UC 577,1; 61 ISSN 0354-3447

Jugoslov Med Biohem 25: 211–220, 2006

Revijski rad
Review paper

APPLICATION OF 2 D-HPLC SYSTEM FOR PLASMA PROTEIN SEPARATION

Isabella Levreri[1], Luca Musante[2], Andrea Petretto[3], Davide Cuccabitta[3], Giovanni Candiano[2] and Giovanni Melioli[1]

[1]U.O. Laboratorio Centrale di Analisi
[2]Laboratorio di Fisiopatologia dell'Uremia, U.O. di Nefrologia
[3]Laboratorio di Spettrometria di Massa, Core facility,
Instituto Scientifico Giannina Gaslini, Genova, Italy

Summary: The ProteomeLab™ PF 2D protein fractionation system is a rapid, semi-automated, 2 D-HPLC instrument that uses two different methods to separate plasma serum proteins: ion-exchange chromatography using a wide range of pH in the first dimension and non-porous reverse-phase chromatography in the second dimension. Because this methodology has only very recently been introduced in proteomic laboratories, little is known about the characteristics of PF 2D fractionation of human serum proteins. To evaluate the system's application in a clinical laboratory setting, the characteristics of the ion-exchange chromatography-based separation were analyzed. Following fractionation of human serum proteins on a linear gradient of pH (ranging from 8.5 to 4.0), each fraction was collected in a cool module of the instrument. Different fractions obtained from the first dimension were then pooled together and loaded on classic 2D gel electrophoresis instrumentation. The different spots obtained were then checked against the Swiss-Prot Database. A total of 36 human serum proteins were identified in different PF 2D-generated fractions. Some important features of the separation system were observed. Different eluted fractions contained different proteins, thus demonstrating the reliability of the fractionation system. The proteins were also fractionated according to the theoretical isoelectric point (p*I*). This was consistent with the evidence that the vast majority of immunoglobulins, characterized by an alkaline p*I*, were not retained by the column and were eluted in the unbound fraction. This outcome also underlies a practical advantage: fractions eluted from pH 8 to pH 4 contained virtually immunoglobulin-depleted serum proteins. This finding supports an immediate use of the PF 2D system in a clinical setting, where abundant proteins should be clearly identified in order to enable evalutation of other less abundant, but potentially relevant, species.

Key words: 2 D gels, plasma protein fractionation, ProteomeLab™ PF 2D, pI value

Introduction

Proteins have very well-defined 3-D structures. A stretched-out polypeptide chain has no biological activity, and protein function arises from the conformation of the protein, which is the 3-D arrangement or shape of the molecules in the protein. The native conformation of a protein is determined by a number of factors, and the most important are the 4 levels of structure found in proteins. Primary, secondary and tertiary refer to the molecules in a single polypeptide chain, and the fourth (quaternary) refers to the interaction of several polypeptide chains to form a multi-chained protein (*Figure 1*).

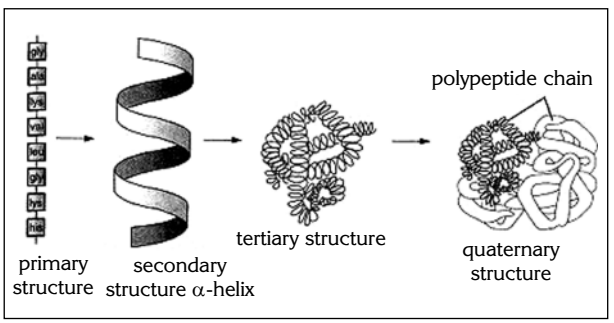

Figure 1 Protein structures

Address for correspondence:
Isabella Levreri, BD,
Laboratorio Centrale di Analisi
Instituto G. Gaslini, Largo Gerotamo
Gaslini 5, 16147 Genova, Italy
e-mail: i_levreri@yahoo.com

Primary structure

Primary structure refers to the number and sequence of amino acids in the protein or polypeptide chain. The covalent peptide bond is the only type of bonding involved at this level of protein structure (1). Thus they are sometimes called the »covalent structure« of proteins because, with the exception of disulfide bonds, all of the covalent bonding within proteins defines the primary structure. In contrast, the higher orders of proteins structure (i.e. secondary, tertiary and quartenary) involve mainly noncovalent interactions. The sequence of aminoacids in a protein is dictated by genetic information in DNA, which is transcribed into RNA, which is then translated into protein. So protein structure is genetically determined. Determination of primary structure is an essential step in the characterisation of a protein.

Secondary Structure

The next level of protein structure refers to the amount of structural regularity or shape that the polypeptide chain adopts. A natural polypeptide chain will spontaneously fold into a regular and defined shape. Two main types of secondary structure have been found in proteins namely α-helix, and β-pleated sheet (2).

