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Two Dimensional Electrophoresis Method for Additional Characterization of Paraproteins in Serum

Clinical application of two dimensional electrophoresis, I.

By A. Lapin, A. T. Endler, E. O. Ogunyemi and F. Gabl

Institut für Klinische Chemie und Laboratoriumsdiagnostik, Universität Wien

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Summary: High resolution two dimensional electrophoresis, one of the most potent methods for the analytical separation of proteins, is still used only in specialized laboratories.

The present paper describes a two dimensional electrophoresis method, which permits characterization of abnormal serum proteins (paraproteins) and is suitable for application in a clinical laboratory. Both steps of the electrophoresis are performed by the "flat bed technique". The critical transfer from the isoelectric focusing step to the gel of the second dimension is facilitated by use of specially prepared foil stencils.

The practicality of the procedure was considerably increased by using commercially available materials such as gels for isoelectric focusing and the silver stain kit. The analysis can be calibrated by measurement of the pH gradient and by using molecular weight protein standards. Moreover, two different samples can be analysed simultaneously in the same gels of the first and the second dimension. This permits a direct comparison of the sample of interest with the sample of reference. Serum samples showing an abnormal band ("M gradient") in routine methods such as agarose gel electrophoresis, were analysed by the described method. Some "M gradients" consisted of single abnormal proteins. Another sample showed a "M gradient" composed of a number of abnormal protein spots in our method.

Questions concerning pathobiological and clinical evaluation of the findings justify further studies using this method.

Zweidimensionale Elektrophorese-Methode für eine zusätzliche Charakterisierung von Paraproteinen Klinische Anwendung von zweidimensionaler Elektrophorese, I.

Zusammenfassung: Die hochauflösende zweidimensionale Elektrophorese, eine der effizientesten Methoden in der Eiweißanalytik, ist derzeit nur in spezialisierten Forschungslaboratorien einsetzbar.

Die vorliegende Arbeit beschreibt eine zweidimensionale Elektrophoresemethode, die eine Charakterisierung von abnormen Serumproteinen (Paraproteinen) erlaubt und im klinischen Laboratorium einsetzbar ist. Beide Elektrophoreseschritte wurden in der „Flachbett-Technik“ ausgeführt. Die Übertragung des Gels der isoelektrischen Fokussierung in das Gel der zweiten Dimension wurde durch speziell angefertigte Schablonen vereinfacht.

Eine entscheidende Steigerung der Praktikabilität wurde durch den Einsatz von käuflichen Materialien wie Gel-Platten für die isoelektrische Fokussierung bzw. Kit für die Silberfärbung erreicht. Die beschriebene Methode erlaubt neben einer Kalibrierung durch die pH Gradientmessung bzw. Verwendung von Molekulargewichtsstandardproteinen auch eine simultane Analyse von zwei verschiedenen Proben in den gleichen Gelen der ersten und der zweiten Dimension. Dadurch kann eine zu analysierende Probe genau mit einer Referenzprobe verglichen werden. Serumproben, die zuvor durch eine Routinemethode wie Agarosegel-

Elektrophorese selektioniert wurden, in der sie eine abnorme Bande („M-Gradient“) gezeigt hatten, wurden mit der beschriebenen Methode analysiert. Manche der „M-Gradienten“ wurden durch ein einziges Protein gebildet. Eine andere Serumprobe zeigte einen „M-Gradienten“, der eine Mehrzahl abnormer Proteinspots in unserer Methode ergab.

Fragen betreffend die pathobiologische und klinische Interpretation dieser Resultate rechtfertigen weitere Studien unter Verwendung dieser Methode.

Introduction

High resolution two dimensional electrophoresis is one of the most potent analytical methods in protein chemistry, permitting the separation of more than one thousand protein components from just a few microliters of biological sample (1). Two dimensional electrophoresis combines isoelectric focusing with SDS-electrophoresis, thus enabling the resolution as well as characterization of proteins based on two physico-chemical criteria: the isoelectric point and the molecular weight. Using special staining procedures, such as the silver stain, some proteins can be detected in nanogram amounts (2).

The importance of two dimensional electrophoresis in clinical chemistry lies in its ability to detect minute changes in protein patterns in body fluids. Correlation of such findings provides information on the genesis of certain diseases and/or establishes new indicators for their diagnosis (3). However, the high resolution two dimensional electrophoresis which allows separation of more than one thousand proteins still represents a „virtuoso technique“ (3). Such an application is rather reserved for specialized research laboratories. The pattern recognition of proteins in trace amounts is of primary interest in clinical chemistry. Moreover, determination of molecular weight, basicity and the number of subunits of an already detected abnormal protein, can also provide a relevant approach to the pathobiochemistry, diagnosis and control of treatment of given disease.

