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Review

Human blood plasma preparation for two-dimensional gel electrophoresis☆

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Abstract

Human plasma consists of mainly large proteins, which vary in terms of both composition and concentration with the physiological state of the individual. Alterations in protein concentrations reflect the current state of the individual's health and thus may be utilized as valuable biomarkers for a specific biological process or disease. Two-dimensional gel electrophoresis (2-DE) has proven to be a valuable method for the separation and comparison of complex protein mixtures, for example, from disease and healthy states, as this method provides information regarding the variation, relative quantities, and structures of the intact proteins. The procedures utilized for the preparation of samples for 2-DE are critical to the acquisition of high-quality results for the discovery of biomarkers. The objective of this study was to review the preparation methods of plasma for 2-DE, particularly those designed to improve the detection of proteins in low abundance in plasma on 2-DE. The use of anticoagulants and protease inhibitors during the collection of blood, the removal of abundant proteins using multicomponent immunodepletion system, and desalting procedure allow us to compile profiles of proteins occurring in low concentrations in the plasma and to improve the pattern generated during 2-DE.

Keywords: Human blood plasma; Two-dimensional gel electrophoresis (2-DE); Proteomics

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1. Introduction

Human plasma is a rich source of biochemical products which can function as indicators of the physiological or clinical status of patients [1–3]. It also evidences high-protein content, including proteins that are synthesized and secreted, shed, or lost from body cells and tissues [4,5]. These protein concentrations in plasma are controlled tightly to balance their physiological functions in areas including immunity, coagulation, small molecule transport, inflammation, and lipid metabolism [6]. Therefore, changes in plasma protein concentrations can be considered reflective of the current state of health [1–5]. Recently, the discovery of biomarkers from blood plasma has become the subject of intensive attention, with the considerable advances that have thus far been made in proteomic research [7–16]. The analysis of large numbers of proteins via proteomic techniques has greatly accelerated our ability to identify new biomarkers for disease or toxicity processes, including cancer [17], diabetes [18], stroke [19], kidney diseases [20], and exposure to aromatic hydrocarbons [11–13]. However, it has been estimated that up to 10,000 proteins may be normally present in plasma, the concentrations of which would vary, from millimolar concentrations for albumin to femtomolar concentrations of low abundant components [1,4]. The majority of potential disease biomarkers may be present at extremely low concentrations within the plasma, and thus might be obscured by the presence of more abundant proteins with similar characteristics [1,4].

As plasma contains such a variety of proteins in a wide and dynamic concentration range, two-dimensional gel electrophoresis (2-DE) is currently the principal technique for the separation of such complex protein mixtures [21–23]. 2-DE is a technique for protein separation that combines two different electrophoretic methods: isoelectric focusing (IEF) in the first dimension (in which proteins are separated according to their isoelectric points [p*I*]) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension (in which proteins are separated according to their molecular weights) [24]. 2-DE not only generates information regarding protein modification and/or expression level changes, but also allows for the isolation of proteins in milligram amounts for further structural analyses, using MALDI-TOF MS, ESI-MS [49–52], or Edman micro-sequencing techniques [25,26]. Owing to these advantages, 2-DE has been used extensively for the discovery of new biomarkers [1,3]. Examples of biomarkers identified via 2-DE are provided in Table 1 [27–39].

When applying 2-DE to complex samples such as plasma for the discovery of biomarkers, it is critical to establish sample preparation procedures in order to obtain high-quality results. The most important component of this procedure is the method selected to (1) prepare plasma while minimizing the loss of plasma proteins, (2) deplete high-abundant proteins, (3) remove contaminants that may interfere with 2-DE analysis, and (4) solubilize the proteins to transform each of them into a physicochemical state suitable for 2-DE analysis. In this review, we have attempted to provide current methods for the preparation of human blood plasma for 2-DE analysis, especially those designed to improve the detection of proteins in low-abundance in plasma.

2. Methodology

The general procedure of plasma preparation for 2-DE analysis is shown in Fig. 1. This procedure involves plasma collection, the depletion of high-abundant plasma proteins, the removal of contaminants that may interfere with 2-DE analysis, and the solubilization of proteins.