Alpha helix. In the alpha-helix the polypeptide folds by twisting into a right handed screw so that all the amino acids can form *hydrogen bonds* with each other. This high amount of hydrogen bonding stabilises the structure so that it forms a very strong rod-like structure. The amino group of each aminoacid is hydrogen bonded to the carboxyl group of the 4th following aminoacid residue, which is on an adjacent turn of the helix. Along the axis of the helix, it rises 0.15 nm per aminoacid residue, and there are 3.6 residues/turn of the helix. The screw-sense of any helix can be RH or LH, but the alpha-helix found in proteins is always RH. The alpha-helix content of proteins of known 3D structures is highly variable. In some e.g. myoglobin and haemoglobin, a substantial amount of the polypeptide chain folds into alpha-helix. While in the protease enzyme chymotrypsin chain there is almost no helix. The ability of a protein to fold into helix is influenced by the amino acids and the R side chains that they possess. A prolyl residue tends to destabilise the alpha-helix structure, because its alpha-N is in a ring system, and cannot participate in the H-bonding. So pro residues tend to be found at bends in the alpha-helix, where helix destabilisation can allow a change in direction. A sequence of asp and or glu residues together can also destabilise the helix because they are highly charged, and repel each other. The forces of repulsion are stronger than the H-bonding. Also a cluster of ile residues with their large bulky R groups tends to disrupt the alpha-helix structure by disrupting the H-bonding.

Beta pleated sheets. In this case more H-bonding is achieved by stretching out the polypeptide chain, and laying it side by side to form H-bonds between lengths of polypeptide chain. Thus providing both inter and intra-H bonds. Called a beta-pleated sheet because of zig zag appearance when viewed from the side. The H-bonds are formed from amino and carboxyl groups as for alpha-helix, but bonding also occurs between different stands of a polypeptide. The stands can run in opposite directions to give antiparallel beta-pleated sheet or they can run in same direction to give parallel beta-pleated sheets. Beta sheets occur in variable amounts in the polypeptide chains of globular proteins e.g. lysozyme and carboxypeptidase, but more commonly associated with fibrous proteins such as silk and keratin.

Protein Loop

Protein loops are polypeptides connecting more rigid structural elements of proteins like helices and strands. Protein loops have high structural flexibility and diversity (3). Length of loop varies from a few to as many as 30 residues, though the majority of loops have less than 12 residues. Modeling the conformation of protein loop is one of the open problems in structural biology.

Tertiary Structure

The tertiary structure of a polypeptide chain is the next level of conformation or shape adopted by the alpha-helices or beta-pleated sheets of the chain. Most proteins tend to fold into shapes that are broadly classified as globular in arrangement, and some, particularly structural proteins form long fibres. These are the main forms of gross tertiary structure. Like secondary structure, the tertiary structure of a protein is stabilised by mostly *non-covalent forces* although tertiary structure can also be stabilised by *covalent bonds:* (a) electrostatic interactions non-covalent, (b) H-bonds non-covalent, (c) hydrophobic interactions non-covalent, (d) disulphide bridges covalent bond. The reason to adopt such intricate and complicated shapes by protein polypeptide chains is that the shape is related to function e.g. Hb has a different function from lysozyme so different shape requirement. Polypeptide chain folds into helices and sheets what produces the special shape to give the proteins their characteristic functions.

Quaternary structure

The fourth level of protein structure is concerned with the interaction of two or more polypeptide chains to associate to form a larger protein molecule. Proteins with more than one polypeptide chain are said to be *oligomeric*, and the individual chains are called subunits or monomers of the oligomer. The

geometry of the molecule is its quaternary structure. Single subunit or polypeptide chain is called a *monomer*, two subunits a *dimer*, three a *trimer*, 4 a *tetramer* etc. The subunits (polypeptide chains) may be identical e.g. muscle creatine kinase is a dimer of 2 identical subunits or non-identical e.g. haemoglobin is a tetramer and contains two alpha + two beta subunits. A considerable range of quaternary structure is found in proteins (4). From dimeric creatine kinase to octomeric tryptophanase, and ribulose diphosphate carboxylase, which has 16 subunits. Arrangement of subunits in the oligomeric structure can also vary. The forces that stabilise a quaternary structure are much the same as those that stabilise the secondary and tertiary structure. The non-covalent interactions is the tendency for hydrophobic groups to combine so as to exclude water. If a polypeptide chain has a face or region that is largely hydrophobic then the two faces tend to attract each other, in order to exclude water from both faces. The tertiary structure has a pleated sheet core (through centre), and when it forms a dimer, the two subunits stack back to back on each other in such a way that the pleated sheet is continued from one subunit to the other. The subunits are joined not only by association of the hydrophobic regions, but also by the H-bonding that now creates a continuous pleated sheet through both of the subunits.

Oligomeric structure

The advantage of association rather than staying as monomers is that in some proteins the subunit alone is not active, so biological activity depends on intact oligomeric structure. However, in other oligomeric proteins the single subunit is biologically active, and appears to act independently of the oligomeric structure. So stability is not the only factor involved. Another advantage of multiple subunits is greater flexibility of activity e.g. haemoglobin and many enzymes show cooperativity (5). In the case of tetrameric Hb, one subunit binds oxygen then stimulates neighbour subunits to bind oxygen more readily and so on through the four subunits so the subunits cooperate to ensure rapid and effective binding of oxygen. If there were no cooperativity then it is likely that competition between the subunits for binding oxygen would be overall less efficient. Cooperativity is mediated through intersubunit contacts. Also subunits provide an advantage in regulation of protein activity. In proteins and enzymes containing identical subunits it is found that the subunits contain special sites called allosteric sites located away from the active site of the enzyme or protein. Allosteric sites bind small molecules such as sugars and nucleotides, and these cause intersubunit changes in shape that regulate the activity at the active site so giving a fine control over the biological activity. Not all enzymes or proteins have allosteric sites, many do not e.g. lactate dehydrogenase (LDH) is a tetramer, and has no known mechanisms of regulation.

