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Review

Strategies for revealing lower abundance proteins in two-dimensional protein maps

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Abstract

One of the most challenging contemporary research endeavors is the mapping of proteins and establishing their linkages to normal and pathological conditions. The availability of current proteomics technologies has greatly facilitated the separation and identification of proteins in a complex protein mixture by standard two-dimensional gel electrophoresis and subsequent MALDI-TOF mass spectrometry. Due to the huge differences in the distribution of proteins in complex proteomes of humans, the detection and identification of proteins expressed in low copy number is a major challenge. The low abundance of important physiologically relevant proteins has rendered their analyses almost impossible without some means of prior purification and enrichment from tissue lysates or biological fluids. It is the current limits of detection of the methods that are used that prevents the detection of these proteins not the proteins themselves. More importantly, considering the frequency at which post-translational modifications of proteins occur, the separation of protein isoforms is essential to understand biological changes, and two-dimensional gel electrophoresis remains the only technique that can offer sufficient resolution to address this issue at a functional level. Cellular fractionation techniques followed by specific affinity probes for tracking target proteins have been developed to deplete the proteome of high abundance proteins in order to increase the sample loading for achieving greater sensitivity for proteins present in low abundance. Those applications can entail the removal of one protein or a class of proteins that interferes with the resolution of proteins in a 2-DE map. Moreover, the use of better solubilizing detergents in combination with an overlapping narrow immobilized pH gradients, results in higher resolution by stretching the protein pattern in the first dimension. In this review we will discuss strategies to remove high abundance proteins that can result in the visualization and detection of low abundance proteins in biological samples. The potential use of these strategies, as a means of developing diagnostic tools for early screening of diseases and identification of drug targets for therapeutic intervention, will also be discussed.

Keywords: Low abundance protein; MALDI-TOF; Gel electrophoresis

Contents

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1. Introduction

The study of human proteomes, their diversity and their relationship to genome has raised great interest. The Human Genome project provides evidence for approximately 30,000–40,000 genes in the human proteome [1], a range that is not much in excess of the gene numbers in the fruit fly *Drosophila melanogaster*. The seemingly small number of genes in the human genome compared to other less complex organisms has evoked scientific debate about the extent of complexity and diversification of the human genome and its differences with other organisms such as bacteria, yeast, flies, other mammals, etc. It is apparent that the paradigm of 'one gene encoding a single protein' is no longer applicable because of differential RNA processing such as alternative RNA splicing, *trans*-slicing RNA events, overlapping transcription events, etc. [2]. The end result of these processes includes post-translational protein modifications resulting in multiple protein products for a single encoded gene. Hence, for every eukaryotic genome studied to date, the actual number of distinct proteins formed in the differing proteomes during the development far exceeds the number of transcription units that encoded them. Additionally, protein degradation and turnover can significantly influence the intracellular concentration of active protein molecules. It has been estimated, that the average number of proteins per gene is one or two in bacteria, three in yeast and three to more than six in human [3]. Hence, the extent of diversity and complexity resulting from post-translational modification and degradation is tremendous and can only be understood by qualitative and quantitative analysis of gene expression at the level of functional protein. Therefore, a direct measurement of protein expression in different proteomes is essential to analyze and understand biological processes during development, in disease and in normal conditions. The interface between protein biochemistry and molecular biology for the global analysis of gene expression and subsequent post-translational modification of product per se is termed 'proteomics' [4]. Proteomics is a technology-based science that studies in a high-throughput mode the expression of different proteins in a proteome, modifications and interaction of proteins that occur due to changes in the proteome during the developmental process, disease-state or exposure to an external stimuli. The core element of proteomics analysis is to combine the separation of proteins in a two-dimensional map together with mass spectrometry (MS) or tandem MS (MS–MS) protein identification. The major advantage of this technique is that it enables the simultaneous separation, visualization and identification of hundreds of unknown proteins at different modification states. No other method is able to achieve this at the present time. The technology has successfully been applied to gain understanding of the protein profile of simple organisms such as *M. genitalium* [5], *E. coli* [6], yeast [6], etc. Characterizing the proteome of a complex organism such as humans however, challenges the limitations of currently available technologies.

One of the challenges in identifying proteins in a complex proteome such as human is the presence of proteins in wide dynamic concentration ranges from very high levels for albumin to low for a specific hormone or a regulatory protein. The differences in the concentration of such proteins may be over thousands to a million times. Hence, a small sample volume (about 10–100 μl) usually applied for proteomics analysis, results in the acquisition of data dominated by high abundance proteins leaving a large percentage of the expressed proteins with insufficient quantities undetected. This large percentage of protein expressed in lower concentration constitutes of low abundance protein and includes proteins required for important regulatory processes or may represent proteins with potential biological marker possibility or may be a likely target for drug intervention. To detect these low abundance proteins of greater biological importance there is an urgent need to develop methods that will remove and deplete the relevant proteome of high abundance proteins, resulting in the analysis of 3–5-fold more proteins present in lower concentration. Strategies to effectively remove high abundance proteins to identify proteins of low abundance therefore have gained much interest.

2. Strategies for the enrichment and identification of low abundance proteins

As discussed above, proteomics is an area of research that seeks to define the function and relative expression profiles of proteins encoded by a given genome at a given time in a given cellular location. The technology separates, identifies, and characterizes the proteins expressed, retained, secreted or released by a cell or tissue in order to establish their function(s) and potential relationship during developmental phase and or onset or progression of diseases, as well as relapse and/or response to therapy. The first step for proteomic analysis requires an adequate sample preparation that will enable visualization and identification of relevant proteins of low abundance. The process is equally important for biological samples such as cell lysates as well as biological fluids such as serum, plasma, saliva, cerebospinal fluids, uterine washings, amniotic fluids, urine, etc. Several factors have an impact on the detection of low abundance proteins in these samples. The first is the dynamic range of protein concentration in a sample. A distinction must be made between the dynamic range of protein concentration in a cell and in biological fluids such as plasma. Corthals et al. [7] predicts that the dynamic range of protein concentration in plasma could be 12 orders of magnitude as the presence of albumin represents over 50% of total plasma protein. In a cell, this situation is less extreme and the most abundant proteins in yeast represent 4% of the total protein [8]. Hence, strategies for low abundance protein separation in cellular situation are slightly different to that applied to biological fluids. As contribution of high abundance proteins in biological fluids is greater than that in tissues or cell preparations, visualization and detection of biologically/clinically low abundance proteins in biological fluids mandates the selective removal of high abundance proteins. This review will focus separately on methods applied for the removal of high abundance proteins in cells/tissue homogenates as well as biological fluids.

