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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

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A rapid, non-gel-based, on-line, two-dimensional separation method is introduced for proteome analysis. Protein fractionation was carried out by first exploiting the differences in their respective isoelectric points (pI) in a Teflon capillary using isoelectric focusing (IEF), followed by a molecular weight (MW)-based separation in a hollow fiber by flow field-flow fractionation (FlFFF). The method developed here (CIEF−HFFlFFF) may be a powerful alternative to two-dimensional polyacrylamide gel electrophoresis, which is currently used for the separation and purification of proteins. In CIEF−HFFlFFF, proteins can be collected as a fraction of a certain pI and MW interval without being denatured. Additionally, the ampholyte solution is simultaneously removed during separation in the hollow fiber, and the overall process time is significantly reduced. This method was applied to a human urinary proteome sample, leading to the identification of 114 proteins with the subsequent off-line use of nanoflow liquid chromatography−tandem mass spectrometry after the tryptic digestion of each collected protein fraction.

The analysis of a proteome requires a comprehensive and systematic approach that may include high-performance separation methods, mass spectrometric analysis, or bioinformatics. The characterization of a protein complex mixture is always complicated since there are frequently a large number of proteins that differ widely in molecular weight (MW), isoelectric point (pI), and their hydrophilic or hydrophobic natures depending on the cellular states. The proper separation of proteins/peptides is required prior to mass spectrometric analysis. A need for high-performance separation techniques for proteins is increasing due to the importance of low-abundant proteins, which are expressed at extremely low levels.[1,2]

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE or 2DE) is widely utilized in proteomics due to its simplicity and ability to resolve more than 1000 protein spots from mixtures based on the differences in MW and pI.[3−5] Since separation in the 2DE technique is carried out for two orthogonal dimensions based on the different physicochemical properties of proteins, the separation power of the two-dimensional separation method can be greatly increased by multiplication of the peak capacity of each single method.[6] While 2DE is widely used in proteomic study, it is quite labor-intensive and there are difficulties in handling hydrophobic proteins due to differences in protein solubility.[7−9] In terms of sensitivity, 2DE is relatively poor such that it cannot cover proteins of low abundance.[10,11] Moreover, the isolated proteins are in a denatured form and are trapped within the gel matrix, which can make it difficult to collect proteins in an intact form for further biological applications when necessary. In addition, the entire processes including the 2DE procedure, spot isolation, and sample preparation for mass spectrometric analysis is time-consuming and difficult to fully automate. For these reasons, a non-gel-based, fully automatable, rapid multidimensional separation technology is needed to overcome the limitations of the current separation methods.

Capillary isoelectric focusing (CIEF) is a high-resolution separation method based on the pI of the proteins.[12] Since separation in CIEF is performed in capillary tubing, the sensitivity can be increased in such a way to handle low-abundance proteins, while maintaining the capability of high-resolution protein separation with a pI difference as small as 0.005 pH unit.[12,13] However, the resolution of CIEF has not been high enough to cover complicated protein mixtures. To improve the separation power, numerous efforts have been made to hyphenate CIEF with other separation techniques. The on-line combination of CIEF with various chromatographic separation methods initially began with

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size exclusion chromatography and some model proteins were separated.[14] Capillary reversed-phase liquid chromatography (CRPLC) was added later, and CIEF−CRPLC was applied for the separation of protein digests in the soluble fraction of the Drosophilia proteome with an overall peak capacity of ~1800, out of a total 8 h of operation,[15] and was utilized for the separation of yeast tryptic peptides. This resulted in a decrease in the minimum loading amount.[2] Capillary gel electrophoresis was coupled on-line with CIEF using a dialysis interface for the separation of hemoglobin.[16] A number of efforts to improve the detection limit have been applied to CIEF by coupling it directly to MS;[17−19] however, the removal of the ampholyte used in CIEF was a necessary step prior to analysis with mass spectrometry (MS). MS was further integrated with on-line CIEF−RPLC−MS for a high-resolution, two-dimensional protein/peptide separation and detection method.[3,20] The latter[3] demonstrated the capability of detecting proteins at low-femtomolar levels with little or no interference from the ampholyte by using a microdialysis membrane-based cathodic cell. However, the RPLC separation requires the use of an organic solvent, which changes the protein conformation and offers protein/peptide fractionation only on the basis of hydrophobicity. In addition, possible protein interactions with the column packing materials may incur a loss or a shear induced degradation of the proteins.

Hollow fiber flow field-flow fractionation (HFFlFFF), a variant of FlFFF subtechniques, is a separation method applicable for biological macromolecules such as proteins, DNA, and cells.[21−25] FlFFF, when compared to chromatographic methods, is an alternative technique for protein separation that is based on the hydrodynamic diameter. It bypasses the potential problems of shear degradation or the adsorption of proteins onto the column packing materials. Furthermore, it is capable of fractionating proteins of large molecular weight (>~10^5 Da), in which case, gel electrophoresis is hardly effective. Separation in HFFlFFF is carried out in a hollow fiber (HF) membrane with a rate control of axial flow, which is along the fiber axis, and radial flow, which exits through the pores of membrane wall. The radial flow plays a role of driving sample components in the vicinity of the membrane wall. When radial flow is applied, proteins form steady-state equilibrium distributions close to the wall having mean layer thicknesses related to their diffusion coefficients. According to the basic FlFFF theory,[26,27] a smaller MW component that has a faster diffusion is located at an equilibrium position further away from the channel wall than the larger one. When axial flow with a parabolic flow profile in the HF is applied, the smaller MW components elute at a higher flow velocity than larger ones, and therefore, components are separated with respect to increasing MW. Recently, HFFlFFF has been gaining interest as an alternative to the conventional FlFFF channel system since it can be developed into a disposable channel, which offers the advantage of reducing carryover problems that are possible when handling biological samples. Additionally, HFFlFFF also performs separations comparable to conventional rectangular channel systems.[23,28] Since the first report of HFFlFFF,[29] it has been used to separate submicrometer- to supramicrometer-sized particles, proteins, bacteria, cells, and polymers.[23,24,30−33] The on-line hyphenation of HFFlFFF and electrospray ionization time-of-flight (ESI-TOF) MS has shown its potential toward the direct characterization of proteome samples at the protein level.[25] Recently, protein separation by HFFlFFF at microflow rates was demonstrated using microbore HF (450-μm i.d.), and the detection limit was decreased to 0.45 pmol of bovine serum albumin (BSA).[34]