The aim of this study was to establish a method, suitable for characterization of serum paraproteins, and practicable in a clinical laboratory.

Materials and Methods

Apparatus

Both steps of the described two dimensional electrophoresis method, the isoelectric focusing and the SDS polyacrylamide gel electrophoresis, were performed in the horizontal mode, using the „Multiphor System“ with a constant power supply and „Multi Temp Thermostatic Circulator“ supplied by LKB Produkter AB (Bromma Sweden).

Chemicals and materials

Acrylamide (99,9% pure) was obtained from Bio-Rad (Richmond, Calif., USA). „Ultrapure“ urea was supplied by Bethesda Research Laboratories (Gaithersburg, Maryland, USA), and Nonidet NP 40 by Fluka AG (Buchs SG, Switzerland). N,N'-methylene diacrylamide (BIS), sodium dodecylsulphate (SDS), Tris-(hydroxymethyl)aminomethane (TRIS), Coomassie Brilliant Blue R 250 and all other reagents and solvents of analytical purity, except where otherwise stated, were obtained from Merck (Darmstadt, FRG).

Ampholine pH 3.5–0.5, „PAG plates“ pH 3.5–9.5 for isoelectric focusing, „Multiphor surface pH electrode“ and „2D Gradient Gel Kit“, containing a gradient mixing device, supporting plastic foils and other materials were supplied by LKB Produkter AB (Bromma, Sweden).

For the molecular weight calibration, „HMW and LMW calibrator kit proteins“ (marker proteins) from Pharmacia Fine Chemicals A.B. (Uppsala, Sweden) were used.

The silver-stain procedure was carried out using the „Bio Rad Silver Stain Kit“ from Bio Rad (Richmond, Calif., USA).

Blood samples

Venous whole blood samples obtained by puncture of the cubital vein were taken in glass tubes and immediately centrifuged at 1700 g. The sera were then frozen at -20°C and stored until analysis. Storage of samples for up to one month at -20°C caused no observable change in the two dimensional electrophoresis protein pattern, compared with that obtained with the same samples analysed immediately after centrifugation. In particular, the zones corresponding to investigated abnormal proteins (paraproteins) remained unchanged.

Agarose gel electrophoresis

The routine screening agarose gel electrophoresis was performed according to the procedure described elsewhere (4). Following the electrophoresis run, gels were stained for 15 min with a solution containing 3 g Coomassie Brilliant Blue R 250 in 500 ml methanol, 200 ml acetic acid and 900 ml of distilled water. The background was destained using several changes of the same solution without Coomassie Blue R 250.

Isoelectric focusing

The separation in the first dimension of the two dimensional electrophoresis was performed on commercially available „PAG plates“. In order to realize the dissociation of proteins into their subunits and to prevent precipitation during the focusing, the „PAG plate“ and samples were incubated at room temperature in a solution containing 9 mol/l urea, 0.7 mol/l β -mercaptoethanol, 20 g/l Nonidet NP 40 and 50 g/l of Ampholine pH 3.5–9.5 (1). The „PAG plate“ was incubated for 30 min. The serum samples were simply diluted 1 to 10, with the above described solution.

Bands of interest in the agarose gel electrophoresis were cut out, washed with distilled water and incubated for 20 min in the same solution. The "PAG plate" was prefocused on the "Multiphor unit" for 15 min at 4 °C with power set at 22 Watts (Multiphor voltage and current at maximum)¹⁾.

Afterwards, samples were applied near the anodic side of the "PAG plate" using "applicator pieces" for diluted serum samples as described in the "PAG plate" instructions. A volume of 20 µl of 1 to 10 diluted serum was applied. Cut agarose bands were applied directly. Focusing was then performed at 10 watts for a further 40 min. Applied pieces were removed and the isoelectric focusing continued for 40 min at 22 watts, until the voltage reached 2200 volts.

Immediately after the run, the pH gradient was measured with a "Multiphor surface pH electrode". The "PAG plate" was stained with Coomassie Brilliant Blue R 250 as described for agarose gel electrophoresis, and stored in the destaining solution until the analysis in the second dimension.

