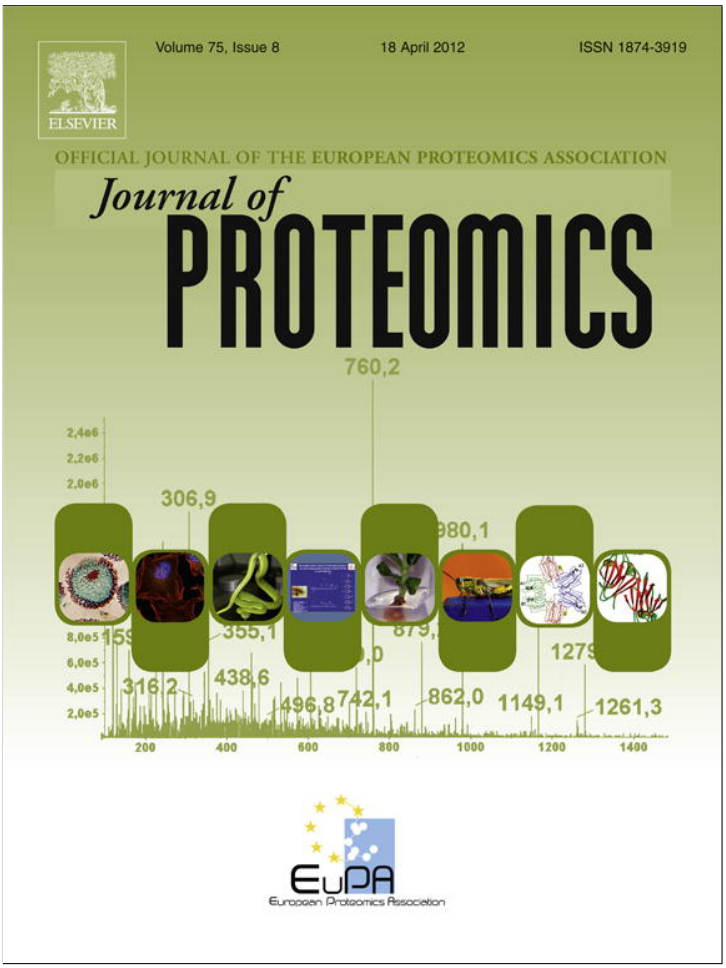

JOURNAL OF PROTEOMICS 75 (2012) 2297–2305

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/jprot

Two dimensional (pI & d_s) separation of phosphorylated proteins by isoelectric focusing/asymmetrical flow field-flow fractionation: Application to prostatic cancer cell line

Ki Hun Kim[a], *Jin Yong Kim*[a], *Myeong Ok Kim*[b], *Myeong Hee Moon*[a,*]

[a]*Department of Chemistry, Yonsei University, 50 Yonsei-Ro, Seodaemun-Gu, Seoul, 120-749, South Korea*
[b]*Division of Life Science and Applied Life Science (BK21), Gyeongsang National University, Jinju, 660-701, South Korea*

ARTICLE INFO

Article history:
Received 28 December 2011
Accepted 29 January 2012
Available online 11 February 2012

Keywords:
2D protein separation
Separation of phosphoproteins
IEF-AF4
Isoelectric focusing
FFF
Prostate cancer cell

ABSTRACT

This study demonstrates the use of on-line isoelectric focusing/asymmetrical flow field-flow fractionation (IEF-AF4), a non-gel based high speed two dimensional (isoelectric point and hydrodynamic diameter) protein separation device used for the isolation/separation of phosphoproteins. IEF-AF4 performance was evaluated by first fractionating α-casein molecules at different pIs and sizes. Collected proteins were analyzed by nanoflow liquid chromatography–tandem mass spectrometry (nLC–MS^n) to determine various isoforms of the phosphopeptides as well as the relative ratio of phosphorylated and unmodified peptides. A narrow pH cut (ΔpH = 0.5) of carrier ampholyte was used in IEF-AF4 to finely resolve phosphoproteins by pI. When the channel lane of multilane AF4 became acidic, the relative ratio of phosphorylated to unmodified or less phosphorylated peptides increased. The current method was applied to prostate cancer cell lysates to demonstrate that IEF-AF4 can examine the relative abundances of specific phosphoproteins, known as biomarkers, in prostate cancer. While affinity-based enrichment methods remove unmodified peptides, IEF-AF4 offers intact phosphoprotein separation at the protein level without removing unmodified proteins. IEF-AF4 enables quantitative analysis without isotope labeling.

1. Introduction

Protein phosphorylation is a reversible modification in which a phosphate group is bound to serine, threonine, and tyrosine. This process is essential in cellular signaling, molecular recognition, and protein folding. In addition, it regulates cell proliferation and the cell cycle [1–5]. Phosphorylation is not only a widespread post-translational modification, it is also closely associated with certain human diseases such as cancer, diabetes, and neurodegenerative disorders. A fundamental understanding of phosphorylation and its location in a protein is essential to analyze the control of many biological systems. Current studies are focused on discovering the phosphoprotein biomarkers of specific diseases, including cancer and leukemia [6–8]. Recent advances in MS have facilitated the highly sensitive analysis of complicated biological molecules, including phosphorylated peptides [9,10]. However, the direct analysis of a phosphorylation site by mass spectrometry (MS) is difficult because the number of involved phosphopeptides is extremely small. In addition, the ionization efficiency of phosphopeptides is poor compared to that of unmodified peptides. Moreover, the ability to localize a phosphorylation site by collision induced dissociation (CID) is hampered by the neutral loss of a phosphate group [11–14]. The preliminary separation or isolation of phosphoproteins and phosphopeptides is critical for any quantitative phosphoproteome analysis.

High resolution (pI < 0.01) phosphoprotein separation at the protein level can be achieved with two-dimensional poly-

* *Corresponding author at:* Department of Chemistry, Yonsei University, Seoul, 120-749, South Korea. Tel.: +82 2 2123 5634; fax: +82 2 364 7050.
E-mail address: mhmoon@yonsei.ac.kr (M.H. Moon).

