Preparative 2-D Purifies Proteins for Sequencing or Antibody Production

Contributed by Jean Charles Sanchez, Nicole Paquet, Graham Hughes and Denis Hochstrasser Medicine & Biochemistry Depts., Geneva University.

Abstract

We report here a new preparative two-dimensional (2-D) electrophoresis system for purification of proteins. The system is based on the same isoelectric focusing and gel electrophoresis principles as analytical two-dimensional electrophoresis. The procedure, which combines the Rotofor® preparative isoelectric focusing (IEF) cell and the Model 491 Prep Cell for preparative gel electrophoresis (PAGE), is applicable to a wide range of biological samples. This preparative 2-D system purifies individual proteins from crude, complex mixtures for detailed compositional analysis and antibody production and is especially advantageous for isolating proteins present in low concentrations in the specimen.

Introduction

Analytical two-dimensional gel electrophoresis is now a routine procedure for reproducible separation of proteins in complex biological samples.[1,2,3] Over 3,000 tissue proteins and more than 1,000 plasma proteins can be resolved by this method. However, analytical 2-D electrophoresis procedures are incapable of supplying sufficient amounts of low abundance proteins for further characterization. It has been necessary to recover proteins from several gels for sequence analysis[4], assay, or antibody production.[5]

In preparative 2-D electrophoresis the first step fractionates proteins into defined pH ranges by liquid-phase isoelectric focusing in the Rotofor cell. The Rotofor cell is capable of 500-fold, or more, purifications of proteins from complex mixtures. Proteins are concentrated in discrete liquid fractions at their respective isoelectric points. In the second purification step, preparative polyacrylamide gel electrophoresis (PAGE) in the Model 491 Prep Cell, individual proteins are isolated on the basis of their size differences.

Samples such as plasma pose a particular problem for electrophoretic techniques due to the presence of high concentrations of albumin and immunoglobulins, which together make up more than 65% of the total plasma protein. The high protein load severely limits the volume of plasma that can be purified by conventional electrophoretic means. The preparative 2-D method circumvents this problem. This method is illustrated with purification of a 70 kd dimeric (34 and 36 kd) apolipoprotein (Apo J) and a 49 kd uncharacterized protein, which in previous blotting experiments appeared to have a blocked amino-terminus. Both have glycosylated isoforms with pIs ranging from 4.9 to 5.3. Apo J, which is in the 0.05 mg/ml concentration range, represents less than 0.15% of total plasma protein. The low plasma concentration of Apo J, and the 49 kd uncharacterized protein is evident from analytical 2-D PAGE of whole plasma (Figure 1) where they are barely visible with silver staining.

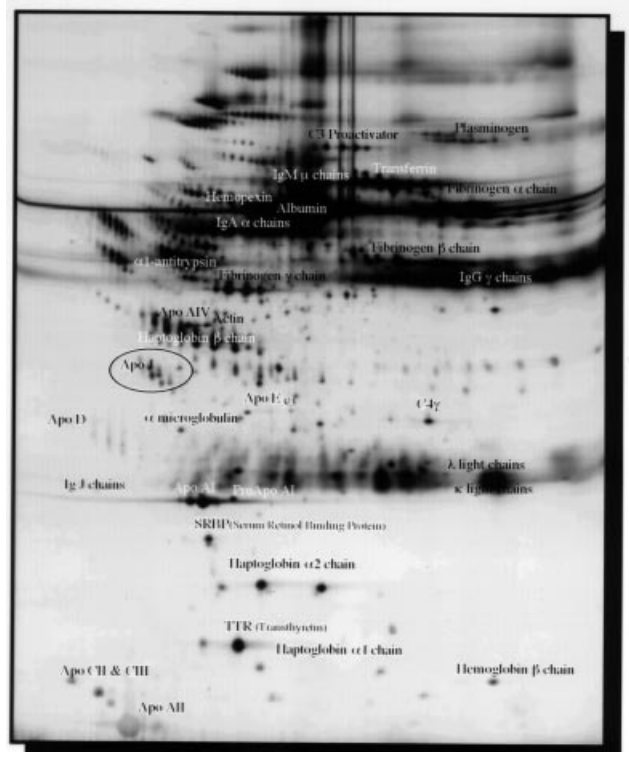

Fig. 1. Analytical 2-D gel map of whole human plasma. This silver stained second-dimension gel demonstrates the complexity of the starting sample. The positions of the glycosylation-induced isoforms of Apo J and the uncharacterized 49 kd protein of interest, purified by preparative 2-D electrophoresis, are indicated.

