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A Chloroform-Assisted Protein Isolation Method Followed by Capillary NanoLC-MS Identify Estrogen-Regulated Proteins from MCF7 Cells

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Abstract

Most commonly reported non-commercial protein isolation methods require the use of detergents and/or chaotropes for better yield, and they all need additional purification or clean-up steps for subsequent mass spectrometry analysis. Moreover, there is no simple procedure available for obtaining both soluble and membrane proteins from the same sample. Here we describe a simple and detergent-free chloroform-assisted protein isolation (ChlAPI) method for mammalian cells and tissues, and demonstrated its suitability to mass spectrometry based proteome analysis. In this single-step method, cultured cells or grounded tissue were mixed in 10% chloroform in ammonium bicarbonate buffer to separate whole cell proteome into biphasic layers. Total number of aqueous phase proteins, as assessed by 2DE, was comparable to the proteins isolated with commonly used detergent containing buffer. Shotgun proteomics analysis of the aqueous and organic phase proteome fractions of MCF7 cells by LC-MS/MS resulted in identification of a total of 752 and 593 proteins, respectively from IPI human protein database. Among the total of 1134 distinct and non-redundant proteins, 29.5% were predicted to be membrane localized; 78% of them were identified from organic phase fraction. Application of this new procedure to estrogen-treated MCF cells lead to the identification of previously known as well as unknown estrogen-responsive gene products. These findings suggest that the simple and inexpensive ChlAPI method described here is suitable for protein isolation from mammalian samples, and is readily compatible with 2DE and LC-MS/MS analyses.

Keywords: Proteomics; Chloroform; Protein isolation; MCF-7 cells; Estrogen; 2DE; nanoLCMS/MS

Abbreviations: E2: 17β-estradiol; MS: Mass spectrometry; MS/MS: Tandem Mass Spectrometry; IPI: International Protein Index; ACN: Acetonitrile; FA: Formic acid; TFA: Trifluoroacetic Acid; DMSO: Dimethyl sulphoxide; PBS: Phosphate Buffered Saline

Introduction

Several proteomic strategies have been developed to identify constituent proteins from various organisms and cell types. These approaches rely on different separation methods coupled to mass spectrometric techniques such as matrix assisted laser-desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS). Global bottom-up proteomic profiling approaches, such as the multidimensional protein identification technology (MudPIT) (Wolters et al., 2001), isotope coded affinity tagging (Smolka et al., 2001), and isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al., 2004) mass spectrometry are now the widely used procedures that can potentially identify and quantify both high and low abundant proteins in complex biological mixtures. Recently, the intact protein based top-down proteomics is also gaining momentum for high throughput on-line analysis of complex samples (Parks et al., 2007). However, one of the key factors to the success of these mass spectrometry based analyses is the availability of efficient as well as simple sample preparation procedures that minimize ion suppression by impurities.

Detergents and chaotropes are commonly used in protein isolation protocols as solubilizing and stabilizing agents. Non-ionic and ionic detergents such as NP-40, Triton X100, and SDS are compatible with conventional biochemical methods, but they interfere with both chromatographic separation and mass spectrometric ionization (Loo et al., 1994) resulting in suppression and obscuring of peptide signals as well as in the formation of adducts. Efforts to remove these surfactants require multiple purification steps which lead to significant sample losses (Barnidge et al., 1999; Blonder et al., 2002).

Organic solvents such as methanol and chloroform have been used in the isolation of proteins from cells or tissues of diverse species from virus to human (Abramsky and London, 1975; Fillingame, 1976; Tsai et al., 1985). These studies originally focused on isolating selected lipoproteins, hydrophobic proteins, or removing lipid contaminants from protein preparations. Use of these solvents have been extended to proteomic studies of hydrophobic and membrane proteins. For example, membrane proteins from *E. coli* were isolated using a chloroform/methanol mixture, followed by solubilizing in urea and detergents, and subjected to 2-dimensional electrophoresis (Molloy et al., 1999). Similarly, detergent-solubilized bacteriorhodopsin was extracted into chloroform/methanol and analyzed by LC-MS (Barnidge et al., 1999). Ferro and colleagues (Ferro et al., 2000) used chloroform/methanol mixture to selectively isolate membrane proteins from chloroplasts, while Goshe and co-workers applied

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methanol/ammonium bicarbonate to solubilize bacterial membrane proteins prior to MS analysis (Goshe et al., 2003). In another example, over 110 mitochondrial membranes proteins were identified through chloroform/methanol extraction, and alkaline/saline treatments followed by LC-MS/MS (Brugiere et al., 2004). A multi-step, chloroform and methanol/water protocol was used for delipidation of proteins in order to enrich the identification of membrane proteins by mass spectrometry (Mirza et al., 2007). Zhang et al have recently reported the isolation of membrane proteins from red blood cells by the use of methanol and TFE for shotgun analysis (Zhang et al., 2007b). Thus, these alternate solubilization techniques have been used either for solubilize proteins or to enrich membrane proteins.

Here we describe the use of chloroform to disrupt mammalian cells and create an organic/aqueous phase partitioning, leading to the isolation of both aqueous soluble and hydrophobic proteins. We applied this single-step chloroform-assisted proteome isolation (ChlAPI) method to isolate proteomes of human breast cancer cells and mouse liver tissue. Two dimensional gel electrophoresis (2DE) was used to assess the efficiency of protein extraction in comparison with detergent aided protein extraction, and protein isolation by French Pressure Cell Press. LC-MS/MS analysis of breast cancer (MCF-7) cell proteome isolated with and without estrogen (E2) treatment revealed known as well as previously unknown estrogen-regulated proteins.

Materials and Methods

Chemicals and column materials

Strong cation exchange (SCX) column (ThermoHypersil-Keystone, Biobasic; dimension: 100 x 0.32 mm; particle size: 5 μ) was purchased from Thermo Fisher Scientific (San Jose, CA, USA) and the C18 particles (particle size: 5 μ) were obtained from Michrom Bioresources, Inc. (Auburn, CA, USA). Fused silica nanospray emitter tips (Picofrit: 75 μm i.d.; 360 μm o.d.) were purchased from New Objective Inc. (Woburn, MA, USA). Sequencing grade trypsin was from Promega (Madison, WI, USA). Trifluoroacetic acid and formic acid were purchased from sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI, USA), and high purity water was from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA).

