	0.2587	2.35
4	17.32	17.30	17.32	17.31	0.0096	0.056
5	20.57	20.42	20.58	20.50	0.0918	0.45
6	22.83	22.70	22.98	22.84	0.1417	0.62

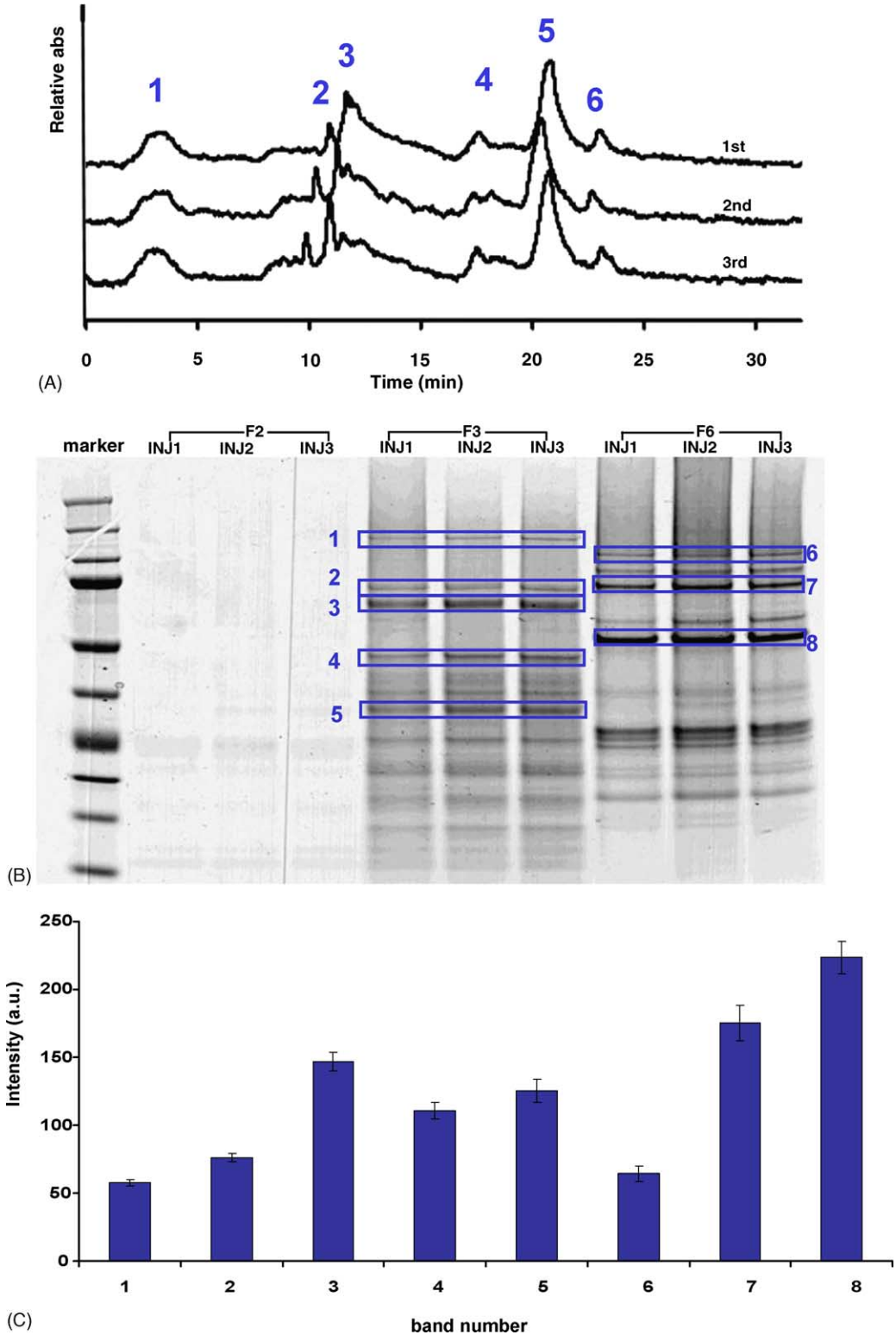

Fig. 5. Multiple analyses of HEK 293 cell lysates with SAX HPLC and gel separation of fractions: (A) UV chromatograms, marked peaks are used to evaluate the reproducibility of retention time (in Table 1); (B) 4–12% SDS-PAGE gel (Invitrogen) image of individual fractions from triplicate analyses and (C) quantitative data (average and standard deviation) of sets of gel bands marked in part (B).