In this study, microbore HFFlFFF was hyphenated with CIEF as a non-gel-based 2D separation technique for intact proteins. The CIEF was carried out in Teflon tubing instead of a silica capillary to minimize electroosmotic flow (EOF). In this experiment, the CIEF segment adopted the microdialysis membrane-based cathodic cell method that was recently reported.[3] After CIEF, the fractionated proteins were pushed to the injection loop of the HFFlFFF with an anolyte by using a syringe pump that could very accurately control the injection volume, and protein separation was then achieved according to MW by HFFlFFF. The experimental setup reported in this study can be fully automated. Since the HFFlFFF separation is carried out with a buffer solution that is free of an organic solvent or surfactant, the proteins can be collected in their intact forms. The developed technique provides a distinct advantage by removing the ampholyte solution simultaneously during the HFFlFFF separation of the protein bands, since it may be easily removed through the membrane wall of the HF along with radial flow. Initial evaluations were made with the separation of several protein standards using an ampholyte with pI values ranging from 3 to 10. The separation efficiency was examined with respect to variations in the ampholyte concentration on the basis of reproducibility and sample recovery. The developed method was used for the fractionation of a human urinary proteome, and the collected fractions were subjected to a tryptic digestion for the shotgun analysis of the peptides by nanoflow liquid chromatography/tandem mass spectrometry (LC−MS−MS). This resulted in the identification of 114

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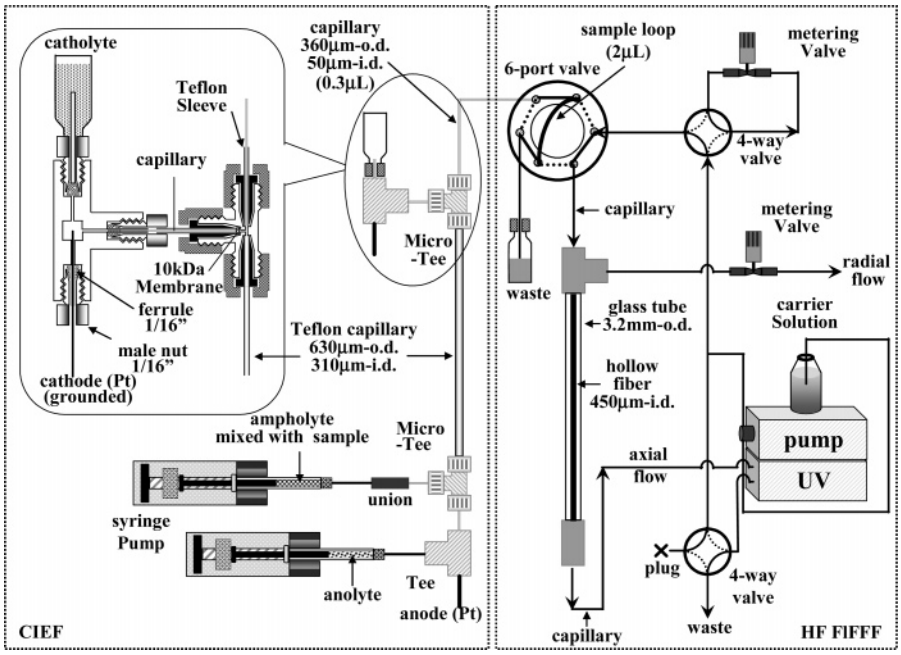

Figure 1. Schematic of the on-line integration of CIEF with HFFlFFF. For details, see the Experimental secction.

proteins including well-known biomarkers of acute phase reactive proteins.

EXPERIMENTAL SECTION

Materials and Reagents. Teflon tubing (310-μm i.d., 610-μm o.d.) used for the CIEF was obtained from Cole-Parmer Inc. (Vernon Hills, IL). Fused-silica capillaries with three different dimensions (50-, 75-, and 200-μm i.d., 360-μm o.d.) from Polymicro Technologies (Phoenix, AZ) were used for tubing connections. HF with a dimension of 450-μm i.d., 720-μm o.d. and a molecular weight cutoff of 30 kDa was obtained from Kolon Central Research Institute (Yongin, Korea). Ammonium bicarbonate and sodium hydroxide were supplied by Sigma (St. Louis, MO), and phosphoric acid was obtained from Merck (Darmstadt, Germany). The ampholyte solution (Fluka Ampholyte High-Resolution pH 3−10) for CIEF and all protein standards tested in this study were acquired from Sigma. All solutions of anolyte and catholyte were prepared with ultrapure water (>18 MΩ·cm) and filtered through a membrane filter (0.10 μm) before use. The human urine sample used in this study was the morning midstream urine collected from a patient with an informed consent and ethics approval by the Royal Melbourne Hospital Ethics Committee. The extraction of proteins from the urine sample was carried as follows. A 60 mL of the urine sample was resuspended with a protease inhibitor cocktail tablet, Complete EDTA-free, from F. Hoffmann-La Roche Ltd. (Basel, Switzerland), and the mixture was centrifuged at 1500*g* for 10 min at 4 °C to remove cell debris. The supernatant urine solution was filtered with an Amicon-30 membrane kit (MWCO of 30 kDa) from Millipore (Bedford, MA) at 5000*g* for 1 h, and the filtrate was stored in a polystyrene tube on ice. The retentate was washed by 30 mL of 25 mM ammonium bicarbonate buffer at 3000*g* at 4 °C for 20 min to remove remaining salts and any interfering materials against further analysis. The retentate was retrieved by centrifuging the cartridge in an inverted way. Protein concentration was measured by Bradford assay from Bio-Rad (Hercules, CA), and the resulting protein solution was stored at −80 °C.

Construction of On-Line CIEF−HFFlFFF. CIEF was performed in a 9.5-cm-long Teflon tubing (310-μm i.d.), both ends of which were connected with a micro-tee from Upchurch Scientific (Oak Harbor, WA), as shown in the left side of Figure 1. At the anodic side of the Teflon tubing, an ampholyte solution mixed with proteins was loaded into the CIEF Teflon tubing by a syringe pump through the micro-tee while the other port of the micro-tee, located at the bottom of Figure 1, was connected via silica capillary tubing to a hand-tight Delrin tee from Upchurch Scientific. The tee at the bottom of Figure 1 was contacted with a Pt electrode as the anode at one port. The other tee port was connected with the tubing from the syringe pump for the delivery of the anolyte (20 mM H_3PO_4) to the Teflon CIEF tubing so that the anolyte could push the isoelectrically focused protein bands toward the HFFlFFF system. The cathodic side of the Teflon tubing was connected to another micro-tee of which one port, in the upper portion of Figure 1, led to the sample loop (2 μL) of the six-port valve from Rheodyne (Cotati, CA) via fused capillary tubing (50-μm i.d./360-μm o.d.). The perpendicular port, located in the left side of Figure 1, was in contact with a small piece of 10-kDa membrane for microdialysis and was also in contact with a capillary tubing (200-μm i.d./360-μm o.d.) fitted with a Delrin tee from Upchurch Scientific to create an electrical contact as shown in the inset of Figure 1. One port of the Delrin tee in the inset diagram was connected to a Pt electrode (cathode), which was electrically grounded; the other port was connected with a small reservoir containing the catholyte (20 mM NaOH) in the vertical direction. With this configuration, any bubbles generated around the cathode could be absorbed by the catholyte reservoir.

