MOLECULAR & CELLULAR TOXICOLOGY, Vol. 1, No. 2, 87-91, June 2005

Capillary Size-exclusion Chromatography as a Gel-free Strategy in Plasma Proteomics

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Accepted 10 June 2005

Abstract

Although 2D-PAGE has been widely used as the primary method for protein separation, difficulties in displaying proteins with an extreme values of isoelectric point (*p*I), molecular size and hydrophobicity limit the technique[1]. In addition, time consuming steps involving protein transfer and extraction from the gel-pieces can result in sample loss. Here, we describe a novel protein separation technique with capillary size-exclusion chromatography (CSEC) for rapid protein identification from human plasma. The method includes protein fractionation along with molecular size followed by in-solution tryptic digestion and peptide analysis through reversed phase liquid chromatography (RPLC) coupled to nanoflow electrospray-tandem mass spectrometry (ESI-MS/MS)[2-4]. Tryptic peptides are applied on a 100 μm i.d. × 10 mm length pre-column and then separated on a 75 μm × 200 mm analytical column at ~100 nL/min flow rate. Proteins were identified over the wide ranges of *p*I (3.7-12.3) when this technique was applied to the analysis of 1-2 μL of human plasma. This gel-free system provides fast fractionation and may be considered a complementary technique to SDS-PAGE in proteomics.

Keywords: size-exclusion chromatography, mass spectrometry, plasma, proteomics

Plasma is a complex body fluid but serves useful biological information in disease diagnosis and therapeutic monitoring because it contains many tissue proteomes as subsets[5]. Thus Sample preparation is one of the most critical issues in proteomic applications. Rapid and accurate identification of proteins with or without their post-translational modification, is necessary, because it may affect reproducibility as a result of the heterogeneity of proteins derived from cell populations[6]. Approach of chromatographic have eliminated some of the disadvantages of gel electrophoresis[7]. Acidic and basic proteins and hydrophobic proteins, which are not easily observed on 2D gels, could be isolated.

To realize gel-free proteomics, the comprehensive systems have been developed to achieve adequate resolution of complex protein mixtures, however relatively little attention has been paid to potential of size-exclusion technique in proteomics to date[8-10]. In this study, CSEC was conducted due to minor deviations from the ideal behavior, which are probably caused by interactions of small proteins and polar groups with the SEC matrix when larger column diameters are applied[11].

Results and Discussion

A protein mixture, containing 6 different molecular sizes, was monitored during 15 experiments over a period of 3 weeks to estimate reproducibility of retention times in SEC column. The good chromatographic separation of 6 proteins shown in Fig. 1 were obtained with the reproducibility of elution time (RSD < 8.2) within the mass range of 3.2 (66.5 kDa. In gel based size-exclusion protein separation, a denaturing step is required by reducing agents due to conformational effects. However, the denaturing process was not necessary in this gel-free technique. The retention time and peak width of denatured proteins were slower and broader than the undenatured (data not shown). These results are also shown in capillary electrophoretic and nanofabricated chip separation systems[12,13]. The dispersed size of unfoleded proteins may have irregular access to the pores due to various levels of denaturing, and it also make broad peaks.

Size-based chromatographic separation is possible only when is no interaction was shown between the solutes and the column media. Most stationary phases in SEC are weakly negatively charged and slightly hydrophobic, resulting in solute/media interactions

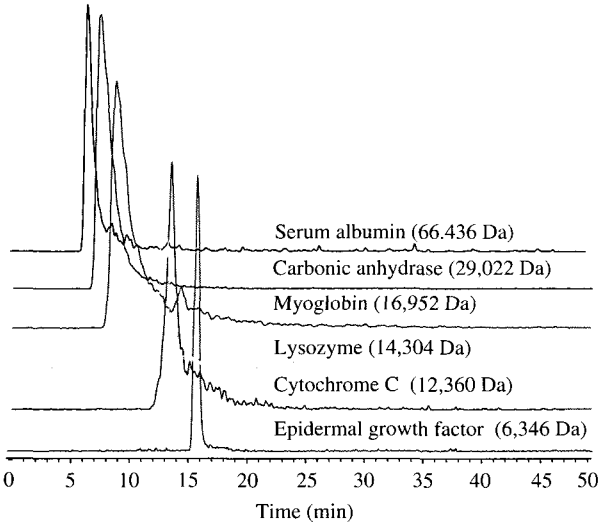

Fig. 1. Capillary size-exclusion chromatograms. (A) Extracted ion chromatograms of a protein mixture used, separated on an agarose based 325 μm i.d. × 250 mm length capillary size-exclusion column with 1.5 μL/min flow rate. Each protein was present at amounts of 1 or 2 pmol on a column. (B) Total ion chromatogram of human plasma injected into CSEC column. Plasma sample was depleted with albumin and immunoglobulins, and the depletion efficiency was confirmed by SEC and SDS-PAGE (data not shown).