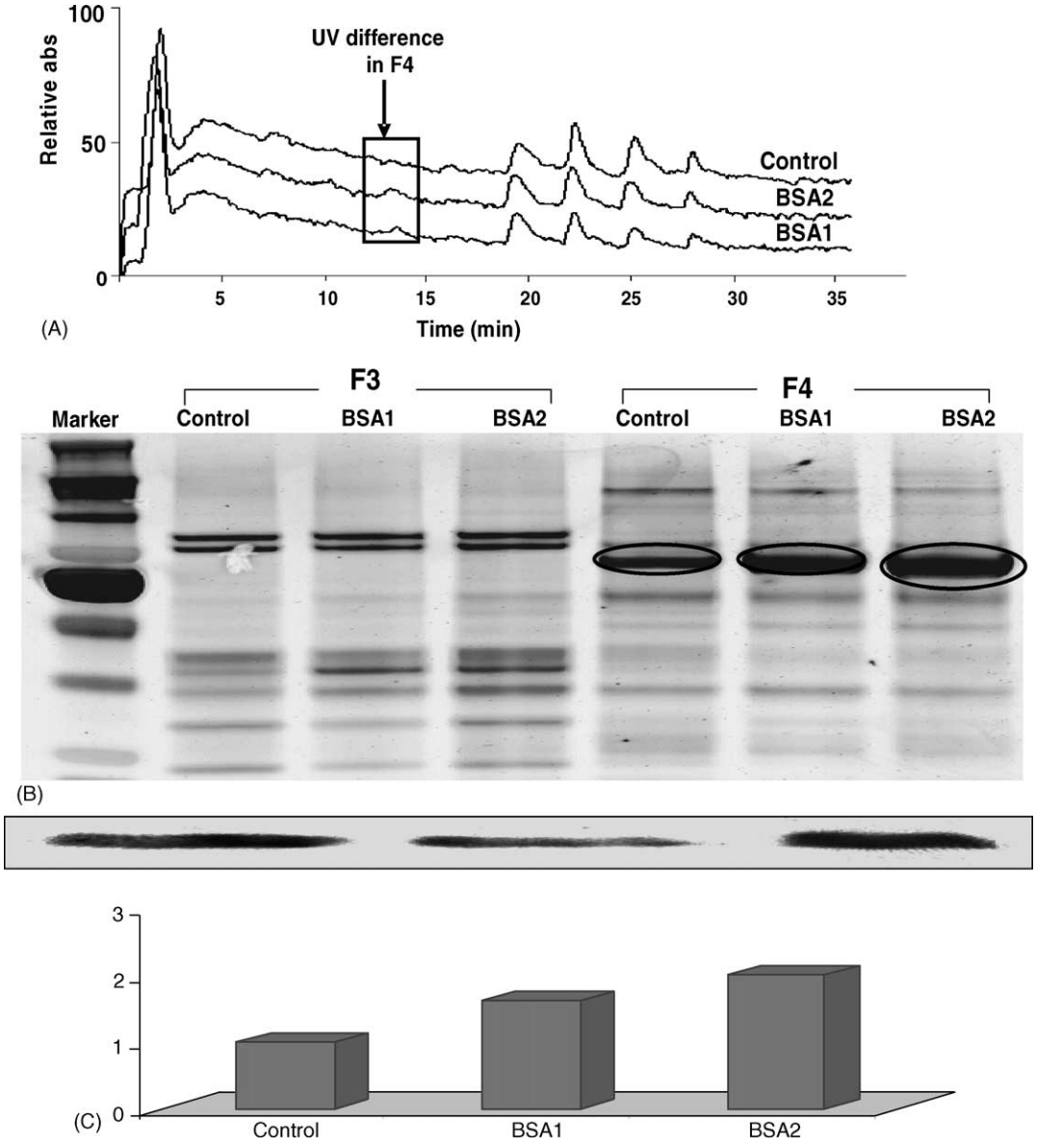

Fig. 6. BSA spiking in control HEK 293 cell lysates: (A) UV chromatograms; (B) 4–12% SDS-PAGE gel (Invitrogen) image of individual fraction and (C) intensity ratio after normalization by Western blot of internal control beta-action. "BSA1" refers to cell lysate spiked 10 μg BSA and "BSA2" refers to cell lysate spiked 20 μg BSA.