The microbore HFFlFFF module was constructed in our laboratory as reported previously.[34] The module consisted of a 25-cm-long microbore HF (made of polysulfone), which was inserted into a piece of glass tubing with dimensions of 3.2-mm o.d. and 1.6-mm i.d. Both ends of the hollow fiber inside the glass tubing were connected with silica capillary tubing (100-μm i.d./360-μm o.d.) by means of a union at one end and a Delrin tee at

the other end. Such connection was made without using glue, and additional details are explained elsewhere.[34] Since the hollow fiber extended through the tee connector, the radial flow penetrating the HF membrane surface could exit via the tee. For the HFFlFFF separation of protein bands from the CIEF, each 1−2 μL protein band was delivered to the sample loop of the six-port valve by a syringe pump with the anolyte. Then the sample was introduced to the hollow fiber by an SP930D solvent delivery pump from Young-Lin Instrument (Seoul, Korea). The carrier solution was used with a 10 mM NH_4HCO_3 solution that was prepared from ultrapure water (>18 MΩ·cm) and filtered with a membrane filter with a pore size of 0.22 μm prior to use. The HFFlFFF operation was divided into two steps: (1) sample injection, shown by the dotted line connection of all valves at Figure 1, while two flow streams were introduced at both the channel inlet and outlet, and (2) separation, shown by the solid line connections. In HFFlFFF, the first step (called the focusing/relaxation procedure for the injected sample) is necessary to establish the equilibrium states of the sample components prior to separation. To carry out the focusing/relaxation in HFFlFFF, the pump flow was divided into two parts (1:9 ratio). One part (1/10) entered the fiber inlet at one-tenth of the total flow rate by adjusting the metering valve connected at the four-way valve (upper right side of Figure 1) as shown by the dotted line configuration. The rest of flow (9/10) entered through the fiber outlet. During this step, the sample components were expected to reach equilibrium positions against the inner wall of the fiber at the 1/10 position of the fiber length. After allowing 60 s for the sample to enter the fiber inlet and for focusing/relaxation, all valve configurations were switched back to the solid line connection (Figure 1) so that flow from the pump was directed toward the fiber inlet only and initiated separation. During HFFlFFF separation, the control of the ratio of the outflow rate to the radial flow rate was accomplished using another metering valve at the radial flow outlet. For the HFFlFFF separation of intact protein bands from the CIEF runs, flow rates of 0.6 mL/min for the inlet flow and 60 μL/min for the outlet flow were used. During the HFFlFFF separation, the protein bands of the next pI intervals that were kept inside the CIEF tubing under an electrical field (300 V/cm) were sequentially pushed to the sample loop for the next HFFlFFF runs. This process could be repeated for as many pI intervals as desired.

CIEF−HFFlFFF of Human Urinary Proteome. When the human urinary proteome sample was fractionated by CIEF−HFFlFFF, ~40 μg of protein extracts (mixed in the ampholyte solution at a concentration of 5.5 μg/μL) was loaded into the CIEF tubing. After isoelectric focusing, six pH band fractions were sequentially loaded into the HFFlFFF system for MW-based separation. During each HFFlFFF separation of the six CIEF urine protein bands, four fractions were collected after a 5-min collection period at the end of the HF module. A total of 24 fractions were tryptically digested for nanoflow LC−MS−MS analysis of the peptides.

Digestion of Urine Protein Fractions. Protein fractions collected during the HFFlFFF runs were quantified using the Bradford method. Each fraction was dried by an Autospin 314U vacuum centrifuge (BioTron, Seoul, Korea) and resuspended in a solution of 8 M urea, 0.1 M NH_4HCO_3, and 10 mM dithiothreitol. After 2 h of incubation, the thiol group was alkylated with iodoacetamide at a total concentration of 20 mM for 2 h at 0 °C in the dark. Then excess cysteine (40×) was added to treat the excess iodoacetamide, and the mixture was diluted into 1.0 M urea. A proteomics grade trypsin (Sigma) was added at a ratio of 50:1 (trypsin/protein), and the mixture was incubated for 24 h at 37 °C. After digestion, TLCK was added to stop the digestion at a slight excess to the number of moles of peptides. The digested mixture was finally desalted using an Oasis HLB cartridge (Waters, Milford, MA), dried, and resuspended in 2% CH_3CN in water for nanoflow LC−MS−MS analysis.

Nanoflow LC−ESI-MS−MS. The nanoflow LC−ESI-MS−MS experiment was carried out using a CapLC equipped with a Q-TOF Ultima mass spectrometer (Waters) with a homemade pulled tip capillary column (75-μm i.d., 360-μm o.d., 15 cm) and an end frit at the tip. The pulled tip column was packed with a methanol slurry of 5-μm, 100-Å Magic C_{18AQ} (Michrom BioResources Inc. Auburn, CA), and the detailed procedures were explained in refs 35 and 36. For on-line sample desalting, a trapping column was made with silica tubing (200-μm i.d., 360-μm o.d.) in which the end frit (2 mm in length) was prepared by a sol−gel preparation and was packed with 5-μm, 200-Å Magic C_{18AQ} for 1 cm. The trapping column and the analytical column were connected via a PEEK microcross, and a platinum wire was used as an electrode to supply the electrospray ionization voltage and was described elsewhere.[37] For the nanoflow LC−MS−MS experiments of each urine proteome fraction, 1.0 μL (100−500 ng for each fraction) of digested peptide mixture from each fraction was injected via an autosampler to the trapping column. After loading, a binary RP gradient elution (mobile-phase composition of (A) 3% CH_3CN in water and (B) 95% CH_3CN in water, both containing 0.1% HCOOH) was pumped through the column and the effluent was fed into the mass spectrometer via the ESI method. The eluant was added in a gradient that began with 5% B (from 2% B at default) for 5 min and was increased to 12% for 25 min and then to 22% B for 60 min. It was then ramped to 80% B over 3 min and was maintained at this level for 10 min, after which it was decreased to 5% B over 2 min and maintained at this level for at least 25 min for column reconditioning. The flow rate during the gradient separation remained at 200 nL/min, and the eluted peptides were directly electrosprayed into the mass spectrometer with a spray voltage of 2.0 kV in the positive mode of ionization. Peptide ions were detected in the data-dependent analysis mode with an MS precursor scan (200−1800 amu) followed by three data-dependent MS−MS scans. For data analysis, the collected raw MS/MS spectra were analyzed with the Mascot Search program using both Swiss-Prot and NCBI human databases. The mass tolerance used to accept was 1.0 amu for both molar masses of the precursor peptide and peptide fragment ions. For screening the search data, only peptides yielding larger than a minimum Mascot score of 30 were accepted as an extensive homology.