1874-3919/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2012.01.034

acrylamide gel electrophoresis (2D-PAGE), which requires sequential phosphoprotein staining [15]. Recently, phosphospecific gels with blot staining methods and metal-affinity gels with SDS-PAGE have been developed for the quantitative analysis of phosphoproteins [16–19]. The gel based methods provide some advantages. They can determine the time course quantitative ratio of phosphorylated to unphosphorylated proteins and can separate phosphoprotein isotypes that have the same number of phosphate groups. However, gel based methods have some drawbacks, including limited sample loading capacities. In addition, gel based methods pose difficulties in isolating proteins with extreme pI or poor solubility, in retrieving phosphoproteins completely without digestion, and in automation.

The processes used for phosphopeptide isolation prior to MS analysis are either chemical or affinity methods. Chemical methods are based on beta-elimination of the phosphate group from phosphoserine and phosphothreonine followed by a Michael addition reaction that assigns the phosphorylation site [20]. Chemical methods can also consist of the selective coupling of phosphopeptides to solid supports using phosphoramidate chemistry (PAC) [3,21,22]. Several affinity methods use immobilized metal ion affinity chromatography (IMAC) with metal ions (Ga, Fe, or others) [9,11,23,24] and resins containing TiO_2 or ZrO_2 [12,25,26]. Strong cation exchange chromatography (SCX) [10,27,28] and hydrophilic interaction chromatography (HILIC) [13,29] have been added prior to phosphopeptide enrichment by IMAC. Affinity based enrichment methods are convenient and powerful tools for the global analysis of phosphorylated proteins. They are particularly useful when the concentration of phosphopeptides is much lower than the concentration of unphosphorylated peptides. However, most affinity based methods require in advance proteolysis and in some cases they limit the differentiation of phosphoprotein isoforms.

Flow field-flow fractionation (FlFFF) [30–33] is a non-gel based separation method for bio-macromolecular species, such as proteins, DNA, and cells. FlFFF separation is carried out by hydrodynamic diameter in an unobstructed rectangular channel space by the orthogonal movement of two liquid flow streams (channel flow for sample migration and cross-flow for sample retardation). Size-based separation results from the differential distribution of molecules according to their diffusion against the cross-flow force acting toward the channel wall. The smaller molecules diffuse further away from the channel wall than the larger ones. When a parabolic flow velocity is applied to sample components, the smaller molecules elute earlier than the larger ones. The resulting separation is in order of increasing hydrodynamic diameter. Since FlFFF operates in typical buffer solutions, biological molecules can maintain their intact states without deforming. This makes FlFFF suitable as a pre-fractionation device for proteomics and lipidomics research of bacteria [34], mitochondria [35,36], exosome [37], membrane proteins [38], and lipoproteins [39]. Recently, the on-line hyphenation of capillary isoelectric focusing (CIEF) with hollow fiber FlFFF (CIEF-HF5) [40] enabled the two-dimensional (2D) separation of a proteome sample by isoelectric point (pI) and hydrodynamic diameter (d_s). The protein fractions from FlFFF were collected for proteomic analysis using nanoflow liquid chromatography–tandem mass spectrometry (nLC–ESI–MS–MS). Later, 2D intact protein separation with FlFFF was performed by introducing a multilane channel system. In this system, protein separation by pI values is carried out in an open flat channel by isoelectric focusing (IEF) in the first dimension. Simultaneous size separation is performed at six asymmetrical FlFFF (AF4) multilane channels (IEF-AF4) [41,42] in the second dimension. Previous studies demonstrated that the IEF-AF4 separation of intact proteins can be achieved in less than 30 min. IEF-AF4 exhibited the advantage that ampholyte solution can be removed simultaneously during AF4 separation through the channel wall (layered with porous membrane). In addition, eluted proteins maintain their intact states without denaturation.

In this study, the IEF-AF4 multilane channel system was applied for the separation of phosphorylated proteins. A typical phosphoprotein, α-casein, was used along with a proteome sample from a prostate cancer cell line. To separate phosphoproteins and their isoforms by small pI differences (less than pI=0.1 per phosphate group addition), a narrow pH cut of carrier ampholyte (ΔpH=0.5) was prepared in advance by fractionating a commercial ampholyte solution in the IEF channel at a desired pH interval. The fractionated carrier ampholyte was utilized so that proteins with different degrees of phosphorylation would elute at different AF4 channel lanes. They could then be collected for nLC–ESI–MS–MS analysis of the degree of phosphorylation and the phosphorylation site. The developed method was applied by examining phosphoprotein biomarkers from DU 145, a human refractory prostate cancer (HRPC) cell line, and PrEC, normal prostate epithelial cells.

2. Experimental

2.1. Reagents and materials

α-Casein from bovine milk was used as the phosphoprotein standard and was obtained from Sigma (St. Louis, MO, USA). DU145 HRPC and PrEC cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and grown in the laboratory. Sequential grade modified trypsin was purchased from Promega Corp. (Madison, WI, USA). Fluka Ampholyte High-Resolution pH 3–6 (40%) was used for isoelectric focusing and was purchased from Sigma. The water used in this study was ultrapure (>18 MΩ·cm) and was filtered with a nitrocellulose membrane filter (0.22 μm) from Millipore (Danvers, MA, USA) prior to use.

Silica capillary tubes with inner diameters (I.D.) of 20, 75, and 100 μm (360 μm-O.D. for all) were obtained from Polymicro Technology LLC (Phoenix, AZ, USA) and were used to prepare the capillary LC column. Magic C18AQ (5 μm–100 Å for the capillary column and 5 μm–200 Å for the trapping column) from Michrom Bioresources Inc. (Auburn, CA, USA) was used as the packing material.

2.2. Cell culture and protein extraction

Cells were cultured in triplicate plates with RPMI-1640 from HyClone (Logan, UT) under humidified conditions containing 5% CO_2 at 37 °C for 96 h. Culturing media were supplemented

with 10% heat-inactivated fetal bovine serum from Gibco (Grand Island, NY, USA), 1% antibio-antimicotic solution containing 10,000 units/mL penicillin, and 10 mg/mL each of streptomycin, HEPES, sodium bicarbonate, insulin, and *p*-aminobenzoic acid. The cells were then subcultured (average cell concentration of 1×10^5/mL) with their respective culture media in 60 mm plates (Nunc; Denmark). The cultured cells were harvested after 24 h and centrifuged at 500 *g*. Each cell pellet was washed twice and diluted with 0.1 M PBS solution (pH 7.4) containing Complete® protease inhibitor cocktail tablets from F. Hoffmann-La Roche Ltd (Basel, Switzerland) and phosphatase inhibitor cocktail I (Sigma, St. Louis, MO) for cell lysis. The cells were disrupted by ultrasonication using a model CP130 Ultrasonic Processor from Cole Parmer Instrument Co. (Vernon Hills, IL, USA) at a pulse mode with a 0.5 s interval under 15 W. Cell debris were removed by two consecutive centrifugations at 5000 *g* for 30 min.