particular gradient step which elutes this peak. The deviation of peak elution time can have some impact on the band intensity in the SDS-PAGE gel. However, since we use relatively long elution time (3 min) for each step, this impact should be relatively small. Fig. 5B shows the gel image of individual fraction comparison of these triplicate samples. We also evaluated the reproducibility of the gel band intensity. Fig. 5C demonstrates the quantitative data (average intensity and standard deviation) of the marked gel bands in Fig. 5B. After comparison of both UV chromatograms and SDS-PAGE images, we found both components showed excellent reproducibility. These results show that step-gradient fractionation can provide excellent reproducibility. We also did continuous gradient fractionation (data not shown here), but that approach turned out to be more difficult to get comparable reproducibility since slight changes in conditions, such as buffer pH or timing of fraction collection, can cause the whole elution pattern to systematically shift. Step-gradient fractionation significantly decreased the variability of continuous gradient fractionation, resulting in significantly improved performance.

3.4. Standard protein spiking experiments

We spiked BSA at two levels into control cell lysates and injected half of the protein mixtures onto the SAX column.

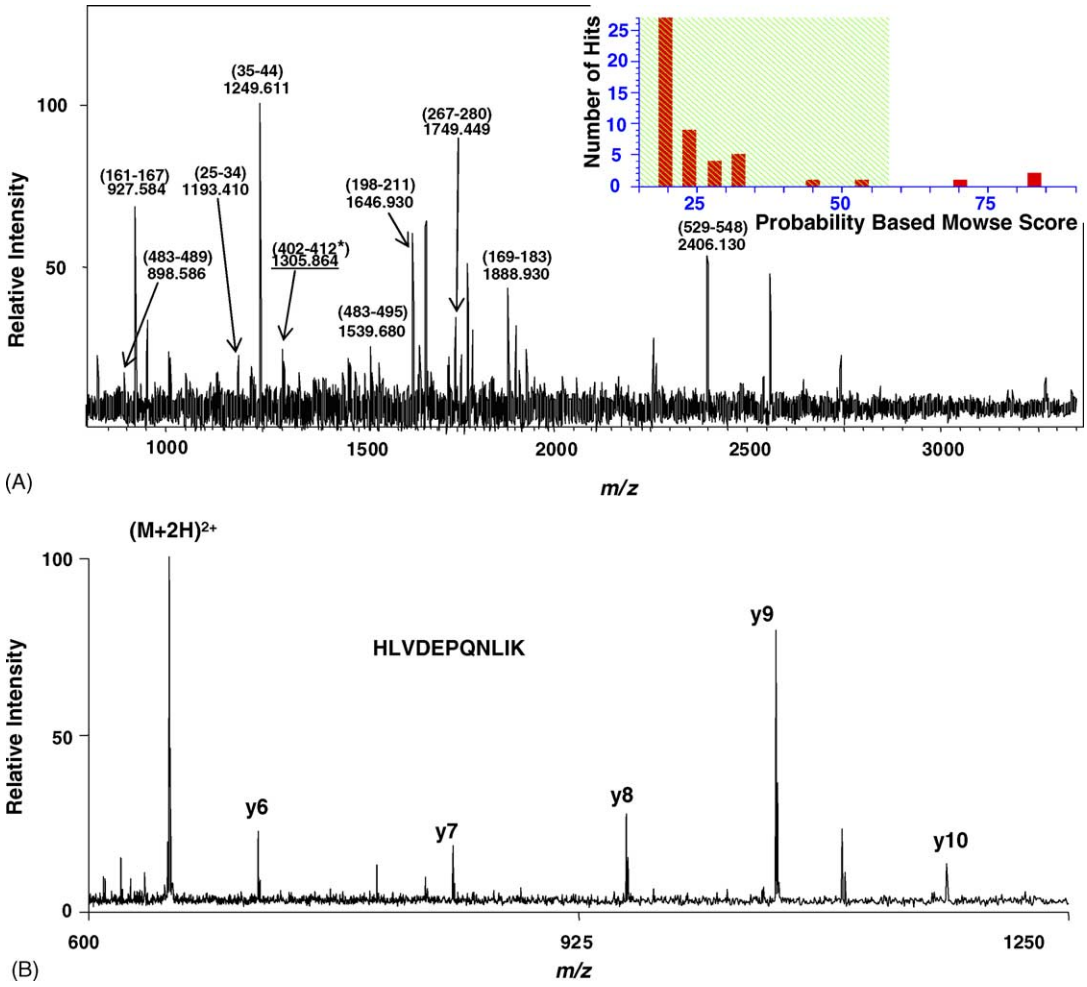

Fig. 7. Mass spectra from in-gel tryptic digestion of band circled in Fig. 6B "BSA2" sample. (A) MALDI-TOF-based peptide mass fingerprint; the identified peptides are labeled with the *m/z* corresponding to the $(M + H)^+$ and sequence range. (B) ESI-FTICR-MS/MS spectrum of the +2 charge state ions of the peptide denoted with an asterisk in part (A). The observed y-series ions confirm the sequence of this BSA tryptic fragment.

