TWO-DIMENSIONAL IMMUNOELECTROPHORESIS OF HUMAN SERUM VERY LOW DENSITY APOLIPOPROTEINS

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1. Introduction

On polyacrylamide disc-gel electrophoresis of delipidated human serum very low density lipoprotein (VLDL) 5 major zones are obtained corresponding to the apolipoproteins C-I, C-II, C-III-1, C-III-2 and E, the latter also denoted arginine-rich apolipoprotein [1,2]. This separation is achieved only in the presence of 8 M urea or other hydrogen bond dissociating agents.

In order to test objectively the purity of isolated apolipoproteins and the specificity of antisera directed against the different apolipoproteins, the two-dimensional agarose gel immunoelectrophoretic technique (crossed immunoelectrophoresis) in [3] seemed necessary.

However this technique is not applicable in the presence of 8 M urea which completely prevents the gelatinization of the agarose.

Furthermore the combined polyacrylamide gel antibody-containing agarose gel electrophoresis technique is hampered by the very slow and incomplete diffusion of protein from the polyacrylamide gel and by differences in endo-osmosis between this gel and agarose making it difficult to maintain electrical contact between the gels [4].

This paper describes a method for the two-dimensional immunoelectrophoresis of VLDL-apolipoproteins, where cellulose acetate gel [5] is used for the electrophoretic separation of the apolipoproteins prior to electrophoresis in an antibody-containing agarose gel.

2. Experimental

Cellulose acetate gel strips, 5.7 × 14 cm (Cellogel 009) were purchased from Chemetron, Milano and agarose (Indubiose 37A) from L'industrie Biologique Française.

Very low density lipoprotein $d < 1.006$ g/cm^2 was isolated from fasting human serum by ultracentrifugation at uniform density in a fixed angle rotor [6] followed by two successive centrifugations.

Delipidated mixtures of VLDL-apolipoproteins were prepared from VLDL by selective extraction with methylsulfoxide and isopropanol as in [7].

Apolipoprotein C-II, C-III-1 and C-III-2 were further purified by preparative flat bed isoelectric focusing [8] and C-I and E by DEAE-ion exchange chromatography as in [1].

Antibodies against the purified apolipoproteins were raised in rabbits in our own laboratory and the immunoglobulin fraction of the rabbit serum was prepared by established techniques [9].

2.1. *Polyacrylamide disc-gel electrophoresis*

Polyacrylamide disc-gel electrophoresis of VLDL-apolipoproteins was run as in [10] omitting tetramethylurea and reducing solutions. The protein samples were made 4 M with respect to urea before application onto the gels. Protein zones were stained with Coomassie brilliant blue R [11].

2.2. *Polyacrylamide gel isoelectric focusing*

Isoelectric focusing of purified apolipoprotein E and staining was performed as in [12]. The protein sample was dialyzed against 8 M urea and mixed with a solution of the acrylamide reagents containing (per ml): 0.5 g urea; 5–20 μg riboflavin; 0.05 ml Ampholine (pH 3.5–10) (LKB-Products, Stockholm).

Photopolymerization was performed for 30–60 min at room temp.

2.3. *Cellulose acetate gel electrophoresis*

Cellulose acetate electrophoresis was performed in the following way. The Cellogel strip was soaked overnight in 0.05 M barbital buffer (pH 8.6) in 8 M urea containing 0.04% EDTA and 0.02% azide or 0.1 M acetate buffer (pH 4.5) in 8 M urea.

After removal of excess liquid by blotting papers the strip was placed on a spraywater-cooled glass plate in an electrophoretic tank essentially as in [13].

After application of wicks (Whatman 3MM) from the buffer reservoirs which contained the buffers in level, 2 μl samples were applied 4 cm from the cathodic end of the strip.

Electrophoresis was for 0.5–2 h at a true potential of 10–15 V/cm, 4–6 mA, measured by placing electrodes on the strip, at 5–8°C. Staining of strips was done with Ponceau S as in [14].