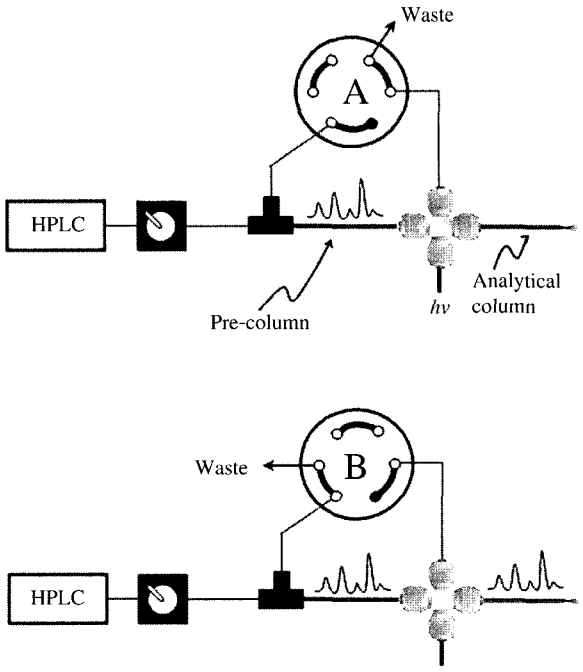

Fig. 2. Tandem RPLC combined to MS/MS analysis system used for peptide identification. (A) Digests (0.5 μL) injected into pre-column with 0% buffer B at unsplit flowrate 2.5 μL/min are desalted and concentrated for 2 min, and then (B) a valve switching enables a split flow (~100 nL/min) into both pre-column and analytical column for further separation. The total retention time for a single run was 120 min from sample injection to end of the run. All procedures used including sample loading, valve-switching, and solvent gradient are fully automated and controlled by ChemStation (Agilent Technologies).

and such unwanted interactions are difficult to predict and may result in poor reproducible retention. Ionic interaction may be minimized by the addition of salts to the mobile phase[9]; however, high concentrations of salt can also cause hydrophobic interactions of proteins with the media. Another important issue in SEC mobile phase is sample solubility because analytes will only separate efficiently if they are completely solvated. Therefore, a mixture of 20% of acetonitrile and 5% methanol was used to suppress hydrophobic interactions and dissolve hydrophobic proteins. In addition, the elution system containing 20% acetonitrile responsible for disrupting any potential protein-protein interactions, which result in a more identified low molecular proteins[14]. Mobile phase in this study also consisted of acetic acid, because this solution was found to be the most efficient for the separations at a desired sensitivity levels in protein/peptide analysis. Other conditions for tryptic digestion were also tested with protein standards: existence of acetonitrile and acetic acid in fractionated samples, and these conditions resulted in no negative effects for sequence coverage (data not shown).

After optimizing of CSEC, the plasma samples, which are depleted with albumin and immunoglobulins, were injected into CSEC column. Alternatively, the fractionated protein sample was digested with trypsin and the peptides separated by a capillary tandem RPLC system, which is conducted with pre- and analytical columns, and analyzed by a fully automated nanoflow ESI-MS/MS system (Fig. 2). In many cases, proteins should be digested and stored in non-volatile salt buffers and other solubilizing agents. This apparent incompatibility is resolved by desalting with Zip-Tip purification prior to MS/MS analysis, but the off-line method is time-consuming and can result in considerable peptide losses. The samples were directly injected into the tandem RPLC system and peptides were first loaded into a pre-column to remove inorganic salts and other hydrophilic compounds, and then introduced into analytical column in series. Separated peptides from analytical column were identified and analyzed by a quadrupole-TOF mass spectrometer. This in-line desalting system is responsible for sample clean-up and rapid sample

injection[15].

The various chromatographic protein fractionation resulted in markedly improved profiles with the gel technique[16]. Human plasma proteins identified in this study shows wide range of *p*I value (3.7-12.3) containing some transmembrane domain fragments and cellular proteins. An improved enrichment of 2 proteins of less than 3 kDa (pepsin-like proteinase and T cell receptor α chain CDR3) were detected in this study while the gels can not visualized resulted by running off the bottom of the gel. The chromatography based pre-fractionation and gel-based techniques agreed at the level of high-abundant proteins but showed different at the low-abundant proteins[16].

Conclusion

For high-throughput proteomic analysis, this technique may be useful and it is imperative that any MS techniques are robust, routine, and minimizing off-line sample losses. The in-line coupled pre-columns resulted in increase not only number of sample injection into the analytical column but also sensitivity[15]. To maximize the number of peptides analyzed, each scan-cycle collects MS/MS data for the three most abundant masses detected in the full scan. The aim of the study was to separate proteins from complex mixtures in a short time, and this gel-free technique was applied to a complex biological specimen human plasma because it is useful diagnostic tools and alteration of the expression of some proteins may be indicative of disease. Using this technique along with in-solution digestion greatly reduces the processing time of the protein identification. To apply this technique in tumor biomarker discovery, combination with quantitative methods are in progress.