Table 1
Examples of biomarkers identified by 2-DE

Context	Biomarker(s) identified	Refs.
Benzen exposure	T cell receptor β-chain, FK506-binding protein, matrix metalloproteinase-13	[12]
Polycyclic hydrocarbon exposure	Putative capacitative calcium entry channel	[13]
Strock	Fatty acid binding protein (FABP)	[19]
Ovarian cancer	Haptoglobin-α	[28,34]
Gastric carcinoma	Annexin V, carbonic anhydrase, prohibitin, fibrin beta, fibrinogen fragment D	[29]
Cardiovascular	Mature Cathepsin D	[30]
Severe acute respiratory syndrome	Peroxiredoxin II	[31]
Severe acute respiratory syndrome	Truncated α_1-antitrypsin	[32]
Lung cancer	Haptoglobin, HGF	[33]
Pregnancy	Clusterin	[35]
Acute myeloid leukemia	α-2-HS glycoprotein, complement-associated SP-40, RBP4, lipoprotein C-III, haptoglobin, immunoglobulin heavy-chain variant, proteosome 26S ATPase subunit 1, haptoglobin-1	[36]
Myocardial infarction	Antitrypsin isoform 1	[37]
Cardiac allograft rejection	α-Crystallin, tropomyosin	[38]
Relapsing polychondritis	Tubulin α ubiquitous, vimentin, alpha enolase, calreticulin, colligin-1	[39]

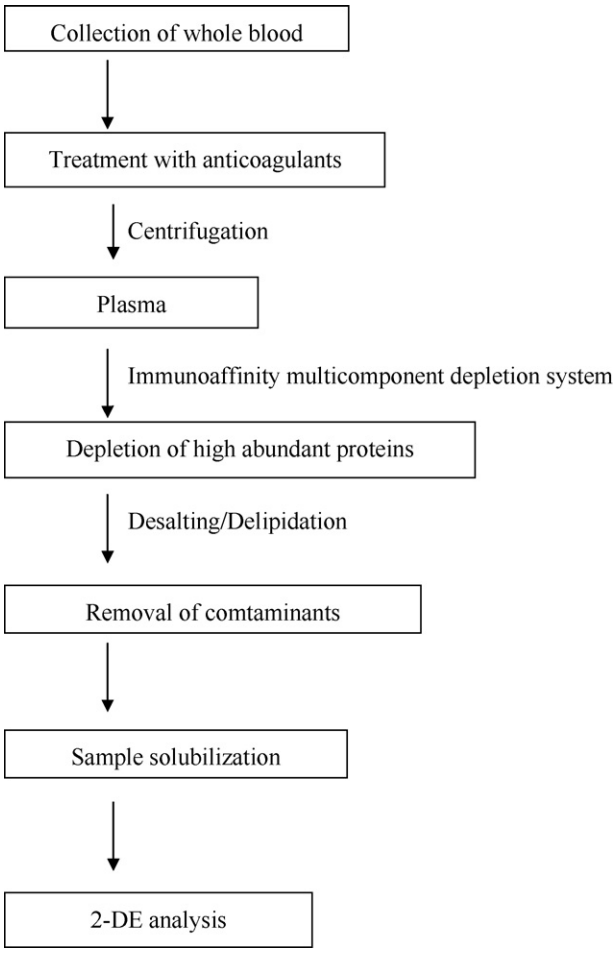

Fig. 1. Representive procedure for plasma preparation for 2-DE analysis.

2.1. Preparation of plasma and serum

The method for the collection of the plasma samples is of great import, as the composition and concentration of plasma proteins varies with the collection method [40,41]. Blood samples can be collected within a blank tube without additives, or drawn in with a syringe via vacuum into a tube containing an anticoagulant, e.g., ethylenediamine tetraacetic acid (EDTA), heparin, or citrate; they may also be either stored at room temperature or refrigerated [42,43]. The type of anticoagulant and its concentration, the plasma storage method (i.e., storage duration or temperature), treatment with a protease inhibitor cocktail (PIC), the timing of supernatant decanting after centrifuging the sample, the number of freeze–thaw cycles, etc., will all contribute to the quality of the blood sample and, thus, affect the proteome in the plasma. Plasma collected with PIC can withstand temporary storage at 4 °C, temporary storage at −20 °C, or at least a single freeze–thaw cycle [42]. It is also vitally important to acquire the supernatant quickly after centrifugation. In addition, preanalytical variables, including sample handling, can alter the low-molecular-weight proteome within the plasma [44]. It was also recommended that at least 30–60 min of clotting time prior to processing would minimize the differences inherent to the overriding effects of coagulation, on the basis of the use of activator tubes, which promote rapid clotting [44]. Marshall et al. have determined that phenylmethylsulfonyl fluid (PMSF) and ethylenediamine tetraacetic acid can affect the stability of the serum samples, as shown by MALDI-MS [42]. In particular, evaluations of the differences in proteins between serum and plasma treated with different anticoagulants (EDTA, citrate, heparin) have been published by several researchers [41–43,45].