Cell culture and harvest

Human breast cancer cell line MCF-7 (ATCC No. HTB-22) was maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% standard fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C with 5% CO_2. At 80% confluence, the above medium was replaced with phenol red free DMEM/F12 medium containing charcoal treated fetal calf serum (Hyclone) after washing the cells with sterile PBS. After 40 hours, the cells were treated with 100 nM of 17β-estradiol (Sigma-Aldrich) in DMSO for 45 minutes. Control cells were treated only with DMSO. Cells were washed with PBS and harvested after 5 min incubation at room temperature with 0.025% trypsin, 0.5% ETDA. The cells were centrifuged at 2400 rpm at 4°C, and the cell pellet was washed with PBS before protein isolation.

Protein isolation

Unless otherwise stated, the protein isolation was performed either on ice or at 4°C. Cells obtained from five 150 x 25 mm cell culture plates (~1 X 10^8 cells) were resuspended in 6 ml of ammonium bicarbonate sample buffer (50 mM ammonium bicarbonate, 10 mM ammonium chloride, 2 mM PMSF, pH 8.0) in a 15 ml polypropylene tube. Chloroform was added to achieve 10% (v/v) final concentration of the cell suspension and incubated in a rotary shaker for 10 minutes. Subsequent centrifugation at 5,000 rpm for 15 minutes resulted in biphasic separation into a top aqueous and bottom organic layers, and the two fractions were collected separately. In order to obtain a clear phase separation, one ml of chloroform was added to the sample before centrifugation. The aqueous fraction was further centrifuged at 10,000 rpm for 10 minutes to remove debris, and PMSF was added freshly. Chloroform was evaporated completely from the organic fraction using a speedvac system (SAVANT instruments, Inc., Holbrook, NY, USA), and the sample was subjected to digestion. Mouse liver tissue of about 1g wet weight was ground in liquid nitrogen using Pestle and Mortar, and sample buffer was added to obtain a cell suspension. Proteins were extracted using 10% chloroform as described above.

Cells dissolved in ammonium bicarbonate sample buffer was poured into the sample holder of FRENCH pressure cell press (Thermo Spectronic ITS 40K; Thermo IEC, MA, USA) and were broken by applying a pressure gauge level of 1000 psi as per the manufacturer's instructions. The lysate was centrifuged, and soluble proteins were collected for 2DE. Detergent aided lysis was carried out by resuspending the cells in RIPA buffer (50mM Tris-HCl buffer (pH 8.0) containing 150mM NaCl, 10 mM EDTA, 0.2% Triton X100, 0.5% NP40 and 2mM PMSF), and subsequent sonication of the crude lysate by using an ultrasonic processor (Cole-Parmer Instrument Company, Illinois, USA) with 20% amplitude, 45 seconds pulse and cooling on ice for 60 seconds. Lysate was centrifuged at 10,000 rpm for 10 minutes and the aqueous phase proteins were obtained for 2DE. Protein estimation of various samples were done using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Two-dimensional electrophoresis and protein spot analysis

The quality of the proteome isolation with the above three methods were assessed by 2DE, and three replicates were performed for each method. For 2DE proteome separation, first dimension isoelectric focusing (IEF) was done as described earlier (Gorg et al., 2000). Briefly, 18 cm Immobiline™ Dry strips (Amersham Biosciences Corporation, Piscataway, NJ, USA) with nonlinear pH gradients from 3-10 were used. One hundred microgram of protein was mixed in sample buffer (6M urea, 2M thiourea, 4% (w/v) CHAPS, 10mM DTT, trace amount of Bromophenol blue), and 7.5 μl of IPG Buffer (pH: 3-10), and the total volume was made up to 350 μl. Strips were loaded onto Ettan™ IPGphor™ horizontal electrophoresis system (Amersham Biosciences Corporation) and rehydrated at 20°C for 10 hrs. The strips were focused for overnight with voltage gradients starting from 10 V to 8,000 V in stepwise and until reaching 50,000 V hrs. The focused strips were equilibrated for 30 minutes in equilibration solution (30% glycerol, 2% SDS, 2% DTT, 50 mM Tris-HCl pH 8.8 and 0.005% w/v bromophenol blue). Second dimension separation was performed on a 12% SDS-polyacrylamide gel using PROTEAN II XL Cell apparatus (Bio-Rad Laboratories). Electrophoresis was carried out at 300 V for 1 hr and then at 600 V until the dye front reached the end of the gel. Silver staining was performed using a commercial silver staining kit as per the manufacturer's instructions (SilvestQuest™ Silver staining kit, Invitrogen Corporation). The stained gels were scanned using Image scanner (Amersham pharmacia) and the protein spots were counted using Compugen Z3 desktop version 3.0 software. Manual verification of protein spots was performed after making a high quality print of the image and counting the spots after dividing the image into multiple sectors.

Digestion of proteins

Soluble proteins in sample buffer was diluted to a concentration

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of 2 mg/ml and mixed with an equal volume of 100 mM ammonium bicarbonate, pH 8.0. Proteins were reduced by 10mM DTT and alkylated with 50mM iodoacetamide before adding trypsin at a trypsin: protein ratio of 1:50 (w/w), and incubated at 37°C for 16 hrs. Second aliquot of trypsin was added and digestion continued for another 4 hrs, and the enzyme reaction was stopped by acidifying the solution with 0.5% TFA. The organic phase proteins were digested separately following the protocol described by Washburn et al. (2001). Briefly, the dried sample pellet was acidified by formic acid and cleaved by cyanogen bromide. After adjusting the solution pH with solid ammonium bicarbonate and water, the sample was digested by trypsin as described above. Trypsin digested peptides were desalted by a peptide trap of 200 µg capacity (Michrom Bioresources, Inc., Auburn, CA, USA). Peptide trap was conditioned with buffer B (70% ACN, 0.1% FA in HPLC water), and equilibrated with buffer A (0.1% FA, 3% ACN in HPLC water) prior to sample loading using a syringe pump. After washing with 1.2 ml of buffer A, peptides were eluted using buffer B. Eluted peptides were dried using speedvac and dissolved in buffer A for LC-MS/MS analysis.

