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High Speed Two-Dimensional Protein Separation without Gel by Isoelectric Focusing−Asymmetrical Flow Field Flow Fractionation: Application to Urinary Proteome

Ki Hun Kim and Myeong Hee Moon*

Department of Chemistry, Yonsei University, Seoul, 120-749, Korea

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An online multilane channel system for isoelectric focusing and asymmetrical flow field-flow fractionation (IEF-AF4) is utilized for the two-dimensional separation (2D: isoelectric point, p*I*, and hydrodynamic diameter, d_s) of a human proteome sample followed by the shotgun proteomic analysis using nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS-MS). IEF-AF4 was recently developed to carry out nongel-based high speed two-dimensional protein separation [Kim, K., et al. *Anal. Chem.* **2009**, *81*, 1715]. In IEF-AF4, proteins are separated according to p*I* along an IEF channel located at the head of six AF4 channels, and then the fractionated protein bands are directed to multilane AF4 channels for size-based separation. In this report, the original IEF-AF4 system has been modified to avoid the possible adsorption of proteins onto the membrane wall of IEF segments during isoelectric focusing by isolating the IEF channel segments from the multilane AF4 channels. The performance of the modified IEF-AF4 system was tested with protein standards and was further applied for the 2D fractionation of the human urinary proteome sample under two ampholyte solutions with different pH ranges (pH 3−10 and 3−6). The entire 2D separation was achieved in less than 30 min. The collected protein fractions were digested for peptide analysis using nLC-ESI-MS-MS, resulting in the identification of 245 total urinary proteins, including 110 unique proteins that are not yet reported in literature. Our experiments also showed a higher efficiency in the identification of urine proteins using ampholyte solution in the narrower pH range.

Keywords: asymmetrical flow field-flow fractionation • isoelectric focusing • IEF-AF4 • 2D-protein separation • urinary proteome • nLC-ESI-MS-MS

Introduction

Proteome analysis often involves an extensive use of separation methods to fractionate complicated protein mixtures prior to analysis with mass spectrometry (MS). Among the currently available separation methods such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), HPLC including ion exchange or size exclusion chromatography, electrophoresis used with or without a gel matrix, free-flow electrophoresis, and ultracentrifugation, 2D-PAGE is widely and routinely utilized due to its outstanding effectiveness in separation resolution.[1−3] Online two-dimensional separations have been reported involving capillary isoelectric focusing (CIEF) coupled with size exclusion chromatography (CIEF-SEC),[4] reversed-phase liquid chromatography (CIEF-RPLC),[2,5] capillary zone electrophoresis (CIEF-CZE),[6] capillary gel electrophoresis (CIEF-CGE),[7] and capillary nongel sieving electrophoresis (CIEF-CNGSE) using a hollow fiber membrane interface.[8] Most of these methods are based on the use of packing media or gel matrix which may result in unwanted sample loss during separation, and the use of organic solvents or surfactants which induce protein denaturation. In addition, if using IEF, it is often necessary to perform a separate dialysis procedure to remove ampholyte solution prior to MS analysis.

Recently, an on-line coupling method to bypass the above-mentioned weaknesses was introduced through the combination of CIEF and hollow fiber flow field-flow fractionation (CIEF-HF5),[9] in which proteins can be separated in two dimensions (p*I* and hydrodynamic diameter). HF5, a cylindrical version of FlFFF (or F4), is capable of separating proteins or macromolecules according to hydrodynamic size by controlling the flow rates of radial flow (exiting through the porous inner wall of the hollow fiber) and axial flow along the fiber axis.[10−15] The FlFFF,[16−18] which typically adopts a rectangular channel system, has very recently shown its capability as a prefractionation device for proteomics research.[19] The use of FlFFF and an off-line combination of nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS-MS) demonstrated size-based prefractionation and protein characterization of mitochondria,[20] exosome,[21,22] and membrane proteins.[23] Since separation in FlFFF is carried out in an empty channel space either with a hollow fiber or with a conventional rectangular channel, unwanted sample interactions with packing materials, often seen in chromatography,

* Corresponding author: Myeong Hee Moon, Department of Chemistry, Yonsei University, Seoul, 120-749, Korea. Phone, (82) 2 2123 5634; fax, (82) 2 364 7050; e-mail, mhmoon@yonsei.ac.kr.