2.1.1. Serum

Serum is obtained by allowing the blood to clot prior to centrifugation. Many of the proteins involved in the coagulation process are also removed during the clotting process. In serum, prothrombin is cleaved into thrombin, fibrinogen is removed to facilitate clot formation, and a limited series of other protein changes (including proteolytic cleavages) occur during the clotting process [4]. Although many of the proteins involved in the coagulation process, including prothrombin, will disappear during these procedures, many of the peptides that are absent in plasma but are detectable in the serum can be identified after coagulation [45]. In studies of the differences between human serum and plasma using an orthogonal 3D intact protein analysis system (IPAS), several proteins, including alpha, beta, and gamma fibrinogens, as well as vitronectin, have been shown to be attributable to coagulation-associated proteins [43]. In addition, proteins which were expected to be depleted during the coagulation process were generally much lower in the serum samples, as compared with the EDTA-plasma or citrate-plasma. Tammen et al. reported in their peptidomic analysis of human blood specimens that a variety of artificial peptides are derived from peptides released during coagulation processes or from other types of enzymatic activity (plasma kalikrein) [45]. Due to the high levels of artificial peptide signals, they concluded that the use of serum samples is not appropriate for peptidomic studies of mono/oligo biomarker discovery.

2.1.2. Plasma

In order to study the plasma proteome, the blood samples from which the plasma is obtained must be treated with anticoagulants [4]. A treated blood sample can then be centrifuged in order to remove the blood cells, thereby leaving the plasma for subsequent studies. Although plasma compositionally resembles serum, it is preferred over serum for proteome studies by many (although not all) laboratory investigations, as its constituents reflect patient pathological status more closely than do the serum constituents. In addition, some changes in the blood constituents can be avoided via the careful selection and control of the types and concentrations of the employed anticoagulants [41]. The most popular anticoagulants are EDTA, heparin, and sodium citrate. There have been several reports of changes in protein expression patterns occurring in conjunction with anticoagulant treatment [41,44]. Citrate and EDTA inhibit coagulation and other enzymatic processes via the chelate-formation of ion-dependent enzymes, in contrast to heparin, which functions via the activation of antithrombin III. In general, the maintenance of hemostasis and the balance between coagulation and fibrinolysis essentially requires a series of enzymatic reactions, which cause sequential activation and the inactivation of protease, and

Table 2
Comparison for depletion methods of high-abundant proteins in plasma

Classification	Methods	Advantage	Disadvantage
Affinity based	Cibracon Blue and related dye	-Low cost -High loading capacity	-Lack of specificity
	Protein A/G	-High affinity for immunoglobulins	-Possible to remove only immunoglobulins -Not bind all of the immunoglobulin subgroups
	Peptide-based ligands	-Low cost -High reproducibility -Sterilizable -Reactivity across species	
Size based	Centrifugal ultrafiltration	-Convenient -Low cost	-Low effectiveness -Low reproducibility
Antibody based	Monoclonal antibody Polyclonal antibody	-High specificity	-Fail to remove the related fragments of target proteins
	IgG-based system	-Depletion of multiple proteins in one step -High affinity and high specificity	-Lack of reactivity across multiple mammalian species -High cost -Low sample capacity
	IgY-based system	-Depletion of multiple proteins in one step -High affinity and high specificity -High reactivity across multiple mammalian species	-High cost -Low sample capacity

platelet activation causing clot formation. Accordingly, the concentrations of plasma proteins are dependent on platelet stability [44]. Platelets tend to be most stable in citrate anticoagulants. EDTA-treated blood is only slightly less stable. However, over longer time periods, marked changes become apparent as the elapsed time prior to centrifugation increases [45].