2.4. *Agarose gel electrophoresis*

Antigen–antibody electrophoresis in agarose gel was done following [3] on 10 × 10 cm glass plates using 1% agarose as in [15] omitting the sample holes.

The antibody concentration of the agarose gel varied between 1–10 μl/cm^2. Electrophoresis was at 1–2 V/cm for 18 h at 10°C.

After completed cellulose acetate electrophoresis a 1–4 mm broad strip, depending on the concentration of the separated apolipoprotein was cut out with a razor blade. This strip was directly when still wet put onto the antibody-containing agarose gel plate in the electrophoretic tank. The separating porous surface of the cellulose acetate strip was placed in contact with the agarose gel. After completed run the strip was removed and the plate was dried and stained with Coomassie brilliant blue R and destained as in [15].

3. Results and discussion

The major human serum very low density apolipoproteins C-I, C-II, C-III-1, C-III-2 and E (arginine-rich lipoproteins) were separated by polyacrylamide disc-gel electrophoresis [12] (fig.1A,B,D).

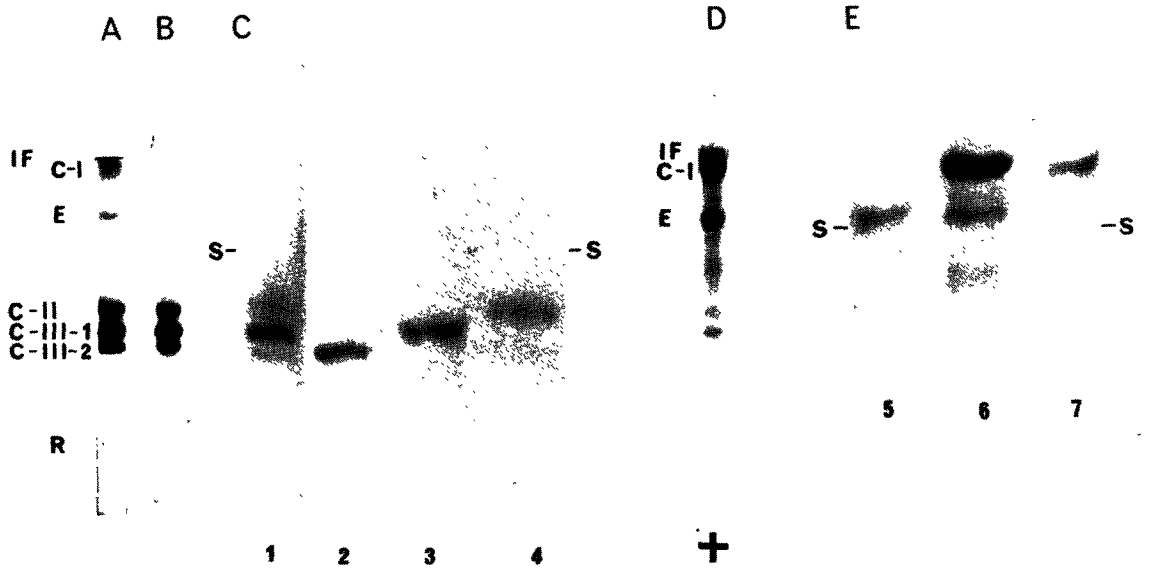

Fig.1. Polyacrylamide disc-gel (A,B,D) and cellulose acetate gel (C,E) electropherograms of VLDL-apolipoproteins. For experimental details see section 2. IF denotes the interface between the sample gel and the separation gel and R the riboflavin marker. S indicates the sample application line. The same relative scale of the electropherograms is maintained. Samples for electrophoresis. (A) Polyacrylamide: VLDL-apolipoproteins solubilized by isopropanol according to [7]. (B) Polyacrylamide: Apolipoprotein C-II, C-III-1 and C-III-2 extracted from VLDL by methylsulfoxide according to [7]. (C) Cellulose acetate: 1, Apolipoprotein C-II, C-III-1 and C-III-2 extracted from VLDL by methylsulfoxide (7); 2, apolipoprotein C-III-2; 3 apolipoprotein C-III-1, apolipoprotein C-II. (D) Polyacrylamide: Apolipoprotein C-I and E extracted by isopropanol from the lipid residue remaining after extraction of VLDL by methylsulfoxide [7]. (E) Cellulose acetate: 5, Apolipoprotein E; 6, apolipoprotein C-I and E extracted by isopropanol from the lipid residue remaining after extraction of VLDL by methylsulfoxide according to [7]; 7, apolipoprotein C-I.