Spiked BSA was eluted in fraction 4 and was observed in both the UV chromatogram signals and the SDS-PAGE images of SAX fractions (Fig. 6). The bands circled were cut and digested with trypsin. We identified BSA from the band circled in Fig. 6B from the "BSA2" sample using MALDI-TOF peptide fingerprint mapping (Fig. 7A) and ESI-FTICR-MS/MS. Fig. 7A illustrates a MALDI-TOF spectrum and peptide fingerprint mapping using MASCOT. Fig. 7B shows the ESI-FTICR-MS/MS of *m/z* 653.3 identified as BSA tryptic digest peptide using the same in-gel digestion solution, which confirms the protein identification using MALDI peptide fingerprint mapping. The average mass measurement accuracy of these identified fragments is 3.8 ppm.

We used another half of the samples for the second SDS-PAGE gel and performed Western blot analysis of the internal control protein, beta actin, as shown in Fig. 6C. Band intensity was measured with ImageQuant software. Fig. 6C also shows the relative intensity ratio of the three bands circled in Fig. 6B after normalization with the internal control beta actin. Since the employed cell culture medium contained bovine serum albumin, we also identified the band in the control cell lysate as BSA. We used the control band intensity as background and subtracted it from bands observed in the BSA spiked samples. After subtraction, we found that the relative intensity ratio between 10 μg BSA spiked sample and 20 μg BSA spiked was about 1:1.9, in good agreement with the expected ratio of 1:2.

Since control samples contained relatively large amounts of BSA, we chose another standard protein ovalbumin, to demonstrate the range for the quantitative study. Fig. 8A is the SDS-PAGE image of SAX fractions which contained ovalbumin. Spiked ovalbumin also eluted in fraction 4. Four different spiking amounts of ovalbumin were chosen: 1 μg/mg cell lysate, 5 μg/mg cell lysate, 10 μg/mg cell lysate, and 20 μg/mg cell lysate. Intensity of gel bands corresponding to ovalbumin increased when spiked ovalbumin concentration increased, as shown in box 1 in Fig. 8A. Also,