3. Depletion of high abundance protein in tissue homogenates

3.1. Sample preparation and selection

An optimized sample preparation procedure for the visualization of low abundance protein in tissue samples includes preparation and purification of the cell type of interest. This will result in optimal cell lysis and solubilization of protein to gain optimal yield of low abundance protein. Sample selection is of paramount importance, for example use of fresh samples for 2-DE analysis was reported to be better than working with frozen samples [9]. Prolonged storage of samples may result in the modification of proteins resulting from storage leading to misleading results [10]. Another challenge to the analysis of tissue proteomes is tissue heterogeneity. The sample to be analyzed should be pure and relevant. For example, in case of cancer proteome analysis, the sample should be free of stroma, blood, serum, etc and represent only tumor. In that case, the use of cancer cell lines derived from tumors is ideal but the representation of the cell line model to the in vivo situation and the transfer of in vitro results to in vivo conditions are still debatable.

3.2. Enrichment of low abundance proteins by affinity methods

Different methods have been developed to deplete tissue samples of non-relevant cell types in order to enrich the tissue homogenate of relevant low abundant protein. The separation of epithelial cells using Dynabeads from colon crypts isolated by mechanical preparation has been shown [11]. Using such techniques significant changes in the protein expression profile between normal mucosa and colorectal cancer were observed using 2-DE gels [11]. Others have used antibody-based purification strategies to purify cells of interest in whole tissue [12]. Other investigators have used tissue homogenates prepared from pieces of tumor without purification of tumor cells for 2-DE analysis [13]. As stromal components are part of a tumor and many genes are differentially expressed in stroma related to the tumor, the usage of whole tumor tissues without purification is justified. In this context, one can consider the example of metalloproteinases that are often expressed by tumor surrounding stroma but not by tumor cells [14]. Solublization of protein in such mixed tissue lysates, however, can be cumbersome and can result in complex patterns due to the presence of several cell types making the interpretation of proteomics study difficult.

Another method of determining low abundance protein in biological samples is radioisotope labeling of cells before 2-DE analysis. Such labeling technique can be performed by ^{35}S-methionine or ^{32}P-phosphorus [15] labeling of cells in culture but it may not always be convenient to radioactively label proteins in tissues. Radioisotopic labeling techniques provide a quantitative measure of a protein spot in response to external stimuli or different physiological conditions.

3.3. Low abundance protein enrichment by laser micro-dissection and other mechanical methods

Different methods have been developed to deplete tumor cells of the surrounding medium. Recently, the laser capture micro-dissection (LCM) technology has gained prominence in separating defined populations of cells in a malignant tissue sample [16]. This method of sample preparation has been successful in prostate cancer and has resulted in preparation of samples with high tissue heterogeneity [17]. A comparative analysis of 2-DE profile obtained from whole tissues and LCM has recently been shown [18]. A high degree of similarity between the different samples but with more enrichment of some proteins in LCM derived samples was observed. Other groups with different malignancies reported

similar result [19–21]. Even though the LCM technique offers benefits for protein profiling of a homogenous population of cells the technique has several drawbacks. The technique is low throughput as only small number of samples can be processed at a time. Moreover, sample misappropriation due to misleading sample staining can be a problem. Hence, a high quality of dissected material can only be guaranteed if the LCM is performed by or under the supervision of a trained pathologist.

Another method of collecting tumor cells of the same entity is to perform mechanical cell extraction either by fine needle aspiration (thyroid and breast tumors) [22] or by surface scraping of tumor tissues (prostate and colon tumors) [23]. As the tumor cells are loosely attached to the connective tissue and will be preferentially released by mechanical force the method generally results in pure tumor cell population.

3.4. Enrichment of low abundance protein by sub-cellular fractionation method

For the enrichment of low abundance proteins in biological sample biochemical protein-enriching approaches are used. The original protein mixture is separated into fractions of different cellular organelles by density-dependent ultracentrifugation procedures resulting in fractions enriched in organelle-specific low abundance protein. This approach improves resolution and enables one to increase the number of protein spots that can be resolved and identified with the probability of identifying low abundant proteins associated with a particular organelle. Such techniques have been successfully applied to identify low abundant membrane protein [24]. However, such fractionation procedures require larger quantities of biological samples, which in all cases is not always possible.

Further enrichment of low abundant proteins separated into organelle fractions can be achieved by selective fractionation, chromatography or electrophoretic procedure [25]. Preparative electrophoresis is a general method of protein purification. The electrophoretic method may comprise separating protein mixtures by preparative electrophoresis on the basis of protein size, usually in the presence of ionic detergents (Prep Cell, Bio-Rad Laboratories) [26], or by isoelectrofocusing on the basis of protein charge either in the presence of ampholines (Rotofor system, Bio-Rad Laboratories) or with the use of multi-compartment electrolyzers with isoelectric immobilized pH gradient (IPG) membranes [27].