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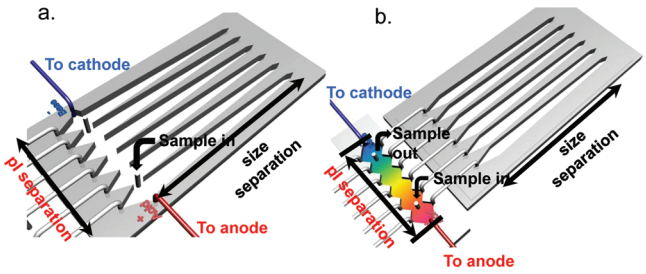

Figure 1. Schematics of IEF-AF4 channels with (a) an IEF segment embedded in a multilane AF4 channel and with (b) a separate IEF channel connected to a multilane AF4 channel. The connection between each electrode and IEF segment was made with Teflon tubing filled with electrolyte solution, leading to each electrolyte reservoir.

can be avoided. The ampholyte solution used for the preceding IEF operation can be removed through the channel wall during the FlFFF operation and eluted proteins can maintain their conformation without being denatured by using a biologically compatible buffer solution as the FlFFF carrier solution.

The original concept of the CIEF-HF5 method was later implemented for use with rectangular type multichannel systems by adapting the conventional asymmetrical FlFFF (or AF4) channel design to carry out IEF-AF4 as shown in Figure 1a in order to enlarge the sample throughput and separation speed.[24] In an IEF-AF4 channel, IEF operation is carried out in the first dimension in a thin, rectangular channel and is then followed by size fractionation in six miniaturized, multilane AF4 channels in the second dimension. Protein separation in each AF4 channel is achieved by the differential migration of protein molecules determined by the force balance of the diffusion coefficients and crossflows, which move across the channel membrane layered at the bottom of the AF4 channel. Under the influence of crossflow, which drives sample components toward the channel wall, protein molecules of smaller sizes (fast diffusion) extrude farther away from the channel wall and elute earlier than those with larger diameters, thus separation is obtained by an increasing order of the hydrodynamic diameter.

In this study, we have modified the IEF-AF4 multilane channel system (Figure 1a) by isolating the IEF segment out of the multilane channel in order to bypass the often experienced protein clogging at the channel membrane during IEF. The modified IEF-AF4 channel, illustrated in Figure 1b, conducts IEF in a separate sawtooth shaped channel; the separated protein bands are transferred to an AF4 channel at six different p*I* intervals via Teflon tubing. This study shows the performance of the modified IEF-AF4 multilane channel for the separation of standard protein mixtures using ampholyte solutions with narrow pH ranges. The procedure was applied to the human urinary proteome sample to demonstrate the potential utility in proteome analysis for the first time. 2D-fractionation of urinary proteome was carried out by varying the pH ranges of the ampholyte solutions. The protein fractions collected after IEF-AF4 were analyzed by the shotgun method using nLC-ESI-MS-MS for protein identification after digestion.

Materials and Methods

Reagents and Materials. The protein standards used were fetuin from fetal calf serum (48 kDa, p*I* 3.3), amyloglucosidease (48 kDa, *p*I 3.6), glucose oxidase (66 kDa, p*I* 4.2), bovine serum albumin (BSA, 66 kDa, p*I* 4.8), apoferritin (444 kDa, p*I* 5.4), carbonic anhydrase (CA, 29 kDa, p*I* 5.8), and alcohol dehydrogenase (ADH, 150 kDa, p*I* 6.2) from Sigma (St. Louis, MO). The human urine sample was obtained from a healthy volunteer (29, male). Two protease inhibitor cocktail tablets (Complete) from F. Hoffmann-La Roche Ltd. (Basel, Switzerland) were added to 50 mL of the human urine. Cell debris was removed by 3000*g* centrifugation and the supernatant urine solution was filtered with a centrifugal kit (Amicon Ultracel-3K: MWCO of 3 kDa) from Millipore (Bedford, MA) at 3000*g* to a final volume of 4 mL. The filtrate was stored in a polystyrene tube on ice. The retentate was washed in 30 mL of HPLC water at 3000*g* at 4 °C for 20 min to remove any remaining salts and interfering materials. Finally, the solution was reconcentrated with an Amicon centrifugal kit to a 1 mL volume. The retentate was retrieved by centrifuging the cartridge upside down. Protein concentration was measured using a Bradford assay, and the resulting protein solution was stored at −30 °C.

The sequential grade modified trypsin was purchased from Promega Corp. (Madison, WI). Ammonium bicarbonate was used for the preparation of the carrier solution of AF4, 10 mM phosphoric acid was used for the anolyte solution, and 20 mM sodium hydroxide was used for the catholyte solution. For IEF, two different ampholyte solutions (Fluka Ampholyte High-Resolution pH 3−10 and pH 3−6) were also purchased from Sigma. The water used in this study was ultrapure (>18 MΩ·cm) and filtered with a nitrocellulose membrane filter (0.22 μm) (Millipore, Danvers, MA) prior to use.