BIO-RAD

Methods

ANALYTICAL 2-D PAGE

Five microliters of whole human plasma were diluted with 10 microliters of dithioerythritol (DTE, 1% w/v), containing SDS (10% w/v). After a 5 minute incubation at 95 ˚C the sample was diluted to 500 microliters with DTE (1% w/v), CHAPS (4% v/v), urea (9 M) and ampholytes (pH range 9–11, 5% v/v). Aliquots of 30 microliters (containing 18 micrograms of protein) were used for analysis on 2-D gels (see Figure 1). Protein-containing fractions obtained from the Rotofor cell were similarly treated.

SAMPLE PREPARATION FOR PREPARATIVE 2-D ELECTROPHORESIS

Whole plasma (20.0 ml) was first dialysed (2 hours, M_r cut off 10,000) against distilled water. Following dialysis, urea (21 g, final concentration 7 M), CHAPS (1.0 g, final concentration 2% w/v) and DTE (0.232 g, final concentration 30 mM), were added. After stirring for 15 minutes, carrier ampholytes [Bio-Lyte® ampholytes; pH range 3–10 (2.5 ml) and pH range 5–7 (0.5 ml)] were added and the volume was brought to 50 ml with distilled water.

PREPARATIVE ISOELECTRIC FOCUSING

The sample (50 ml) containing 1.2 g of total protein, was loaded into the Rotofor cell for initial fractionation in a wide-range pH gradient (pH 3–10). Constant power (10 W) was applied for 5 hours with the system cooled to 4 ˚C. Runs were terminated when the voltage had stabilized (1,500 V) for about 30 minutes. Twenty Rotofor fractions were collected. Selected fractions were analyzed by 2-D PAGE. Rotofor fraction 5 (pH 4.3) was substantially free of the bulk plasma proteins, albumin, and immunoglobulins, and it was highly enriched for Apo J and the unknown protein. This step provided approximately 500-fold purification of the protein of interest (Figure 2A).

REFRACTIONATION

Rotofor fractions 4, 5, and 6 were collected, pooled, and refractionated in the Rotofor cell without additional ampholytes. The total protein load was 50 milligrams. Upon refractionation, an overall 1,000-fold purification of Apo J and the unknown protein was obtained in Rotofor fraction 11 (Figure 2B).