Protein characterization and fractionation

Protein type is usually determined by separating and isolating the individual proteins from a complex mixture of proteins, so that they can be subsequently identified and characterized. Proteins are separated on the basis of differences in their physicochemical properties, such as size, charge, isoelectric point adsorption characteristics, solubility and heat-stability. The choice of an appropriate separation technique depends on a number of factors, including the reasons for carrying out the analysis, the amount of sample available, the desired purity, the equipment available, the type of proteins present and the cost. One of the factors that must be considered during the separation procedure is the possibility that the native three dimensional structure of the protein molecules may be altered. A prior knowledge of the effects of experimental conditions on protein structure and interactions is extremely useful when the most appropriate separation technique must be selected. Firstly, because it helps to determine the most suitable conditions to use to isolate a particular protein from a mixture of proteins (*e.g.,* pH, ionic strength, solvent, temperature *etc*.) (6), and secondly, because it may be important to choose conditions which will not affect the native molecular structure of the proteins.

Methods Based on Different Solubility Characteristics

Proteins can be separated by using differences in their solubility in aqueous solutions. The solubility of a protein molecule is determined by its amino acid sequence because this determines its size, shape, hydrophobicity and electrical charge. Proteins can be selectively precipitated or solubilized by altering the pH, ionic strength, dielectric constant or temperature of a solution (7). These separation techniques are the most simple to use when large quantities of sample are involved, because they are relatively quick, cheap and are not particularly influenced by other food components. They are often used as the first step in any separation procedure because the majority of the contaminating materials can be easily removed.

Salting out

Proteins are precipitated from aqueous solutions when the salt concentration exceeds a critical level, which is known as *salting-out*, because all the water is »bound« to the salts, and it is therefore not available to hydrate the proteins. Ammonium sulfate [$(NH_4)_2SO_4$] is commonly used because it has a high water-solubility, although other neutral salts may also be used, *e.g.,* NaCl or KCl. Generally a two-step procedure is used to maximize the separation efficiency. In the first step, the salt is added at a concentration just below that necessary to precipitate out the protein of interest. The solution is then centrifuged to re-

move any proteins that are less soluble than the protein of interest. The salt concentration is then increased to a point just above that required to cause precipitation of the protein. This precipitates out the protein of interest (which can be separated by centrifugation), but leaves more soluble proteins in solution. The main problem with this method is that large concentrations of salt contaminate the solution, which must be removed before the protein can be resolubilzed, *e.g.,* by dialysis or ultrafiltration.

Solvent Fractionation

The solubility of a protein depends on the *dielectric constant* of the solution that surrounds it because this alters the magnitude of the electrostatic interactions between charged groups. As the dielectric constant of a solution decreases the magnitude of the electrostatic interactions between charged species increases. This tends to decrease the solubility of proteins in solution because they are less ionized, and therefore the electrostatic repulsion between them is not sufficient to prevent them from aggregating. The dielectric constant of aqueous solutions can be lowered by adding water-soluble organic solvents, such as ethanol or acetone. The amount of organic solvent required to cause precipitation depends on the protein and therefore proteins can be separated on this basis. The optimum quantity of organic solvent required to precipitate a protein varies from about 5 to 60%. Solvent fractionation is usually performed at 0 °C or below to prevent protein denaturation.

Electrophoresis

Electrophoresis relies on differences in the migration of charged molecules in a solution when an electrical field is applied (8). It can be used to separate proteins on the basis of their size, shape or charge. In *non-denaturing electrophoresis*, a buffered solution of native proteins is loaded onto a porous gel, usually polyacrylamide and a voltage is applied across the gel. The proteins move through the gel in a direction that depends on the sign of their charge, and at a rate that depends on the magnitude of the charge, and the friction to their movement:

$$\text{mobility} = \frac{\text{applied voltage} \times \text{molecular charge}}{\text{molecular friction}}$$

Proteins may be positively or negatively charged in solution depending on their isoelectic points (pI) and the pH of the solution. *A protein is negatively charged if the pH is above the pI, and positively charged if the pH is below the pI*. The magnitude of the charge and applied voltage will determine how far proteins migrate in a certain time. The friction of a molecule is a measure of its resistance to movement through the gel and is largely determined by the relationship between the effective size of the molecule, and the size of the pores in the gel. The smaller the size of the molecule, or the larger the size of the pores in the gel, the lower the resistance and therefore the faster a molecule moves through the gel. Smaller pores sizes are obtained by using a higher concentration of cross-linking reagent to form the gel. In non-denaturing electrophoresis the native proteins are separated based on a combination of their charge, size and shape.