2.3. *Preparation of a narrow pH cut of carrier ampholyte*

A carrier ampholyte solution with a narrow pH interval was customized by fractionating the commercial ampholyte solution (pH 3–6) from Fluka using the IEF channel unit constructed with a plastic block [42]. The IEF channel is a simple flat space with six inlets and outlets and is illustrated at the left of the multilane AF4 channels in Fig. 1. To isoelectrically focus the original ampholyte (40% concentration), the concentrations of both electrolyte solutions were adjusted to 40 mM phosphoric acid for the anolyte solution and 25 mM sodium hydroxide for the catholyte solution. For each IEF of ampholyte, the 40% ampholyte loading amount was limited to 100 μL. The applied electrical voltage was 1 kV (lower than a typical IEF voltage ~2 kV for proteins) to avoid bubble generation or a thermal gradient. Fractionated ampholyte solution was collected at six outlets by pumping HPLC grade water through the six inlets of the IEF channel. To test α-casein for IEF-AF4 separation of phosphoprotein, a pH 3–6 ampholyte was utilized to obtain a narrow pH cut of carrier ampholyte with the pH interval of 4.5–5.0, which is also the pI interval of α-casein. The fractionated ampholyte solution collected at each IEF channel outlet had ΔpH=0.5. The fractionated ampholyte solution was concentrated by SpeedVac into a yellow colored gel-like solution and then stored. The concentrated ampholyte solution was diluted prior to protein separation.

2.4. *IEF-AF4 separation*

IEF-AF4 multichannel operation, shown in Fig. 1, was similar to the procedure reported in the previous study [42]. The Teflon tube connecting each electrolyte reservoir and IEF segment with electrolyte was filled by pumping both catholyte and anolyte solutions in by air pressure using a disposable 10 mL syringe. The electrolyte reservoirs of each electrode were the same as those used in the previous report, [42] and were made of acryl block with 20 cm^3 cylindrical chambers. After filling each tube with electrolyte, the IEF channel space was washed with ultrapure water using an HPLC pump connected to six inlets of the IEF channel. α-Casein or cell lysate in the narrow pH cut of ampholyte solution was loaded into the IEF channel via a KDS100 syringe pump (pump 3 in Fig. 1) from KD Scientific (Holliston, MA, USA). A model 7125 loop injector with a 20 μL loop from Rheodyne (Cotati, CA, USA) was set up through a separate inlet at one end of the IEF segment while the outlet was left open, as shown in Fig. 1. The protein and ampholyte solution injection volume was fixed at 20 μL. Electric voltage was applied through Pt wires in both reservoirs at 2 kV for 10–15 min using a model 205B-10R High Voltage Power Supply from Bertan (Hicksville, NY). When IEF was achieved for proteins, the electric current decreased from ~300 μA to ~50 μA.

After IEF, the fractionated protein bands were relocated to six different AF4 channels and AF4 separation was carried

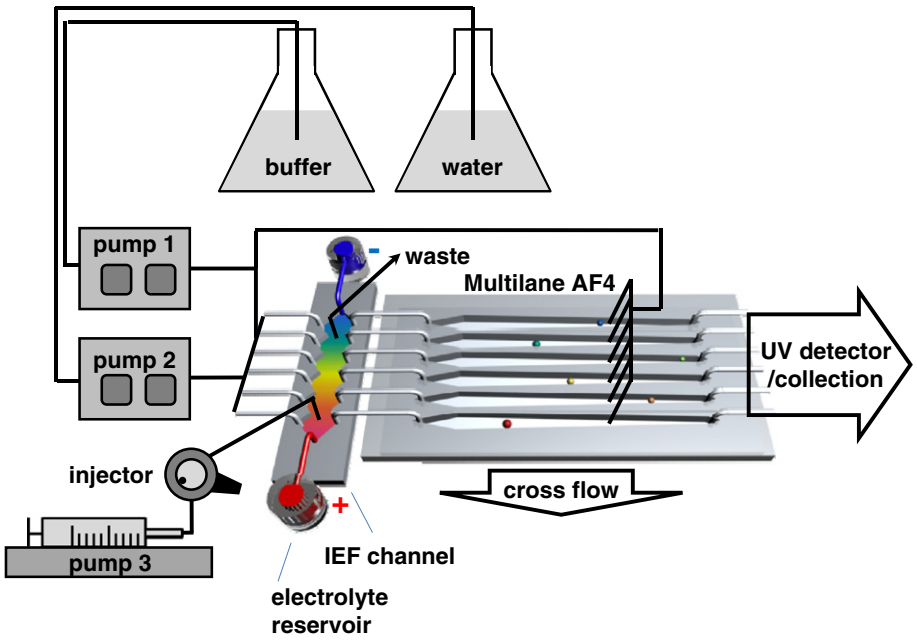

Fig. 1 – Schematics of IEF-AF4 channel for the 2D (pI & d_s) protein fractionation.

out at each lane. The AF4 separation process of the multilane AF4 channels was nearly the same as that of a typical AF4 system. The tip-to-tip channel length of an individual AF4 channel in the multilane AF4 system was 11.0 cm with an initial breadth of 0.7 cm and a final breadth of 0.3 cm. The AF4 channel thickness was 300 μm cut from Teflon spacer. PLCGC (MWCO, 20 kDa) from Millipore Corp. (Danvers, MA) was used for the AF4 channel membrane to prevent sample penetration by crossflow. IEF protein fractions had to achieve sample relaxation by a focusing process before AF4 separation occurred. IEF protein fractions were transferred to the beginning of each AF4 channel (~ 0.5 cm from the AF4 channel inlet) by focusing two opposite flow streams (one from the IEF channel outlet leading to the AF4 channel inlet and the other from the middle of the AF4 channel). The two streams exited through the channel membrane as cross-flow. The sample transfer and consecutive focusing/relaxation took place in 90 s. After focusing/relaxation, the incoming flow stream from the channel inlet was applied at a desired rate to successfully separate target proteins in increasing order of hydrodynamic diameter. This was accomplished by controlling outflow and cross-flow rates. 10 mM of NH_4HCO_3 was used as carrier solution for the AF4 separation. Eluted proteins were monitored at 280 nm by a series of model M720 UV detectors from Young-Lin (Seoul, Korea). They were also collected at different time fractions for MS analysis. Protein fractions collected from AF4 runs were filtered with Ultracel-3 K centrifugal filter devices to remove detergent molecules. Filtered proteins were washed with water and re-suspended in a solution of 8 M urea, 10 mM dithiothreitol, and 25 mM NH_4HCO_3 for tryptic digestion. The digestion procedure was the same as has previously been reported [42].