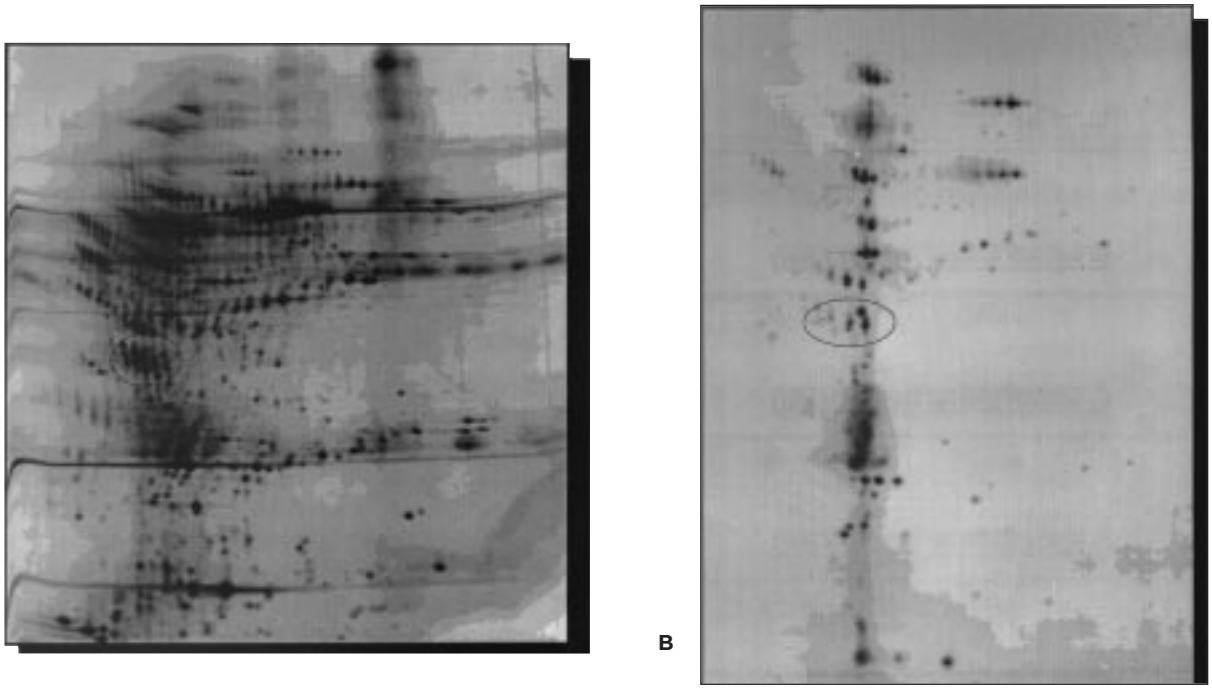

Fig. 2. Analysis of Rotofor fractions by 2-D gel electrophoresis. A. Initial Rotofor fractionation provided enrichment for the proteins of interest in Rotofor fraction 5, shown here. B. Refractionation of Rotofor fraction 5 shown here resulted in 1,000-fold purification of Apo J and the unknown 49 kd protein by comparison to the starting sample in Figure 1. Analytical 2-D gels were silver stained.

PREPARATIVE SDS-PAGE

For preparative gel electrophoresis, the discontinuous buffer system of Laemmli was used.[5] The total acrylamide concentration (%T) of the separating gel was optimized at 12%.

The sample (Rotofor fraction 11) contained approximately 2.5 mg of total protein dissolved in 2.0 ml of sample buffer (see Table 1). After a 5 minute incubation at 95 ˚C, the sample was loaded onto the prep cell and the gel run for 16 hours. Running buffer was pumped through the elution chamber at a rate of 0.5 ml per minute.

Table 1. Model 491 Prep Cell Running Conditions

Resolving gel	12% acrylamide/ 2.6% C (PDA crosslinker)			
Resolving gel length	8 cm in 37 mm gel tube			
Resolving gel buffer	Tris-HCl (0.375 M) pH 8.8			
Stacking gel	4% T/2.6% C (PDA crosslinker)			
Stacking gel buffer	Tris-HCl (125 mM) pH 6.5			
Running buffer	Tris-Glycine-SDS (25 mM–192 mM-0.1%)			
Elution buffer	Tris-Glycine-SDS (25 mM–192 mM-0.1%)			
Sample buffer A	10% SDS + 2.32% DTE			
Sample buffer B	1% g DTE + 4% CHAPS + 9 M urea + 5% ampholytes pH 9–11			
Sample	Rotofor fraction 11 was dialyzed and freeze dried then dissolved in 100 microliters of sample buffer A. Then 1,900 microliters of sample buffer B was added.			
Elution rate	0.5 ml/min			
Power	start	50 mA	177 V	8 W
	finish	50 mA	301 V	12 W