Here the separation of these apolipoproteins was achieved by cellulose acetate gel electrophoresis, using 0.05 M barbital buffer (pH 8.6) in 8 M urea (fig.1C,E).

In the polyacrylamide system at pH 9 apolipoprotein C-I after completed electrophoresis normally remains as a diffuse band in the sample gel or just below its interface (fig.1A,D).

Apolipoprotein E (the arginine-rich lipoprotein) has been reported to be composed of polymorphic forms with identical amino acid composition which occasionally separate on polyacrylamide disc-gel electrophoresis [16] or on isoelectric focusing in urea-containing buffers [17]. The purified apolipoprotein E used in the present investigation produced one band on polyacrylamide disc-gel electrophoresis at pH 9 and two bands on isoelectric focusing in the pH 3.5–10 gradient (fig.2).

On cellulose acetate electrophoresis of apolipoprotein C-I and E the former at pH 8.6 migrated as a sharp band in the cathodic direction, whereas apolipoprotein E remained close to the starting position (fig.1E).

In 0.1 M acetate buffer (pH 4.5) in 8 M urea, both C-I and E migrated in the anodic direction. However, E yielded a diffuse zone and was not separated from residual amounts of C-protein in the apolipoprotein preparation (fig.3).

The resolution of the polyacrylamide disc-gel and cellulose acetate electrophoretic techniques at the separation of apolipoprotein C-II, C-III-1 and C-III-2 seemed equivalent (fig.1B,C) but the cellulose acetate electrophoresis was much more rapid. A drawback of the cellulose acetate technique is that the concentration of the sample must be high as the sample volume has to be kept small, in contrast to the polyacrylamide technique where relatively large volumes of diluted sample can be applied onto the gel.

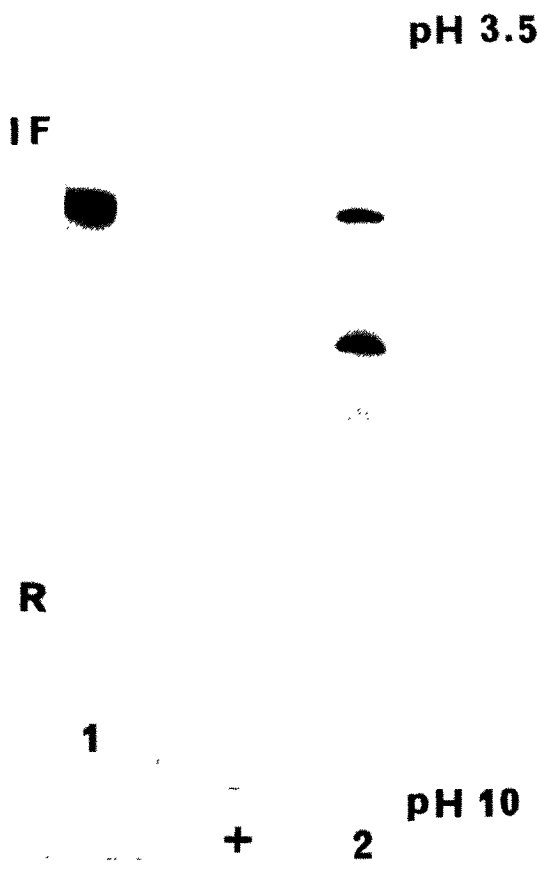

Fig.2. Polyacrylamide disc-gel (1) and isoelectric focusing (2) electropherograms of purified apolipoprotein E. IF denotes the interface between the sample gel and the separation gel and R the riboflavin marker.