Liquid chromatography and mass spectrometry

A Surveyor LC system (Thermo Fisher Scientific, San Jose, CA, USA) with an in-house built solvent splitter was coupled to a LCQ™ Deca XP plus mass spectrometer (Thermo Fisher Scientific) for LC-MS/MS analysis. Digested peptide samples was separated online by an SCX column and then followed by a C-18 reversed phase (RP) capillary column. The RP column with nanospray emitter was packed with 5 µm C18 particles under 400 psi of helium using a pressure bomb. For each experiment (estrogen treated and untreated), peptides purified from 200 µg digested proteins were injected onto the SCX column using an auto sampler, and fractionated using 9 salt steps of increasing concentration of (0, 10, 20, 30, 50, 70, 90, 120, and 400 mM) NH_4Cl solutions. Peptides from each salt elution were trapped in an online peptide trap and desalted by 3% ACN and 0.1% FA solution (solvent A). The online desalted peptides was eluted to and separated by the C18 analytical column using a 160 min. chromatographic gradient, and 200 nL/min flow rate. The gradient was ramped from 5% B (97% CAN in H_2O, 0.1% FA) at 5 min. to 40% B in 130 min., then to 80% B in 145 min. After an additional 5 min. in 80% B for it is ramped back to 5% B. Eluted peptides were ionized by a nanospray emitter that is arranged in line with the inlet of the LCQ. The transfer capillary temperature and spray voltage of the emitter tip were set at 180°C and 1.6 kV, respectively. Normalized collision energy was fixed at 35% for MS/MS. For the detection of peptides, one full (MS) scan (m/z range of 400-2000) was followed by fragmentation (MS^2) scan on five most abundant ions.

Informatics and data analysis

Raw data were obtained from mass spectrometer for aqueous and organic phase protein fractions using Xcalibur (Thermo Fisher Scientific) software. The Raw files were converted into mzXML files for SEQUEST (Eng et al., 1994) search against the IPI Human database version 3.16 (62322 entries). Search parameters used were: precursor mass tolerance of 3 Da, peak extraction with average mass, semi-tryptic search with two or fewer missed cleavages, and fixed carbamidomethylation of cysteine residues. Cleavage at methionine was added for the organic phase samples that have undergone cyanogen bromide treatment. Peptide and protein assignments were validated using PeptideProphet (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003) tools which are available as a part of the Trans-Proteomic Pipeline (www.proteomecenter.org). List of protein identifications was filtered using a 0.9 probability threshold, which corresponds to less than 1% estimated false discovery rate (FDR). The data sets acquired on control_aqueous, control_organic, E2_aqueous, and E2_organic were analyzed separately. Protein groups in each set with indistinguishable protein accession numbers were collapsed into a single group.

For comparative analysis, two data sets from control and E2 treatments were combined, and proteins belonging to common, control-specific, and E2 treatment-specific were obtained using in-house software. This software analysis allowed removal of ambiguities arising from protein isoforms and multiple accessions. The resulting combined dataset was used for spectral count analysis (Vellaichamy et al., 2009). Total number of MS^2 spectra that were assigned to peptides was calculated and the dataset was further normalized to account for differences in the total number of peptides identified. Subsequently, ratio of normalized spectrum counts was calculated for proteins identified in both experiments, and then log-transformed. Resulting distribution was fitted using a robust Gaussian distribution fitting procedure with 10% outlier removal, and the mean and standard deviation (SD) were determined. Subsequently for proteins identified in common, two-SD threshold was applied to derive the list of differentially expressed proteins. In addition, proteins were designated as differentially expressed if they were identified only in either the control (down-regulated) or E2-treated sample (up-regulated) with four or more spectra.

Gene ontology (GO) searches for cellular localization of the proteins using the gene identifiers were performed at the SOURCE web-site (http://smd.stanford.edu/cgi-bin/source/sourceSearch). Transmembrane domain analysis of the proteins was performed using the TMHMM server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Results and Discussion

Proteome isolation by chloroform treatment

Suspension of MCF-7 cells in the aqueous ammonium bicarbonate buffer containing chloroform resulted in the rupture of cell membrane which was observed by microscopic examination. Preliminary experiments were conducted by varying the amount of chloroform and the incubation time on constant number (~1x 10^8) of cells to optimize lysis conditions. A 10% chloroform mixture, and 8-10 minutes of incubation time resulted in complete cell lysis. Upon centrifugation, the lysate partitioned into a biphasic organic and aqueous layers with soluble proteins in the aqueous layer, and presumably the hydrophobic and membrane proteins in the chloroform layer. The disintegration of cell membrane is considered to be due to the dissolution of membrane lipids by chloroform during the mixing process as the chloroform is known to act on hydrophobic membrane lipids (Barnidge et al., 1999; Mirza et al., 2007). Herein we denote this isolation protocol as 'chloroform-assisted protein isolation (ChlAPI)' method.

To check the quality of sample preparation and efficiency of the isolation method, we used 100 µg of the soluble proteins and performed 2DE. Silver staining of the 2DE gel for MCF-7 cell proteins obtained through ChlAPI method showed that proteins are distributed within a wide range of pI (pH 3-10) and molecular weights (Figure 1A). There were a total of 1362 protein spots that were sharp and distinct (Figure 1A). The appearance of these clearer 2DE gel spots with ChlAPI isolated proteins were probably due to the removal of lipids and hydrophobic impurities by chloroform (Mirza et al., 2007).

To directly compare ChlAPI method with conventional detergent-based protein extractions, MCF-7 cells were ruptured by non-ionic