2.2. Depletion of high-abundant plasma proteins

Many proteins that could function as good biomarkers are detected at relatively low concentrations [1–4]. One of the challenges in the identification of specific biomarker proteins is the removal of proteins with high concentrations, which interfere severely with the ability to detect proteins at lower concentrations in the plasma [46]. The most abundant plasma proteins constitute approximately 97% of the total plasma proteins [4]. The core plasma protein, albumin, comprises approximately 50% of plasma protein content, and is present at high concentrations (on the order of 30–50 g/L). The other principal plasma proteins (present on the order of g/L) are immunoglobulins (Igs), including both heavy and light chain-fibrinogen, transferrin, haptoglobin, and lipoproteins. These proteins would constitute principal candidates for removal prior to the performance of a proteomic analysis of low-abundant proteins. A considerable number of attempts have been made to remove albumin and other abundant proteins from the plasma.

The primary approaches for the depletion of albumin or high-abundant proteins have been reported as follows, and summarized in Table 2: an affinity-based method, a size-based method and an antibody-based depletion method are assessed [47,49,51–53,55–66].

2.2.1. Affinity-based methods

The affinity-based methods are predicated on the affinity of albumin for Cibracon Blue (CB) and associated dyes [52,56,57,59]. CB dye has not been used expressly for binding albumin, but also for the binding of NAD, FAD, and ATP binding sites of proteins, via ionic and hydrophobic interactions, which allows CB dye to bind to a large number of other proteins, resulting in the potential loss of other proteins [52,53]. This non-specific binding probably includes both proteins that bind to the dye as well as some minor proteins that bind albumin [59,67]. Despite this high degree of non-specific protein binding, this method has several advantages, including high-loading capacity and low cost [65]. Due to their relatively low cost, they are disposable, which prevents any cross-contamination between different samples [66].

Generally, IgG removal is commonly achieved by the immobilization of protein A or protein G onto the affinity resins, which bind to the Fc region of the IgG [51,53]. However, Proteins A and G may not bind to all of the immunoglobulin subgroups, thereby leaving a portion of these heterogeneous abundant proteins behind. Other methods involve the use of a proprietary polypeptide affinity matrix and a peptide-based affinity medium that removes albumin [51–53]. The peptide with modified sequences of the Fc region was produced by protein engineering in an effort to increase albumin binding capacity, which has been employed for the depletion of abundant proteins from plasma [51]. The advantages of these peptide-based ligands include low cost, reproducibility, sterilizability, a completely defined product, and nonbiological origin.

2.2.2. Size-based methods

Another depletion method involves the use of a centrifugal ultrafiltration device for the separation of proteins by size. The effectiveness of this approach, however, has been reported to be limited [59,61]. As ultrafiltration membranes have a normal pore-size that represents the average of a normal distribution of smaller and larger pores, it is somewhat common to observe suboptimal retention rates particularly for high concentrations, as in serum samples [61], thereby highlighting the limitations in reproducibility.

2.2.3. Antibody-based depletion methods

Antibody-based depletion methods have recently been developed and described by many researchers [47,49,50,60,62–66]. Individual antibody methods have proven more specific in the depletion of targeted proteins, and resulted in a more complete removal of abundant proteins [48,49,51,60]. Monoclonal antibodies appear to represent a promising option for the depletion of high-abundant proteins, and have been used to target a specific epitope of these proteins, thereby resulting in the removal of the targeted proteins. However, this technique may not be appropriate for the recognition of all forms of the targeted protein, due to the presence of proteolytic fragments, post-translational modifications (PTMs) such as glycosylation, phosporylation, the disruption of the antigen-binding site in partially or completely unfolded proteins, or the occlusion of the epitope as the result of protein-protein interactions [48].

Polyclonal antibodies, on the other hands, are more likely to deplete multiple structural forms of a protein. They are desirable for the depletion of as many high-abundant proteins as possible, while minimizing the incidental losses of nontargeted proteins. Recently, the depletion systems using several polyclonal antibodies with high specificity have been developed, which can remove multiple proteins simultaneously, in a rapid and efficient manner [50,62–66]: immunodepletion systems based on IgG and IgY antibodies [48,50,62–65]. The relative popularity of these methods may be resultant from this polyclonality of the immunoglobin ligands.