Teflon tubing with 0.020 in. i.d. was used for the connection between the IEF channel and the AF4 channel; silica capillary tubing (i.d. 100 μm and o.d. 360 μm) was used to control the outflow rate of each AF4 channel. A silica capillary column with an i.d. of 75 μm was used to prepare capillary LC column. Two 7-port manifolds from Upchurch Scientific (Oak Harbor, WA) were utilized for the flow splitting into both the six IEF channel inlets and the six focusing flow stream inlets of the AF4 channels.

Modification of the Online IEF-AF4 Channel. The online IEF-AF4 channel system employed in this study was modified into two separate channel blocks (Figure 1b). The system was based on the original channel design (Figure 1a) which had an IEF segment embedded into the multilane AF4 channel, as reported in the previous study.[24] IEF was carried out in a separate channel, as shown in Figure 1b, with the multilane AF4 channel connected to the IEF channel by Teflon tubing. All of the tubing connections were made with 1/16 in. PEEK hand-tight fittings from Upchurch Scientific (Oak Harbor, WA). The IEF channel segment was constructed by clamping two nonfritted plastic blocks together with a Teflon spacer 6.0 cm long, 0.5 cm wide, and 0.030 cm thick. As shown in Figure 1b, the spacer in the IEF channel has a sawtooth shape to help the carrier solution enter and exit the IEF channel laterally, thus, avoiding the intermixing of like-pH protein bands during transfer to each multilane AF4 channel.

The tip-to-tip channel length, L_{tt}, of each AF4 channel shown in Figure 1b is 11.0 cm, with an initial breadth of 0.7 cm, decreasing to a final breadth of 0.3 cm. The lengths of the triangles at both the inlets and outlets are 0.7 and 0.3 cm, respectively. A channel spacer for AF4 is cut with the same Teflon spacer (0.030 cm thick). At the bottom of the multilane AF4 channel, a sheet membrane, PLCGC (MWCO, 20 kDa) from Millipore Corp. (Danvers, MA), is layered above a water-permeable, 5 μm pore ceramic frit embedded in a plastic channel block, allowing for crossflow passage. The upper wall

of the channel is a plain plastic block, the same as in a conventional AF4 channel system.

IEF-AF4 Operation. Before IEF, both catholyte and anolyte solutions were injected from each reservoir to the IEF channel in order to fill the connecting tubing with each electrolyte solution. Electrolyte reservoirs, the same as used in the previous report,[24] were made of plastic blocks with 20 cm^3 cylindrical chambers. After filling each tubing connection with electrolyte, the IEF channel was washed with HPLC-grade water using an HPLC pump connected to six inlets of the IEF channel. Protein mixtures (40−120 μg) in ampholyte solution were then loaded into the IEF channel (shown in Figure 1b) via a KDS100 syringe pump from KD Scientific (Holliston, MA) through a separate inlet located at one end of the IEF segment, while the outlet was left open. The injection volume of protein solution with ampholyte was fixed at 20 μL. During IEF, 2 kV of electric voltage was applied for 5−10 min through Pt wires immersed in both reservoirs using a model 205B-10R High Voltage Power Supply from Bertan (Hicksville, NY). A decrease of electrical current from ~300 to ~50 μA was observed during the isoelectric focusing of proteins, indicating that IEF had been achieved.

After IEF, separated protein bands were transferred to the AF4 channels through six lateral IEF outlets by delivering 10 mM of NH_4HCO_3 carrier solution from an external pump toward the multilane AF4 channels. The procedures for AF4 separation are similar to those of a typical AF4 system. When protein bands from the IEF channel were loaded at the beginning of each AF4 channel, another carrier flow stream from the outlet side of each AF4 channel was delivered to provide a focusing/relaxation procedure for incoming sample components, while the crossflow exits the AF4 channels through the membrane located at the bottom of the channel. The ratio of incoming flow rate to focusing flow rate was adjusted to 1:5. During this focusing/relaxation step, each protein fraction reaches its equilibrium state through the balance of two forces: crossflow field and size diffusion. The focusing/relaxation time period, including sample delivery from the IEF channel to the AF4 channels, lasted for 90 s. After the focusing/relaxation process, the focusing flow stream from each AF4 channel outlet was stopped and only the flow stream from each AF4 channel inlet (through the IEF channel) was delivered at the increased rate required for AF4 separation. During AF4 separation, proteins were separated in an increasing order of hydrodynamic diameter, as controlled by the balance of crossflow and outflow rates. Protein components eluting at each channel outlet were either monitored at 280 nm by multiple UV detectors, UV M720 from Young-Lin (Seoul, Korea), or collected at different time fractions for proteomic analysis without passing through detectors.