2.5. *nLC–ESI–MS–MS*

The nanoflow LC separation was performed in a homemade pulled tip capillary column (17 cm × 360 μm O.D., 75 μm I.D.) packed with 5 μm–100Å Magic C18AQ resin from Michrom BioResources Inc. (Auburn, CA) using a model 1200 capillary LC system from Agilent Technologies (Waldbronn, Germany). The capillary column was directly interfaced to a LTQ Velos ion trap MS or LTQ FT MS from Thermo Finnigan (San Jose, USA) with electrospray ionization (ESI). A trapping column (2 cm × 360 μm O.D., 100 μm I.D.) for desalting peptides packed with 5 μm–200 Å Magic C18AQ was connected before the analytical column through a PEEK microcross from Upchurch Scientific. The packing procedures for the analytical and trapping columns were explained in the previous reports [42]. A binary gradient elution was applied for LC separation. Mobile phase A consisted of 98/2 water/acetonitrile and mobile phase B consisted of 95/5 acetonitrile/water. Both solutions were added with 0.1% formic acid. Sample injection into the trapping column was performed by an autosampler with injection amounts of 0.5–1 μg by 5% mobile phase B for 10 min. The gradient elution began with an initial increase to 15% B for 1 min with a gradual increase to 32% B for 70 min. To clean the column, mobile phase B was increased to 80%, maintained for 10 min, and then returned to 5% for 3 min. At least 20 min were allowed to recondition the column before the next injection was made. The nLC effluent flow rate was adjusted to 200 nL/min by controlling the length of capillary tubing connected at the microcross. Peptides eluted from the nLC column were injected to MS via ESI at 2.0 kV for a MS precursor scan (m/z 300–1800). The MS^2 and MS^3 experiments using CID were followed by two-step data dependent MS/MS scans at 35% normalized collision energy. From each MS precursor scan, five predominant ions were selected for MS^2 analysis. When MS^2 detected a neutral loss of phosphate group from a precursor ion, the resulting fragment ion (−98 Da) was dissociated again for MS^3 fragmentation using a LTQ FTMS. Only MS^2 experiments were carried out with the LTQ Velos MS instrument. Both MS instruments were programmed with the same capillary voltage, collision energy, and other run parameters.

The Proteome Discoverer search program and TurboSEQUEST mechanism from Thermo Finnigan (San Jose, CA, USA) were used with the NCBI database to identify proteins. The mass width was set to 1.0 u for both precursor and fragment ions. Searched data screenings were made with the following threshold: 0.1 for the minimum delta-correlation (ΔCn) scores, 1.5, 2.0, and 2.5 for the minimum cross-correlation (Xcorr) values for single, double, and triple charged ions, respectively.

3. Results and discussion

3.1. *Performance of IEF-AF4 for the separation of phosphorylated proteins*

The ability to fractionate phosphorylated proteins by IEF-AF4 was evaluated with α-casein, a typical phosphoprotein standard. Since α-casein pI values are in the pH range of 4.5–5.0, depending on the degree of phosphorylation, a narrow pH cut (pH 4.5–5.0) of carrier ampholyte solution was utilized for the IEF-AF4 separation of intact α-casein molecules. The carrier ampholyte was fractionated in our lab from a commercial carrier ampholyte solution (pH 3.0–6.0) by a separate procedure described in the experimental section. 10 μL of the mixture of α-casein (1 mg/mL) and the carrier ampholyte (pH 4.5–5.0) was loaded to the IEF channel and it was allowed to reach equilibrium for 10–15 min until the electric current dropped under 50 μA, indicating that IEF separation was complete. Each protein fraction separated by IEF was then transferred to each of six AF4 channels by pump flow and was fractionated ($\dot{V}_{in}/\dot{V}_{out}$ = 0.84/0.24 mL/min at each lane) by differences in hydrodynamic diameters. A void peak at the beginning (1–2 min) of each fractogram was followed by the elution of proteins monitored by UV detector, as shown in Fig. 2. Small molecules contained in the sample mixture, including ampholyte and CHAPS micelles (added to help solubilize hydrophobic proteins and prevent them from precipitation during IEF), were supposed to have been removed through the channel membrane during the focusing/relaxation procedure. However, due to incomplete removal some remaining species eluted along with the void peak. The α-casein IEF-AF4 fractograms obtained at all six AF4 channel lanes are shown in Fig. 2, with distinct elution peaks at the first three channel lanes (lanes 1–3). The other channel lanes (lanes 4–6) did not demonstrate clear elution of α-casein molecules. The pH interval of the first

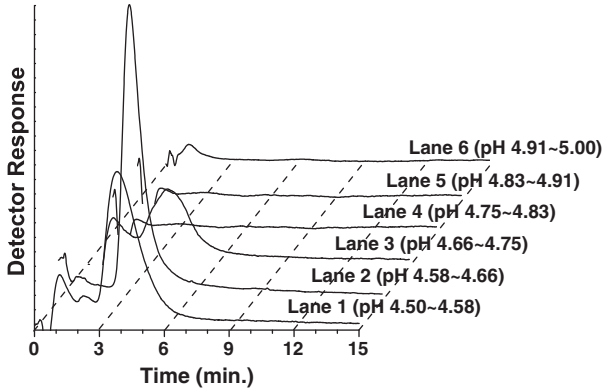

Fig. 2 – Six fractograms of α-casein by IEF-AF4 multichannel. IEF was carried out at 2 kV in 10 min. AF4 separation was performed at $\dot{V}_{in}/\dot{V}_{out}$ = 0.84/0.24 in mL/min in each lane.

three channel lanes was 4.50–4.75, which is lower than the theoretical pI value (4.98) of α-casein. α-Casein molecules were observed in lanes with pH lower than the expected theoretical pH, showing that α-casein molecules were phosphorylated to some degree. (In typical phosphoproteins, the pI of a protein changes by 0.05 pH units per phosphorylation.) The different peak intensities of different lanes implied a possible distribution in the degree of phosphorylation, since the pI value of a protein decreases with increases in phosphorylation.