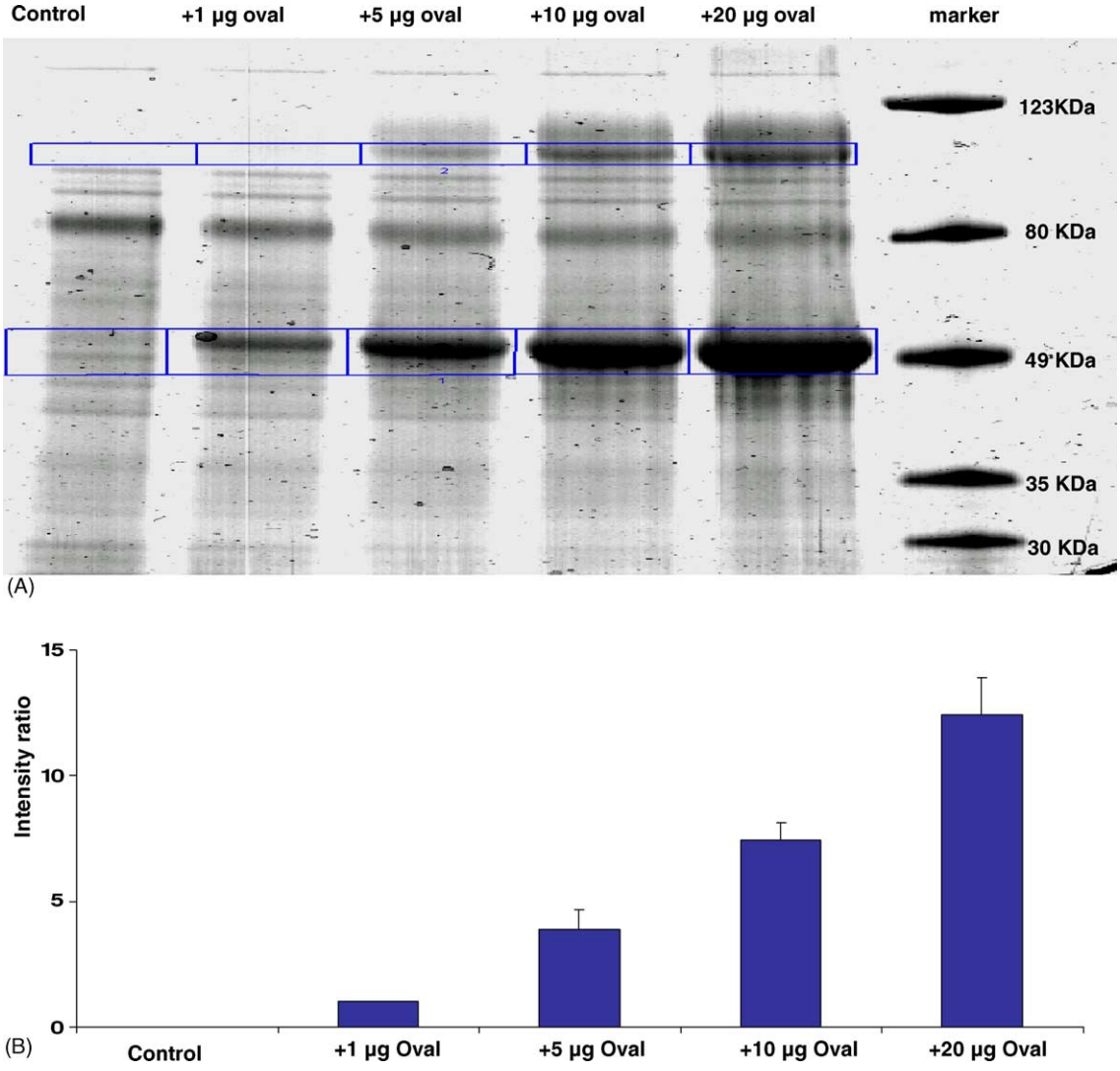

Fig. 8. Ovalbumin spiking in control HEK 293 cell lysates: (A) 4–12% SDS-PAGE gel (Invitrogen) image and (B) intensity ratio after subtracting control as the background. Quantitative data are shown here with average values and standard deviations.

another set of band intensities (box 2 in Fig. 8 A) increased corresponding to the spiked concentration. The MW of this set of bands is about two times larger than the MW of ovalbumin, which probably contain ovalbumin dimer. Here we only quantitatively analyzed the bands in box 1. Band intensities were measured with ImageQuant software. We used the control band intensity as background and subtracted it from bands observed in the ovalbumin spiked samples. After subtraction, we calculated the relative intensities of each band compared to the intensity of 1 μg/mg cell lysate spiking sample. The relative intensity comparison is shown in Fig. 8B by average value and standard deviation. The data in Fig. 8B show that in general, the relative intensities of the detected bands correlated well with the amount of ovalbumin protein that was spiked into the cell lysates. The relatively low observed intensity of the band resultant from spiking 20 μg/mg ovalbumin may be resultant from overloading of ovalbumin on the gel. Overall, the results from spiking experiments suggest that this method can be used for quantitative comparison.

3.5. SAX-HPLC analysis of BACE, BACE2 overexpressing cells and vector control cells

The following study focused on the following three cell lines that include BACE overexpressing HEK cells, BACE2 overexpressing HEK cells, and cells transfected by pcDNA3 vector only as control. The SAX-HPLC separation of these three different cell lysates demonstrated different UV chromatogram profiles (Fig. 9A). All three of these chromatograms showed a majority of proteins eluted in the range of 20–50% buffer B. With comparison of these chromatograms, we found that they showed similar peak patterns but reproducible differences were observed. For example, as compared with control cell lysates, BACE and BACE2 overexpressing cell lysates have greater similarity.