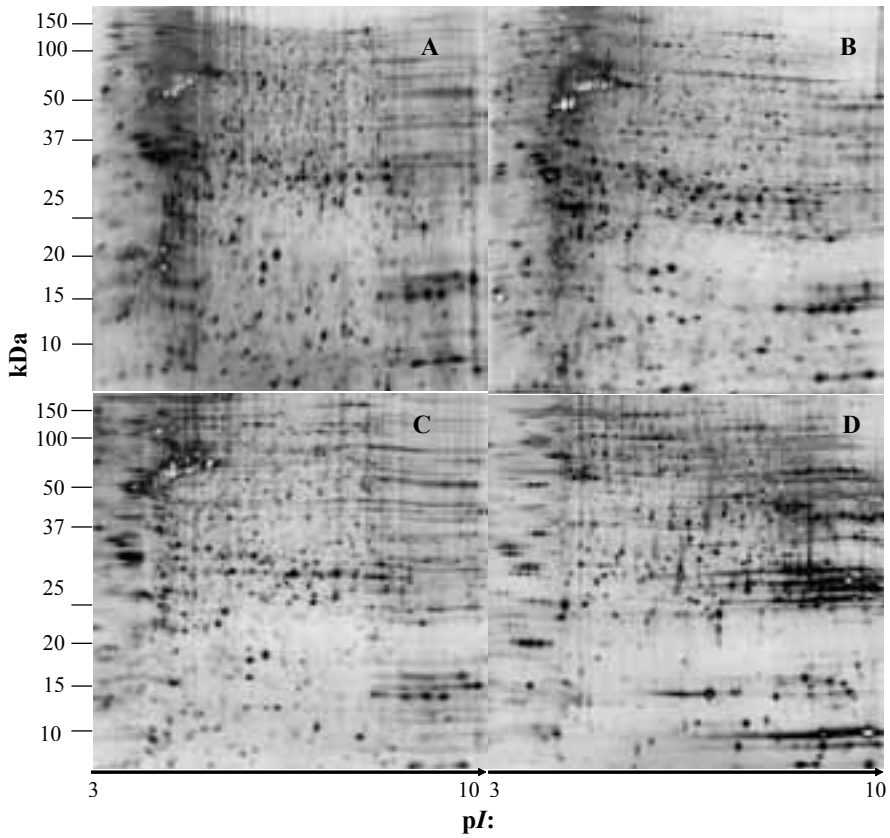

Figure 1: Two-dimensional gel electrophoresis images of MCF-7 cell and mouse liver tissue proteomes isolated by different protein isolation methodologies. Aqueous phase proteins isolated by using chloroform-assisted protein isolation (ChlAPI) method from MCF-7 cells (A) and mouse liver tissue (D). MCF-7 cell total soluble proteome isolated by detergent lysis followed by sonication (B), and by French Pressure Cell Press(C).

detergents IGEPAL (NP-40) and Triton X100 followed by ultra-sonication to enhance cell lysis. The 2DE image of the detergent-extracted soluble proteins (Figure 1B) showed more or less the same distribution of the proteins as those obtained by the ChlAPI method (Figure 1A), and the total number of spots counted was1333. We also tested French pressure cell press aided cell lysis to isolate proteins, and subsequently obtained similar results (Figure 1C; 1286 protein spots). This indicated that the ChlAPI method of protein isolation is comparable to the commonly used method of protein isolation, however avoids the use of detergents.

Previous publications on the 2DE gel analysis of breast cancer cells report similar number of proteins. For example, Canelle and colleagues documented 1249 proteins from 150 μg of whole cell extracts of MCF cells using a 2DE lysis buffer containing urea, thiourea, CHAPS, and Triton X-100 (Canelle et al., 2006). Similarly, using 2DE lysis buffer extracted 800 μg of MCF cell proteins, Bianchi and others were able to identify an average of 1700 proteins from the silver stained 2DE gels (Bianchi et al., 2005). Although, these studies have resolved proteins at a pI scale of 3-10, they have obtained the above number of protein spots using whole cell lysates prepared in 2DE buffer whereas comparable numbers of protein spots were observed here with only the aqueous fraction of ChlAPI method. This suggests that protein isolation using ChlAPI method could potentially leads to higher proteome coverage if both fractions were analyzed. ChlAPI method was also tested on intact tissue samples such as mouse liver tissue. The total number of protein spots obtained with 100 μg protein extract was 1127. In addition, the spot distribution pattern on the 2DE gel including the many linearly arranged protein spot cluster across the pI dimension (Figure 1D) was observed to be similar to the previously published 2DE images of mouse liver proteome available at the Swiss-Prot 2D PAGE database (http://au.expasy.org/ch2d/2DHunt) ; images can be retrieved through text search using 'liver_mouse'). Thus, it is evident that the ChlAPI method can also be extended to isolate proteins from mammalian tissue samples.

ChlAPI method is compatible with LC-MS/MS

After the 2DE based verification of the complexity of proteins isolated using ChlAPI method, we next asked whether we could apply this procedure for shotgun LC-MS/MS (MudPIT) based interrogation of the whole cell proteome of MCF-7 cells treated with and without estrogen (17β-estradiol a.k.a E2). MCF-7 cells express high levels of ER α which is known to exert pleiotropic cellular responses upon binding to estrogens (Klinge, 2000; Moggs and Orphanides, 2001). As described in the methods section, aqueous and organic phase proteome fractions were isolated from control and E2-treated MCF7 cells using ChlAPI method. Proteins present in the aqueous phase are presumably hydrophilic which were subjected to trypsin digestion; whereas the organic fraction was speculated to contain a higher amount of hydrophobic and membrane proteins, and they were udergone pre-cleavage by cyanogen bromide. Accordingly, the automated MudPIT LC-MS/MS runs were conducted for four samples (control_aqueous, control_organic, E2_aqueous, E2_organic).

SEQUEST search analysis of all the spectra obtained from control_aqueous sample identified a total of 752 unique protein groups from

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the IPI human database with the minimum protein- p value of 0.9 which corresponds to a false discovery rate (FDR) of <1%. The protein groups consisted of IPI accessions that matched to the same set of peptides. Data analysis of the control_organic sample spectra resulted in the identification of 593 unique protein groups. Combining the results of control_aqueous and control_organic samples showed the presence of a total of 1134 non-redundant proteins (Table 1). Similarly, SEQUEST searches of the E2_treated samples identified a total of 1145 proteins with FDR of <1% (Table 1).

Comparative analysis of data obtained from control and E2-treated samples, and subsequent spectral count analysis revealed proteins dysregulated by estrogen in MCF7 cells. An in-house software was used for combining database search results from control and E2-treated data and lead to global identification of proteins that are common (887 proteins), unique to control (255 proteins), and unique to E2-treatment (266 proteins) (Figure 2A). After the conversion of IPI accessions to entrez gene accessions, final list of non-redundant accessions obtained for common, specific to control, and specific to E2-treated were 879, 255, and 266, respectively. Additionally, MS^2 spectra based normalization and statistical analysis was performed (see Material and Methods section) for assessing differential protein expression. Distribution of protein expression (log) ratios obtained for proteins identified in both control and E2-treated sample is given in Figure 2B. Proteins that pass the threshold cut-off, and considered up and down- regulated by estrogen are colored in red and green, respectively (Figure 2B). Based on the normalization and statistical analysis, proteins identified only in control and only in E2-treated samples were also further trimmed (see Material and Methods section). Consequently, totals of 90 and 74 proteins were considered up- and down- regulated respectively, by estrogen in MCF7 cells (Figure 2C). These estrogen-responsive gene products are further discussed in later sections.