In *denaturing electrophoresis* proteins are separated primarily on their molecular weight. Proteins are denatured prior to analysis by mixing them with *mercaptoethanol*, which breaks down disulfide bonds, and *sodium dodecyl sulfate* (SDS), which is an anionic surfactant that hydrophobically binds to protein and causes them to unfold because of the repulsion between negatively charged surfactant headgroups. As proteins travel through a gel network they are primarily separated on the basis of their molecular weight because their movement depends on the size of the protein molecule relative to the size of the pores in the gel: smaller proteins moving more rapidly through the matrix than larger molecules. This type of electrophoresis is commonly called *sodium dodecyl sulfate – polyacrylamide gel electrophoresis,* or *SDS-PAGE.* After the electrophoresis is completed, the proteins are made visible by treating the gel with a protein dye such as Coomassie Brilliant Blue or silver stain. Denaturing electrophoresis is more useful for determining molecular weights than non-denaturing electrophoresis, because the friction to movement does not depend on the shape or original charge of the protein molecules. A modification of electrophoresis, is another technique called *Isoelectric Focusing Electrophoresis* in which proteins are separated by *charge* on a gel matrix which has a pH gradient across it. Proteins migrate to the location where the pH equals their isoelectric point and then stop moving because they are no longer charged. This methods has one of the highest resolutions of all techniques used to separate proteins. Available gels can cover a narrow pH range (2–3 units) or a broad pH range (3–10 units). Isoelectric focusing and SDS-PAGE can be used together to improve resolution of complex protein mixtures. This means to perform the Two Dimensional Electrophoresis. Proteins are separated in one direction on the basis of *charge* using isoelectric focusing, and then in a perpendicular direction on the basis of *size* using SDS-PAGE.

Separation due to Different Adsorption Characteristics – Chromatography

Chromatographyc technique involves the separation of compounds by selective adsorption-desorption at a solid matrix that is contained within a column through which the mixture passes. Separation is based on the different affinities of different proteins for the solid matrix (9).

Ion Exchange Chromatography

Ion exchange chromatography relies on the reversible adsorption-desorption of ions in solution to a charged solid matrix or polymer network. This technique is the most commonly used chromatographic technique for protein separation. A positively charged matrix is called an *anion-exchanger* because it binds negatively charged ions (anions). A negatively charged matrix is called a *cation-exchanger* because it binds positively charged ions (cations). The buffer conditions (pH and ionic strength) are adjusted to favor maximum binding of the protein of interest to the ion-exchange column. Contaminating proteins bind less strongly and therefore pass more rapidly through the column. The protein of interest is then eluted using another buffer solution which favors its desorption from the column (*e.g.,* different pH or ionic strength).

Affinity Chromatography

Affinity chromatography uses a stationary phase that consists of a *ligand* covalently bound to a solid support. The ligand is a molecule that has a highly specific and unique reversible affinity for a particular protein. The sample to be analyzed is passed through the column and the protein of interest binds to the ligand, whereas the contaminating proteins pass directly through. The protein of interest is then eluted using a buffer solution which favors its desorption from the column. This technique is the most efficient means of separating an individual protein from a mixture of proteins, but it is the most expensive, because of the need to have columns with specific ligands bound to them.

Size Exclusion Chromatography

This technique, sometimes known as *gel filtration*, also separates proteins according to their size. A protein solution is poured into a column which is packed with porous beads made of a cross-linked polymeric material (such as dextran or agarose). Molecules larger than the pores in the beads are excluded, and move quickly through the column, whereas the movement of molecules which enter the pores is retarded. Thus molecules are eluted off the column in order of decreasing size. Beads of different average pore size are available for separating proteins of different molecular weights. Manufacturers of these beads provide information about the molecular weight range that they are most suitable for separating. Molecular weights of unknown proteins can be determined by comparing their elution volumes Vo, with those determined using proteins of known molecular weight: a plot of elution volume versus log (*molecular weight*) should give a straight line.

Dialysis

Dialysis is used to separate molecules in solution by use of semipermeable membranes that permit the passage of molecules smaller than a certain size through, but prevent the passing of larger molecules. A protein solution is placed in dialysis tubing which is sealed and placed into a large volume of water or buffer which is slowly stirred. Low molecular weight solutes flow through the bag, but the large molecular weight protein molecules remain in the bag. Dialysis is a relatively slow method, taking up to 12 hours to be completed. It is therefore most frequently used in the laboratory (6). Dialysis is often used to remove salt from protein solutions after they have been separated by salting-out, and to change buffers.

Ultrafiltration

A solution of protein is placed in a cell containing a *semipermeable membrane,* and pressure is applied. Smaller molecules pass through the membrane, whereas the larger molecules remain in the solution. The separation principle of this technique is therefore similar to dialysis, but because pressure is applied separation is much quicker. Semipermeable membranes with cutoff points between about 500 to 300,000 D are available. That portion of the solution which is retained by the cell (large molecules) is called the *retentate,* whilst that part which passes through the membrane (small molecules) forms part of the *ultrafiltrate.* Ultrafiltration can be used to concentrate a protein solution, remove salts, exchange buffers or fractionate proteins on the basis of their size.

ProteomeLab

Compared to traditional fractionation techniques, e.g., SDS-PAGE, that analyze modified proteins derived from strong chemical treatments such as reduction with 1,4-dithiothreitol (DTT) and alkylation with iodoacetamide, a relatively new method of fractionation of intact proteins is the liquid, two-dimensional system provided by Beckman Coulter, the ProteomeLab PF2D. This system allows us to separate proteins according to the isoelectric point (pI) and hydrophobicity. Thus, PF2D appears to offer a new platform tool to be integrated with other proteomic techniques for the fractionation of proteins, and thereby contribute to broaden our knowledge, particularly in the study of the human serum proteome (*Figure 2*).