3.5. Affinity chromatography for the enrichment of low abundance protein

Affinity chromatography has recently been established as a method for enriching proteins of low abundance. The technique is based on the specific interactions between immobilized ligands and target proteins. These applications involve the removal of a specific protein or a class of protein that might interfere with 2-DE resolution enabling the visualization of low abundance proteins in gel. The process is termed ‘immunoaffinity chromatography’ when an immune protein is used as the ligand to target a specific protein. The technique has successfully been used to visualize erythropoietin receptor that is moderately expressed in cultured cell lines [28]. Under normal circumstances this protein cannot be visualized in whole-cell extracts but can be enriched by antibody-based affinity purification to yield a silver-stained band [28]. One of the commonly used methods for phosphorylated proteins or phosphopeptides is immobilized metal affinity chromatography (IMAC) using gallium or iron-chelated affinity columns that can selectively bind negatively charged phosphate groups [29,30]. Affinity chromatography can only enrich a single low abundance protein or a group of proteins at a time. Therefore, multiple affinity columns and purification steps are required to achieve the desired result. On the other hand, using group specific ligands such as heparin [31], triazine dye [32] and metal ions (Cu^{2+}, Ni^{2+} and Zn^{2+}) [33] one can separate proteins into different groups. For example, using immobilized metal ion affinity columns before 2-DE analysis, the resolution of mouse liver proteins classified as metal binding and non-binding proteins was increased [33]. Similarly, separated profiles of mitochondria calcium binding proteins, glycoproteins and hydrophobic membrane proteins were obtained by enriching these proteins using calcium chelated, Con A and phenyl immobilized mini-spin affinity columns fraction before 2-DE and MS analysis [34]. These studies indicate that sub-cellular fractionation followed by affinity chromatography based on non-specific interactions such as ionic or hydrophobic interaction can be successfully used to enrich a proteome for low abundance proteins of diversified functional role.

3.6. Tracking low abundance cell signaling proteins using proteomics approaches

Ten to fifteen percentage of the proteins encoded by the human genome constitutes proteins involved in intracellular signaling. These proteins are expressed at a level that is several hundred to thousand-fold lower than structural proteins and proteins involved in metabolic pathways. This has created an immense challenge for cell biologists to study specific cell signaling proteins, either individually or in a group. The difficulty in the analysis of signaling proteins can be exemplified by the study of protein kinases involved in the reversible phosphorylation of proteins, a major form of post-translational events in cells. Nearly one third of proteins inside the cells are subjected to such phosphorylation, at multiple sites, approximately an average of 10 sites per protein [35]. This permits a massive and complex cascade of signaling networks inside the cell. The study of protein phosphorylation by 2-DE methods has relied heavily on ^{32}P-phosphate labeling of cells before resolution of proteins by 2-DE map. However, incubation of cells in culture in the pres-

ence of millicurie amounts of ^{32}P-phosphate in low phosphate containing medium to ensure sufficient labeling of intracellular pool of ATP has been an issue. Both the high amount of radioactive ^{32}P required for labeling and low phosphate concentrations in the medium can induce stress response in some cells, which can down regulate certain signaling pathway enzymes making it difficult to evaluate the actions of external stimuli. To study low abundant signaling events in cells, some researchers have resorted to Western blot analysis followed by 2-D PAGE. But this technique is completely reliant on the availability of highly specific and potent antibody that in many cases is not feasible. Some researchers have resorted to the use of phosphosite specific antibodies immobilized to metal affinity columns prior to 2-DE analysis [36,37]. However, to track signaling proteins for their expression and activation states in high throughput fashion it is necessary to develop affinity probes as specific as potent antibodies that can be linked to solid support such as beads, membranes of other matrices [35]. Many companies are exploring the usage of such binding peptides to track signaling proteins (e.g., Affibody, www.affibody.se). Recently, the use of peptide antibody mimetics (PAM) has been introduced by Kinexus Bioinformatics Corporation, in collaboration with Biomime Corporation [35]. PAMs are short peptides 15–20 amino acids in length against a target protein directly synthesized on a membrane. Usage of PAMs has the potential of detecting low abundance signaling protein after 2-D PAGE.

Recent introduction of rapid affinity-capture of signal transduction proteins (GRASP) has introduced a new phase in molecular medicine by which the activity of signaling pathways from patient-derived carcinomas can be compared to benign epithelial surfaces [38]. In this method, epithelium from a carcinoma (benign or malignant) is scrapped without the lysis of the basement membrane. Epithelial lysates are prepared and the target protein is captured by immunoprecipitation and then resolved by 2-DE and analysed by Western blotting [38]. This technology can be used to determine the activation status of a signal transduction pathway by capturing an appropriate target protein, either in an active or an inactive state. GRASP represents an advance in proteomic approaches as it detects protein–protein interaction present in cells as they exist in vivo in their native tissue microenvironment.

3.7. Enrichment of low abundance hydrophobic and basic proteins

Hydrophobic proteins are usually lost during sample preparation or during entry of proteins into the IPG gels or during focusing when the proteins reach their isoelectric points. Recently, improvements in the solubilization of proteins for the first dimension have been achieved by using more effective reducing agents [39] and powerful chaotropes and surfactants [39,40] than were previously used. Moreover, sequential extraction procedures that incorporate these solubilizing agents and hydrophobic chromatographic techniques have allowed the separation and detection of hydrophobic proteins in cell lysates [41].

Sample pretreatment for the enrichment of basic protein followed by focusing on narrow p*I* range strips beyond the usual pH 10 endpoint of commercial IPG strips have allowed the visualization and detection of basic proteins in cell lysates [42]. The focusing of histones in a pH 10–12 IPG range has successfully been shown [42]. Since then, the use of broad range pH 3–12 and pH 4–12 IPGs have been shown to successfully focus alkaline protein [43].

4. Strategies to enrich low abundance protein in biological fluids

The introduction of new technologies for the detection of disease specific biomarkers in the biological fluids of patients will have an important impact on the health sector. This need is particularly urgent in cancer and other diseases where early diagnosis dramatically improves patient outcome [44].