SDS-electrophoresis

The preparation of the second dimension electrophoresis gel was performed according to the method of Görg et. al. (5) with some modifications: The SDS polyacrylamide gel was prepared containing a linear density gradient and with dimensions of 1 × 90 × 230 mm. Mixing chambers of the gradient mixing device contained 14 ml of "heavy" and "dense" solution, respectively. Both solutions contained 1 g/l SDS, and were buffered by 375 mmol/l TRIS-HCl to pH 8.8. The heavy solution contained 219 g/l acrylamide, 6 g/l BIS and 160 g/l-glycerol, while the light solution contained no acrylamide, BIS or glycerol. The SDS polyacrylamide gel was then formed in order to obtain a linear density gradient with acrylamide concentration ranging from 48.6 to 219 g/l. For the transfer of the sample from the first into the second dimension electrophoresis, two specially prepared foil stencils were used. The first stencil of 130 × 130 mm in size had a slot of 90 × 4 mm. The second foil stencil of the same size as the SDS-electrophoresis gel (230 × 110 mm) had two 90 × 4 mm slots parallel to its longer side. Between these, two small slots of 2 × 0.5 mm were made, which functioned as an application mask for marker proteins (fig. 1). The gel from the first dimension was placed on a glass-plate (gel side up) and covered by the first foil stencil, so that the slot of the stencil enclosed the best sample track. The gel was cut out with a scalpel in order to obtain a gel strip of 90 × 4 mm.

The stencil was lifted and the gel surrounding the cut sample track was removed with tweezers. Finally, the foil supporting the gel was cut out with a margin of 5 mm around the gel strip. In this arrangement the gel strip could be easily handled by carrying it on the piece of the cut foil. Before the transfer to the SDS-electrophoresis gel, the first dimension gel strip was incubated for 2 min in electrode buffer as mentioned below.

At the same time the second foil stencil was layed on the prepared SDS-polyacrylamide gel in order to cover the whole gel surface, placing two slots of 90 × 4 mm near and parallel to the cathode side. By cutting the SDS gel enclosed by the stencil slots, gel slots were prepared into which the first dimension gel strips (gel side down) were lowered (fig. 1). By pressing along the backing foil of the first dimension gel strip, the gel to gel contact along the edges was established. Then the backing foil was removed leaving the first dimension gel strip in the slot of the second dimension gel. After application of marker proteins, using the two small slots, the stencil was then removed and the SDS electrophoresis gel was transferred to the Multiphor unit. The described transfer procedure prevents the danger of touching or damaging one of the gels

Fig. 1. (A) Foil stencil for the gel transfer from the isoelectric focusing stage to the SDS electrophoresis gel. Dimensions in millimeters.

(B) Transverse section showing the transfer of the gel strip from the isoelectric focusing separation (a) to the gel slot of the SDS electrophoresis gel (d); (b) and (e) are supporting foils of the gels of the first and second dimensions respectively; (c) is the foil stencil.

of the first or second dimension. The SDS-electrophoresis was performed at 5 °C using an electrode buffer of 25 mmol/l TRIS, 192 mmol/l glycine with 1 g/l SDS (pH 8.3). In the first hour of the run the voltage was set at 250 volts (current and power at maximum). During this time, the Coomassie Blue dye which was contained in the first dimension gel strip was focused to a sharp front on the anodic edge of the first dimension gel.

The voltage was then increased to 800 volts. After 40 min, as the dye front moved at least 1 cm into the SDS gel, the first dimension gel strip was removed and the gel slot was filled with the electrode buffer.

The electrophoresis was then performed for another two hours until the Coomassie Blue dye front moved out from the gel towards anodic side. the gel was removed and stained according to the procedure as stated in the "Bio Rad Silver Stain" product description. Stained gels were sealed in polyethylene bags and stored at 4 °C for documentation.

Results and Discussion

The "flat bed electrophoresis technique" used in both dimensions of the described two dimensional electrophoresis method has several advantages (5). One of these is its high degree of practicability. Additionally, using commercially available materials like gels for the isoelectric focusing and the silver stain procedure, we were able to analyse more than twelve samples per week. The analyses can be performed by one person only. The calibration of gels of both dimensions by measuring the pH gradient after isoelectric focusing and by the use of marker pro-

¹⁾ During the isoelectric focusing the power only was set on a fixed level. The voltage increased and the current decreased continuously to final values of 2000 V and 20 mA respectively.

teins for the molecular weight axis guaranteed an exact adjustment of two dimensional electrophoresis coordinates to each spot in the protein map. The possibility of a simultaneous analysis of two different samples under identical conditions, using the same gel for the first and the second dimensions respectively permits a correct comparison of proteins from a sample of interest with a sample of reference.