The PF2D instrument consisted of a double HPLC interfaced by a refrigerated fraction collector. Provided by Beckman Coulter, two buffers were used to perform the chromatofocusing chromatography: Buffer 1, containing the proprietary mixture of urea, n-octylglucoside and triethanolamine, which is adjusted to a pH of 8.5 with saturated iminodiacetic acid; and Buffer 2, which is a proprietary mixture of urea, n-octyl-

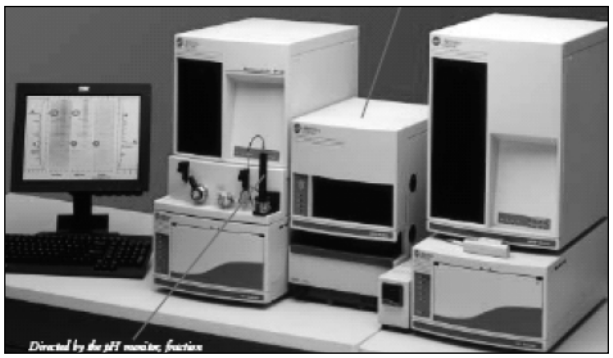

Figure 2 ProteomeLab

glucoside and ampholytes prepared to a pH of 4.0. After about 130 min of equilibration on a 2.1 × 250 mm HPCF-1D column charged by a positive matrix, the sample was injected manually through a 2 mL loop at a flow rate of 0.2 mL/min via a single liquid pumping mechanism. Proteins were fractionated on the basis of their pI.

Before injection, 10 mg of human serum samples from healthy donors needed to be desalted and small molecular weight solutes removed. The remainder of the proteins were exchanged with Buffer 1, with cut-off recoveries of macromolecules >5 Kda (PD-10 G25 medium column, Amersham Biosciences, Piscataway, NJ, USA).

The principle of chromatofocusing is based on the generation of a pH gradient inside the column starting at 8.5 and ending at 4.00 that determines the elution of those proteins whose net charge is zero. Proteins have different pIs because of their different amino acid sequences, and tend to aggregate and precipitate at their pI because there is no electrostatic repulsion keeping them apart. Each protein fraction is collected at each 0.3 pH variation point; in the first 20 min of analysis excessively basic proteins are eluted, while at the end of the pH gradient, both excessively basic and excessively acidic proteins are collected every 5 minutes.

Proteins are detected by absorbance at 280 nm by a UV detector, principally due to the presence of aromatic amino acids (tryptophan, tyrosine, and phenylalanine) and disulfide bonds.

Following analysis in the first dimension, the column was washed with 1 mol/L NaCl and deionized water. Then, once the default method was started, the instrument automatically injected 0.2 mL of each fraction collected in a 96 deep microwell plate into the second dimension reverse phase chromatography, which separates proteins based on their hydrophobicity.

Unlike chromatofocusing, the HPRP module of the second part of PF2D involved a binary pump system that simultaneously circulated two different solvents related in a linear gradient between buffer A (deionized water and 0.1% TFA) and buffer B (acetonitrile and 0.08% TFA). The bases of separation was the absorption-desorption of proteins which derived from the first dimension of PF2D. The stationary phase of the column, a 1.5 mm C18 non-porous silica bead 4.6 × 33 mm HPRP column at 50 °C, was non polar, and the mixing of buffers of different polarity determined the elution of protein characterized according to *degree of hydrophobicity,* which is calculated by the percentage content of non-polar amino acids. Various »hydrophobicity« scales exist to evaluate the polarity of proteins on the bases of physicochemical properties of amino acids. A more positive value indicates a stronger hydrophobicity. Hydrophilic amino acids have negative values. In a protein, hydrophobic amino acids are more likely to be located in the protein interior, whereas hydrophilic amino acids are more likely to interface with the aqueous environment.

When proteins eluted at the flow rate of 0.75 mL/min, they were monitored at 214 nm, the necessary wavelength to detect the amide bond.

In this work, we analyzed the fractions of the first dimension of PF2D using classic 2D SDS-PAGE gel as a reference method to identify proteins eluted at specific pH values and to evaluate whether the theoretical pI is representative of the pH at which proteins elute in this instrument.

Results and Discussion

Human sera were loaded onto the ProteomeLab™PF2D system and, following protein interactions with the ion exchange column in the first dimension, when pH gradient was created, the elution of proteins started as soon as all charges on their surface were neutralized. The obtained fractions were pooled and seven 2D gels were performed and analyzed (10–12). *Figure 3* represents the unfractionated se-

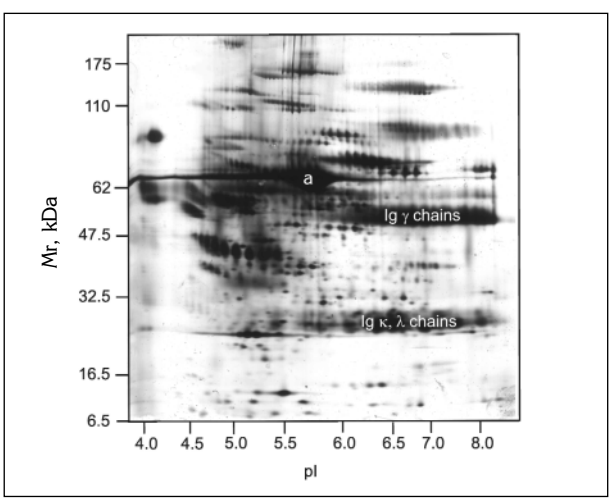

Figure 3 2D gel of the unfractionated serum before elution on the PF 2D system.
a, albumin; Ig, immunoglobulins