RESULTS AND DISCUSSION

Validation of CIEF−HFFlFFF with Protein Standards.. An evaluation of the CIEF−HFFlFFF method was performed through

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the separation of standard protein mixtures: myoglobin (15 kDa, pI 6.8), typsinogen (24 kDa. pI 9.3), carbonic anhydrase (29 kDa, pI 5.85), BSA (66 kDa, pI 4.8), and yeast alcohol dehydrogenase (YADH; 150 kDa, pI 6.23). At the beginning of the CIEF run, an ampholyte solution (5% v/v) mixed with protein mixtures (125, 200, 50, 180, and 250 ng, respectively, at the above) was loaded to the CIEF Teflon tubing having a volume of 7.2 μL using a syringe pump as shown in Figure 1. Isoelectric focusing was performed with an electric field of 500 V/cm for 20 min until the focusing was completed, after which the field was reduced to 300 V/cm and maintained at this level until the end of the HFFlFFF separation. After isoelectric focusing, a small volume of the anolyte solution (20 mM H_3PO_4) was delivered to the CIEF tubing with the strict control of the delivery volume using another syringe pump such that a very accurate volume of a focused protein band could be injected into the sample loop of the HFFlFFF system. For standard proteins, four different fractions (pH intervals of 3−5, 5−6, 6−8, and 8−10) were sequentially separated by HFFlFFF. After the first fraction (pH 8−10, ~2 μL) was loaded in the sample loop of the six-port valve for HFFlFFF separation, the valve configuration (including two 4-way valves) was changed as represented by the dotted line connection in Figure 1 so that the sample was injected to the HF. This is discussed in greater detail in the Experimental Section. After the sample band was injected into the HF module, all valve configurations were returned to the solid line configuration shown in Figure 1 and the separation was initiated. The fractogram (UV detector signal) of the HFFlFFF run of the first fraction (Figure 2a) showed the elution of the trypsinogen (pI 9.3) within 6 min. The carrier solution used for the HFFlFFF experiments was 10 mM NH_4HCO_3. The small peak at the beginning of the HFFlFFF run represents the void peak caused by a pulse during the valve conversion. It was found in the FlFFF fractogram of the second CIEF fraction (pH 6−8) that two protein standards (myoglobin and YADH) were well resolved with a shoulder peak that was presumably from dimers of myoglobin that eluted after peak 1. The other two CIEF fractions appeared to be well resolved according to their MW. When the same protein mixtures were run by HFFlFFF without an initial CIEF, components 1−3 were not completely resolved at the flow rates used and the fractogram (marked as "without CIEF") is shown in Figure 2a. Especially for components 1−3, a complete separation was not achieved since their values were too close to be resolved by HFFlFFF alone. Interestingly, as seen in the HFFlFFF fractogram of the second CIEF fraction, YADH (peak 5) eluted without dissociating into subunits. This was confirmed in two ways. The retention times of the YADH (peak 5) observed in the two fractograms with or without CIEF were nearly the same, which indicated that the molecular conformation of YADH was not altered during CIEF. A 2DE experiment for the same protein mixtures (a total of 50 μg) revealed that YADH was detected at a location between 36.5 and 55 kDa according to the marker proteins in the right side of the gel shown in Figure 2b, while all of the other proteins appeared at locations corresponding to their MWs. Thus, the subunits of YADH were presumed to be dissociated under the SDS solution used for the 2DE. This demonstrates that the CIEF−HFFlFFF method developed here can allow for the separation of intact proteins and allow for the collection of protein fractions in their intact forms without SDS or an ampholyte

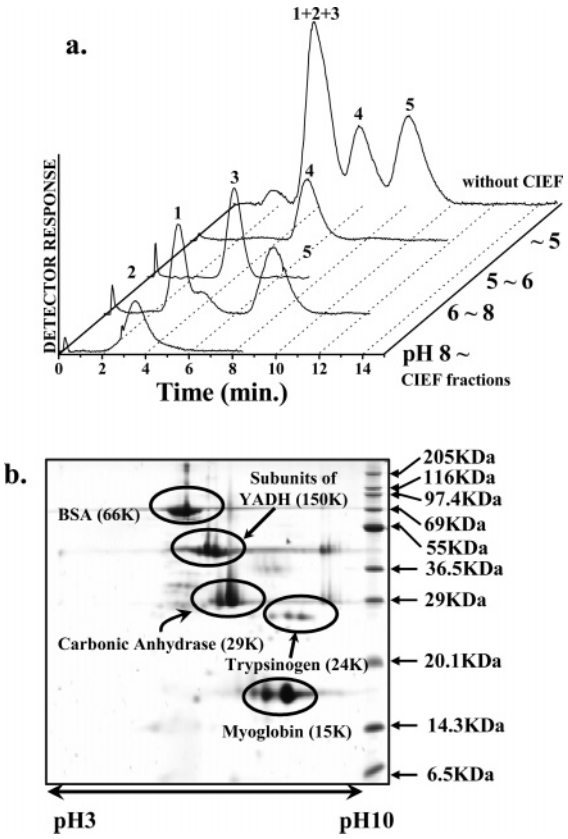

Figure 2. (a) HFFlFFF fractograms of various proteins without CIEF and after CIEF: (1) horse myoglobin (16.9 kDa, pI 7.2), (2) trypsinogen (24 kDa, pI 9.3), (3) carbonic anhydrase (29 kDa, pI 5.85), (4) BSA (66 kDa, pI 4.8), and (5) YADH (yeast alcohol dehydrogenase, 150 kDa, pI 6.23). Flow rates were 0.6 mL/min for the inlet flow and 60 μL/min for the outlet flow. After CIEF, protein bands were injected at four consecutive pH intervals (indicated as pH 3−5, 5−6, 6−8, and 8−10) and HFFlFFF separation after each injection of the protein band from CIEF was repeated. Flow rate conditions for all HF FlFFF runs were the same at 60 and 540 μL/min for the outflow rate and the radial flow rate, respectively. (b) The scanned image of 2D-PAGE for five proteins. The gel was stained with Coomasie Blue. It is noted that YADH appeared as dissociated subunits.

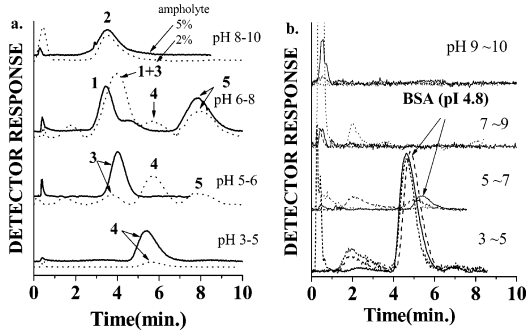

Figure 3. (a) Effect of ampholyte concentrations (2 and 5%) on the separation efficiency of CIEF was represented with the HFFlFFF fractograms of the four CIEF fractions. (b) The reproducibility of the CIEF−HFFlFFF separation of BSA. Flow rate conditions used for the entire HF FlFFF separation were the same as given in Figure 2a.

solution. The ability to conduct the on-line purification of protein fractions was another advantage of using CIEF−HFFlFFF since the ampholyte solution and other salts contained in the protein solution could be removed through the HF membrane pores during the HFFlFFF separation. In addition, the entire fraction-

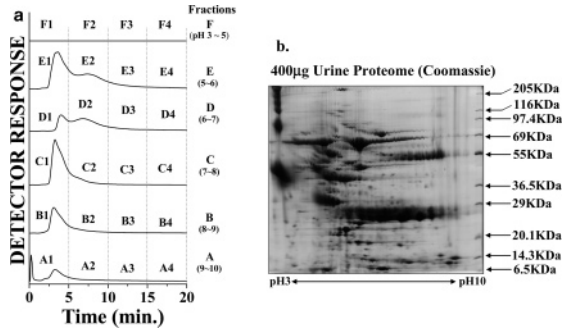

Figure 4. (a) CIEF−HFFlFFF fractionation of a human urinary proteome sample. The HFFlFFF fractograms for the six CIEF fractions of the urine proteins (~40 μg). (b) A scanned image of the 2D-PAGE for the same urine proteome sample (~400 μg).