The immunodepletion system based on IgG is capable of simultaneously removing six of the most abundant proteins in human plasma, albumin, immunoglobulin G (IgG), immunoglobulin A (IgA), haptoglobin, transferrin, and alpha-1-antitrypsin, using high-specificity rabbit polyclonal antibodies. This method is capable of the depletion of high-abundant proteins, including different molecular forms and many proteolytic products of these proteins, coupled with low nonspecific losses of other proteins, rapidly and efficiently, in a single purification step [50,58,62,65,66]. An immunodepletion system based on IgY employed chicken antibodies, which orient the antibody with the antigen-binding site facing outward, with a constant portion of the antibodies covalently coupled to a solid phase material. This permits maximum antigen binding efficiency. This system also allows for the depletion of human albumin, IgG, IgA, IgM, fibrinogen, and the transferrin, and depletion of albumin in a variety of mammalian species [64]. The principal disadvantages of antibody-based depletion include its relatively high cost and low sample capacity. Fortunately, antibodies are highly robust proteins, it appears likely that such systems will last for many purification cycles if appropriate care is taken to minimize both proteolysis and column clogging. Multicomponent immunoaffinity columns have recently become commercially available [54]. Although these commercial kits are relatively expensive, they are also quite convenient and useful for small samples, such as those that can be applied to electrophoretic gels.

With regard to format, approaches for the depletion of albumin or high-abundant proteins, using spin-column format and chromatography format, have been previously utilized. In the spin-column format, the reagent can be regenerated many times to deplete the same sample or multiple samples after proper column washing. In addition, the polyclonal antibody resin also functions effectively in a spin column format, which allows for the parallel processing of multiple samples or aliquots and requires no complex instrumentation. The liquid chromatography column format is also amenable to automation on a high-throughput scale. In order to analyze the clinical samples, speed and reproducibility are crucial for the accumulation of statistically reliable information.

Depletion is one method that allows the screening of low-abundant proteins in plasma, but depletion can also result in a risk of losing proteins of interest other than those specifically targeted for depletion. As albumin is a plasma transport protein that binds with a large number of compounds (including hormones, cytokines, lipoproteins, and amino acids) [68], its removal from plasma may also result in the removal of low-abundant proteins. Attempts to recover low abundant proteins from an immunoaffinity column have previously involved the use of a mild solvent, salt-out preparation, molecular sieve filtration, and a peptide-based affinity medium [49,51,69,70].

2.3. Inactivation or removal of interfering compounds

The 2-DE analysis of plasma has been shown to be especially difficult due to the many components that bind with proteins, the hydrolysis of the proteins into fragments, and the interference of other charged molecules used in the analysis. In addition, the plasma fractions depleted of high-abundant proteins may be diluted by the high-salt buffer. Therefore, if possible, such substances-principally proteases, salts, lipids, polysaccharides, and nucleic acids-should be eliminated and plasma proteins should be concentrated into small volume, prior to the application of electrophoresis.

2.3.1. Proteases

Proteases in plasma must be inactivated in order to prevent the degradation of proteins, during the whole procedure of plasma preparation for 2-DE, which might result in artificial electrophoresis spots. Protease inhibitors have been extensively used to inactivate proteases; in fact, the general protocol for plasma samples includes the application of a protease inhibitor cocktail (PIC). Hulmes et al. reported that the inclusion of a PIC in the plasma sample resulted in stable and reliable human plasma samples, which yielded reproducible results on proteomic analysis [41]. However, protease inhibitors are not always recommended, as they may modify protein structures and induce changes in the charge of the protein molecule. Proteases also are less active at lower temperatures; therefore, it is recommended that the plasma be prepared at as low a temperature as possible.

General methods for the inhibition of protease activity include the boiling of the plasma sample in SDS buffer in the absence of urea, including a high-pH buffer such as tris-base, the inactivation of the protease using low pH, or the precipitation of proteases using ice-cold 20% TCA solution. The majority of sample buffers have denaturing activity sufficient to inhibit protease activity in the plasma [71]. TCA/acetone precipitation has

proven quite useful for the minimization of protein degradation by proteases and for the removal of contaminants [72]. However, incomplete precipitation, protein resolubilization, or both may result in a partial loss of proteins of interest.

2.3.2. Salt ions

Salt ions may interfere with electrophoretic separation and, in some cases, may induce the modification of the protein structure [73]. During electrophoresis, salt ions may compete with proteins in migration toward the electrode, thereby interfering with protein focusing. Salts may also increase the conductivity of the IEF gel with increasing current. As protein focusing will not occur until these ions have moved to the end of the IEF gel, the time required for IEF will be prolonged. Salt concentrations in excess of 100 mM may induce electroendosmosis, resulting in an uneven distribution of water within the gel, and the subsequent formation of dehydration and over-hydration zones [74].