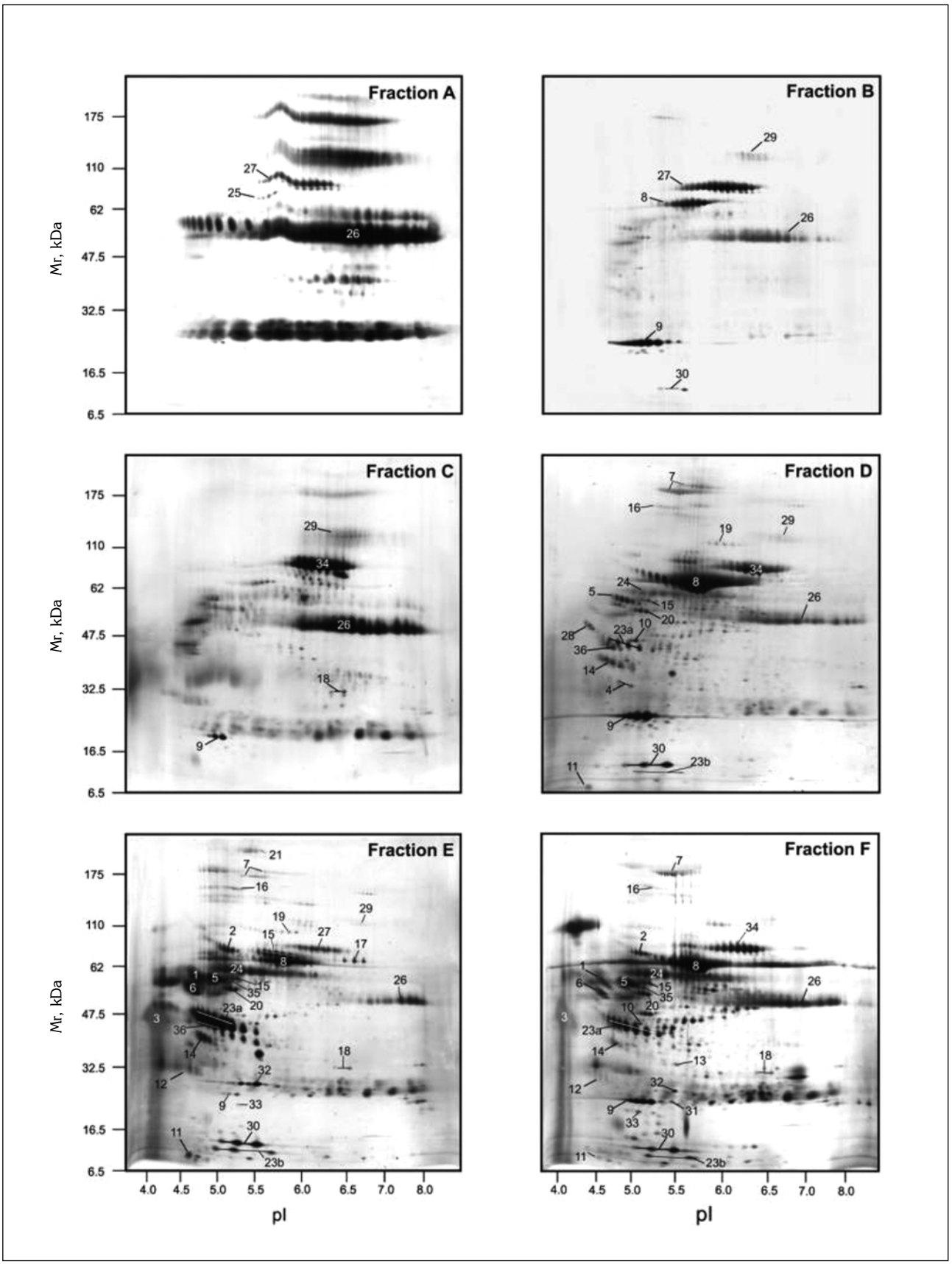

Figure 4 2D gels of pooled fractions eluted from the PF 2D first dimension. Numbers indicate the positions of different proteins as shown in *Table I*, identified using the Swiss-Prot Database as reference.

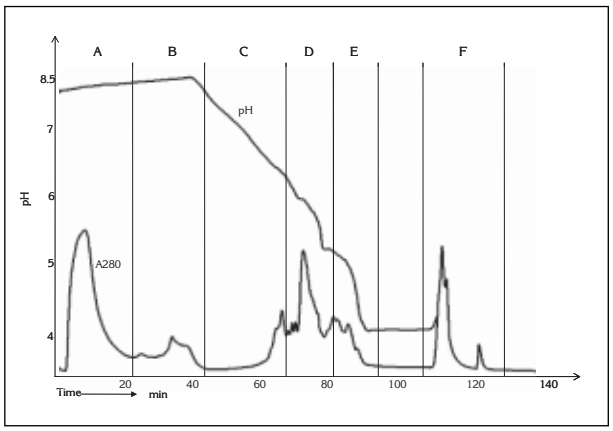

Figure 5 Representation of the pH gradient and UV absorbance at 280 nm of the separation of human serum proteins using the ion-exchange column (first dimension) of the PFD system. Retention times in minutes is on the horisontal axis.