Digestion of Collected AF4 Protein Fractions. Protein solutions were filtered using Ultracel-3K centrifugal filter devices for the proteome analysis of urinary protein fractions collected from IEF-AF4. Retrieved proteins were washed with water, resuspended in a solution of 8 M urea, 10 mM dithiothreitol, and 0.1 M PBS, and incubated at 37 °C for 2 h to reduce the disulfide bonds. The solution was then treated with 20 mM of iodoacetamide (IAM) in an ice bath for 2 h in the dark, to alkylate the remaining thiol groups. After removing the remaining IAM by adding cysteine (40 times excess to IAM), the solution was diluted with PBS to a final concentration of 1 M urea. The solution was then digested with sequential grade trypsin for 24 h at 37 °C, followed by the deactivation of trypsin with the addition of TLCK (*N*-α-para-tosyl-L-lysine chloromethylketone hydrochloride) at a concentration 10 times that of trypsin. The digested protein solution was desalted using an Oasis HLB cartridge from Waters (Milford, MA), and dried with a SpeedVac for storage.

nLC-ESI-MS-MS. The nanoflow LC-ESI-MS-MS experiment was carried out using the model 1200 capillary LC system from Agilent Technologies (Waldbronn, Germany) using a home-made capillary pulled tip column (17 cm × 75 μm i.d., 360 μm o.d.) interfaced to a LCQ Deca XP MAX ion trap mass spectrometer from Thermo Finnigan (San Jose, CA) with electrospray ionization. The capillary column was packed in methanol with 5 μm-100 Å Magic C18AQ resin from Michrom BioResources, Inc. (Auburn, CA). To desalt the urinary peptide samples, a trapping column (3 cm × 200 μm i.d., 360 μm o.d.) packed with 5 μm-200 Å Magic C18AQ is connected via a PEEK microcross from Upchurch Scientific prior to the pulled tip analytical column, in which one end of the microcross was connected with a Pt wire (applied with 2.0 kV) as an electrode for electrospray ionization.

The nanoflow LC separation was carried out in the pulled tip capillary column with binary gradient separation. The mobile phase compositions were as follows: 98/2 H_2O/CH_3CN for mobile phase A and 95/5 CH_3CN/H_2O for B, both added with 0.1% formic acid. The peptide mixture was injected to the trapping column first from the autosampler with injection amounts of 0.5−1 μg using 5% mobile phase B for 10 min. After sample loading, the gradient elution began with an increase to 15% B for 1 min, then ramped to 32% B for 49 min. For column cleanup, the gradient increased to 80% B for 3 min, maintained at 80% B for 10 min, then returned to 5% B for 3 min where it was held for at least 15 min for column reconditioning. The nLC flow rate was maintained at 200 nL/min during gradient separation, and the eluting peptides were directly sprayed to MS by ESI under 2 kV with an MS precursor scan (300−1800 amu) followed by three data-dependent MS/MS scans (28% normalized collision energy).

Protein identification from the raw MS/MS data was achieved using the TurboSEQUEST search program from Thermo Finnigan (San Jose, CA) using the NCBI database. The mass tolerance for both the precursor peptide ion and the fragment ion was set to 1.0 u between the measured monoisotopic mass and the calculated mass. Data screening for homology was made by selecting peptides yielding the minimum delta-correlation (ΔCn) scores of 0.1 and cross-correlation (Xcorr) values larger than 2.0, 2.5, and 3.3 for single, double, and triple charged ions, respectively.

Results and Discussion

The operation of the modified IEF-AF4 channel shown in Figure 1b is similar to the original multilane channel system (Figure 1a) described in Materials and Methods, with the exception of a slightly longer period of time to transport fractionated sample bands from the IEF channel to the multilane AF4 channel. The IEF-AF4 system was evaluated with mixtures of seven protein standards. Figure 2 shows the two-dimensional separation of seven proteins eluted at different channel lanes according to p*I* values in the first dimension, and separated by size in the second dimension. IEF was carried out at 2.0 kV for 500 s under 1.0% ampholyte solution with a pH range of 3.0−6.0, and flow rate conditions of $V_{in}/V_{out} = 0.84/0.24$ mL/min. The subscripts "in" and "out" of each flow rate represent each AF4 channel inlet and outlet, respectively, thus, the total influx rate for the multilane channel was 5.04 mL/