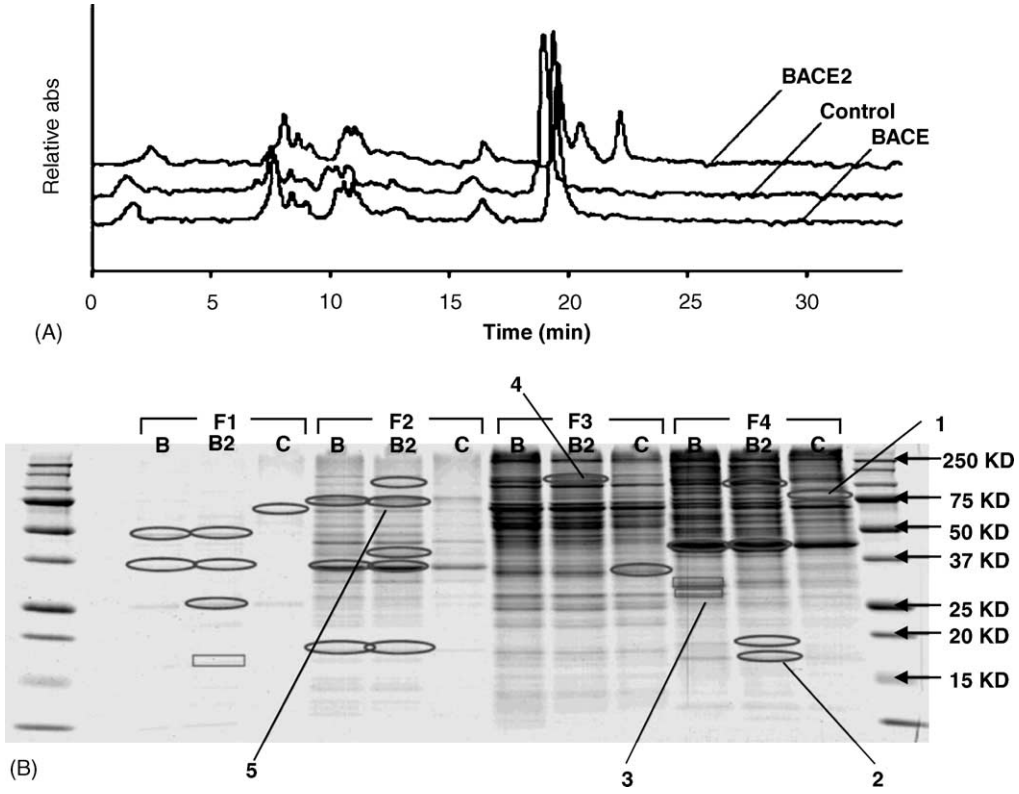

Fig. 9. Comparison of BACE and BACE2 overexpressing cells with pcDNA3-transfected control cells. (A) UV chromatograms and (B) 8–16% SDS-PAGE gel (Bio-Rad) image of individual fractions (B = BACE, B2 = BACE2, C = control). The bands marked with numbers are the identified proteins shown in Table 2.

At present, these data are currently used to produce a relatively qualitative picture of protein separation, but will be used in future studies to optimize fractionation and determine which fractions are subjected to additional LC methods. A better comparison of proteins present in each cell lysate is shown with gel separations described below.

3.6. SDS-PAGE gel comparison

The SDS-PAGE gels were used to further separate proteins present in fractions resultant from SAX. The corresponding fractions of different cell lysates showed similar band patterns. In fact, a majority of protein bands appeared identical among samples. However, we were also able to visually identify many differences among the samples. In Fig. 9B, bands marked with circles are found up-regulated in both BACE and BACE2 overexpressing cell lysates relative to vector-only control cell lysate. These differences are resultant from overexpression of BACE and BACE2, and are targets for protein identification as described below. There are also differences between BACE and BACE2 overexpressing cells (marked as rectangular boxes). For example, bands marked with rectangular boxes were found in fraction 1 in BACE, but not BACE2 nor the control samples and those in fraction 4 were found in BACE2, but not BACE nor the control samples. Since BACE but not BACE2 has been identified as being expressed at high levels in brain [29,31], these bands may shed light on the different functional roles held by these very similar proteases.