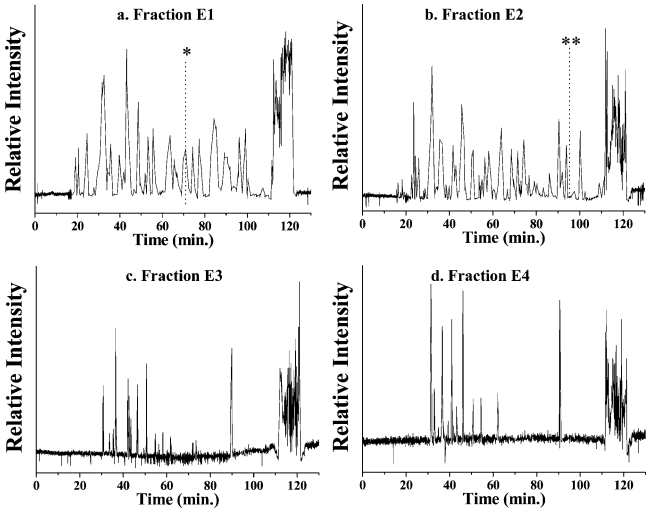

Figure 5. BPCs of the four CIEF−HFFlFFF fractions (E1−E4) by nanoflow LC−MS−MS after the tryptic digestion of each protein fraction. For a detailed explanation of the binary gradient conditions of the RPLC run, see the Experimental Section in the text.

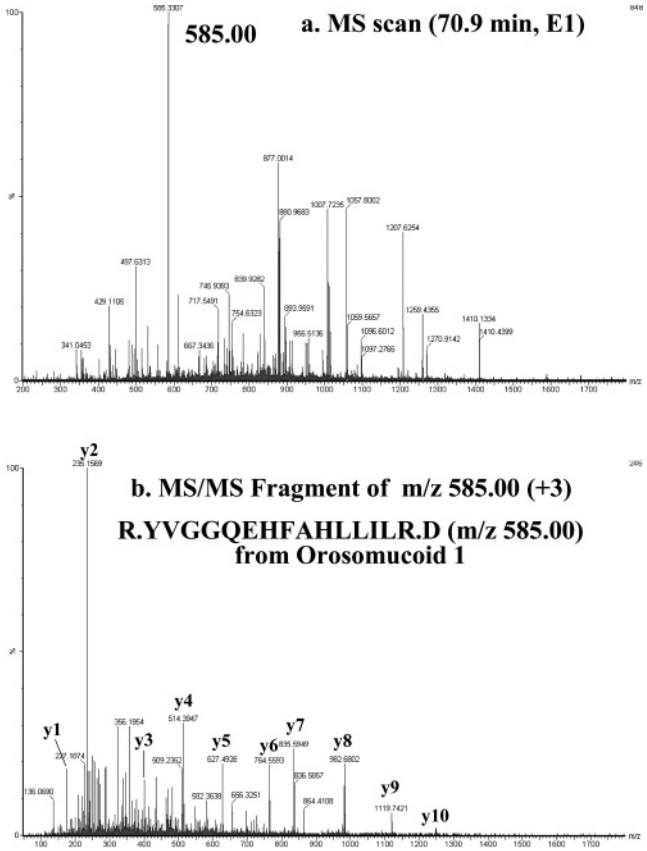

Figure 6. (a) MS spectrum for the precursor scan at 70.9 min (marked as * at Figure 5a) of the LC effluent for the peptide mixtures from the CIEF−HFFlFFF fraction E1 and (b) the CID spectrum of *m*/*z* 585.00 (triply charged), which was identified as a peptide from orosomucoid 1.

ation time can be greatly reduced compared with the 2DE method, which normally requires ~1.5 days.

To optimize the CIEF run condition, the efficiency of CIEF was tested at different ampholyte concentrations. While the concentration of the ampholyte solution used for the results shown in Figure 2 was 5% (v/v), resolution of the CIEF at a lower concentration was not good compared to that shown in Figure 2a. Figure 3a shows the efficiency of the CIEF run by comparing the HFFlFFF fractograms obtained at two different concentrations of ampholyte: 5 (solid lines) and 2% (dotted lines). It was shown that the second CIEF fraction (pH 6−8) with 2% ampholyte contained the components 3 and 4, which were expected to be in the third and fourth fractions, respectively. This showed that the CIEF performance was not adequate at a 2% ampholyte concentration. In addition, the separation was not improved when ampholyte concentrations higher than 5% were used. From these results, the ampholyte concentration was fixed at 5% for this length of CIEF tubing.

The reproducibility and recovery efficiency of the CIEF−HFFlFFF system were evaluated by injecting 300 ng of BSA into the system, and the fractograms of these three repeated operations are superimposed in Figure 3b. The average retention time of BSA from the pH 3−5 fraction was calculated to be 4.68 min with 3.0% of RSD (n = 3), and the average recovery value calculated from the peak area was 88.4 ± 0.1% compared to the peak area measured from an HFFlFFF run for BSA without CIEF. There appeared to be some loss in recovery due to the early elution of a few of the BSA species along with the pH 5−7 fraction. This was induced to some degree by EOF, which drove some focused bands toward the cathode. While a Teflon tube was adopted for the current CIEF run in order to minimize EOF, this loss of recovery revealed that contributions of EOF were not completely eliminated.