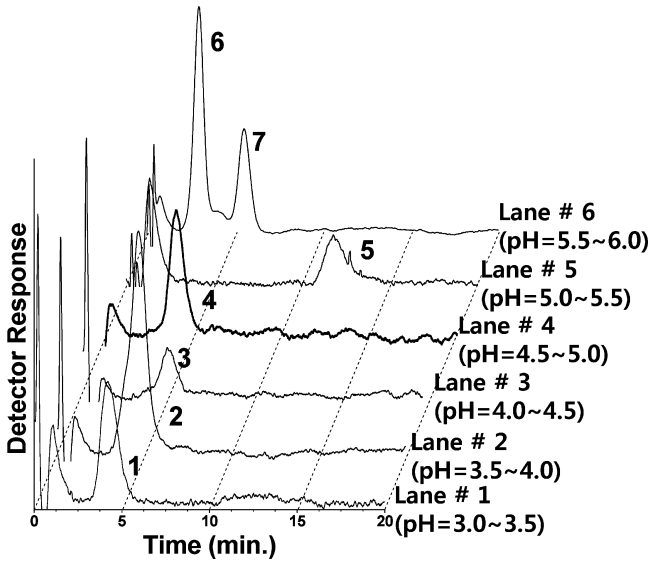

Figure 2. IEF-AF4 fractograms of protein mixtures: peak no. 1, fetuin (48 kDa, pI = 3.3); 2, amyloglucosidase (48 kDa, pI = 3.6); 3, glucose oxidase (66 kDa, pI = 4.2); 4, BSA (66 kDa, pI = 4.8); 5, apoferritin (444 kDa, pI = 5.4); 6, carbonic anhydrase (29 kDa, pI = 5.8); and 7, alcohol dehydrogenase (150 kDa, pI = 5.9). The ideal pH range of each channel lane is expected to be 0.5. IEF was carried out using a 1.0% ampholyte solution (pH 3−6) at 2.0 kV for 500 s. Flow rate conditions for AF4 are V_{in}/V_{out} = 0.84/0.24 in mL/min for each AF4 channel.

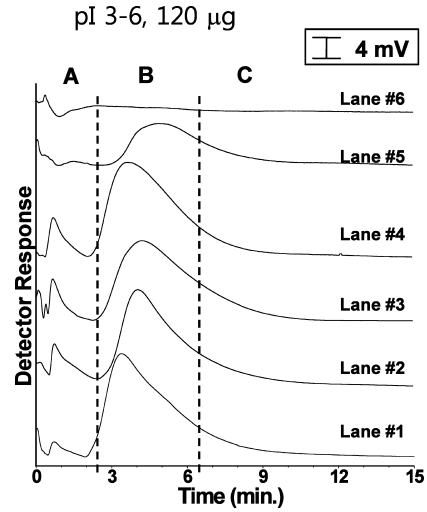

Figure 3. IEF-AF4 separation of human urinary proteome using ampholyte solutions with pH = 3.0−6.0 under the same experimental conditions used in Figure 2. Protein fractions were collected for shotgun proteomic analysis at the end of each channel during the time interval marked in broken lines.

min. From the channel lanes 1−6, it shows individual components belong to the expected pH interval of each channel lane established by the given ampholyte solution (ΔpH = 0.5 for each lane and, i.e., pH of the AF4 channel lane 1 = 3.0−3.5): peak 1, fetuin (48 kDa, pI = 3.3); 2:, amyloglucosidase (48 kDa, pI = 3.6); 3, glucose oxidase (66 kDa, pI = 4.2); 4, BSA (66 kDa, pI = 4.8); 5, apoferritin (444 kDa, pI = 5.4); 6, CA (29 kDa, pI = 5.8); 7, ADH (150 kDa, pI = 5.9). Since the molar mass values of the first four proteins are not significantly different from each other, the first four fractograms (lanes 1−4) appear to elute with only a slight difference in retention times. However, carbonic anhydrase (peak no. 6, 29 kDa, pI = 5.8) and alcohol dehydrogenase (no. 7, 150 kDa, pI = 5.9), eluted at lane 6, show a clear separation according to MW, indicating that AF4 successfully provides the size separation of proteins during elution and that IEF-AF4 resolves larger molar mass proteins (>100 kDa) successfully, as shown in peaks 5 and 7, while 2D-PAGE shows limited capability in resolving larger proteins. The demonstration of IEF-AF4 separation of protein mixtures illustrated in Figure 2 was carried out with an ampholyte with a narrower than normal pH range, and shows the possibility of incorporating a narrow pH range ampholyte solution into the selective isolation of target protein molecules with a small difference in pI values. Moreover, the data in Figure 2 indicates that there is no observable influence of ampholyte solution on either protein separation or detection, indicating that ampholyte solution is already filtered out and fractionated proteins are purified on-line during IEF-AF4 separation. The total injection amount for the 2D separation of protein standards was 70 μg (10 μg each), which shows that sample throughput can be enlarged compared to the limited maximum amount of 40 μg allowed in CIEF-HF5.