During IEF-AF4 separation, α-casein fraction was collected from each channel. Each fraction was digested with trypsin to produce peptide mixtures for nLC–ESI–MS–MS analysis used to identify the location of phosphorylation sites. The experimental approach for distinguishing phosphate group positions has been well developed and uses sophisticated MS methods. MS^3 analysis is used to study particular peptide molecules obtained from the neutral loss of a phosphate group from corresponding precursor ions during the first MS–MS analysis. For instance, the phosphorylation site for a peptide ion as indicated by MS^3 analysis is shown in Supplementary Figure (SF) 1. SF 1a is the base peak chromatogram (BPC) of the digested fraction mixture from lane 1. SF 1b represents a typical precursor MS scan at a time slice of 63.9 min. The fragment ion spectra of the first collision induced dissociation (CID) of the precursor ion m/z 976.5 $[M+2H]^+$ is shown in SF 1c. The neutral loss of phosphate groups (−98.0 Da) from the precursor ions appeared to be a dominant process at the first CID, resulting in the formation of a base peak at m/z 927.7 as $[M+2H-H_3PO_4]^{+2}$. The second CID base peak results are represented in SF 1d. The database search identified a phosphopeptide sequence from α-casein subunit 1 as YKVPQLEIVPNsAEER ($[M+2H^+]^{2+}$ and m/z 977.0). The small letter "s" in the peptide sequence represents the phosphorylation site. This is the typical procedure used in this study to identify the position of a phosphate group at a specific amino acid in a peptide. However, to analyze a real proteome sample, the selective enrichment or isolation of phosphoproteins/phoshphopeptides is often required prior to MS analysis. This is because the ionization of phosphorylated peptides in MS is less efficient than for other unmodified peptides. Selective phosphopeptide isolation based on affinity methods can be limited in examining the degree of phosphorylation of a specific peptide since it removes all unmodified peptides. However, with IEF-AF4 and nLC–ESI–MS–MS, all proteins are eventually subjected to separation in the AF4 channel and all protein fractions can be retrieved. Therefore, the ratio of a phosphopeptide to an unmodified peptide of the same kind can be investigated by examining the peak area of each peptide in a single nLC–ESI–MS chromatogram. In Fig. 3, the degree of phosphorylation of two different peptides was examined by comparing the extracted chromatograms of the phosphorylated and unmodified peptide at different channel lanes from nLC–ESI–MS data: a) peptide of YKVPQLEIVPNsAEER ($[M+3H^+]^{3+}$ and m/z 651.6) from subunit 1 of α-casein and b) NAVPITPTLNREQLsTsEENSKK ($[M+3H^+]^{3+}$ and m/z 879.9) from subunit 2. Channel lane 1 of Fig. 3a represents an extracted chromatogram showing only two peptides: the unmodified peptide with an AA sequence of YKVPQLEIVPNSAEER ($[M+3H^+]^{+3}$ and m/z 625.0) and the phosphorylated peptide, YKVPQLEIVPNsAEER ($[M+3H^+]^{3+}$ and m/z 651.6). These two peptides had different retention times due to the influence of the phosphate group. Fig. 3a shows that the peak area ratio of phosphopeptide/unmodified peptides increases as the lane becomes acidic (from lanes 3 to 1), demonstrating that more phosphorylated proteins elute at the acidic lane. Peaks in Fig. 3 were plotted after area normalization at

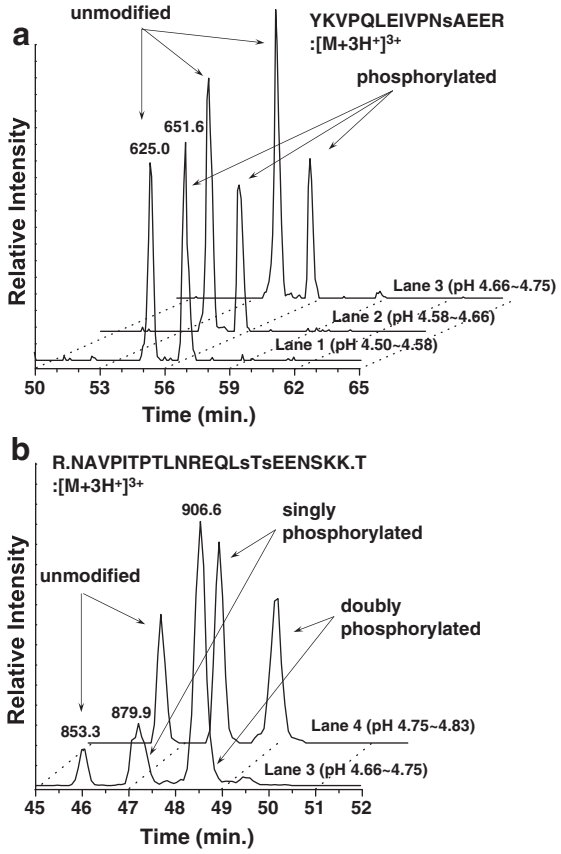

Fig. 3 – The extracted chromatograms of unmodified and phosphorylated peptides of a) YKVPQLEIVPNsAEER from subunit 1 of α-casein obtained at channel lanes 1–3 and b) NAVPITPTLNREQLsTsEENSKK from subunit 2 at lanes 3 and 4. The elution order consists of the unmodified peptide followed by phosphopeptides with increasing phosphorylation.

each channel lane. Table 1 lists the peak area of the unmodified and phosphorylated peptide at each lane calculated from the extracted chromatogram. The phosphopeptide of Fig. 3a listed in the first row of Table 1 shows a significant increase in the relative peak area of singly phosphorylated peptides at the channel lane 1. Though the pH range of each channel differs, the same phosphopeptide is observed at different channel lanes. This is because in most cases, different phosphorylation combinations occur at different phosphorylation sites. Moreover, different phosphoisoforms with the same number of phosphorylations (therefore with similar pI values) can elute at the same channel lane since there are several phosphorylation sites in the two α-casein molecule subunits. However, the chance of finding a phosphate group at every phosphorylation site increases as the channel lane becomes acidic. Similar observations were made with two other singly phosphorylated peptides listed in Table 1.