Blood transports essential nutrients to the cells and carries away metabolic waste products and other substances. Hence, blood proteins are useful diagnostic tools and alteration of the expression of some blood proteins is an early sign of an altered physiology and may be indicative of disease [45]. During a heart attack or in other pathological conditions where muscle degeneration is involved, damaged or dying cells secrete their contents into the bloodstream. The resulting concentration of one such serum protein, creatine-kinase B is a measure of the amount of damaged muscle and is indicative of a diseased state. In routine diagnostic laboratories identification of specific low abundant disease-associated proteins in serum relies heavily on time consuming and expensive radiolabeled or enzyme-linked immunoassay methods (RIA or ELISA) that only have the ability to evaluate a single protein component at a time. Due to the heterogenous nature of most physiological disorders there is a common belief that no single marker is likely to be sufficiently predictive [46] and a few studies have emphasized the need for more than one candidate biomarker to enhance already available diagnostic/prognostic tests [47]. The necessity to develop a panel of multiple diagnostic/prognostic markers can be met by utilizing proteomic approaches to plasma/serum and urine specimens that have the capacity to profile multiple biomarkers [48,49]. However, the application has been limited by the presence of high abundance ‘common housekeeping’ proteins like albumin, immunoglobulins, transferrin, haptoglobin, etc. These proteins constitute approximately 60–97% of the total serum protein. If proteomics technologies are to be used routinely for diagnostic purposes, a rapid, inexpensive and a simple method is required to remove these high abundance proteins.

4.1. Application of affinity chromatograph in biological fluids

For the affinity depletion of high abundance protein in biological fluids, the technique of ‘affinity extraction’ can be

applied to deplete a biological fluid sample of a specific protein or a group of proteins from a sample before analysis with 2-DE method. Examples include the use of immobilized protein A and anti-immunoglobulin support to adsorb selectively immune complexes from patient samples [50]. This method can be used in conjunction with the depletion of albumin from biological fluid samples such as serum, peritoneal fluid or cerebospinal fluid samples by adsorption on affinity resins [51]. Recently, ProtoClear, a patented affinity matrix, based on the small molecule Cibacron Blue has been used to remove albumin and IgG from human serum samples. This led to improved resolution of protein spots in 2-DE gel maps [52]. Recently, we have shown the use of Affi-Gel Blue (another cibacron blue based matrix, Bio-Rad laboratories) and protein A in the depletion of albumin from human serum [51]. This method resulted in the removal of proteins co-migrating at 68 kDa with concomitant visualization of 28 unique spots and enhanced visualization of several common protein spots after Affi-Gel Blue treatment (Fig. 2). A time course studies on human serum after Affi-Gel Blue treatment showed concomitant removal of proteins other than albumin (Fig. 2). Within 1 h of Affi-Gel Blue treatment, significant loss of albumin was achieved with no significant loss of protein profile or number of protein spots [51]. However, 16 h treatment of Affi-Gel Blue resulted in the loss of albumin with approximately 35% loss of total number of protein spots compared to 1 h treatment [51] (Fig. 1). These observations suggest that even though 16 h exposure of human serum to Affi-Gel Blue can result in significant loss of albumin and consequent enhancement of several low abundance proteins, it is also associated with nonspecific removal of serum protein other than albumin. We have recently shown enhanced visualization of potential biomarkers in the serum of ovarian cancer patients using 1 h Affi-Gel Blue treatment method [53]. Other dye-ligands that could alternatively be employed to remove abundant blood proteins include Procion Red He3B, Reactive Blue MRB, Reactive Green H4G, Reactive Green HE4BD, Reactive Yellow M8G and Reactive Brown M4R all of which can be coupled to supports such as Sepharose 4B or 6B [54]. However, the draw back in the usage of these reactive dyes is that the planar ring structure of these dyes through a complex combination of electrostatic, hydrophobic and hydrogen bonding can bind other proteins besides albumin resulting in non-specific removal of proteins [51,52]. Hence, the use of reactive dyes in affinity based depletion methods for the removal of high abundance proteins should be used with caution. However, under optimized conditions the use of reactive affinity dyes in conjunction with a supporting matrix can be a useful tool for the depletion of high abundant proteins from biological fluids [51,52].

In an effort to remove high abundance protein from cerebrospinal fluid to identify markers for Alzheimer's disease, Patterson [55] in combination with Oxford GlycoSciences and Pfizer have developed a method for selective removal of high abundant albumin, IgG, transferrin and haptoglobin by affinity depletion before 2-DE analysis. The study involved 512 samples and resulted in the visualization of 1131 protein spots in 2-DE map and the identification of potential markers of Alzheimer's disease [55]. Recently, Agilent Technologies have introduced "Agilent multiple affinity removal system" that binds and retain six highly abundant protein-albumin, IgG, IgA, transferrin, antitrypsin and haptoglobin in one step (Agilent Technologies Inc., USA). This multiple affinity removal system uses 1100 series HPLC to ensure consistency of results within the samples and the ability to automate sample processing. Hence, the technology combines the specificity of antibody–antigen recognition with the efficiency of standard liquid chromatography instrumentation resulting in substan-

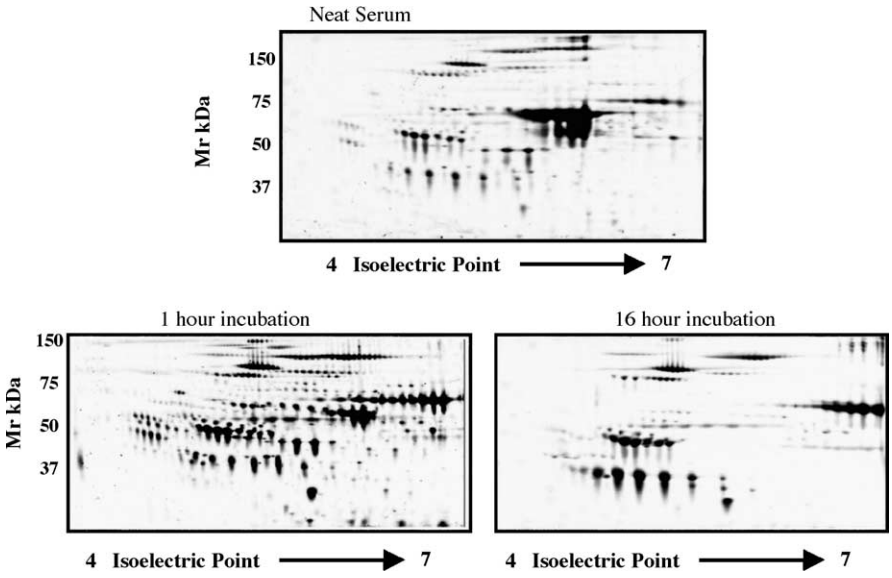

Fig. 1. Time dependent removal of albumin after treatment of human serum with Affi-Gel Blue. Serum sample was treated with Affi-Gel Blue for 0 min, 1 and 16 h before being resolved by 2-D PAGE.