The modified IEF-AF4 channel was employed for the 2D fractionation of urinary proteome from a healthy donor. The intact urinary proteome sample was injected into the IEF-AF4 channel and IEF was carried out under the same run condition using different ampholyte solutions: pH = 3.0−6.0 and pH = 3.0−10.0 as described in Figure 2. The AF4 fractograms shown in Figure 3 were obtained at six channel lanes using pH 3.0−6.0 ampholyte. During IEF-AF4 separation, eluting proteins were collected at three time intervals of 0−2.0, 2.0−6.0, and 6.0−15.0 min as represented by the broken lines in Figure 3, and each fraction was accumulated by repeated runs. In the run using pH = 3.0−6.0 ampholyte, 120 μg of urinary proteome sample was loaded for each set of separations and repeated five times. However, with pH = 3.0−10.0 ampholyte solution, each run was injected with 40 μg and repeated 10 times. Nanoflow LC-ESI-MS-MS analysis of collected fractions after tryptic digestion showed an interesting comparison between the two different ampholyte solutions. A closer examination of the precursor MS scan of peptide mixtures from fraction 4C (representing the AF4 fraction C of channel lane 4, expected pH 4.5−5.0) employed with pH 3.0−6.0 ampholyte solution is comparable to the corresponding fraction 2C (pH 4.2−5.3) employed with pH 3.0−10.0 ampholyte as shown in Figure 4. The precursor scan MS spectra shown in Figure 4a,b were observed at 48.3 min of nLC separation for fraction 4C and at 44.2 min for fraction 2C, respectively. Since two different capillary columns were used for each set of analysis, some difference in retention times was observed; however, the selection of Figure 4b was based on the monitoring of the same peptide ions for both runs. For example, peptide ions of m/z 716.6 and 1106.5 marked in Figure 4a were detected in Figure 4b as m/z 716.6 and 1107.3, respectively. While the peptide ions m/z 1106.5 and m/z 1107.3 were identified in both runs (Figure 4a,b) as K.AMLSGPGQFAENEVNFR.E from transcription elongation factor B polypeptide 1 (pI = 4.78) by data-dependent collision induced dissociation (CID) runs, the CID spectrum of m/z 716.6 ion, identified as R.NTGVISVVTTGLDR.E from E-cadherin (pI = 4.58), was observed only in the run using ampholyte solution under a narrower pH range. The loss of m/z 716.6 in Figure 4b data was due to the weak intensity (4.8×10^5) of the peptide ion peak in the precursor scan of Figure 4b, showing that three other peptide ions (m/z 739.1, 1107.3, and 1690.7) with

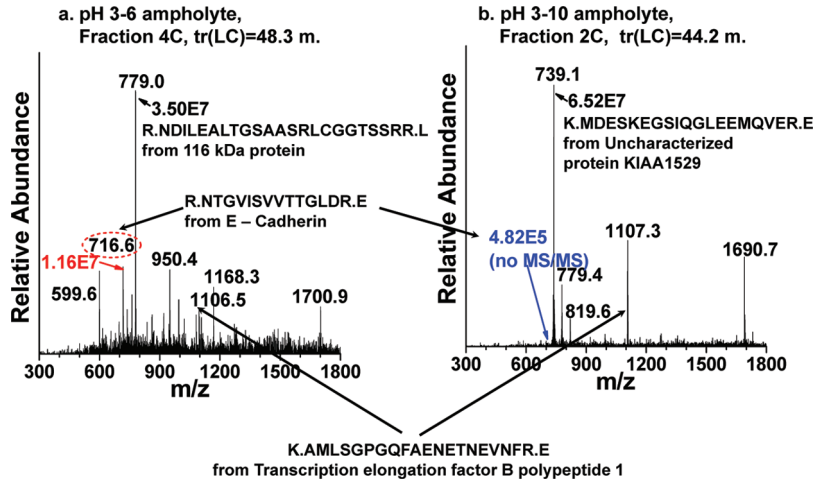

Figure 4. Precursor scan MS spectra of digested AF4 fractions collected by changing ampholyte solutions ((a) pH = 3.0−6.0 and (b) pH = 3.0−10.0). (a) Fraction 4C represents the AF4 fraction C of the channel lane 4 and (b) fraction 2C for the fraction C of the lane 2. The retention time of each spectrum represents the time slice of the corresponding precursor MS scan during nLC-ESI-MS-MS.

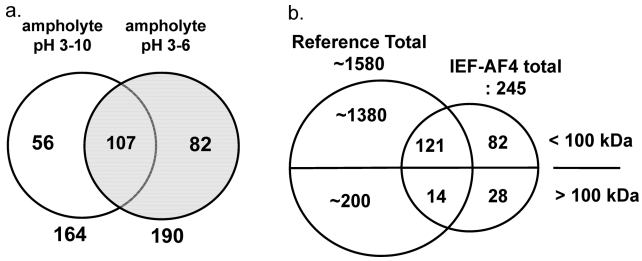

Figure 5. The number of urinary proteins identified from IEF-AF4 separation followed by nLC-ESI-MS-MS analysis of collected fractions after digestion; (a) protein numbers from different ampholyte solutions are compared and (b) from references according to MW values.

relatively high intensities (>∼2×10^7 in intensity) may influence the ionization of ions of relatively low abundance.