Similar observations are shown in Fig. 3b with the doubly phosphorylated peptide AA sequence of NAVPITPTLNREQLsTsEENSKK ($[M+3H^+]^{+3}$ and m/z 906.6), which is from subunit 2 of α-casein. The peak area of each isoform is listed in Table 1. In this case the three different isoforms can be found because there are two phosphorylation sites (marked with a small letter "s"). The extracted chromatogram from channel lane 3 in Fig. 3b shows the consecutive elution and peak area distributions of the unmodified peptide (m/z 853.3), the singly phosphorylated peptide (m/z 879.9), and the doubly phosphorylated peptide (m/z 906.6). These three peptides were observed in four channel lanes and the relative peak area of the doubly phosphorylated peptide was higher in lanes 1–3 than in lane 4. For a peptide with three phosphorylation sites (VNELsKDIGsEsTEDQAMEDIK), only two different types (double and triple) of phosphorylation were observed without the unmodified and singly modified peptide. The fraction from channel lane 1 had a larger population of triply phosphorylated peptide (37.22 vs. 7.70%) than the fraction from channel lane 2. These observations show that the same protein molecules may have the same degree (or number) of phosphorylation but different phosphorylation site patterns.

3.2. *IEF-AF4 phosphoprotein separation from human prostate cancer cell line*

IEF-AF4 was applied for the 2D separation of phosphoproteins from DU145, the hormone-refractive prostate cancer (HRPC) cell line and PrEC, the normal prostate epithelial cell line. CHAPS was added to both the ampholyte and AF4 carrier liquid to increase the solubility of hydrophobic proteins. Fig. 4 shows the six fractograms of the PrEC cell lysate sample. Two eluting protein fractions (t_r=0–5 and 5–10 min) were collected from each channel lane. In the fractograms of channel lanes 1 and 6, the relatively large void peaks followed by protein peaks were observed at the beginning of elution. Since the pH interval for the carrier ampholyte was 5.0–6.0 (ΔpH=0.17/lane), proteins with pI values larger or smaller than this interval accumulated at both extremes of the IEF channel and eluted with relatively large peak signals. The AF4 fractions of each cell lysate sample were washed using the centrifugal filter unit and digested with trypsin. Peptide mixtures of each fraction (12 fractions from each cell line) were analyzed by nLC–ESI–MS–MS. The proteins identified from the TurboSEQUEST database search are listed in Supplementary Table 1. By counting only proteins with multiple peptide hits, 1435 proteins were identified from DU145 and 1749 proteins were identified from PrEC cells. 546 proteins were found in both samples. Some specific phosphopeptides known to be involved with prostate cancer were included on the list of identified proteins. To quantitatively compare the

Table 1 – Peak area ratio of phosphopeptides in α-casein. Each percentage was calculated as the peak area of each peptide/total peak area of all phosphoisoforms eluted at the same lane. Blanks represent the below PQL (Practical Quantification Level).

Sequence	Subunit	Lane #	Ratio of peak area (%, to total area)			
			Unmodified	Single	Double	Triple
YKVPQLEIVPNsAEER	1	1	47.45	52.55		
		2	63.23	36.77		
		3	69.56	30.44		
yLGYLEQLLR	1	1	5.49	94.51		
		2	17.46	82.54		
		3	62.73	37.27		
TVDMEsTEVFTKK	2	1	26.47	73.53		
		2	29.48	70.52		
		3	31.47	68.53		
		4	71.43	28.57		
NAVPITPTLNREQLsTsEENSKK	2	1	4.99	22.94	72.07	
		2	5.74	24.57	69.68	
		3	7.77	19.99	72.24	
		4	22.60	39.28	38.13	
YKVPQLEIVPN$_p$SAEERLHsMK	1	1	3.16	96.12	0.72	
		2	3.53	95.98	0.49	
		3	3.89	95.47	0.64	
		4	6.93	93.07	0.00	
VNELsKDIGsEsTEDQAMEDIK	1	1			62.78	37.22
		2			92.30	7.70

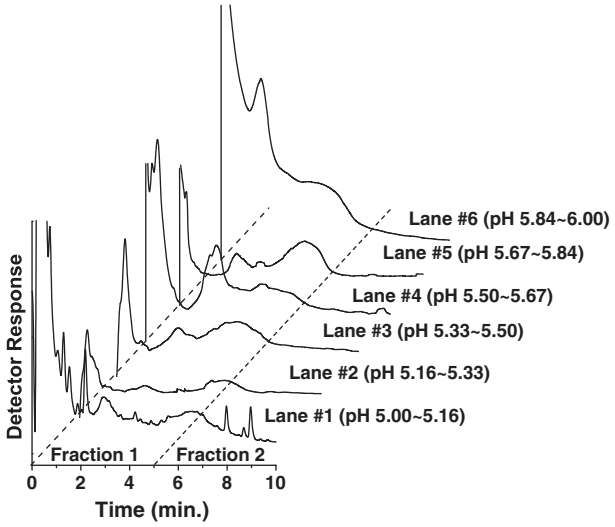

Fig. 4 – IEF-AF4 fractograms of PrEC cell lysates (50 μg injection). At each lane, two fractions were collected for the nLC–ESI–MS–MS analysis after tryptic digestion.