Sample	Total proteins identified (<1% FDR)	No. of proteins annotated by GO	Membrane proteins (%)
Control, aqueous fraction	752	376	11.7
Control, organic fraction	593	356	39.6
E2-treated, aqueous fraction	649	340	15.0
E2-treated, organic fraction	712	403	40.2
Control total (aqueous + organic)	1134	589	29.5
E2-treated total (aqueous + organic)	1145	594	30.5
Common (control and E2 treated)	879	480	29.0

GO = Gene Ontology; FDR = False Discovery Rate

Table 1: MCF7 cell proteins identified after chloroform-assisted protein isolation (ChIAPI) and mass spectrometry.

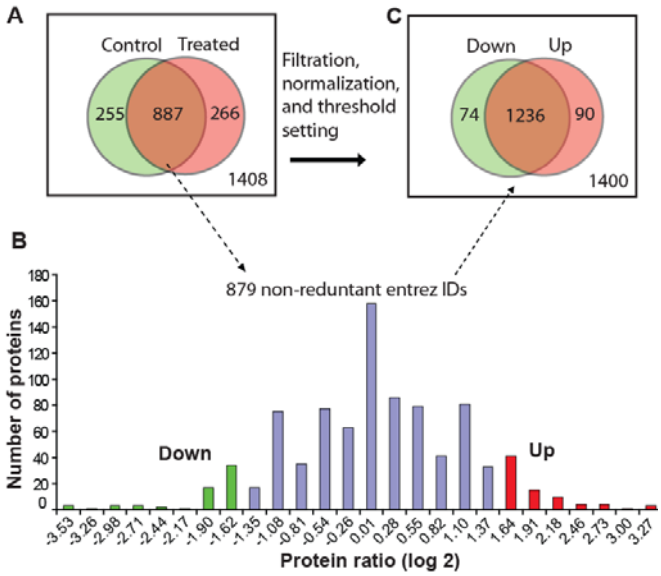

Figure 2: Total and estrogen-regulated proteins identified from MCF-7 cells after ChIAPI and nanoLC-MS/MS. (A) Venn diagram showing the total number of proteins identified by mass spectrometry analysis of aqueous and organic phase samples from control (-E2) and treated (+E2) MCF-7 cells with a false discovery rate of <1%. (B) Protein expression ratio distribution of MCF-7 proteins identified from both control and E2-treated samples. Entrez accessions for common proteins listed in 'A' are further curated to remove redundant identifications (Keen and Davidson). Protein spectral count (log 2) ratios were placed into bins separated by 0.27 and plotted against the number of proteins in each bin. Those that are marked green and red are considered true differentials and are unexpected by chance based on threshold. (B) Venn diagram showing the number of estrogen down- and up-regulated proteins based on the cut-off used. Proteins shown in the middle are considered unchanged based on the threshold used.

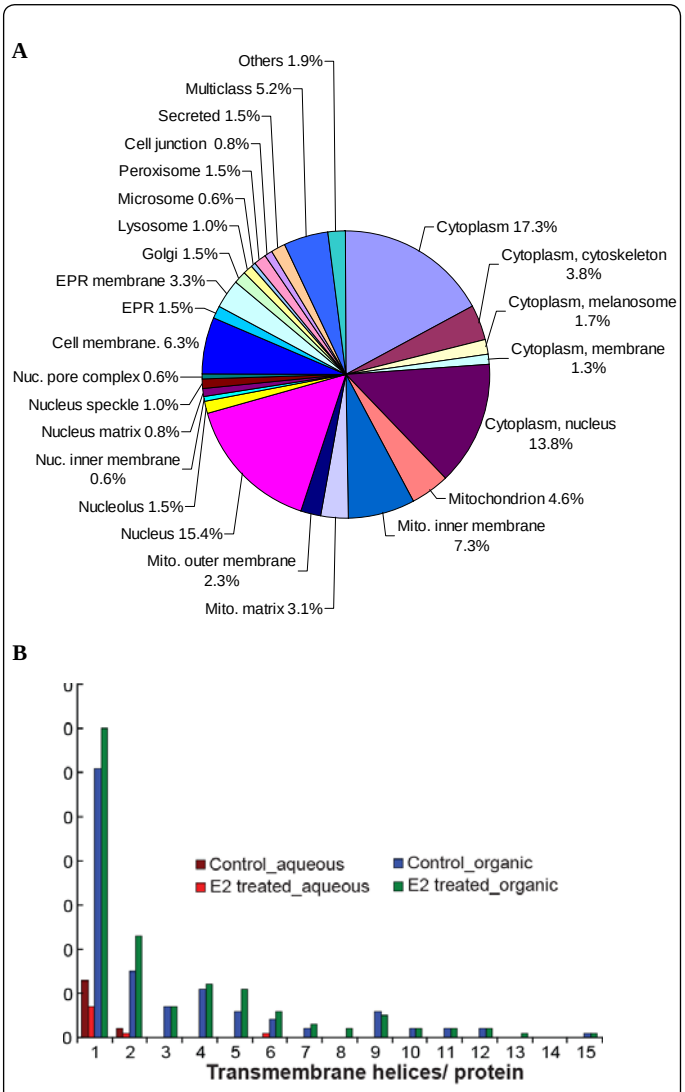

Figure 3: Cellular localization of MCF-7 proteins isolated using ChIAPI method. (A) Cellular localization of MCF-7 proteins identified as common from two LC-MS/MS experiments (control and E2-treated) showing their percentage distribution into each of the 26 categories. Given here are the characterized locations for 879 proteins. (B) Comparison of transmembrane helices on MCF-7 proteins identified from aqueous and organic phases of ChIAPI method.

One of the unique properties of higher organisms is the complexity of cellular organization with compartmentalization. Any phenotypic changes such as the development of cancer in those cells are the result of coordinated and dynamic changes of proteins at these sub-cellular compartments. Therefore, it is important that the protein isolation methodology is able to extract proteins from different sub-cellular compartments so as to help interrogate the changes or perturbations. In addition, a good protein isolation method should be unbiased towards any cellular compartment. Accordingly, ChlAPI method isolated MCF-7 proteins were analyzed for their cellular localizations. As they were identified from two independent ChlAPI experiments, the 879 entrez identifiers that were common to control and E2-treated samples were interrogated to access the reproducibility. Report on cellular location was found for 480 proteins. Search outputs mapped these proteins to more than 50 different types of cellular compartment annotations, and they were further collapsed manually into 26 categories (Figure 3A). As shown in the pie chart, proteins from almost all of the cellular compartments have been isolated with a typical proportion of proteins in the major compartments. Percentage of identified proteins exclusive to the major compartments such as cytoplasm, nucleus, and mitochondria were, 24, 20, and 17.5, respectively, and a total of about 14% annotated to be localized both in nucleus and cytoplasm (Figure 3A). This showed that the ChlAPI method is efficient to obtain proteins from majority of the cellular compartments.