Although the described method was designed as a method for characterization of abnormal serum proteins detected previously, an evaluation of spots and regions usually present in the serum was necessary for broad orientation. For this reason analyses of serum fractions of known composition had to be performed. In the case of routinely used serum agarose electrophoresis it is well known which of the major protein constituents belongs to each band of agarose gel. Thus, by successive two dimensional electrophoresis analyses of separated agarose gel bands in reference to the total serum, some major serum constituents were identified in our serum protein map (fig. 2). The result was verified by comparison with some already published serum protein maps (6–8).

Fig. 2. Protein map of major serum constituents: (a) albumin, (b) transferrin, (c) IgG heavy chains, (d) α_1 antitrypsin, (e) immunoglobulin light chains, (f) apolipoprotein I-A, (g) prealbumin.

Further, we analysed serum samples of patients suffering from paraproteinaemia in comparison with serum from a healthy person. Examples of protein maps from these investigations are shown in figure 3. In each case, the abnormal protein is marked by an arrow. The pathological serum samples were selected by a previous routine agarose gel electrophoresis where an abnormal peak ("M gradient") was detected. The nature of paraprotein was established by immunofixation (9).

The information about the molecular weight of a paraprotein and in particular its isoelectric points is of clinical interest. As recently reported (10, 11) the isoelectric point of a paraprotein may correlate with the failure of renal function. Another case of paraproteinaemia is seen in figure 4. The protein map of the serum shows a system of abnormal spots. Since the previous agarose gel electrophoresis showed a prominent "M gradient", we cut this pathological agarose gel band out and we analysed it together with the total serum of the patient. As can be seen in figure 5 the "M gradient" is composed of a multiplicity of spots grouped into three lines of constant molecular weights of 40 500, 81 000 and more than 150 000 daltons, showing differences in their isoelectric points ranging from 5.9 to 7.4 pH units. Estimating visually the sum of their intensities, it can be assumed, that the "M gradient" is composed solely by these abnormal spots.

As demonstrated by reported two dimensional electrophoresis analyses of serum samples, abnormal bands ("M gradients") in routinely used agarose gel electrophoresis do not necessarily consist of a single protein. In certain cases "M gradients" can include a number of protein components. Further investigations for pathobiochemical and clinical implications of these findings are currently in progress.

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Fig. 3. Protein maps of four serum samples of patients suffering from paraproteinaemia. Each sample showed a single abnormal band ("M gradient") in the agarose gel electrophoresis. The immunological characterization of corresponding paraproteins was established by immunofixation: (A) lambda *Bence Jones* protein, (B) IgG-lambda paraprotein, (C) kappa *Bence Jones* protein, (D) IgG-kappa paraprotein. Identification of abnormal spots in each protein map was performed by simultaneous two dimensional electrophoresis analysis of pathological serum sample and the isolated "M gradients" from the agarose gel electrophoresis. Abnormal spots corresponding to constituents of "M gradients" are marked as follows: λ - lambda light chain, κ - kappa light chain, G - IgG heavy chain. Normally occurring serum constituents (a) to (f) are referred to in figure 2. Running as indicated in fig. 2.

Fig. 4. Protein map from a simultaneous analysis of a serum sample from patient R. M. (left) together with a serum sample from a healthy person (right). The patient's serum shows a group of additional spots (→). The pathological serum sample was analysed previously by agarose gel electrophoresis and showed a prominent "M gradient", which was characterized by immunofixation as a γ heavy chain of IgG immunoglobulin. Normally occurring serum constituents (a) to (f) are referred to in figure 2. A separation of marker proteins for molecular weight determination can be seen in the middle of the gel in the vertical dimension.

Running as indicated in fig. 2.

Fig. 5. Protein map from a simultaneous analysis of the pathological serum sample from the figure 4 (left) and the isolated "M gradient" from its previous agarose gel electrophoresis (right). The protein map of the constituents of the isolated "M gradient" shows a group of spots grouped into three lines of constant molecular weights (40500, 81000 and more than 150000 daltons) with isoelectric points ranging from 5.9 to 7.4 pH units. A separation of marker proteins for molecular weight determination can be seen in the middle of the gel in the vertical dimension.

Running as indicated in fig. 2.

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Dr. Alexander Lapin
Institut für Klinische Chemie
und Laboratoriumsdiagnostik
Universität Wien
Lazarettgasse 14
A-1090 Wien, Österreich