degree of phosphorylation of specific peptides related to cancer, a peptide with the AA sequence of TLEEEAKTHEAQIQEMR ($[M+3H^+]^{+3}$ and m/z 681.7) from cellular myosin heavy polypeptide 9 (226.4 kDa and pI 5.6) was selected as an internal control (IC). This was used to calculate the relative MS peak area of a target phosphopeptide since myosin is a housekeeping gene product [43] found in both channel lanes 4 and 5. For instance, Table 2 lists three phosphopeptides that show a prominent difference in the relative ratio of peak area between the target and the myosin IC peptide. The peak area of a phosphopeptide with an AA sequence of KIssEsLSTCWR ($[M+3H^+]^{+3}$ and m/z 565.4, CID spectra shown in Supplementary Fig. 2) from mitogen-activated protein kinase 14 (MAPK14, collected from the second fraction of channel lane 4; marked as 4-2 in Table 2) was calculated for both DU 145 and PrEC cell samples. MAPK14 is a member of the MAP kinase family. Its activation increases as prostate cancer progresses to androgen-independent or further [44]. The ratio of the phosphopeptide of MAPK14 to the myosin IC peptide was about 50% higher in DU145 than in the normal cell line. Two other phosphopeptide candidate markers also showed a remarkable difference in relative regulation. The phosphopeptide sequence of WtHDKyQGDGIVEDEEEtMENNEEK ($[M+3H^+]^{+3}$, m/z 1056.0, and CID spectra shown in Supplementary Fig. 2) from Bcl2-associated transcription factor 1 (106.1 kDa and pI 9.9) is a transcriptional repressor that promotes apoptosis [45]. The peak area ratio of the target peptide to the myosin IC peptide in the HRPC cancer cell was three times (0.15 vs. 0.05 of ratio a/b in Table 2) higher than in normal PrEC cells. The same comparison was made with another peptide marker, LGKDAVEDLESVGK ($[M+2H]^{+2}$ and m/z 730.7) from a Dermcidin precursor (11.3 kDa and pI 6.5). In this case, a different internal control peptide was used to match the pI value of the target protein: TTGIVMDSGDGVTHTVPIYEGYALPHAILR ($[M+3H]^{+3}$ and m/z 1062.0) of β-actin (41.7 kDa and pI 5.48). The above phosphopeptide from a Dermcidine precursor in the cancer cell line showed a remarkable difference in concentration compared to the same protein that was not found in the normal cells. These peptides are not easily detected when the whole cell lysate is digested and analyzed without preliminary phosphopeptide enrichment. In addition, phosphopeptide enrichment methods simply remove unmodified peptides. A suitable method, such as isotope labeling, should be incorporated to study quantitative variation in phosphorylation. 2D phosphoprotein separation by IEF-AF4 and quantitative target phosphopeptide analysis can be performed by nLC–ESI–MS–MS using a proper internal control peptide. Because this can be done without secondary protein/peptide modification, a combined method can effectively differentiate the relationship between protein phosphorylation and disease status. Furthermore, diagnosis of adult diseases can be performed from a patient's biofluids. To integrate the developed method into an analytical protocol for adult cancer, known phosphopeptide markers should be further studied. New markers should be thoroughly examined using patient samples from the different developmental stages of prostate cancer.

Table 2 – The ratio of the peak area of each target phosphopeptide involved with prostate cancer to that of the internal control (IC) peptide from two housekeeping gene products: a peptide sequence of TLEEEAKTHEAQIQEMR from cellular myosin heavy polypeptide 9 (226.4 kDa, pI 5.6) and TTGIVMDSGDGVTHTVPIYEGYALPHAILR from β-actin. (41.7 kDa, pI 5.48). Small letters in the AA sequence denote the phosphorylated amino acid.

Channel lane — fraction no.	Cell type	Peak area of the target peptide (a)	Peak area of IC (b)	Ratio (a/b)
		KIssEsLSTCWR from MAPK14	Cellular myosin	
4-2	DU145	1.2E6	6.5E5	1.84
	PrEC	1.6E7	1.3E7	1.23
		WtHDKyQGDGIVEDEEEtMENNEEK from Bcl2-associated transcription factor 1	Cellular myosin	
5-2	DU145	1.1E5	7.5E5	0.15
	PrEC	1.8E5	3.5E6	0.05
		LGKDAVEDLESVGK From Dermcidin precursor	β-Actin	
3-1	DU145	9.4E5	4.6E5	2.03
	PrEC	Below PQL	3.2E5	–

4. Conclusions

This study showed that IEF-AF4, a non-gel based high speed 2D (pI & d_s) separation method, can selectively isolate phosphoproteins from a proteome sample. A narrow pH cut of carrier ampholyte was essential for fine phosphoprotein fractionation because a protein's pI only changes slightly after phosphorylation. 2D fractionation of α-casein molecules was followed by shotgun analysis of digested peptide mixtures using nLC–ESI–MS–MS. This demonstrated that various phosphorylation isoforms can be characterized with a simultaneous determination of the relative ratio of phosphorylated to unmodified peptide. The phosphopeptide peak area, compared to unmodified or less phosphorylated peptides, increased as the channel lane became acidic. IEF-AF4 can be used to determine the up/down regulation of some specific phosphopeptides/phosphoproteins that are known as disease markers. Because current methods for phosphopeptide isolation or enrichment are based on affinity methods and ordinary unmodified peptides are readily removed during the enrichment process, quantitative phosphopeptide analysis without isotope labeling is not straightforward. The IEF-AF4 method still requires an improvement in the resolution of separated proteins by using miniature multilane AF4 channels. However, it does provide the unique ability to fractionate a targeted phosphoprotein by combining two liquid based elution techniques. The degree of post-translational modification can then be analyzed with nLC–ESI–MS–MS.

Acknowledgment

This study was supported by grant no. 2011-0016438 and in part by grant KRF-2008-313-C00567 from the National Research Foundation (NRF) of Korea funded by the Korean government (MEST).

Appendix A. Supplementary data

Supporting Information Available CID spectra of phosphopeptides are contained in Supplementary Figs. 1–2. Supplementary Table 1 lists the proteins identified by nLC–ESI–MS–MS from digested peptide mixtures of collected IEF-AF4 fractions. This information is available free of charge via the Internet.

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2012.01.034.