Identified proteins from IEF-AF4 followed by nLC-ESI-MS-MS in both ampholyte solutions are listed in the Supplementary Table 1. Overall, a total of 245 human urinary proteins were identified from the database search; however, only 107 of these proteins were common to both experiments, as shown in Figure 5a. This total number is higher than the 114 proteins identified in our earlier work using CIEF-HF5 and nLC-ESI-MS-MS.[9] The number of proteins exclusively identified from the ampholyte solution with pH 3.0−6.0 was 82, while the number of proteins identified using the broader pH ampholyte solution was only 56. Since urine can be simply obtained without an invasive procedure, urinary proteome analysis offers vast amounts of information, as well as aids in the discovery of noninvasive biomarkers. Recent progress in urinary proteome analyses have led to an accumulation of ∼1580 species of human urine proteins.[25−29] Among the 245 proteins identified in this study, as shown in Figure 5b, 135 species were found in literature leaving 110 unique species which have not yet been reported in the literature. These are marked in Supplementary Table 1. Moreover, it was found that 42 proteins were above 100 kDa and 28 among them were unique species first identified in this report and shown in Table 1. This demonstrates the high efficiency separation of the AF4 channel in recovering larger molecular species.

While most studies on urine proteome analysis utilized gel-based or affinity-based methods prior to MS analysis thus requiring a considerable amount (∼1 L) of urine sample, our results began with only 50 mL of urine. This urine was then processed to only about 600 μg of extracted urinary proteome; the resulting protein fractions were retrieved as intact states in solution when separation was finished. This advantage offers the possibility of collecting a target protein at a specific p*I* and MW regime in its intact condition for comparative studies of protein regulation, post-translational modification, and even structural alterations of proteins by mutations. Our study identified few proteins that were reported to cause inherited diseases when deficient or mutated. For instance, oncostatin-M specific receptor subunit beta precursor, alpha-galactosidase A precursor, cathepsin D precursor, and cublin precursor, which were found in the experiment using the narrow pH ampholyte solution, are known to trigger diseases such as primary localized cutaneous amyloidosis,[30] Fabry disease,[31] human neurodegenerative disorder,[32] and megaloblastic anemia,[33] respectively, when they are deficient or mutated pathogenically. While many studies are based on gene level analysis, the developed IEF-AF4 channel can provide an easy retrieval of a specific protein fraction for disease-related studies at the protein level.

Conclusions

This study demonstrates the use of multilane speedway devices for the 2D separation of proteome in an elution system. Evaluation of the modified IEF-AF4 channel system with protein standards showed that the high speed separation of a human urinary proteome sample can be achieved in less than 30 min, and that the collected fractions can be utilized for proteomic analysis using the shotgun method. Since the IEF-AF4 multilane system utilizes bio- and mass-compatible buffer solutions for second-dimension separation (AF4 separation stage), collected protein fractions are still intact, as well as free from impurities or ampholyte. These advantages can negate the need for additional purification steps for subsequent MS analysis and other biological assays. The off-line analysis of IEF-AF4 and nLC-ESI-MS-MS resulted in the identification of 245 urinary proteins, using a sample of only 50 mL of urine. Among the proteins identified, 110 proteins were unique to the literature list of urinary proteins (∼1580 total proteins). In addition, this study was able to resolve large MW protein

Table 1. Large MW Proteins (>100 kDa) from Urine Proteome Analysis Using IEF-AF4 and nLC-ESI-MS-MS

