Proteomic analysis for the study of amniotic fluid protein composition

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Introduction

Amniotic fluid (AF), routinely used for prenatal diagnosis, contains large amounts of proteins produced by the amnion epithelial cells, fetal tissues, fetal excretions and placental tissues. The amniotic fluid is initially formed from maternal plasma that later crosses fetal membranes from 10 to 20 weeks of gestation. The biochemical composition of AF is modified throughout pregnancy and its protein profile reflects the genotypic constitution of the fetus and regulates feto-maternal physiological interactions (Tsangaris GT et al., 2006).

By looking at the composition of the amniotic fluid, scientists can provide valuable information about the health of the fetus and may indicate potential pathological conditions. Although many amniotic fluid proteins have been identified and are currently used to detect potential fetal anomalies, little is known about the functions of these proteins and how they interact with one another. Identification of changes in the protein content of amniotic fluid, therefore, may be used to detect a particular type of pathology, or to ascertain a specific genetic disorder. In the search of potential biomarkers in the AF, growing interest is cutrrently given to proteomics based aprroach (analysis) that represents a major development in the rapid detection of novel diagnostic markers (Michaels JE et al., 2007). Proteomics diagnostics combine pattern profiling of tissues and body fluid with sophisticated bioinformatic tools in order to identify patterns within the complex proteomic profile that can discriminate between normal, beningn and disease states. Additionally, MS-based proteomics stand to become the preferred platform for routine clinical and medical biomarker detection and have been succesfully used for the early diagnosis of several types of disease.

Despite a variety of new approaches, proteomics still relies heavily on two-dimensional electrophoresis (2-DE) as underlying separation technology. This technique uses the power of both isoelectric focusing (IEF) and SDS-PAGE electrophoresis to separate proteins firstly by their pI and then by their relative mobility (Mr) (Rabilloud T et al., 2002). 2D-PAGE represents undoubtedly one of the most-used techniques for protein separations, however more sophisticated methods are currently employed (Figs. 1 and 2). The other key tool of proteomics is mass spectrometry (MS) (Anderson NL et al., 2000). It is through the integration of 2DE and MS
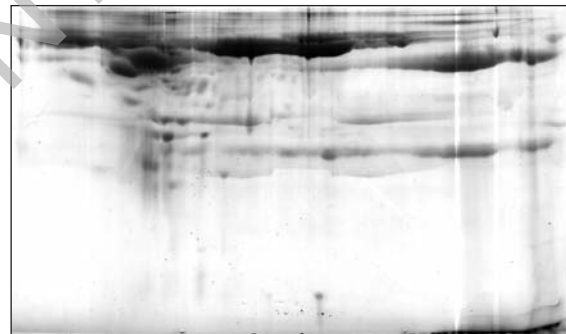

Figure 1. Representative 2-D gel of normal AF sample obtained without removal of albumin and IgGs. Proteins (200 μg) were separated on immobilized pH 3-10 IPG strips followed by separation on an 8-16% gradient SDS-PAGE gels and stained with Biosafe Coomassie.

Figure 2. Representative 2-D gel of normal AF sample after albumin/IgGs depletion. Proteins (200 μg) were separated on immobilized pH 3-10 IPG strips followed by separation on an 8-16% gradient SDS-PAGE gels and stained with Biosafe Coomassie.
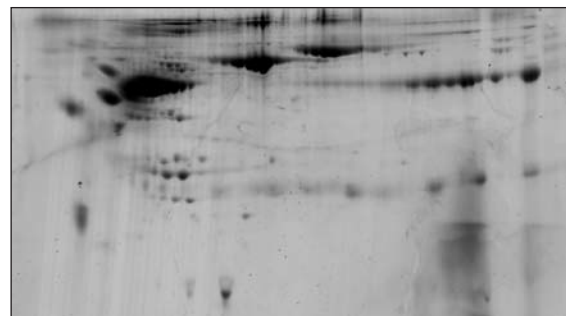
that proteomics achieves its greatest power. First, the gel-separated proteins are digested into peptides by sequence-specific proteases and an eluted peptide mixture is acquired. Then matrix-assisted laser desorption/ionization is performed to produce a mass spectrum or "peptide-mass fingerprint". The second step in protein identification relies on the fragmentation of individual peptides in the mixture to gain sequence information. Both mass spectrum and sequence information can be searched against databases to identify proteins.

Proteomics methods

In the present work we used a proteomic approach, combining 2DE and MS, in order to study the protein composition of AFS. 10 mL of AF samples were obtained, after written informed consent, from women undergoing amniocentesis in the 16-18th week of gestation. Following centrifugation for the collection of amniocytes for cytogenetic analysis, supernatants were aliquoted and frozen at –80 °C. 4 mL aliquots were chosen for proteomic analysis. Women with gestational diseases or pregnancy complications will be excluded from the study and all women used as controls have normal uneventful deliveries at term. The protocol was approved by the local Institutional Ethics Board.