Methods

Materials and Reagents

Six protein standards as markers of molecular size, epidermal growth factor (EGF, 6.3 kDa), cytochrome c (12.4), lysozyme (14.3), myoglobin (16.9), carbonic anhydrase (29.0), and human serum albumin (HSA, 66.4) were purchased from Sigma (St. Louis, MO, USA). Human plasma used in the study was obtained from the healthy male donor. Microcon YM-50 (MWCO: 50 kDa) was purchased from Millipore (Billerica, MA, USA) and sequencing-grade trypsin (specific activity, 16,500 μ/mg) was obtained from Promega (Madison, WI, USA). All aqueous solutions were prepared with freshly-distilled water.

Preparation of Capillary Columns and Plasma Solution

SEC and RPLC analyses were conducted on fused silica capillary columns and they were prepared in-house by following a procedure based on procedures of this experiments[20,21]. The frit slurry solution was prepared by mixing 20 μL of formamide with 80 μL of potassium silicate (Kasil-1, PQ Corp., Valley Forge, PA, USA). Packing slurries were prepared in ratio of 20 mg/mL using 24-40 μm Superdex™ 75 prep grade (Amersham Biosciences, Uppsala, Sweden) for SEC and C18 media (Vydac, Hesperia, CA, USA) for RPLC with slurry liquids of 20% ethyl alcohol and methyl alcohol, respectively. Capillary columns were made up with the unpacked volume and were used without the inlet frit.

Chromatographic Separation Systems

A Rheodyne injector (Rheodyne, Rohnert Park, CA) with a 1.0 μL internal loop was comprised for μ-fractionation. Plasma sample pretreated with Microcon YM-50 was injected into CSEC column (Superdex 75, 325 μm i.d. × 250 mm length) and then separated at 1.5 μL/min with 80% buffer A (0.1% acetic acid in 5% acetonitrile) and 20% buffer B (0.08% acetic acid in 95% acetonitrile contains 5% methanol) in isocratic condition. The 1 min elution segment (1.5 μL) was collected for tryptic digestion. Each CSEC fraction was mixed with 1 mg/mL DTT (0.05 mL) with 50 mM of ammonium bicarbonate. The resulting solution was incubated for 30 min at 70 °C. After being cooled to room temperature, 10 mg/mL IAA (0.1 mL) with 50 mM ammonium bicarbonate was added and kept for 1.5 h at room temperature. Then samples were digested by adding 0.1 mL of 10 μg/mL trypsin solution and incubation for 12-16 h at 37°C. The reaction was quenched by freezing and the sample was dried on a SpeedVac (Savant, Holbrook, NY, USA), and then taken up in distilled water for the next nanoflow ESI-MS/MS analysis.

The digested samples were separated and analyzed using the same eluent system with protein separation. The Rheodyne injector (0.5 μL) was connected with Agilent 1100 capillary HPLC system (Agilent Technologies, Palo Alto, CA, USA). The injected peptides were first loaded onto a pre-column (Peptide C18, 300 Å pore size, 3 μm particle; 100 μm i.d. × 10 mm length) at 2.5 μL/min flow rate for desalting. The peptides were eluted from pre-column and loaded into analytical column (PicoFrit; Everest C18, 300 Å, 5 μm; 75 μm i.d. × 200 mm length; tip 8 μm, New Objective, Worburn, MA, USA), and then peptides

were separated with linear gradient of 0 to 80% buffer B at a flow rate of ~100 nL/min over 90 min, and then ramped back to initial conditions (100% buffer A) over 30 min.

Mass Spectrometry

To identify proteins, the capillary HPLC was coupled to a LC-MSD Trap XCT plus (Agilent Technologies) mass spectrometer equipped with micro-nebulizer. The capillary voltage was 3.8 kV, and the dry gas temperature was 350°C. Each full scan mass spectrum was measured at the range of 500-1,800 μ. An Applied Biosystems (Framingham, MA) QSTAR XL quadrupole-time of flight (TOF) mass spectrometer was used for peptide identification equipped with a nanospray source (Proxeon Biosystems, Odense, Denmark). The electrospray interface design utilizes a micro-tee (Upchurch Scientific, Oak Harbor, WA, USA) with a 1-inch piece of platinum rod, which was inserted into one stream of the micro-tee to supply the electrical connection. Electrospray voltage was typically 2.0-2.7 kV with mass tolerance of 50 mmu. The MS/MS spectra were obtained using collision gas of 6 with doubly, triply, and quadruply charge states. Accumulation time and pulsar frequency were maintained at 3 s and 6.99, respectively. Each full scan mass spectrum was followed by three data dependent MS/MS spectra of the three most intense peaks. Mass spectra obtained by automated ESI-MS/MS analysis were used to identify the corresponding peptides against human database of NCBInr using the Spectrum Mill™ (Agilent Technologies), which assesses MS/MS spectral quality based on sequence tag length (>1) and signal-to-noise (>25) criteria, with protein (>20) and peptide (>10) scores.

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