	name	accession	p*I*	MW (kDa)	peptide	p*I* 3−6	p*I* 3−10	reference
1	117 kDa protein	IPI00333858.3	9.2	117.0	2	V		
2	202 kDa protein	IPI00043516.5	6.9	202.3	3	V		
3	Centrosomal protein 110 kDa	IPI00376383.2	5.1	205.0	2	V		
4	Ciliary dynein heavy chain 5	IPI00152653.3	5.7	528.7	2	V		
5	hypothetical protein LOC286077	IPI00394829.3	6.5	127.0	1	V		
6	Insulin receptor substrate 2 insertion mutant (Fragment)	IPI00464978.1	8.7	137.3	2	V		
7	Isoform 1 of Oncostatin-M specific receptor subunit beta precursor	IPI00022674.2	5.5	110.4	2	V		
8	Isoform 1 of Plectin-1	IPI00014898.1	5.6	531.4	2	V		
9	Isoform 1 of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 3	IPI00339381.3	8.7	113.9	1	V		
10	Isoform 4 of Vacuolar protein sorting-associated protein 54	IPI00163718.4	6.1	109.3	2	V		
11	Isoform A of Alpha-fetoprotein enhancer-binding protein	IPI00014186.3	5.8	404.2	2	V		
12	Isoform Alpha-1B-1 of Voltage-dependent N-type calcium channel subunit alpha-1B	IPI00025477.4	8.4	262.3	1	V		
13	Macrophage mannose receptor 2 precursor	IPI00005707.6	5.5	166.5	2	V		29
14	Nuclear receptor coactivator 2	IPI00018251.1	6.2	159.1	1	V		
15	Protein FAM47C	IPI00445315.2	6.7	115.3	1	V		
16	Similar to testis development protein NYD-SP29	IPI00293098.10	5.5	104.8	1	V		
17	Valyl-tRNA synthetase	IPI00000873.3	7.3	140.4	1	V		
18	106 kDa protein	IPI00293088.4	5.7	105.8	5	V	V	25, 29
19	Basement membrane-specific heparan sulfate proteoglycan core protein precursor	IPI00024284.4	6.0	468.5	7	V	V	25−27, 29
20	Ceruloplasmin precursor	IPI00017601.1	5.4	122.1	1	V	V	25, 27−29
21	Collagen alpha-1(VI) chain precursor	IPI00291136.4	5.1	108.5	4	V	V	25, 29
22	Cubilin precursor	IPI00160130.3	5.0	398.5	3	V	V	25, 29
23	Fibrillin-1 precursor	IPI00328113.2	4.7	312.1	4	V	V	25, 29
24	Fibulin-2 precursor	IPI00023824.1	4.6	126.5	1	V	V	29
25	Isoform 1 of Roundabout homologue 4 precursor	IPI00103871.3	6.2	107.4	2	V	V	25, 29
26	Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4 precursor	IPI00218192.2	6.2	101.1	5	V	V	25, 27−29
27	Lysosomal alpha-glucosidase precursor	IPI00783446.1	5.6	105.3	1	V	V	27, 28
28	Pro-epidermal growth factor precursor	IPI00000073.1	5.5	133.9	5	V	V	25, 29
29	132 kDa protein	IPI00384786.4	6.9	131.5	1		V	
30	Cadherin-4 precursor	IPI00024034.2	4.5	100.2	2		V	
31	Dedicator of cytokinesis protein 6	IPI00184772.5	6.3	229.6	2		V	
32	Heat shock regulated-1	IPI00002127.5	5.6	496.1	3		V	
33	Isoform 1 of Attractin precursor	IPI00027235.1	7.0	158.4	3		V	
34	Isoform 1 of DNA-dependent protein kinase catalytic subunit	IPI00296337.2	6.7	468.8	2		V	
35	Isoform 1 of SLIT-ROBO Rho GTPase-activating protein 1	IPI00376259.1	6.4	124.2	3		V	
36	Isoform 1 of Tenascin-R precursor	IPI00160552.3	4.6	149.5	2		V	29
37	Isoform 2 of DNA-directed RNA polymerase I 135 kDa polypeptide	IPI00026445.3	7.4	121.9	1		V	
38	Isoform 4 of Nesprin-1	IPI00247295.3	5.3	1004.6	1		V	
39	Isoform BI-1-GGCAG of Voltage-dependent P/Q-type calcium channel subunit alpha-1A	IPI00012136.1	9.0	282.2	2		V	
40	Low-density lipoprotein receptor-related protein 2 precursor	IPI00024292.1	4.8	521.6	1		V	25, 29
41	Similar to Protein KIAA0226	IPI00022496.5	6.2	124.0	1		V	
42	Similar to Zinc finger protein 440	IPI00472817.2	9.5	102.2	2		V	

species in which 17% of proteins (42 among 245) were larger than 100 kDa. The developed IEF-AF4 system can be applied to fractionate and selectively isolate target protein molecules to monitor disease biomarkers and for clinical or genetic treatments. When a finer selection of protein p*I* intervals can be made by implementing more AF4 channels (above 10 channels in parallel), it may be possible to separate proteins with or without post-translational modifications at different channel lanes, an alternative speedway for therapeutic and clinical studies. The current system is powerful for protoemics study since it provides high speed separation of even large MW protein species; however, it is relatively weak to apply for differential proteomics work at which a large number of samples are often needed to be scanned. While the current IEF-AF4 system attains higher speed and higher throughput separation compared to the preliminary CIEF-HF5 system, improvement is still needed in the area of high resolution separation.

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Supporting Information Available: List of identified proteins from nLC-ESI-MS-MS of peptide mixtures digested from collected IEF-AF4 fractions is listed in Supplementary Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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