As reported by several workers, mass spectrometry analysis of membrane proteins has been difficult due to the intrinsic nature of the hydrophobicity and difficulty in isolating them with common buffers. Various attempts were made to improve the identification of these biologically important class of proteins (Barnidge et al. 1999; Blonder et al., 2002; Ferro et al., 2000; Mirza et al., 2007; Molloy et al., 1999; Zhang et al., 2007b). It has been documented that about 30% of the mammalian proteome is made up of these membrane proteins (Stevens and Arkin, 2000). Interestingly, total number of membrane proteins detected in this study from two independent ChlAPI experiments, as shown for two sets of samples (control total, and E2-treated total), was close to 30% (Table 1) which matches perfectly to the expected coverage of membrane proteome (Stevens and Arkin, 2000). Moreover, as expected the organic phase samples (either control or treated samples) contained 40% membranes proteins (Table 1) whereas the aqueous phase samples had only 12-15%. An average, 78% of total membrane proteins were identified from organic phase. The efficiency of ChlAPI method to extract higher number of membrane proteins into the organic phase is further supported by the prediction of number of transmembrane domains on the identified proteins (Figure 3B). While there were only few aqueous phase proteins that contained one or two transmembrane domains, large number of organic phase proteins contained three or more transmembrane domains (Figure 3B). It is also noticeable that the organic phase samples still had a major proportion of proteins from other non-membrane locations of the cell (Table 1). This observation apparently indicated that while the ChlAPI method could be used to selectively enrich either membrane or non-membrane proteins, combining the mass spectrometry data from aqueous and organic phases help to increase the total proteome coverage.

Analysis of estrogen-responsive gene products

Estrogens and their receptors are known to play key roles in the genesis, progression, and treatment of breast cancers. MCF-7 cells have been used extensively as an in vitro model for studying the effects of estrogen exposure, estrogen receptor activation and inhibition on hormone-dependent breast tumor cell proliferation (Keen and Davidson, 2003; Sandhu et al., 2005). To determine whether the proteins identified in MCF-7 proteome are involved in estrogen response, 'SEQUEST' search results were normalized and spectral count-based differential protein expression analysis was performed with high confidence as described before. This resulted in the identification of 90 and 74 proteins as estrogen up-regulated and down-regulated, respectively (Figure 2; Tables 2 and 3). Proteomics analysis of MCF-7 proteins has been previously reported by several workers (Bianchi et al., 2005; Canelle et al., 2006; Huber et al., 2004; Kim et al., 2005; Lee et al., 2006; Malorni et al., 2006; Sandhu et al., 2005; Zhu et al., 2008). Some of these studies have performed E2 treatment, and several proteins reported as estrogen-regulated in their studies are identified as estrogen responsive in our analysis. For example, dCTP pyrophosphatase 1 (XTP3TPA/ DCTPP1) which was identified as an estrogen up-regulated protein with highest protein expression ratio in this study (Table 2A) was reported as estrogen up-regulated by Lee et al. (2006). Transcripts of XTP3TPA were found to be affected by E2 metabolites (Kim et al., 2005). Similarly, histone H1 and HSP90 α identified in our study as dysregulated by estrogen were also reported to be up-regulated by other workers (Table 2A). Interestingly, expression of prohibitin 2 (PHB) appear to be down-regulated following E2 treatment in our study (Table 3A) although it was reported earlier to be up-regulated by E2 (Zhu et al., 2008).

IPI accession	Entrez GI	Symbol	Ratio	P value	Ref.
IPI00012197	79077	DCTPP1	8.61	1.00	27
IPI00017855	50	ACO2	8.16	1.00	
IPI00419237	51056	LAP3	8.16	1.00	
IPI00003968	4704	NDUFA9	7.25	1.00	
IPI00295992	55210	ATAD3A	6.35	1.00	
IPI00295400	7453	WARS	6.35	1.00	
IPI00006211	9217	VAPB	5.89	1.00	
IPI00295851	1315	COPB1	5.67	1.00	
IPI00032426	51072	MEMO1	5.44	1.00	
IPI00029764	10946	SF3A3	5.44	1.00	
IPI00217468	3009	HIST1H1B	4.99	1.00	56
IPI00031523	3324	HSP90AA2	4.68	1.00	27,56
IPI00221234	501	ALDH7A1	4.53	1.00	
IPI00554811	10093	ARPC4	4.53	1.00	
IPI00069750	22827	PUF60	4.53	1.00	
IPI00410693	26135	SERBP1	4.53	1.00	
IPI00150057	6601	SMARCC2	4.53	1.00	53
IPI00166785	93380	TMEM32	4.53	0.99	
IPI00028031	37	ACADVL	4.53	1.00	
IPI00147874	54187	NANS	4.08	1.00	
IPI00003377	6432	SFRS7	4.08	1.00	
IPI00008964	81876	RAB1B	3.93	1.00	
IPI00550689	51493	C22orf28	3.63	0.99	
IPI00012837	3799	KIF5B	3.63	1.00	
IPI00017592	3954	LETM1	3.63	1.00	
IPI00016007	55930	MYO5C	3.63	1.00	
IPI00294891	4839	NOL1	3.63	1.00	
IPI00470467	5447	POR	3.63	1.00	
IPI00642211	6051	RNPEP	3.63	1.00	
IPI00218288	9871	SEC24D	3.63	1.00	
IPI00010402	83442	SH3BGRL3	3.63	0.99	
IPI00021326	6464	SHC1	3.63	0.99	55
IPI00221178	7165	TPD52L2	3.63	0.99	8
IPI00072377	6418	SET	3.48	1.00	
IPI00179529	481	ATP1B1	3.32	1.00	
IPI00008274	10487	CAP1	3.26	1.00	
IPI00472675	23165	NUP205	3.17	1.00	
IPI00293276	4282	MIF	3.11	1.00	
IPI00642244	23065	KIAA0090	3.02	1.00	
IPI00024670	7905	REEP5	3.02	1.00	
IPI00164417	30000	TNPO2	3.02	1.00	
IPI00025849	8125	ANP32A	2.90	1.00	
IPI00550069	6050	RNH1	2.90	1.00	

Table 2a: Proteins up-regulated by estrogen in MCF7 cells. Proteins identified from control and E2-treated MCF7 cells.