CIEF−HFFlFFF of a Human Urinary Proteome and Nanoflow LC−MS−MS for Protein/Peptide Identification. The developed CIEF−HFFlFFF method was applied for the fractionation of a human urinary proteome sample. The urine sample was filtered with a membrane filter having a MWCO of 30 kDa, and the protein fraction with sizes larger than 30 kDa was utilized. Approximately 40 μg of the urine protein mixture was loaded into the Teflon capillary for CIEF, and the focused sample bands were injected into the HFFlFFF system with six different volume fractions in a sequential injection order of pH intervals 9−10, 8−9, 7−8, 6−7, 5−6, and 3−5. The protein amount loaded was the maximum for the current CIEF experimental setup. Since human urinary proteome sample contains some high-abundant proteins such as albumin, a considerably large amount of protein mixtures is examined in order to analyze low-abundant proteins. The six pH intervals were chosen arbitrarily. When it is

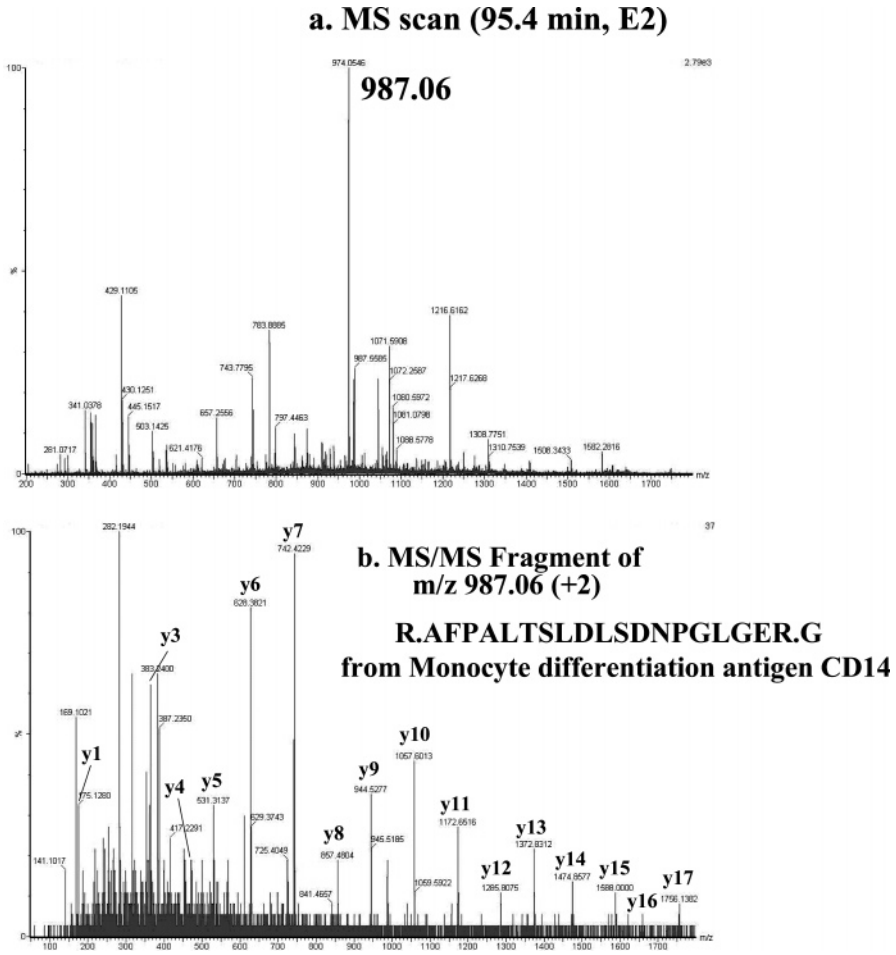

Figure 7. (a) MS spectrum of the precursor scan at 95.4 min (marked as ** at Figure 5b) of the LC effluent for the peptide mixtures from the CIEF−HFFlFFF fraction E2 and (b) the CID spectrum of *m*/*z* 987.06 (+2), which was identified as a peptide from monocyte differentiation antigen CD14.

needed to examine a fine pH interval of a protein fraction, it can be divided into more intervals as needed. However, efficiency of a fine pH interval was not evaluated in this study. The six pH fractions were resolved by HFFlFFF, and the corresponding fractograms are shown in Figure 4a along with the time interval of each subfraction collected during the HFFlFFF run. As the pH of the CIEF fraction decreased, the intensity of the peak observed below 5 min of retention time appeared to increase significantly (from CIEF fractions A−C), and the secondary peak after 5 min began to appear below pH 6−7. This indicated that the pI values of proteins with MW larger than 100 kDa were smaller than 7. However, apparently no significant signal was observed in the HFFlFFF fractogram of CIEF fraction F. This may be a result of the EOF, which drove some proteins toward the cathodic side (toward the fraction E), since the lowest pH fraction remained in the CIEF tubing the longest time. The presence of the low pH fractions was confirmed with a few spots at the pH 3 regime of the 2DE plate shown in Figure 4b, which was conducted with 400 μg of the same urine proteome sample. It was shown that a considerable amount of protein was distributed in the upper left of the 2DE gel plate, which corresponded to the low pH and higher MW regime. Therefore, the HFFlFFF fraction, E2, may contain these proteins due to the EOF. This may be one of the drawbacks of CIEF−HFFlFFF. However, while the entire operation of 2DE took ~36 h to obtain a complete separation, not including the time required for the identification of each protein spot, protein separation was achieved in less than 3 h with CIEF−HFFlFFF including all six HFFlFFF runs and channel reconditioning. For protein identification after the 2DE experiment, each spot in the 2DE plate must be removed, enzymatically digested, and cleaned up to remove surfactants and salts prior to mass spectrometric analysis.

In this study, each HFFlFFF fraction was collected over 5-min intervals followed by a digestion with trypsin and the resulting peptide mixtures were analyzed by nanoflow LC−MS−MS. The LC−MS chromatograms obtained from the four HFFlFFF fractions (E1−E4) are shown in Figure 5. Each chromatogram is presented with the base peak chromatogram (BPC) from a precursor scan of the MS experiment. While fractions E1 and E2 showed numerous peptide peaks, fractions E3 and E4 appeared to have only a few peptide peaks. Each eluting peptide was examined further with collision-induced dissociation (CID) employing the data-dependent MS−MS method, and the two CID spectra are shown in Figures 6 and 7. The mass spectrum of the precursor scan at 70.9 min (marked as * in Figure 5a) of fraction E1 is shown in Figure 6a. The CID spectrum of $m/z = 585.00$ (+3, triply charged) shown in Figure 6b yielded an identification of the peptide R.YVGGQEHFAHLLILR.D from orosomucoid 1. Figure 7 shows the results identified as a monocyte differentiation antigen CD14 with a peptide sequence of R.AFPALTSLDLSDN-

Table 1. Identified Human Urinary Proteins by CIEF−HFFIFFF and Nanoflow LC−ESI-MS−MS[a]