tial removal of high abundance protein with subsequent increased loading of low-abundance proteins onto gels and enhanced detection of low-abundance serum proteins in a high throughput fashion. In a similar fashion, the minor proteins in human milk were analyzed by using immuno-adsorbents to remove three major proteins (α-lactalbumin, lactoferrin and secretory immunoglobulin A) [56]. In another study, an immunoaffinity column that contained β-casein and bovine IgG-specific immobilized sepharose was used to remove major proteins and low abundance bovine milk proteins were identified [57]. In addition to these affinity methods, an affinity spin tube filter method that relies on protein G-antibody binding has been used either to remove high abundance protein or to enrich the proteome of specific low abundance proteins [58]. These studies indicate that immunoaffinity based methods are effective in selective removal of high abundance proteins and are gaining considerable importance for the identification of low abundance proteins in biological fluids.

4.2. High abundant protein depletion strategies using multiple chicken IgY antibodies

Recently, the use of chicken IgY antibodies raised against high abundant proteins such as albumin, transferrin, fibrinogen, IgA, IgM, IgG covalently linked to a solid support has gained prominence in the depletion of human plasma of high abundance protein before 2-DE PAGE (Charles River Proteomic Services, Worcester, MA). This method allows high throughput depletion of abundant proteins from serum/plasma of multiple mammalian species. Due to greater evolutionary differences between birds and mammals, there is a greater probability of producing specific and potent antibodies against evolutionary conserved high abundant mammalian antigens by immunizing chickens. The yolk of eggs laid by an immunizied chicken is an abundant source of polyclonal antibodies. The avian equivalent of IgG, usually referred as IgY, is significantly different from IgG. In addition to having different sequence, IgY has higher molecular weight, higher electrophoretic mobility and lower isoelectric pH. Moreover, ionic detergent has no effect on the affinity of IgY for different antigens but can inhibit the interaction of IgG with some antigens. The hinge region present between the Fab pieces of the immunoglobulin is absent in IgY. This hinge region renders IgG less stable than IgY, making IgY more suitable to be covalently linked to solid-phase support. Moreover, chicken IgY does not cross react with mammalian IgG nor does it bind bacterial or mammalian receptors, reducing nonspecific binding and eliminating the need for cross-species immunoadsorptions.

Recently, we have been successful in developing ‘first and second generation chicken antibodies’ capable of depleting human plasma of high abundance protein (Fig. 2, Rice et al., unpublished data). The ‘first generation antibody’ was raised by immunizing chicken with multiple doses of whole plasma. The ‘second-generation antibody’ on the other hand, was raised against plasma that had already been subjected to affinity depletion by Affi-Gel Blue and immunodepletion using chicken IgY obtained from the first round (Fig. 3). Such method of immunodepletion before 2-D PAGE drastically reduced the visualization of high abundance protein making it possible to detect protein of low abundance (Fig. 3).

4.3. Low abundance protein enrichment in biological fluids by sample pre-fractionation

Pre-fractionation of a protein pool prior to 2-DE can create discrete sample pools allowing better separation and identification of low abundance proteins. Current pre-fractionation methods include sequential extraction with increasingly stronger solubilization solution [59,60], sub-cellular fractionation [61,62], and selective removal of high abundance protein by using affinity chromatography methods. Recently, pre-fraction methods based on isoelectric trapping methods have been developed. The method relies on the use of multicompartment electrolyzer with an isoelectric membrane that separates plasma proteins into three main p*I* fractions, an acidic fraction, a basic fraction and an albumin fraction with a p*I* between 5.6 and 6.1 [27]. This method of fractionation procedure enables increased protein loads and eliminates protein precipitation during IEF and increases the number of more acidic proteins visualized on narrow range 2-DE gels compared to un-fractionated plasma [27]. However, major draw back in this method is the loss of protein close to the p*I* 5.6 due to protein precipitation on the membrane. Others have used a miniaturized form of an IEF device or a flat bed granulated Sephadex gel [63] or SDS–PAGE-based size fractionation techniques [64] to enrich biological fluids of low abundance proteins.

Gradiflow method that relies on protein separation based on molecular size by selection of specific separation membrane cut off size and charge on the protein by adjusting the pH of the system has also been used [65]. Horvath et al. [66] reported the use of cibacron blue affinity matrices in the Gradiflow system to enrich the majority of serum proteins through the depletion of albumin. While others have reported the combination of only charge and size separation capacity of Gradiflow to deplete plasma of albumin [66]. Using the Gradiflow method, one can separate proteins in a biological fluid at high ionic strength to help maintain the solubility of proteins near their isoelectric point without the use of denaturants [65]. As the method does not use IEF membranes for the charge separation of proteins, protein aggregation and precipitation common in conventional IEF-based membrane separation system can be minimized. On the other hand, Gradiflow’s ability to separate proteins by molecular weight enables the separation of serum proteins away from albumin.