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IPI accession	Entrez GI	Symbol	Spectra	P value	Ref.
IPI00166205	196374	KRT78	17	1.00	
IPI00002557	26958	COPG2	10	1.00	
IPI00007927	10592	SMC2	7	1.00	
IPI00397526	4628	MYH10	7	1.00	
IPI00005162	10094	ARPC3	6	1.00	
IPI00020042	5704	PSMC4	6	1.00	
IPI00016457	1384	CRAT	6	1.00	
IPI00031397	2181	ACSL3	6	1.00	
IPI00215805	55770	EXOC2	6	1.00	
IPI00375380	5719	PSMD13	6	1.00	
IPI00007847	7335	UBE2V1	6	1.00	
IPI00028055	10972	TMED10	5	1.00	
IPI00221083	5910	RAP1GDS1	5	1.00	
IPI00009790	5214	PFKP	5	1.00	10
IPI00303207	6059	ABCE1	5	1.00	
IPI00384280	51449	PCYOX1	5	1.00	
IPI00002972	7625	ZNF74	5	0.92	
IPI00003856	529	ATP6V1E1	5	1.00	
IPI00004503	3916	LAMP1	5	0.98	
IPI00006379	51602	NOP5	5	1.00	
IPI00013219	3611	ILK	5	1.00	2
IPI00019269	80349	WDR61	5	1.00	
IPI00033151	51426	POLK	5	0.90	44
IPI00217051	89795	NAV3	5	1.00	
IPI00218922	11231	SEC63	5	1.00	
IPI00301323	8886	DDX18	5	1.00	
IPI00303318	51571	FAM49B	5	1.00	
IPI00479905	4716	NDUFB10	5	1.00	
IPI00234252	6599	SMARCC1	5	0.99	53
IPI00178667	7153	TOP2A	4	1.00	53
IPI00005904	11218	DDX20	4	0.99	
IPI00013623	11000	SLC27A3	4	1.00	
IPI00023542	54732	TMED9	4	0.98	
IPI00026215	2237	FEN1	4	1.00	40
IPI00029631	2079	ERH	4	1.00	
IPI00069817	9031	BAZ1B	4	1.00	
IPI00219729	8402	SLC25A11	4	1.00	
IPI00291939	8243	SMC1A	4	1.00	
IPI00383754	10352	WARS2	4	0.92	
IPI00414168	54708	MARCH5	4	0.99	
IPI00444704	2926	GRSF1	4	1.00	
IPI00555956	5692	PSMB4	4	1.00	
IPI00014232	23204	ARL6IP1	4	0.99	
IPI00022758	8139	GAN	4	0.99	
IPI00028275	9793	CKAP5	4	0.99	
IPI00151358	81037	CLPTM1L	4	1.00	
IPI00218546	573	BAG1	4	1.00	

Table 2b: Proteins up-regulated by estrogen in MCF7 cells. Proteins identified from E2-treated MCF7 cells only.

Recently, the PHB which is also an estrogen receptor co-regulator was shown to play a repressive role in estrogen signaling in MCF-7 cells (He et al., 2008), supporting our observation. In addition to the above examples, many proteins that were previously reported to be directly linked to estrogen and estrogen receptor such as TPD52, SHC, ILK, TOP2A, FEN1, CSTB, and GRB2 are indicated in Tables 2 and 3 along with the references (Acconcia et al., 2006; Al-Gubory et al., 2008; Byrne et al., 1996; Canesi et al., 2007; Deroo et al., 2004; Kolar et al., 1989, Moggs et al., 2005; Schultz-Norton et al., 2007; Song et al., 2002; Suzuki et al., 2004; Tang and Wade, 2009; Toffolatti et al., 2006; Verma et al., 2004; Walker et al., 2007; Zhang et al., 2007a; Zhang et al., 2004).

For integrative analysis of transcriptomic and proteomic data, we compared the list of all identified proteins with results from a gene expression profiling study performed using high density DNA microarrays to detect hormone-responsive changes in transcript levels (Finlin et al., 2001). Using Locus Link reference accession numbers as common identifiers between the two datasets, we observed an overlap of 84 proteins (Supplementary file 1) out of a total of 1400 proteins detected by mass spectrometry analysis, and 694 estrogen-responsive genes identified in the microarray study (including the previously reported genes) (Lin et al., 2004). The estrogen responsive proteins that pass the stringent threshold set in our analysis are indicated in Supplementary file 1. Transcript levels of seven (XTP3TPA, SEC24D, BAG1, FEN1, TOP2A, PHB2, and ATP6V1A) of the 11 E2-regulated proteins showed concordant trend in expression, where as four of them (SET, ARHGAP1, MCM3, and MCM6) showed the opposite. The discordance between protein expression and their transcript levels points to a possible involvement of E2-dependent post-transcriptional control mechanism for such proteins in MCF-7 cells. The proportion of estrogen responsive proteins in the MCF-7 proteome (6%; 84/1400) is comparable to that of the responsive genes (4%; 694/17735) detected in the transcriptome by microarray analysis (Lin et al., 2004).

It is noteworthy that several proteins that are previously unknown to be regulated by estrogen are designated as estrogen-regulated (Tables 2 and 3) in our analysis, and are interesting candidates for future study. For example, histidine triad nucleotide-binding protein 1 (HINT1) is found as down-regulated by estradiol (Table 3A). Very recently, the tumor suppressor HINT1 is reported to inhibit the phosphorylation of p27 by Src (Cen et al., 2009), an event leading to increased cell proliferation (Chu et al., 2007). The inhibition of p27 phosphorylation involved down-regulation of Src (Cen et al., 2009). Interestingly, it was previously known that estradiol bound ER activates Src, culminating in cell proliferation (Migliaccio et al., 1996). Thus, it is conceivable that estradiol regulate HINT1 negatively, leading to the up-regulation of Src which in turn is responsible for increased cell proliferation. Identification of HINT1 as estrogen down-regulated in our proteomics analysis supports this hypothesis. Overall, the above results indicate that the ChIAPI method described in this report and