Therefore, salts must be removed prior to the loading of the IEF gel. Salts can be removed from plasma via dialysis, gel filtration, TCA/acetone precipitation, or the use of centrifugal filter devices for the concentration of proteins (e.g., Centricon columns; Millipore, Bedford, MA, USA) or the PlusOne 2-DE clean up kit (Amersham Biosciences, Uppsala, Sweden) [75]. Another way to overcome salt interference is the dilution of the sample to below a critical concentration of salt. The salt concentrations for immobilized pH gradient (IPG) strips should be lower than 10 mM in cases in which the sample is subjected to in-gel rehydration [76].

2.3.3. Lipids

Plasma turbidity is normally caused by its lipid components (triglycerides, phospholipids, and sterols), the concentrations of which can vary in accordance with the health and diet of the blood donor. Lipids can also interfere with immunodepletion procedures, the IEF current, and the 2-DE resolution. The removal of lipids from blood plasma has been generally accomplished using a solubilization buffer containing a strong denaturing agent throughout the sample preparation procedure [77], acetone precipitation with four volumes using 100% cold acetone at −20 °C for at least 2 h, or high-speed centrifugation [47].

Lipids bind to proteins via hydrophobic bonds, which affect their charges and molecular weights. These bonds can be broken using a charged compound which disrupts hydrophobic interactions. Detergents act on hydrophobic bonds by enabling the stable dispersion of hydrophobic residues within a hydrophilic environment [78]. SDS and dithiothreitol (DTT) are routinely utilized to treat plasma, followed by 5 min of heating at 95 °C. However, negative SDS charges may affect the p*I* of the sample. In order to minimize these adverse effects, the SDS concentration should be diluted to 0.25% (w/v) via the addition of an excess amount of ionic or nonionic detergent (e.g., Nonidet P-40 (NP-40), Triton X-100 or 3-3 [cholamidoprophy] dimenthlammonio-1-propanesufonate [CHAPS]) prior to IEF [75]. Through the lipid extraction using organic solvents (e.g., ethanol or acetone), many organic-soluble contaminants, including detergents and lipids will be retained in the organic phase. However, extraction methods can result in a severe loss of proteins, either because certain proteins are soluble in organic solvents, or because the precipitated proteins are not consistently resolubilized.

2.3.4. Nucleic acids and polysaccharides

Nucleic acids can interact with proteins via electrostatic interactions, thereby increasing the viscosity of the sample solution, clogging the pores of the polyacrylamide gels, preventing IEF, and eventually resulting in 2-DE patterns with horizontal streaks. When the gels are stained following 2-DE separation, the nucleic acids in the gel will also be stained, resulting in a background smear [79]. Nucleic acids can be removed by digestion with a mixture of protease-free RNase and DNase [80], by adding carrier ampholytes with subsequent ultracentrifugation [81], or by precipitation with a basic polyamine at a high-pH value [82]. TCA/acetone precipitation (typically 20% TCA in 100% acetone) can also be employed in the removal of nucleic acids, although this is associated with a risk of protein loss.

Polysaccharides can also interfere with IEF via the obstruction of gel pores. Ultracentrifugation can be used to remove high-molecular-weight polysaccharides. Precipitation in TCA, ammonium sulfate, or phenol/ammonium acetate effectively removes low-molecular-weight polysaccharides.

2.4. Solubilization of proteins

After the contaminants were removed, plasma proteins must be solubilized and kept soluble within a particular pH throughout 2-DE separation. The solubilization process includes the denaturation of the proteins to break noncovalent bonds and disulfide bonds within and among the proteins, in addition to noncovalent bonds between proteins and nonproteins, including lipids or nucleic acids, while maintaining the native charge and molecular weight of soluble proteins until the end of the 2-DE process [78]. The hot SDS method is the classic sample solubilization method. This method can be problematic, however, as the anion SDS may interfere with protein IEF [75]. The protein solubilization process has been amply reviewed by Show and Riederer [74].