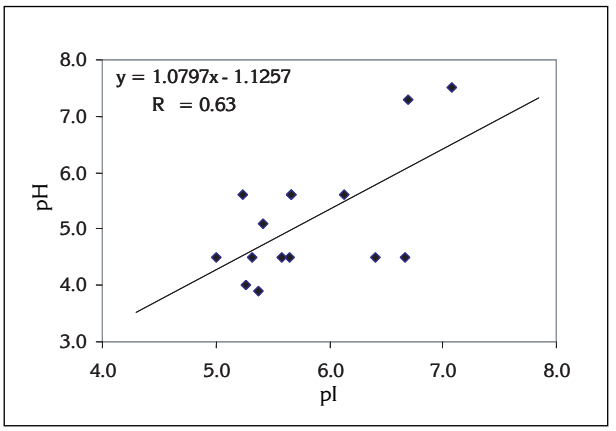

Figure 6 Correlation between the pI calculated for denaturated proteins (horizontal axis) and the pH of elution of each protein corresponding to the experimental pI (vertical axis)

rum before running on the PF2D. All the scattered spots were identified using Swiss Prot Database, a two-dimensional polyacrylamide gel electrophoresis database that contains data on proteins identified on various 2-D PAGE and SDS-PAGE reference maps (13). The other gels in *Figure 4* were the result of pooled fractions eluted from first dimension of PF2D ranging from 8.50 to 4.00 (Fractions B, C, D, E) (*Figure 5*). Over the pH gradient, the »unbound« Fraction A, which included all the proteins characterized by an alkaline pI, and the high strength anionic wash out with 1 mol/L NaCl, Fraction F, which comprised all of the most acidic proteins, were considered. All the proteins identified are numbered in the figures and reported in a *Table I* where a semi-quantitative assessment was carried out for each fraction. In the first sample, Fraction A, we found all the proteins that are characterized by a pI > 8.5, such as Immunoglobulin D, G, M. This important finding allowed us to focus attention on other abundant and less abundant proteins present in all the fractions generated by first dimension of PF2D. Indeed, many biochemical tests allow more suitable and specific investigation of Ig.

Most serum proteins were eluted in Fraction E, which showed an experimental pH range between 5.12 and 3.92, and in Fraction F, the wash out step of analysis, which gathered all acidic proteins present in the serum, i.e., those that remained inside the column for a longer time because of an abundance of negative charges on their surface.

A total of 36 human serum proteins were identified in different PF2D-generated fractions.

Comparing theoretical pI of identified proteins, obtained from Swiss Prot Database, and the experimental pH at which they eluted during chromatofocusing, a significant correlation ($rs=0.60$, $p<0.01$) was observed between the two parameters when only monomeric proteins were plotted (*Figure 6*).

The ProteomeLab PF2D system represents a valuable tool that clinical pathology laboratories may well increasingly make use of for diagnostic purposes. Even if it is an expensive platform, it could be more readily applied in the near future, not only because it allows identifying a very large number of proteins in a single sample, but also because it enables the detection of specific disease-related protein patterns. However, despite its promise as an automated method for the virtually complete analysis of different circulating proteins, the characteristics of such fractionation require further validation before the system can be fully implemented in a clinical setting. Indeed our future plans foresee the characterization of main serum proteins in order to classify them on the bases of their pI and hydrophobicity (14, 15), and as such construct a sort of two dimensional »geographic map« of the human serum proteome under given physiopathologic conditions. This will be possible through the use of other powerful techniques, such as mass spectrometry, which is able to detect and identify the whole protein.

Table I Plasma proteins identified using 2D gels from pooled PF2D fractions

			Fraction					
Spot	Protein	%	A	B	C	D	E	F
1	α_1-anti-chimo-trypsin	0.08					+++	++
2	α_1-glycoprotein	0.01					+++	++
3	α_1-acid glycoprotein	1					+++	++
4	α_1-microglobulin	<0.1				++		
5	α_1-anti-trypsin	3				+	+++	+
6	α_2-HS glycoprotein	2					+++	++
7	α_2-macroglobulin	4					+	+
8	Albumin	56		++		+++++	+	+++
9	APO A1	<0.1		++ +	++	+++	+/–	++
10	APO AIV	<0.1				+		+
11	APO C II	<0.1				+	++	+
12	APO D	<0.1					+	+
13	APO E	<0.1						+
14	Apo J (Clusterin)	<0.1				++	+++	+
15	AT3	<0.1				+	+	++
16	Ceruloplasmin	0.08				+	++	+
17	Complement C3	1.01					++	
18	Complement C4	0.07			+		+	+
19	Complement factor B	<0.1				+	+	+
20	Fibrinogen G	<0.1				+	+	++
21	Fibronectin	<0.1					++	
22	Haemopexin	1.01					++	
23a	Haptoglobin beta chain	4				+	++++	++
23b	Haptoglobin alpha chain	4				+	++++	++
24	IgA	5					+++	+++
25	IgD	<0.1	+					
26	IgG	11	++++	++	+++	+	+	++
27	IgM	4	++++	+++			++	+
28	Leucin rich glycoprotein	<0.1				++		
29	Plasminogen	<0.1		++	++	+	+/–	
30	Pre-albumin (trans thyretin)	0.01				+++	++++	++
31	Pro APO A1	<0.1						+
32	Serum amiloid P	<0.1					+++	+
33	Serum retinol-binding protein	<0.1					+	+
34	Transferrin	3			++++	+++		++
35	Vitamin D binding protein	<0.1					++	++
36	Zn-α_2-glycoprotein	<0.1				+	++	

%: indicative concentration of each protein in plasma proteome.
A, B, C, etc.
+/-, +, ++, etc: indicate a semiquantitative value of each protein derived from 2D gel spots.