AC no.	identified proteins	pI	MW	no. pept
CIEF Fraction A				
gi_20372502	anti-acetylcholine receptor immunoglobulin κ light chain	9.34	12 002	1
gi_230581	chain H	8.46	50 444	1
Q99988	growth/differentiation factor 15	9.79	34 660	1
P69905	hemoglobin α chain	8.73	15 174	1
gi_229585	Ig A1 Bur	9.24	74 642	1
P01834	Ig κ chain C region	5.58	11 773	1
P80362	Ig κ chain V−I region WAT	5.08	11 844	1
P01619	Ig κ chain V−III region B6	9.34	11 628	1
P01842	Ig λ chain C regions	6.92	11 401	1
gi_510844	IgM	9.06	11 929	1
gi_1322200	immunoglobulin κ	10.19	11 981	1
P61626	lysozyme C	9.38	16 982	3
P15586	*N*-acetylglucosamine 6-sulfatase	8.6	62 840	2
O75594	peptidoglycan recognition protein	8.92	22 116	2
gi_229528	protein Len	9.23	24 499	2
gi_5031925	proteoglycan 4	9.53	152 195	1
Q13891	transcription factor BTF3 homologue 2	10.35	7 601	1
CIEF Fraction B				
O14639	actin-binding LIM protein 1	8.88	87 588	1
Gi_5360679	anti-Entamoeba histolytica immunoglobulin κ light chain	8.26	23 576	1
Gi_3721651	anti-HBsAg immunoglobulin Fab κ chain	8.3	23 783	3
P02749	β-2-glycoprotein I	8.34	39 584	1
P00751	complement factor B	6.67	86 847	1
Q15828	cystatin M	8.31	16 785	1
Q08495	dematin	8.94	45 600	1
Q15054	DNA polymerase δ subunit 3	9.38	51 653	1
P02675	fibrinogen β chain	8.54	56 577	1
P01857	Ig γ-1 chain C region	8.46	36 596	3
P01771	Ig heavy chain V−III region HIL	9.43	13 671	1
P01765	Ig heavy chain V−III region TIL	9.24	12 462	1
P01603	Ig κ chain V−I region Ka	9.01	12 006	1
P18135	Ig κ chain V−III region HAH	7.74	14 178	1
P01620	Ig κ chain V−III region SIE	8.7	11 882	3
gi_P01700	Ig λ chain V−I region HA	9.07	12 003	1
Q9UL16	nasopharyngeal epithelium specific protein 1	9.99	46 253	1
P41222	prostaglandin-H2 D-isomerase	7.66	21 243	1
Q9Y252	RING finger protein 6	9.16	78 444	1
gi_16554039	unnamed protein	8.4	65 755	5
CIEF Fraction C				
P68871	hemoglobin β chain	6.81	15 971	4
P02790	hemopexin	6.55	52 385	2
31873233	hypothetical protein	8.13	56 994	1
P01859	Ig γ-2 chain C region	7.66	36489	3
P01861	Ig γ-4 chain C region	7.18	36 431	2
7438712	Ig κ chain NIG93	7.85	23 726	6
P01621	Ig κ chain V−III region NG9	6.29	10 836	2
P01625	Ig κ chain V−IV region Len	7.92	12 746	2
P04208	Ig λ chain V−I region WAH	6.29	11 832	1
21669331	immunoglobulin κ light chain VLJ region	7.6	29 191	1
Q5G863	neutrophil defensin 1	6.54	10 531	1
Q9NRM7	serine/threonine protein kinase LATS2	8.44	120 194	1
P02787	serotransferrin	6.81	79 280	12
P49815	tuberin	7.06	202 732	2
21410211	unknown	7.59	25 350	1
Q9H0A0	UPF0202 protein KIAA1709	8.5	116 543	1
CIEF Fraction D				
P01023	α-2-macroglobulin	6	164 600	4
P02760	AMBP protein	5.95	39 986	6
11275302	anti TNF-α antibody light chain	6.19	23 787	1
11118905	anticardiolipin immunoglobulin light chain	6.45	10 396	1
410564	β-trace	6.13	2 891	1
P27708	CAD protein	6.02	245 167	1
Q9UBR2	cathepsin Z	6.7	33 868	1
CIEF Fraction D				
P13987	CD59 glycoprotein	6.02	14 177	1
223130	fibrinogen β B 1−118	6.17	12 891	1
P06396	gelsolin	5.9	86 043	1
P00738	haptoglobin	6.13	45 861	8
34785974	HP protein	6.06	25 727	1
6808233	hypothetical protein	6.33	27921	1
P01876	Ig α-1 chain C region	6.08	38 486	1
P01766	Ig heavy chain V−III region BRO	6.45	13 332	1
P01602	Ig κ chain V−I region HK102	6.07	12 931	1
P01714	Ig λ chain V−III region SH	6.02	11 500	1
P01717	Ig λ chain V−IV region Hil	6.04	11624	1
42760294	immunoglobulin λ-1 variable region	6.56	11 479	1
Q14624	inter-α-trypsin inhibitor heavy chain H4	6.51	103 522	2
P02750	leucine-rich α-2-glycorotein	6.45	38 382	3
P98160	membrane-specific heparan sulfate proteoglycan core protein	6.06	479 248	2
P02753	plasma retinol-binding protein	5.75	23 337	2
Q9UPQ9	protein KIAA1093	6.57	183 444	1
P02768	serum albumin	5.92	71 317	9
Q96B97	SH3-domain kinase binding protein 1	6.24	73 253	1
P52735	Vav-2 protein	6.67	102 446	1
CIEF Fraction E				
P19652	α-1-acid glycoprotein 2	5.03	23 873	1
P01009	α-1-antitrypsin	5.37	46 878	2
P02765	α-2-HS-glycoprotein	5.43	40 098	1
P15144	aminopeptidase N	5.27	109 711	1
999108	anti-CD19 antibody light chain variable region	5.54	12 468	1
P61769	β-2-microglobulin	6.06	13 820	2
P53004	biliverdin reductase A	6.06	33 692	1
10835794	chain C	5.75	23 552	7
58222074	chain J	5.39	15 862	1
P02792	ferritin light chain	5.51	19 933	1
P02671	fibrinogen α/α-E chain	5.7	95 656	3
P02679	fibrinogen γ chain	5.37	52 106	
P01877	Ig α-2 chain C	5.71	37 283	
33318894	Ig heavy chain variable region	5.19	12 822	1
P01593	Ig κ chain V−I region AG	5.67	12 099	1
P01614	Ig κ chain V−II region Cum	5.28	12 782	1
P01617	Ig κ chain V−II region TEW	5.69	12 422	2
P04434	Ig κ chain V−III region LOI	5.63	12 863	2
P80748	Ig λ chain V−III region LOI	4.95	12 042	1
12655763	immunoglobulin λ chain variable region	5.58	11 276	1
Q92985	interferon regulatory factor 7	5.69	55 042	1
P01042	kininogen	6.34	71 945	1
P05451	lithostathine 1 α	5.65	19 118	4
Q9UHC7	makorin 1	5.05	54 697	1
P08571	monocyte differentiation antigen CD14	5.84	40 678	2
P59666	neutrophil defensin 3	5.71	10 580	1
P12270	nucleoprotein TPR	5.01	265 601	1
539611	perlecan	6.05	468 525	2
Q9NQC1	PHD finger protein 15	6.36	63404	1
190981	regenerating protein	5.65	19 132	1
P16499	rod cGMP-specific 3′,5′-cyclic phosphordiesterase	5.48	100 294	1
P25311	zinc-α-2-glycoprotein	5.57	34 079	5
CIEF Fraction F				
183955	hepatitis B surface antigen antibody	4.46	11 835	1
P04433	Ig κ chain V−III region VG	4.85	12 681	2

[a] Values for the pI and MW for each protein are based on the database.