REFERENCES

[1] Hubbard MJ, Cohen P. On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem Sci 1993;18:172–7.
[2] Hunter T. Signaling—2000 and beyond. Cell 2000;100:113–27.
[3] Zhou H, Watts JD, Aebersold R. A systematic approach to the analysis of protein phosphorylation. Nat Biotechnol 2001;19:375–8.
[4] Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science 2002;298:1912–34.
[5] Thingholm TE, Jensen ON, Larsen MR. Analytical strategies for phosphoproteomics. Proteomics 2009;9:1451–68.
[6] Yu LR, Issaq HJ, Veenstra TD. Phosphoproteomics for the discovery of kinases as cancer biomarkers and drug targets. Proteomics Clin Appl 2007;1:1042–57.
[7] Jaffe JD, Keshishian H, Chang B, Addona TA, Gilette MA, Carr SA. Accurate inclusion mass screening: a bridge from unbiased discovery to targeted assay development for biomarker verification. Mol Cell Proteomics 2008;7:1952–62.
[8] Deguchi A, Soh JW, Li H, Pamukcu R, Thompson WJ, Weinstein IB. Vasodilator-stimulated phosphoprotein (VASP) phosphorylation provides a biomarker for the action of exisulind and related agents that activate protein kinase G. Mol Cancer Ther 2002;1:803–9.
[9] Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, et al. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat Biotechnol 2002;20:301–5.
[10] Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villén J, Li J, et al. Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci U S A 2004;101:12130–5.
[11] Black TM, Andrews CL, Kilili G, Ivan M, Tsichlis PN, Vouros P. Characterization of phosphorylation sites on Tpl2 using IMAC enrichment and a linear ion trap mass spectrometer. J Proteome Res 2007;6:2269–76.
[12] Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jørgensen TJD. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol Cell Proteomics 2005;4:873–86.
[13] Albuquerque CP, Smolka MB, Payne SH, Bafna V, Eng J, Zhou H. A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol Cell Proteomics 2008;7:1389–96.
[14] Boersema PJ, Mohammed S, Heck AJR. Phosphopeptide fragmentation and analysis by mass spectrometry. J Mass Spectrom 2009;44:861–78.
[15] Wu J, Lenchik NJ, Pabst MJ, Solomon SS, Shull J, Gerling IC. Functional characterization of two-dimensional gel-separated proteins using sequential staining. Electrophoresis 2005;26:225–37.
[16] Steinberg TH, Agnew BJ, Gee KR, Leung WY, Goodman T, Schulenberg B, et al. Global quantitative phosphoprotein analysis using multiplexed proteomics technology. Proteomics 2003;3:1128–44.
[17] Inoue SI, Kinoshita T, Matsumoto M, Nakayama KI, Doi M, Shimazaki KI. Blue light-induced autophosphorylation of phototropin is a primary step for signaling. Proc Natl Acad Sci U S A 2008;105:5626–31.
[18] Lee BS, Lasanthi GD, Jayathilaka P, Huang JS, Gupta S. Immobilized metal affinity electrophoresis: a novel method of capturing phosphoproteins by electrophoresis. J Biomole Tech 2008;19:106–8.
[19] Kinoshita E, Kinoshita-Kikuta E, Koike T. Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. Nat Protoc 2009;4:1513–21.
[20] Oda Y, Nagasu T, Chait BT. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. Nat Biotechnol 2001;19:379–82.
[21] Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, et al. Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. Nat Methods 2005;2:591–8.
[22] Bodenmiller B, Mueller LN, Mueller M, Domon B, Aebersold R. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat Methods 2007;4:231–7.
[23] Neville DCA, Rozanas CR, Price EM, Gruis DB, Verkman AS, Townsend RR. Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and

matrix-assisted laser desorption mass spectrometry. Protein Sci 1997;6:2436–45.
[24] Posewitz MC, Tempst P. Immobilized gallium(III) affinity chromatography of phosphopeptides. Anal Chem 1999;71: 2883–92.
[25] Thingholm TE, Larsen MR, Ingrell CR, Kassem M, Jensen ON. TiO_2-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. J Proteome Res 2008;7:3304–13.
[26] Kweon HK, Håkansson K. Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. Anal Chem 2006;78:1743–9.
[27] Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 2006;127:635–48.
[28] Li X, Gerber SA, Rudner AD, Beausoleil SA, Haas W, Villén J, et al. Large-scale phosphorylation analysis of α-factor-arrested *Saccharomyces cerevisiae*. J Proteome Res 2007;6:1190–7.
[29] McNulty DE, Annan RS. Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. Mol Cell Proteomics 2008;7:971–80.
[30] Giddings JC. Field-flow fractionation: analysis of macromolecular, colloidal, and particulate materials. Science 1993;260:1456–65.
[31] Ratanathanawongs-Williams SK. Field-flow fractionation handbook. In: Schimpf ME, Caldwell KD, Giddings JC, editors. New York: Wiley-Interscience; 2000. p. 271–8.
[32] Wahlund KG. Field-flow fractionation handbook. In: Schimpf ME, Caldwell KD, Giddings JC, editors. New York: Wiley-Interscience; 2000. p. 279–94.
[33] Reschiglian P, Moon MH. Flow field-flow fractionation: a pre-analytical method for proteomics. J Proteomics 2008;71: 265–76.
[34] Reschiglian P, Zattoni A, Cinque L, Roda B, Dal Piaz F, Roda A. Hollow-fiber flow field-flow fractionation for whole bacteria analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 2004;76: 2103–11.
[35] Kang D, Oh S, Reschiglian P, Moon MH. Separation of mitochondria by flow field-flow fractionation for proteomic analysis. Analyst 2008;133:505–15.
[36] Kim KH, Kang D, Koo HM, Moon MH. Molecular mass sorting of proteome using hollow fiber flow field-flow fractionation for proteomics. J Proteomics 2008;71:123–31.
[37] Kang D, Oh S, Ahn SM, Lee BH, Moon MH. Proteomic analysis of exosomes from human neural stem cells by flow field-flow fractionation and nanoflow liquid chromatography–tandem mass spectrometry. J Proteome Res 2008;7:3475–80.
[38] Kang D, Yoo JS, Kim MO, Moon MH. A soft preparative method for membrane proteome analysis using frit inlet asymmetrical flow field-flow fractionation: application in a prostatic cancer cell line. J Proteome Res 2009;8:982–91.
[39] Lee JY, Min HK, Choi D, Moon MH. Profiling of phospholipids in lipoproteins by multiplexed hollow fiber flow field-flow fractionation and nanoflow liquid chromatography–tandem mass spectrometry. J Chromatogr A 2010;1217:1660–6.
[40] Kang D, Moon MH. Development of non-gel-based two-dimensional separation of intact proteins by an on-line hyphenation of capillary isoelectric focusing and hollow fiber flow field-flow fractionation. Anal Chem 2006;78: 5789–98.
[41] Kim KH, Moon MH. Development of a multilane channel system for nongel-based two-dimensional protein separations using isoelectric focusing and asymmetrical flow field-flow fractionation. Anal Chem 2009;81:1715–21.
[42] Kim KH, Moon MH. High speed two-dimensional protein separation without gel by isoelectric focusing-asymmetrical flow field flow fractionation: application to urinary proteome. J Proteome Res 2009;8:4272–8.
[43] Eisenberg E, Levanon EY. Human housekeeping genes are compact. Trends Genet 2003;19:362–5.
[44] Gioeli D, Mandell JW, Petroni GR, Frierson Jr HF, Weber MJ. Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res 1999;59:279–84.
[45] Kasof GM, Goyal L, White E. Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. Mol Cell Biol 1999;19:4390–404.