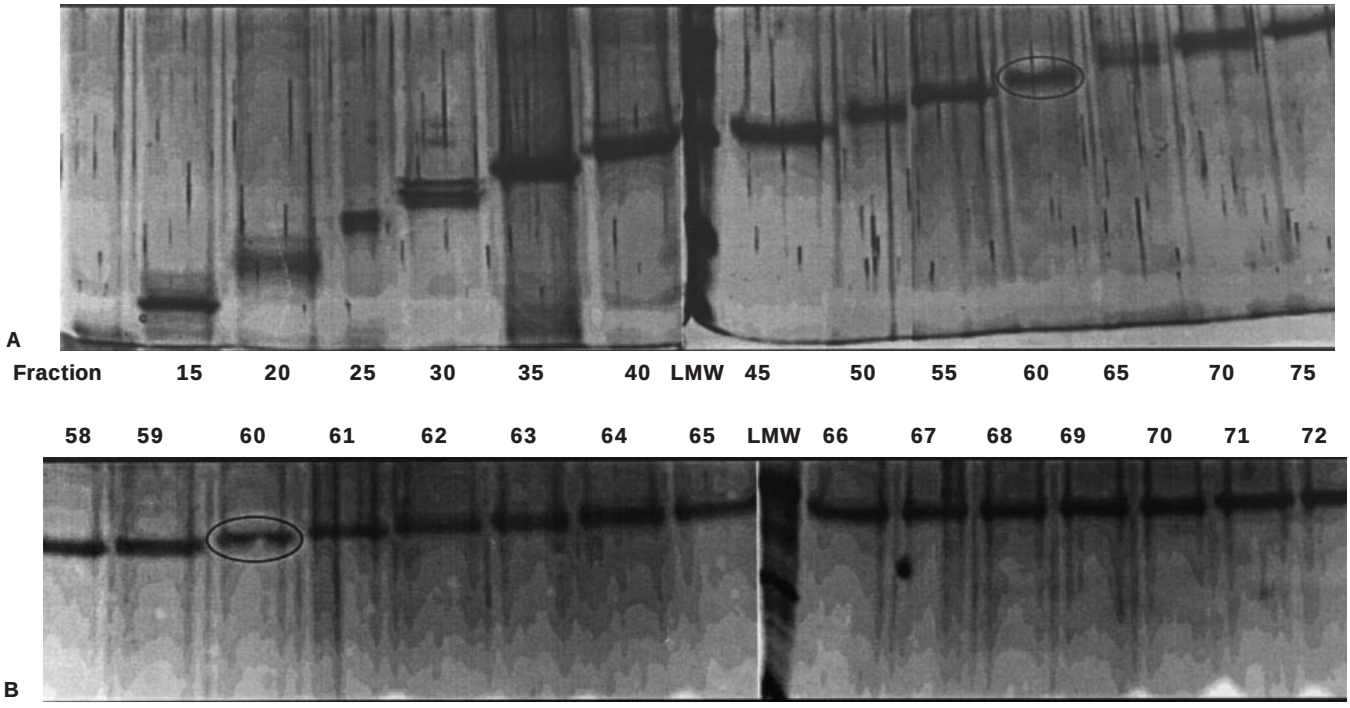

Fig. 3. Analysis of protein fractions eluted from the prep cell. A Aliquots from every fifth Prep cell fraction were analyzed on silver stained SDS-PAGE gels. The elution position of Apo J was fraction 60 and the 49 kd protein eluted in fraction 70 B. Every prep cell fraction near the peak of eluted Apo J and the 49 kd protein was then analyzed. The fractions containing Apo J were 60, 61, and 62. and the ones containing the 49 kd protein were 71, 72, and 73.

FRACTION COLLECTION AND ANALYSIS

The elution chamber outlet of the Model 491 Prep Cell was connected to a fraction collector (Bio-Rad Econo System) and 80 5-ml fractions (5 ml each) were collected. Fraction number one was the first fraction containing visible amounts of the bromophenol blue marker dye. To locate the fractions containing Apo J and the 49 kd uncharacterized protein, 30 microliters from every fifth fraction were analyzed by SDS-PAGE (Figure 3A). Once the elution positions of the Apo J and 49 kd protein were determined, 30 microliters of every fraction near the peak of the eluted proteins of interest were analyzed by SDS-PAGE (Figure 3B).

ANTIBODY PRODUCTION

Rotofor fraction 11 containing Apo J was assayed for protein[6] and frozen at -20 °C until used. Polyclonal antibodies were raised in rabbits against proteins purified as described above using conventional immunization procedures.[7] Polyclonal antibodies were specific for Apo J and did not cross-react with other plasma proteins. Using preparative 2-D electrophoresis (combined Rotofor and Model 491 Prep Cell) we were able to obtain a pure preparation of Apo J, free of cross-contamination, despite the presence of a series of presumably glycosylation-induced isomers (Figure 1).