The abundant profile of human urine changes as a result of disease state or drug toxicity. To succeed in identifying the differential protein in human urinary proteome, protein fractionation strategies enriching proteins of molec-

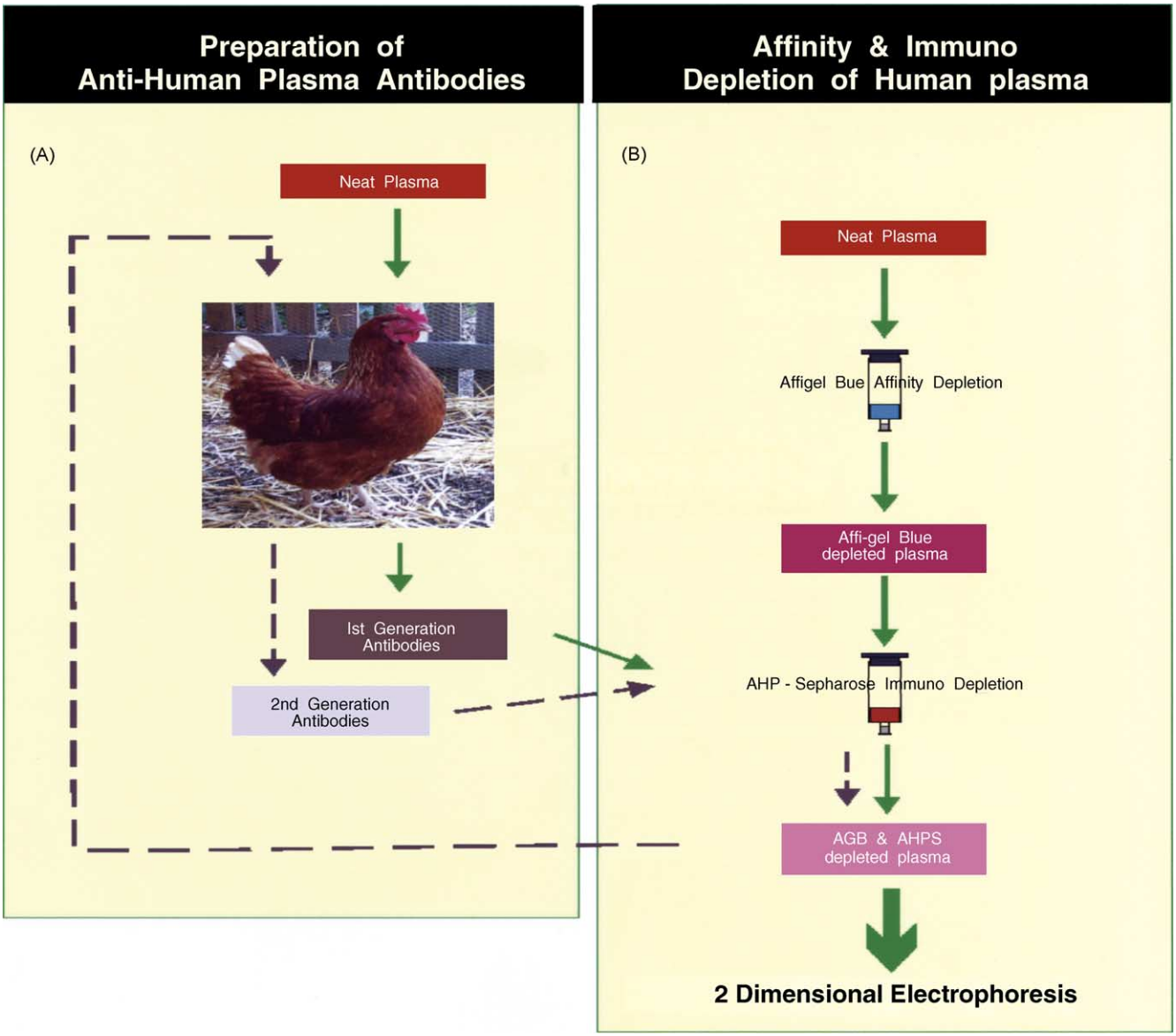

Fig. 2. Schematic representation of a device for the processing of multiple chicken IgY antibodies.

ular masses lower than 30 kDa is required as the kidney restricts passage of plasma proteins from the glomerular filtrate in the Mr range above 40 kDa. Pieper et al. [67] has recently described a protein fractionation strategy separating proteins of molecular mass lower than 30 kDa from larger proteins. The fraction containing proteins with M_r higher than 30 kDa was depleted of high abundant albumin and immunoglobulin G by immunoaffinity subtraction chromatography [67]. Following 2-DE display, superior protein resolution was obtained and the application of mass spectrophotometry to protein spots led to a successful identification of 30% of urinary proteins with M_r values higher than 30 kDa. The fractionation approach used by Piper et al. [67] holds promise for the biomarker discovery of proteins from the urine of patients suffering from different pathological conditions.

4.4. Use of overlapping narrower pI range strips for enrichment of low abundance protein in biological fluids

The use of wide range pH gradient such as 4–7 or 3–11 gives an overview profile of a proteome displaying hundreds of overlapping proteins that are impossible to detect and identify. In order to detect proteins of low abundance it is crucial to simplify such profiling of protein so that individual spot can be visualized. The advantage of using overlapping narrow immobilized pH gradients is to gain higher resolution of protein by stretching the protein pattern in the first dimension. This simplifies the computer aided image analysis and allows the visualization and identification of more proteins. This method has been used for the resolution of a yeast proteome where a total of 2286 yeast proteins were spotted using pH gradients of 4–5, 4.5–5.5, 5–6, 5.5–6.7 and 6.9 compared