3.7. Protein identification

Data from MALDI-TOF-MS were used to search protein databases for protein identification. Twenty-five gel bands were excised as marked in Fig. 9B. Protein identification results were considered significant with $p < 0.05$. Some proteins that were identified in this study using MALDI-TOF peptide mass fingerprint are listed in Table 2.

Our initial efforts have resulted in protein identification for many spots that were changed in abundance between samples. A separate publication is under preparation to present all proteins identified and a discussion of the significance of these new results. However, among initial results, HSP70 (heat shock protein 70) that we observed to be increased in abundance as a result of BACE expression, was also reported previously to be increased in abundance in AD brain tissue [36]. Rab3D, whose up-regulation was only found in BACE but not BACE2 overexpressing cells, is a member of the Ras family of GTPases that is a key regulator of intracellular vesicle transport during exocytosis [37,38]. In addition, several other GTPase proteins have been found previously to be altered in abundance in AD brain tissue [39]. Therefore, both Rab3D and BACE are members of the secretory pathway and the present results suggest that some cooperativity

Table 2
Several identified proteins and their physiological function

Band number	Protein	Observed mass (kDa)	Reported mass (Da)	Accession number	MASCOT score
1	Chain A, heat-shock 70 kDa protein 42 kDa ATPase N-terminal domain	75	41827	gi|6729803	75
2	Mitochondrial ribosomal protein S10	17	23163	gi|16554607	65
3	RAB3D, member RAS oncogene family; similar to zinc finger protein 180 (HHZ168)	26	24267	gi|4759000	107
			24137	gi|30155719	
4	EBNA-2,co-activator (100 kDa)	110	99671	gi|33869218	71

exists between BACE activity and Rab3D expression. EBNA-2 co-activator p100 was found to be increased in abundance in both BACE and BACE2 overexpressing cells. This protein is involved in co-activation of gene expression relevant to Epstein-Barr antigen 2 [40]. Its relationship to BACE or BACE2 overexpression is unclear.

4. Conclusions

The results presented in this paper highlight the utility that new strategies for protein separation hold for *comparative proteomics* research. Additionally, the new approach has shown much greater flexibility for protein separation as well as sample loading. We used SAX chromatography as the first dimension of protein separation, which has a capacity as large as 25 mg. Since much more protein can be loaded on the first dimension, as compared to conventional gel-based IEF separation, the demands on mass spectrometry performance at the protein ID stage are reduced. Furthermore, greater loading also provides increased ability to visualize and identify low abundance proteins by increasing total sample loading amount into SAX column. Also, reverse phase HPLC can be added as another dimension for more separation as needed. Flexibility also exists in the second dimension of our approach. For example, with SDS-PAGE separations, we can get better separation on larger molecular weight samples by increasing gel running time or capture of low molecular weight proteins by decreasing gel running time to keep low molecular weight proteins in the gel. This was useful for the present study; for example, the molecular weights of several proteins are below 30 kDa. For high concentration of polyacryamide gel as normally used to separate low MW proteins, it is more difficult to extract peptides out of the gel, which means larger amounts of protein are required for identification of small MW proteins. Recently more attention was put towards large molecular weight protein identification since it has been difficult to get good separation from 2D gel. This new approach also shows some advantage for identification of large MW proteins.

Reproducibility experiments have shown that step-gradient fractionation can provide excellent reproducibility. An additional advantage of this method is that protein abundance level changes can be easily visualized with side-by-side vertical comparison in one gel. Separation of multiple samples in the same gel significantly reduces run-to-run variation as well, as is shown with DIGE. Since samples are loaded into one gel, it can provide more reliable and reproducible data than is obtained by visual comparison of large numbers of 2D gels. This method allows analysis of virtually any number of samples in parallel, as shown here for control, BACE, and BACE2 samples. Furthermore, we expect that this approach will enable successful identification of much lower abundance proteins and greater dynamic range as a result of the greater sample loading amount. With dynamic range and sensitivity representing two of the most challenging aspects of proteomics research, the present method promises to significantly extend our capabilities to more interesting, less abundant and in some cases, more biologically significant proteins. High performance FTICR-MS can be applied to in-gel digestion sample to resolve more than one protein in a single gel band. Finally, since the first dimension chromatographic step can be tailored to many different types of separation techniques, we anticipate this approach and derivatives resultant from it, will significantly expand proteomics research to classes of proteins not readily tractable by current methodology.

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