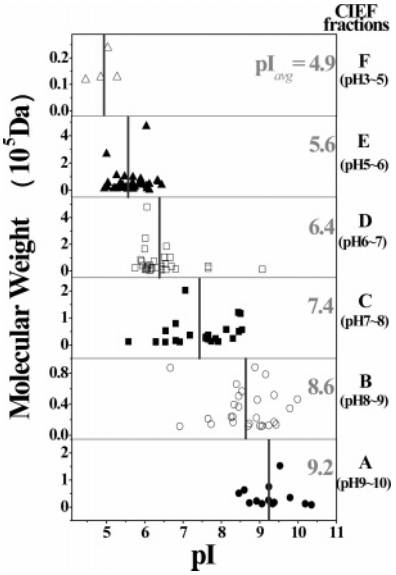

Figure 8. Plots of MW vs pI values for the urine proteins identified at all CIEF fractions. The pI and MW of each protein were based on the values from the search results. The average pI value was marked for each fraction.

PGLGER.G, of which the precursor ion was m/z = 987.06 (+2) at 95.4 min (marked as ** in Figure 5b) from fraction E2. Both proteins were reported as biomarkers for an inflamed pilonidal abscess in the literature.[37] These results demonstrated the outstanding potential for selective protein isolation using CIEF−HFFlFFF.

All fractions (A1−F4) were individually analyzed by LC−MS−MS, and the proteins that were identified by a database search are listed in Table 1. The values of the pI and MW of each protein found from a database (Swiss-Prot and NCBIR) are listed together along with the number of peptides identified. Table 1 showed that the pI values of the identified proteins in each CIEF fraction matched well with the pH interval of each fraction with only a few exceptions: the pI values of some proteins appeared to be slightly higher or lower than each corresponding pH interval. In each fraction, there were 5−7 proteins that appeared in nearby pH fractions. The listed pI values for the proteins in each fraction were plotted with respect to MW in Figure 8. While the pI values of the proteins appeared to be scattered in each fraction to some degree, the average pI value (marked with vertical lines in Figure 8) of the proteins identified in each fraction fell within the corresponding pH interval. These values are listed in Table 2 along with the identified numbers of peptides and proteins in each fraction. The total number of proteins identified from all fractions was 114. This was similar to 113 that was obtained from 2D-PAGE of a human urinary proteome followed by peptide mass fingerprinting only.[38] When the same sample in this study was digested and examined by LC−MS−MS directly without a preliminary fractionation, the identified number of proteins was only 21 at the same LC−MS−MS experimental condition utilized for analyzing each CIEF−HF FlFFF fraction. Since the proteome sample used in this study was filtered with membrane filter having a MWCO of 30 kDa during preparation and only the fraction >30 kDa was utilized, a direct LC−MS−MS experiment for the same sample gave a low number of protein identifications. Relatively low identification of a direct LC−MS−MS experiment could be due to the influence of high-abundant proteins. However, an improvement in protein identification after CIEF−FlFFF separation of proteins followed by nanoflow LC−MS−MS could be due to the isolation of low-abundance proteins from highly abundant ones by the two-dimensional separation method.

(38) Oh, J.; Pyo, J.-H.; Jo, E.-H.; Hwang, S.-I.; Kang, S.-C.; Jung, J.-H.; Park, E.-K.; Kim, S.-Y.; Choi, J.-Y.; Lim, J. *Proteomics* **2004**, *4*, 3485, 3497.

Table 2. Comparison of the Number of Identified Proteins from the Human Urine Sample at Each Fraction Using LC−ESI-MS−MS after Separation with CIEF−HFFIFFF

	fractions of the different pH ranges					
	A (9−10)	B (8−9)	C (7−8)	D (6−7)	E (5−6)	F (3−5)
no. of peptides	22	39	64	71	88	6
no. of proteins	17	27	23	32	39	4
no. of new proteins	17	20	16	27	32	2
average of pI	9.2	8.6	7.4	6.4	5.6	4.9
total	114					

CONCLUSION

The two-dimensional separation technique developed here, CIEF−HFFlFFF, may be a powerful utility in proteomic analyses since the proteins can be fractionated on the order of MW and pI and the eluted protein fractions are in an undenatured, purified form. There are significant advantages to the employment of CIEF−HFFlFFF (for proteins) followed by off-line nanoflow LC−MS−MS (for the digested peptide mixtures) in protein characterization. Compared to the other CIEF hyphenated separation techniques such as with LC or CE, CIEF−HFFlFFF is operated without using an organic solution or surfactant, which would later need to be removed prior to MS analysis. Since proteins are separated under a MS-compatible buffer solution in HFFlFFF with the simultaneous removal of the ampholyte solution used for the CIEF run, they can be directly utilized for biological assays or bottom-up proteomic analyses. While 2DE experiments generally require a considerable amount of proteins in order to view as many spots as possible, not all spots can be identified by MS analysis (either by MALDI-MS or LC−MS after spots are removed for protein digestion) due to low concentration. However, because CIEF−HFFlFFF can be operated with a smaller concentration of proteins, low-abundance proteins can be isolated from the more abundant ones during two-dimensional separations. The latter feature is helpful in bypassing the ionic suppression effect that is caused by high-abundant peptide ions during MS analysis of digested peptides, and it leads to a better chance of identifying low-abundance proteins. Compared to the direct analysis of protein/peptide mixtures by a one-dimensional LC−MS−MS analysis (the so-called shotgun proteomic method) without a preliminary fractionation, CIEF−HFFlFFF followed by LC−MS−MS provides the potential for enlarging the identifiable number of proteins due to a minimization of the influence from the high-abundant proteins.

CIEF−HFFlFFF is a rapid process, and it can be fully automated for the analysis of a protein complex mixture. A possible application of CIEF−HFFlFFF would be a direct interface

to ESI-MS−MS for a top-down proteomic approach. From an earlier study,[25] it was demonstrated that HFFlFFF can be successfully coupled to ESI-TOF MS in an on-line manner for the characterization of intact proteins. A challenging application would be the approach of quantitative analysis by isolating proteins over certain pI and MW ranges that contain target proteins. The ability of simplifying protein complex mixtures by CIEF−HFFlFFF depends on the resolution of the 2D separation. This may be improved by removing the EOF influence in the CIEF separation and through flow rate optimization for the HFFlFFF. The throughput can be increased by optimizing the length and the inner diameter of the tubing used for the CIEF as well as the applied voltage. Since the Teflon tubing used for the CIEF and hollow fibers used for the HFFlFFF are inexpensive and do not require column packing or preconditioning, they can be treated as a disposable component in the procedure if avoiding carryover is critical.

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