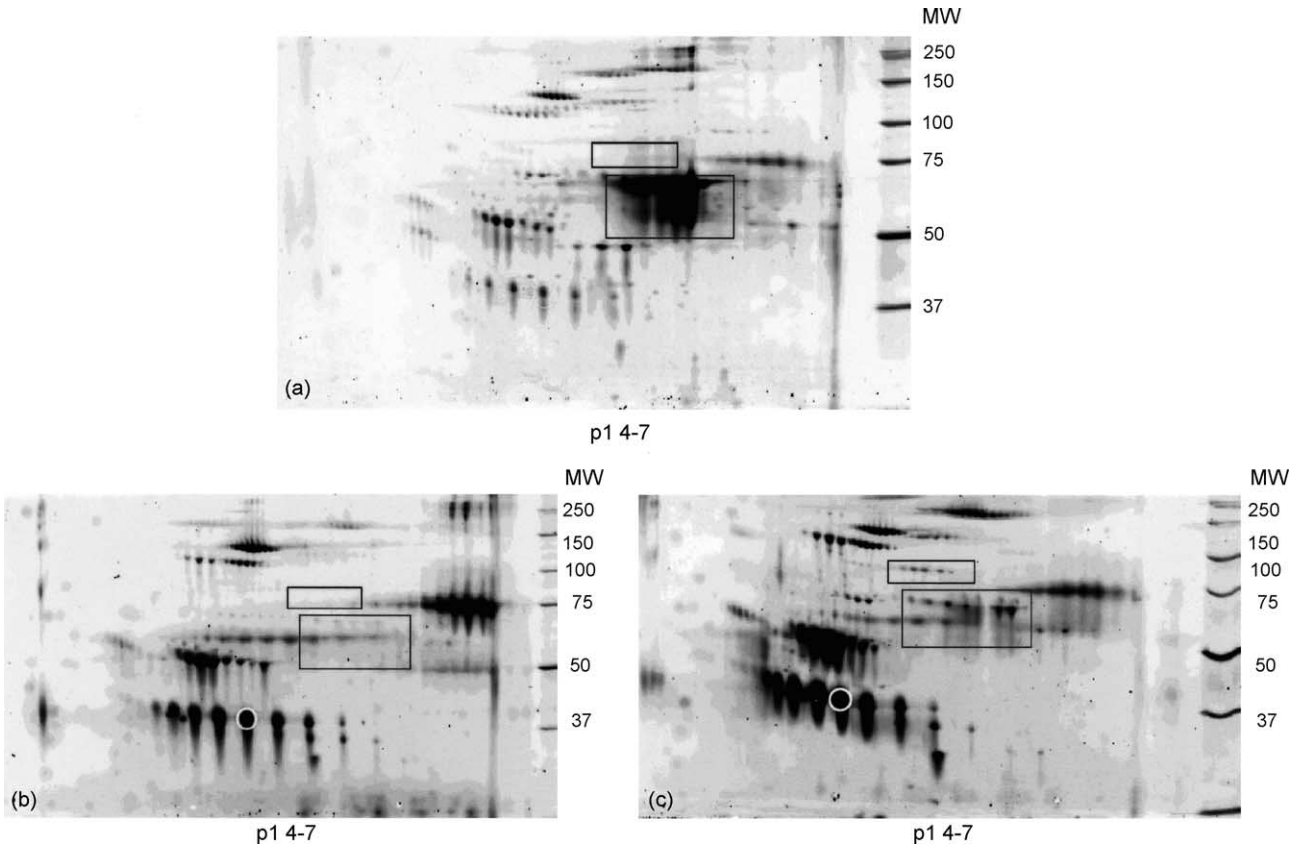

Fig. 3. 2-DE profile of human serum before and after treatment with chicken IgY antibodies: (a) profile of untreated human serum; (b) display of proteins present after Affi-Gel Blue treatment and (c) profile of proteins present after treatment with Affi-Gel Blue and then anti-human serum Sepharose 4B. Differences in the protein profile are indicated by blue, red and yellow colour. In neat untreated serum (a) protein smear outlined in red is albumin.

to 755 protein spots in the pH 3–10 gradient [68]. Hoving et al. [69] have also used this method to visualize a larger proportion of the B-lymphoma proteome to detect proteins present at low copy numbers. Using such over lapping narrow p*I* strips recently we have been successful in identifying low abundant biomarkers in serum from ovarian cancer patients (Ahmed et al., unpublished data). However, overlapping narrower p*I* range strips can be used successfully for acidic and neutral p*I* proteins. Protein separation in the alkaline pH range has recently been optimized [69].

4.5. Enrichment of low abundance protein by localized venous drainage

One of the methods that has evoked recent interest for protein expression profiling for markers of cancer involves collection of venous blood close to the site of tumor growth. Such localized drainage of venous blood allows analysis of concentrated protein mixture without being diluted by the systemic circulation. The underlying rationale behind this approach is that the cells in tumor tissues are likely to over express proteins that may be released directly or shed by 'ectodomain shedding' a process, in which the extracellular domain of transmembrane proteins are proteolytically cleaved from the cell surface and shed into the neighboring blood vessels before being diluted by the systemic circulation. For example, the cancer specific antigen 125 (CA-125) is a serum marker for the diagnosis of ovarian cancer. CA-125 shows wide variation in sensitivity and specificity in the serum of ovarian cancer patient [70], yet high levels of this marker may be detected in ovarian cancer patient's ovarian venous drainage. Due to the multi-factorial genesis of cancer, it is likely that a combination of several markers will be required to effectively predict the biological behavior of a tumor. For example, neoplasms are classified into several subtypes and are divided into primary and metastases tumors originating from the primary site. Hence, in cancer research there is a need to develop markers that will aid in the classification of these different tumors. This is not only required for academic interest but is also crucial for optimal treatment choices. In this context, use of venous blood close to the tumor site may serve as a useful source for the development of markers that may aid in the classification of different tumors.

Apart from the low abundant protein enriching fractionation methods described above, protein–protein interaction in immunoglobulin G-associated minor proteins in biological fluids can be visualized and identified by techniques that employ combination of denaturing and nondenaturing 2-DE gels (1% agarose) [71]. These methods allow the visualization and identification of native protein non-covalently associated in

complexes. Structural analyses of these proteins may provide new aspect on the functions of proteins present in biological fluids.