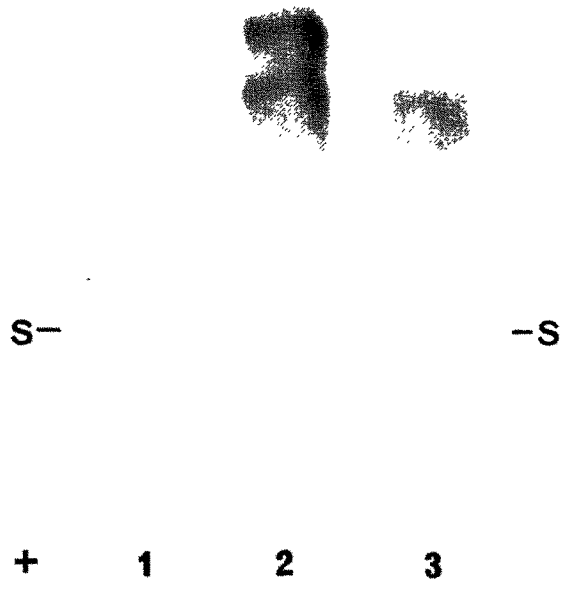

Fig.3. Cellulose acetate gel electropherogram obtained by electrophoresis in 0.1 M sodium acetate buffer in 8 M urea at pH 4.5 of: 1, apolipoprotein C-I; 2, same mixture of apolipoprotein C-I and E as in fig.1D; 3, apolipoprotein E. S indicates the sample application line.