IPI accession	Entrez GI	Symbol	Ratio	P value	Ref.
IPI00023748	4666	NACA	0.07	1.00	
IPI00221091	6210	RPS15A	0.08	1.00	
IPI00002966	3308	HSPA4	0.08	1.00	
IPI00011253	6188	RPS3	0.09	1.00	
IPI00031045	11034	DSTN	0.11	1.00	
IPI00027252	11331	PHB2	0.11	1.00	56
IPI00020017	10974	C10orf116	0.11	1.00	
IPI00008438	6204	RPS10	0.13	1.00	
IPI00301109	27068	PPA2	0.15	1.00	
IPI00059369	9832	JAKMIP2	0.15	0.98	
IPI00026216	9520	NPEPPS	0.16	1.00	
IPI00411706	2098	ESD	0.18	1.00	
IPI00177366	100011218	LOC100011218	0.20	1.00	
IPI00299024	10409	BASP1	0.23	1.00	
IPI00239077	3094	HINT1	0.23	1.00	
IPI00028888	3184	HNRPD	0.23	1.00	
IPI00024661	9632	SEC24C	0.23	1.00	
IPI00020567	392	ARHGAP1	0.23	1.00	
IPI00016613	1457	CSNK2A1	0.23	1.00	
IPI00011416	1891	ECH1	0.23	1.00	
IPI00219077	4048	LTA4H	0.23	1.00	
IPI00179026	28985	MCTS1	0.23	1.00	
IPI00024266	4259	MGST3	0.23	1.00	
IPI00172656	23197	UBXD8	0.23	0.99	
IPI00010796	5034	P4HB	0.25	1.00	
IPI00216298	7295	TXN	0.26	1.00	13
IPI00021828	1476	CSB	0.26	1.00	26
IPI00020956	3068	HDGF	0.26	1.00	
IPI00031691	6133	RPL9	0.26	1.00	
IPI00413324	6139	RPL17	0.26	1.00	45
IPI00030876	1729	DIAPH1	0.27	1.00	
IPI00076042	678754	LOC678754	0.28	0.99	
IPI00290770	7203	CCT3	0.28	1.00	27
IPI00181006	10848	PPP1R13L	0.30	1.00	
IPI00301271	6185	RPN2	0.30	1.00	

Table 3a: Estrogen down-regulated proteins from MCF7 cells. Proteins identified from control and E2-treated MCF7 cells.

IPI accession	Entrez GI	Symbol	Spectra	P value	Ref.
IPI00005589	644907	RPL32P18	17	1.00	
IPI00306959	3855	KRT7	14	1.00	50
IPI00012074	10236	HNRNPR	11	1.00	
IPI00334587	3182	HNRPAB	9	1.00	21
IPI00031517	4175	MCM6	8	1.00	
IPI00299155	5685	PSMA4	8	1.00	
IPI00182289	6235	RPS29	8	1.00	
IPI00024157	2287	FKBP3	7	1.00	
IPI00021347	7332	UBE2L3	7	1.00	49
IPI00008475	3157	HMGCS1	6	1.00	46
IPI00306332	6152	RPL24	6	1.00	
IPI00026271	6208	RPS14	6	1.00	56
IPI00010779	7171	TPM4	5	1.00	
IPI00007682	523	ATP6V1A	5	1.00	
IPI00027397	90861	HN1L	5	1.00	
IPI00167572	283742	FAM98B	5	1.00	
IPI00549467	56954	NIT2	5	0.99	
IPI00170436	79143	MBOAT7	5	1.00	
IPI00219155	6155	RPL27	5	1.00	
IPI00003310	5439	POLR2J	5	1.00	
IPI00009950	10960	LMAN2	5	1.00	
IPI00027285	6628	SNRPB	5	1.00	
IPI00013214	4172	MCM3	4	1.00	33
IPI00021327	2885	GRB2	4	1.00	42
IPI00029012	8661	EIF3A	4	0.99	
IPI00029623	5687	PSMA6	4	1.00	
IPI00294627	7536	SF1	4	1.00	
IPI00550364	55276	PGM2	4	1.00	
IPI00002460	310	ANXA7	4	1.00	
IPI00005160	10095	ARPC1B	4	1.00	
IPI00016862	2936	GSR	4	1.00	3
IPI00022300	25840	METTL7A	4	0.99	
IPI00030939	2778	GNAS	4	1.00	
IPI00215918	378	ARF4	4	0.99	
IPI00328587	2025	ENO1P	4	0.99	
IPI00465361	6137	RPL13	4	1.00	
IPI00008599	10682	EBP	4	1.00	
IPI00029695	6598	SMARCB1	4	1.00	
IPI00149849	25839	COG4	4	1.00	

Table 3b: Estrogen down-regulated proteins from MCF7 cells. Proteins identified from control MCF7 cells only.

LC-MS/MS are effective for identifying and characterizing proteomic constituents that are important for basic breast cancer biology.

Conclusions

High throughput technologies such as transcriptomics and proteomics offer the capacity to find alterations in large scale, and identify previously unknown novel targets in cancer. One of the problems in the widely used mass spectrometry based proteomics is the interference of detergents and other impurities in the sample that arise from the protein isolation procedures. Yet another problem is the tremendous complexity of the biological samples that exceeds the current analytical method and requires fractionation to detect low abundance proteins and also the membrane proteins. Here, we have used a chloroform-assisted, detergent-free protein isolation method (ChlAPI) to isolate and fractionate mammalian proteome into aqueous (hydrophilic) and organic (hydrophobic) fractions. Our result showed that the ChlAPI method is an efficient and unbiased method as revealed by the quality and quantity of proteome detected by 2DE and LCMS/ MS. Application of ChlAPI method is demonstrated for the proteome profiling of breast cancer cell line, MCF-7 with and without estrogen, and subsequently identified both known and previously unknown estrogen-regulated gene products. From a total of 1400 proteins identified, 6% of their transcripts are estrogen responsive in MCF-7 cells, and is comparable with the proportion of estrogen responsive genes from microarray studies. Furthermore, our results show that the ChlAPI method could be used to selectively isolate or enrich membrane proteome that is suitable for mass-spectrometry analysis. Thus, the simple, detergent-free, and inexpensive ChlAPI method described here could be used for mass spectrometry based proteome analysis of mammalian cells or tissues.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

SKS, AV conceived the study, and AV performed the experiments. AV and AIN analyzed the data, AIN performed informatics analysis. TTY helped AV in experiments, and GRK helped in data analysis. AV, CYL, SKS drafted the manuscript, and ETL helped in coordination of the work.

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