PRIMENA 2D-HPLC SISTEMA ZA RAZDVAJANJE PROTEINA

Isabella Levreri[1], Luca Musante[2], Andrea Petretto[3], Davide Cuccabitta[3], Giovanni Candiano[2] and Giovanni Melioli[1]

[1]U.O. Laboratorio Centrale di Analisi
[2]Laboratorio di Fisiopatologia dell'Uremia, U.O. di Nefrologia
[3]Laboratorio di Spettrometria di Massa, Core facility,
Instituto Scientifico Giannina Gaslini, Genova, Italy

Kratak sadržaj: The ProteomeLab[a] PF 2D sistem za frakcionisanje proteina je brz, poluautomatski 2D-HPLC aparat koji koristi dve različite metode za razdvajanje plazma serumskih proteina: jonoizmenjivačku hromatografiju koja koristi širok opseg pH u prvoj dimenziji i neporoznu reverzno-faznu hromatografiju u drugoj dimenziji. Samo zbog toga što je ova metodologija nedavno predstavljena u laboratorijama za proteomikane, malo se zna o karakteristikama PF 2D frakcionisanja proteina u humanom serumu. Da bi se procenila primena sistema u okvirima kliničke laboratorije, analizirane su osobine razdvajanja zasnovanog na jonoizmenjivačkoj hromatografiji. Posle frakcionisanja proteina humanog seruma na linearnom gradijentu pH (u opsegu od 8,5 do 4,0), svaka frakcija je sakupljena u hladnom modulu aparata. Različite frakcije iz prve dimenzije su zatim zajedno objedinjene i stavljene na klasičnu aparaturu za 2D gel elektroforezu. Dobijene različite tačke su zatim proverene preko Swiss-Prot baze podataka. Ukupno 36 proteina humanog seruma je identifikovano u različitim PF 2D proizvedenim frakcijama. Pojedine važne osobine separacionog sistema su bile zapažene. Različite elucione frakcije sadržale su različite proteine što pokazuje pouzdanost sistema za frakcionisanje. Proteini su takođe frakcionisani prema teoretskoj izoelektričnoj tački (p*I*). To je bilo u skladu sa dokazom da velika većina imunoglobulina, okarakterisana sa alkalnim p*I*, nije bila zadržana u koloni i bila je eluirana u nevezanoj frakciji. Ovakav rezultat takođe naglašava praktičnu prednost: eluirane frakcije od pH 8 do pH 4 sadržale su zapravo proteine seruma bez prisustva imunoglobulina. Ovaj nalaz podržava neposrednu upotrebu PF 2D sistema u kliničkim procedurama, gde bi trebalo veliki broj proteina jasno identifikovati da bi se omogućila procena drugih manje prisutnih, ali potencijalno važnih, tipova.

Ključne reči: PF 2D gelovi, frakcionisanje plazma proteina, ProteomeLab[a] PF 2D, p*I* vrednost

References

1. Branden C, Tooze J. Introduction to Protein Structure, 2nd edn. New York: Garland Publishing, 1999.
2. Kyte J. Structure in Protein Chemistry. New York: Garland Publishing, 1995.
3. Mathews CK, van Holde KE & Ahern K-G Biochemistry, 3rd edn. San Francisco: Benjamin Cummings, 2000.
4. Stryer L. Biochemistry, 4th edn. New York: WH Freeman, 1995.
5. Anfinsen CB. Principles that govern the folding of protein chains. Science 1973; 181 (96): 223–30.
6. Deutscher M. (Ed.) Guide to Protein Purification. Academic Press 1997.
7. Voet D. Voet J. Biochemistry John Wiley & Sons, Inc, NY. 1990.
8. Dunn MJ. Quantitative two-dimensional gel electrophoresis: From proteins to proteomes *Biochem Soc Trans* 1997; 25: 248–54.
9. http://www.chromatography-online.org/
10. Musante L, Candiano G, Ghiggeri GM. Resolution of fibronectin and other uncharacterized proteins by two-dimensional polyacrylamide electrophoresis with thiourea. J Chromatogr B Biomed Sci Appl. 1998; 705: 351–6.
11. Bjellqvist B, Ek K, Righetti PG, Gianazza E, Gorg A, Westermeier R, Postel W. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 1982; 6: 317–39.
12. Gorg, A. Postel, W. Domscheit, A. Gunther, S Two-dimensional electrophoresis with immobilized pH gradients of leaf proteins from barley (Hordeum vulgare): method, reproducibility and genetic aspects. Electrophoresis 1988; 9 (11): 681–92.
13. http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN
14. Zhu K, Zhao J, Lubman DM, Miller FR, Barder TJ. Protein p*I* shifts due to posttranslational modifications in the separation and characterization of proteins. Anal Chem 2005; 77 (9): 2745–55.
15. Liotta LA, Ferrari M, Petricoin E. Clinical proteomics: written in blood. Nature 2003; 425 (6961): 905.

Received: August 15, 2006
Accepted: September 15, 2006