Sample solutions for first-dimension separations always involve urea, a neutral chaotrope that denatures proteins via the disruption of noncovalent and ionic bonds between amino acid residues. Urea may be included in the sample solution, at a concentration of 5.0 M to 9.8 M. Urea solutions are, however, somewhat unstable. The spontaneous degradation of urea to cyanate can occur at room temperature. Cyanate ions remove the positive charges on amine and sulfhydryl groups in proteins (a process known as carbamylation), thereby affecting the efficiency of IEF. DTT is an effective cyanate scavenger. Rabilloud et al. reported on the utility of thiourea-containing denaturing mixtures for proteins that are highly prone to aggregation [78,83]. The use of thiourea in combination with urea, for IEF in particular, has been determined to improve the solubilization of hydrophobic proteins [84]. Thiourea is normally used at a 2 M concentration in combination with 5 M to 7 M urea. However, this tends to inhibit SDS-protein binding during the

second-dimensional separation, which results in poor separation on the 2-DE gel [74].

Disulfide bonds are broken via the addition of an excess of a thiol-reducing agent, e.g., 2-mercaptoehanol, DTT, dithioerythritol, tris (2-carboxyethyl) phosphine, and tributyl phosphine (TBP). However, a portion of the thiol-reducing agent will be ionized at a basic pH, enter the basic end of the gel, and ruin the pH gradient, thereby disrupting this region or inducing the reoxidation of the reduced disufide bonds [74,85,86]. In order to inhibit this reoxidation, it was required for the alkylation of the thiol group, which results in the prevention of the precipitation of some proteins and, frequently, in the production of spurious spots in the basic pH region as the consequence of the formation of disulfide bonds. The most frequently employed reductant is DTT, which is administered at concentrations in the range of 20–100 mM; 2-mercaptoehanol is no longer widely used in carrier ampholyte IEF. TBP does not appear to migrate during IEF, and should be useful in the separation of proteins. Unfortunately, it is somewhat difficult to handle [87–89].

Anionic detergent SDS has been traditionally used, but has been determined to interfere with the IEF of proteins, as stated previously [74,75,77]. The zwitterionic detergent, CHAPS, is currently the most common detergent utilized in standard 2-DE procedures, and belongs to the class of linear sulfobetaine surfactants including caprylyl sulphobetaine (SB3–10) and amidosulfobetaine 14 (ASB-14).

Carrier ampholytes also augment protein solubility via the minimization of protein aggregation and the production of more uniform conductivity across the pH gradient without disruption of the IEF or alterations in the shape of the gradient. Carrier ampholytes are small, soluble, amphoteric molecules with a high degree of buffering capacity near their p*I* [90]. When a voltage is applied during IEF, each carrier ampholyte moves into the closest p*I*, resulting in a continuous pH gradient [79]. Ampholytes may be utilized in concentrations as high as 2% (v/v). Carrier ampholytes also scavenge cyanate ions and facilitate the precipitation of nucleic acids during centrifugation [74]. The objective of solubilization processes is to achieve a well-focused first-dimensional separation: IEF. Additional solubility problems frequently arise during the IEF at sample entry and solubility at the isoelectric point. These problems can be solved by the use of a commercial (IPG) gel, which is prepared by the covalent incorporation of a gradient of acidic and basic buffering groups into a polyacrylamide gel, during the gel casting procedure.

In some cases, a buffer or bases are added when basic conditions are required for the promotion of full solubilization or the minimization of proteolysis. Bases or buffers should be diluted to 5 mM or less prior to the loading of the sample onto the IEF.

3. Conclusions

The discovery of protein biomarkers in plasma represents a significant technical challenge. Although a variety of proteomic techniques have been attempted so far, no generally applicable technique has yet been developed for the identification of biomarkers that can replace 2-DE with regard to its ability to separate and display several thousand plasma proteins simultaneously. The selection of an appropriate blood plasma preparation method is important for confident 2-DE results. The use of anticoagulants and protease inhibitors during blood collection increases the chances for consistent results. The removal of abundant proteins using multicomponent immunodepletion systems allows us to compile profiles of proteins occurring in low concentrations in the plasma. Desalting and acetone precipitation improve the pattern generated during 2-DE, although this may result in the loss of several proteins. These techniques harbor promise for more widespread use in the preparation of samples for the identification of biomarkers that can be used in early disease detection, as well as to monitor disease progression and augment the accuracy of the prognosis using human blood plasma.

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