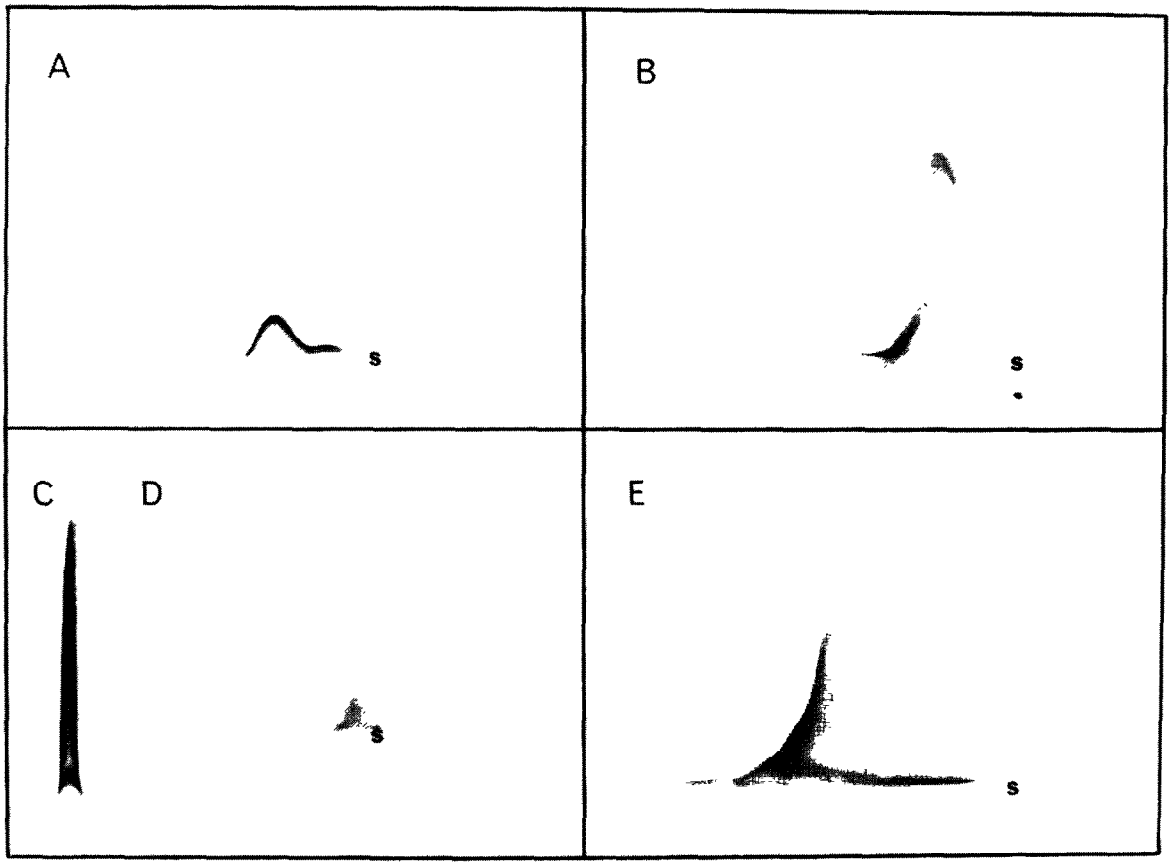

Fig.4. Electropherograms obtained after cellulose acetate gel electrophoresis of a VLDL-apolipoprotein mixture in the first dimension followed by antibody-containing agarose gel electrophoresis in the second dimension. The apolipoprotein mixture was the same as in fig.1 A, containing apolipoprotein C-I, C-II, C-III and E. S indicates the sample application line at electrophoresis in the first dimension. Antibody in the agarose gel. (A) Anti-C-I, 50 μg protein/cm²; (B) Anti-C-II, 100 μg protein/cm²; (C) Anti-E, 500 μg protein/cm² (D) Anti-E, 500 μg protein/cm²; (E) Anti-C-III, 200 μg protein/cm².

After cellulose acetate electrophoresis, separated proteins may normally be quantitatively and rapidly eluted.

Fig.4 shows the results from cellulose acetate electrophoresis of a mixture of apolipoprotein C-I, C-II, C-III and E in the first dimension and antibody-containing agarose gel electrophoresis in the second dimension after placing a strip of the cellulose acetate electropherogram on the agarose gel surface.

The antibodies used were prepared by immunizations with purified apolipoproteins. The experiments with anti-C-I (fig.4A) demonstrated that the faint zone seen between apolipoprotein C-I and E in fig.1E was immunologically equivalent with the main C-I zone. This electrophoretic heterogeneity of C-I may reflect the strong tendency of this apolipoprotein to aggregate.

With anti-C-II one peak was obtained (fig.4B) whereas anti-C-III produced a double peak as expected representing precipitation lines for C-III-1 and C-III-2, respectively, which are known to be immunologically equivalent (fig.4E).

On two-dimensional immunoelectrophoresis of the apolipoprotein mixture against anti-E only a small immunoprecipitation peak was obtained (fig.4D).

It was found that cellulose acetate adsorbed apolipoprotein E at pH 8.6. One-dimensional agarose gel immunoelectrophoresis of the same amount of directly applied apolipoprotein mixture containing apolipoprotein E yielded a rocket as shown in fig.4C. The

fact that cellulose acetate adsorbs E at pH 8.6 in combination with its low electrophoretic mobility makes the present two-dimensional technique less suitable for the direct study of apolipoprotein E. However, its use for the demonstration of the presence or absence of anti-C-I in anti-E preparations is obvious.

These results demonstrate that the combined cellulose acetate–agarose gel electrophoresis is a simple and rapid means for testing the specificity of antibodies directed against different VLDL-apolipoproteins as well as the purity of apolipoprotein preparations.

Isoelectric focusing may be carried out on modified cellulose acetate [18]. The investigation of the polymorphism of the VLDL-apolipoproteins after separation by this electrofocusing method in the first dimension will be the subject of a future study.

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