One of the major difficulties in analyzing the proteome of human AF is the dynamic range of the concentrations of the proteins present in the sample. Human serum albumin (HSA) constitutes around 70% of total protein content with immunoglobulins (Igs) being the second most abundant fraction. Removal of these two proteins alone clears about 75% of the total protein present in AF, thereby allowing the enhanced detection of the remaining proteins that are present in far lower concentration.

Depletion of the most abundant proteins allows: 1) visualization of proteins co-migrating with albumin and IgGs 2) higher sample load for improved visualization of lower abundance proteins. However, the depletion method needs to be inexpensive and reproducible. We describe such a protocol that combines delipidation by centrifugation, IgG removal and HSA depletion with a commercial available KIT commonly used for serum depletion (ProteoPrep™ Blue Albumin Depletion Kit, Sigma Aldrich). The protocol is streamlined to increase reproducibility and is compatible with many proteomic platforms, including two-dimensional gel electrophoresis and high-performance liquid chromatography. The reproducible depletion of lipids, IgG, and HSA permits a higher load of the remaining AF proteins.

The most common method for depletion of albumin is the use of Cibacron Blue resin equilibrated with a buffered salt solution. Cibacron Blue typically has high capacity but displays lower specificity. Low specificity may result in the loss of proteins of interest. Antibody-based albumin depletion resins display higher specificity but have lower capacity and are more expensive.

Lower abundance proteins, which co-migrate with albumin and IgG, are unmasked after depletion. Removing albumin also reduces smearing in the upper portion of the gel, which can obscure the higher molecular weight proteins. The removal of albumin and IgG will lead to re-duced contamination of spots, which can interfere with MALDI identification. In addition, depletion of the high abundance proteins allows for increased (4-5 fold) amounts of sample that can be loaded onto a gel (data not shown). ProteoPrep Blue typically removes >95% human albumin and >80% human IgG from 75 ul serum which allows for subsequent detection of lower abundance proteins.

Experimental procedure

AF samples (5 mL) were precipitated using three volumes of 100% ice-cold acetone at -20 °C overnight and the pellet was resuspended in 0.5 mL MEDIA I (0.32M sucrose, 0.1mM MgCl$_2$, 0.1mM EDTA and 10 mM Tris-HCl pH 8). Preliminary steps required the identification of the maximum protein amount that can be loaded into the column without affecting column efficiency. Different protein concentration were tested before lodiang the sample into the column (2-3-5 mg/mL) and we found that 3 mg/ml (around 100 µL of sample volume) results in a very good albumin/IgGs depletion with a high rate of protein content after column purification steps.

Samples were depleted of the major serum proteins (albumin and IgGs) using PROT-BA depletion kit. For two-dimensional electrophoresis, 200 g of proteins were dissolved with 200 mL of rehydration buffer (8 M urea, 20mM dithiothreitol, 2.0% (w/v) CHAPS, 0.2% Biolytes, 2 M thiourea and bromophenol blue).

For the first-dimension electrophoresis, 200 µL of sample solution were applied to a ReadyStrip™ IPG strip pH 3-10 NL (non-linear) (Bio-Rad Laboratories, Milan, Italy). The strips were soaked in the sample solution for 1 h to allow uptake of the proteins. The strips were then actively rehydrated in Protean IEF Cell Apparatus (Bio-Rad) for 16 h at 50V. The isoelectric focusing was performed at 300V for 2 h linearly; 500V for 2 h linearly; 1000V for 2 h linearly, 8000V for 8 h linearly and 8000V for 10 h rapidly. All the processes above were carried out at room temperature. The focused IEF strips were stored at –80 °C until second dimension electrophoresis was performed.

For second dimension electrophoresis, thawed strips were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodacetamide in place of dithiothreitol. Linear Gradient (8-16%) Precast criterion gels (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad) were run along with the sample at 200V for 65 min. After electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min. Approximately 40 mL of SyrpoRuby Gel Stain (Bio-Rad) were used to stain the gels for 2 h, on a gently continuous rocker. The gels were placed in deionized water overnight for destaining. Images from SYPRO Ruby-stained gels used to measure protein content were obtained with a UV transilluminator (λ_{ex} = 470 nm, λ_{em} = 618 nm; Molecular Dynamics). PDQuest spot detection software is used to compare 2D gels from different groups (for example CTR and pathological samples).

PDQuest software offers powerful comparative analysis and is specifically designed to analyze many gels at once. Powerful automatching algorithms quickly and accurately match gels and sophisticated statistical analysis tools identify experimentally significant spots. After completion of spot matching, the normalized intensity of each protein spot from individual gels is compared between groups using statistical analysis. Statistical significance is assessed by a two-tailed Student's *t*-test, P values < 0.05 are considered significant for comparison between control and experimental data. Selected spots are manually excised from gels and submitted to trypsin proteolysis and subsequently to MS analysis. Both mass spectrum and sequence information can be searched against databases to finally obtain protein identifications (Perluigi et al., 2008 in press).

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