5. Advances and limitations of 2-DE based technology

A number of methodological improvements have been made to 2-DE technology since its introduction by O'Farrell and Klose in 1975. The development of immobilized pH gradients of different ranges has made the technique more reproducible and allowed comparison of results between inter-laboratory groups. This improvement has also resulted in increasing the loading capacity of a sample making the technique more sensitive for the detection and identification of low abundance protein. The resolution of basic and hydrophobic proteins has also been increased by the introduction of narrower basic pH strips [69] and by the introduction of new reagents such as more efficient detergents and chaotropes. The use of novel extraction procedures to deplete a complex proteome of high abundance proteins present in several orders of magnitude have made possible the visualization and detection of low abundance biologically important proteins. However, the extraction procedures currently in use result in the several-fold dilution of protein samples leaving one with inadequate concentration of protein to load on the gel. Hence, to achieve increased protein loading capacity of a proteome subjected to high abundance protein depletion procedures, optimal method of concentrating dilute protein samples is required. The use of precipitation techniques, high molecular weight cutoff centrifugal filters and freeze drying devices has greatly facilitated in concentrating dilute protein mixtures over hundred fold and have thus, aided in overcoming the protein loading problem.

The introduction of some of the more sensitive staining dyes has increased the visualization and made identification of protein spots present in minute amount feasible. Previously, visualization of proteins separated by 2-D PAGE was dependent on Coomassie blue or silver staining. However, the recent introduction of fluorescent dyes offers substantial advantage over these colorimetric detection methods and has increased the sensitivity and quantitative reproducibility of a proteomic profile over a broad dynamic range. Fluorescence two-dimensional differential gel electrophoresis (2-D DIGE) is a new advancement in proteomics technology. In this technique, proteins can be labeled co-valently by two fluorescent dyes prior to isoelectric focusing. The mixture of protein is then run on a single 2-DE gel. This method allows differential expression analysis of protein and significantly reduces the artifact problem introduced in gel matching using standard fluorescent dyes such as Sypro Ruby.

One area within the field of proteomics that has expanded most is the automation of protein characterization in mass spectrometry (MS). With full automation, the matrix assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF-MS) and nano-electrospray quadrupole-quadrupole time of flight mass spectrometry (*n*-ESIQ(q)TOF-MS) is capable of analyzing several hundreds of proteins separated on a gel within a reasonable time period. The introduction of robotic spot cutters and robot guided image analysis software results in high throughput identification of proteins separated on a gel. The improved sensitivity of the MS instrument at present can identify proteins present in picomole quantity [72].

The development in computer-based image analysis systems (e.g., PDQUEST and MELANIE) has allowed the efficient evaluation of hundreds of proteins separated on 2-DE gels. However, the accuracy of gel matching can decline with the heterogenity between samples being compared and if the experiments are run under different conditions. Even though, most of the available 2-DE software has the automated processes of spot detection, quantification, matching and statistical analysis, a limited level of manual editing and re-evaluation is still required. Hence, caution should be taken during manual editing and re-evaluation to eliminate the chances of introducing false positives.

The availability of public databases has made the identification of sequenced proteins easy. These databases are regularly updated and offer access to information on different proteins. These databases can be reached through Web sites like ExPASy server in Geneva or NCBI port in USA. The availability of such databases makes inter-laboratory gel comparisons possible and enhances protein spot identification by gel matching. However, exchange of 2-DE gel patterns between inter-laboratory experiments should be treated with caution as minor variations in sample handling before 2-DE may give rise to protein patterns with specific loss or gain of protein spots. Interesting developments in proteome data acquisition that incorporate the development of laboratory information processing systems (LIPS) [73] such as sample information, clinical diagnosis, analyzed images, protein identification and quantification, etc will increase the possibilities of multivariate analysis of data and will prevent the loss of vital biological information. Such an integrated network of information will result in clinicopathological diagnosis of disease resulting in accurate prognosis and treatment prediction.

6. Future perspectives and conclusions

The focus in biomedical research is to identify early stage markers for the diagnosis and better therapeutic treatment of diseases or to determine the molecular defect that underlines a specific disease or to identify potential targets for drug treatment. Proteomics is used as a technology to solve the underlying basis of all these problems. The basis of the technology is not only to list differentially expressed proteins in a proteome, but the technology acts as a circuit where the intercellular information generated in a proteome is portrayed in a protein profile. The differences in the protein profile

of a diseased proteome compared to that of a normal one could be potentially used as a diagnostic marker. However, the search for a single marker for disease identification or a single target for drug treatment is not likely to be successful for complex diseases such as cancer. Proteomics is the only technology that offers sufficient resolutions to understand the subtle phenotype changes in a diseased proteome with a view to develop better markers and drug targets. However, there is a great need for the development of methods that would simplify complex protein mixtures of a human proteome. The technology is challenged by the presence of as many as 100,000 proteins with a dynamic range estimated between 5 and 12 orders of magnitude (7). Scientists working with human cells have long recognized the need to develop pre-fractionation procedures either at the sub-cellular level or in combination with isoelectric fractionation to deplete the human proteome of abundant proteins so that successful mining for low abundant proteins is achievable. The intense ongoing effort addresses the development of several advanced physicochemical methods for protein fractionation. The use of sub-cellular fractionation protocols, affinity methods, laser micro-dissection technology, fractionation by narrower p*I* range strips and antibody adsorption methods have gained recent interest. In most cases, the use of a single fractionation method is not sensitive to detect the rare low abundant proteins and a combination of sub-cellular and iso-electric fractionation protocols are required to allow quantitative assessment of the enriched minor components. By allowing one to adhere to such multiple fractionation methods there is a possibility that the proteins of interest may show up in one or more fractions. With the development of each fractionation protocol for tracking low abundant proteins new information is being obtained. Procurement of such information is essential for biological/clinical researchers to understand the complex biological processes governing a diseased state and to make progress in the diagnostic and prognostic fronts. But before that can be achieved, a period of exploration and optimization is still required to simplify the human proteome by high abundant protein depletion.

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