SEQUENCE ANALYSIS

Prep cell fractions 71, 72, and 73 containing the 49 kd unknown protein, were pooled and concentrated to 500 microliters by freeze drying. The final concentration of components in the 500 microliter sample was: Tris (200 mM) - glycine (1.6 M) - SDS (0.8%). The sample was then reduced with DTT (2.0 µM, 2 hours at 37 °C) and carboxymethylated with iodoacetic acid (ICH_2COOH, 5 µM, pH 8) for 30 minutes in the dark. Following dialysis against water for 48 hours, the sample was again freeze dried, and the SDS extracted.[8] The protein was then digested with TPCK-trypsin in 4 M urea, pH 8.0. Prior to sequencing, peptides were separated with a Microbore C8 HPLC column (1 x 100 mm) with a 0.1% TFA/Acetonitrile system.[9] Sequence analysis was done on an ABI 473 A Sequenator. We have found no sequences similar to those of the 49 kd protein in data base searches.

Results

This report describes a rapid electrophoretic procedure for purification of proteins from crude extracts in concentrations where comprehensive sequence analyses and antibody production are feasible. Apo J and the 49 kd uncharacterized protein were obtained in a highly purified state. The preparative 2-D procedure typically yields from 20–40 micrograms of the proteins.

The advantages of the primary fractionation step (liquid phase IEF) cannot be over-emphasized, notably with respect to the fractionation of plasma proteins. Here, high plasma concentrations of certain proteins, such as albumin, alpha-1-antitrypsin, immunoglobulins or transferrin limit the volume of plasma that can be processed. Pre-fractionation of plasma with the Rotofor cell confines these proteins to their respective pI ranges. It is then possible to undertake a sequential, detailed analysis of the different Rotofor fractions. Each fraction represents a defined, restricted pI interval, containing an adequate quantity of protein for the preparative PAGE purification step.

This procedure can be considered a viable means of obtaining highly purified preparations of plasma proteins, even those present in low concentrations. Yields are such that comprehensive sequence data can be generated on amino-terminally blocked proteins and antibody production is feasible. The procedure offers great potential as a firstline protein purification procedure, whether applied to plasma or other biological samples.

References

1 *O'Farrel, P. H., High resolution two-dimensional electrophoresis.* J. Biol. Chem., *250, 4007–4021 (1975).*
2 *Anderson, L. and Anderson, N.G., Some perspectives on two-dimensional protein mapping.* Clin. Chem., *30, 1898–1905 (1984).*
3 *Hochstrasser, D.F.* et al., *Methods for increasing the resolution of two-dimensional protein electrophoresis.* Anal. Biochem., *173, 424–435 (1988).*
4 *Bauws G.* et al., Proc. Natl. Acad. Sci. USA, *86, 7701–7705 (1989).*
5 *Hochstrasser, A.-C.* et al., *Preparative isoelectrofocusing and high resolution 2-dimensional gel electrophoresis for concentration and purification of proteins.* Applied and Theoretical Electrophoresis, *1, 265–275 (1990).*
6 *Lowry* et al., *Protein measurements with Folin-phenol reagent.* J. Biol. Chem., *193, 265–275 (1951).*
7 *James R.W.* et al., Arteriosclerosis and Thrombosis, *11, No.3, 645–652 (1991).*
8 *Konigsberg, W.H. and Henderson L.*, Meth. Enzym., *91, 254 (1990).*
9 *Hughes* et al., Biochem. J., *271, 641–647 (1990).*

BIO-RAD

Bio-Rad Laboratories

Life Science Group

Website *www.bio-rad.com* ***U.S.*** *(800) 4BIORAD* ***Australia*** *02 9914 2800* ***Austria*** *(01)-877 89 01* ***Belgium*** *09-385 55 11* ***Canada*** *(905) 712-2771*
China *86-10-62051850/51* ***Denmark*** *45 39 17 99 47* ***Finland*** *358 (0)9 804 2200* ***France*** *01 43 90 46 90* ***Germany*** *089 318 84-0*
Hong Kong *852-2789-3300* ***India*** *(91-11) 461-0103* ***Israel*** *03 951 4127* ***Italy*** *39-02-216091* ***Japan*** *03-5811-6270* ***Korea*** *82-2-3473-4460*
Latin America *305-894-5950* ***Mexico*** *514-2210* ***The Netherlands*** *0318-540666* ***New Zealand*** *64-9-4152280* ***Russia*** *7-095-4585822*
Singapore *65-2729877* ***Spain*** *34-91-661-7085* ***Sweden*** *46 (0)8 627 50 00* ***Switzerland*** *01-809 55 55* ***United Kingdom*** *0800-181134*