Separation of apolipoprotein B species by agarose-acrylamide gel electrophoresis

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Summary Human apolipoprotein (apo) B has been recognized to exist in two different forms designated apoB-100 and apoB-48. The two apoB forms are usually separated by $NaDodSO_4$ gel electrophoresis with a low percentage polyacrylamide gel in a tube gel apparatus. However, the matrix of this low percentage gel is relatively weak, and one can separate the two forms of apoB in a slab gel apparatus only if one utilizes a gradient polyacrylamide gel or a higher percentage polyacrylamide gel which results in a poorer separation of the protein bands. We have developed an agarose-acrylamide gel electrophoretic method to separate the two major apoB forms. The gel is a mixture of 0.5% agarose and 2% acrylamide. The agarose-acrylamide method is fast, has the advantage of being able to be used on an analytical or preparative scale in a vertical slab gel apparatus, and the gel is of sufficient strength to be used in immunoblotting and/or radioautography. —**Gabelli, C., D. G. Stark, R. E. Gregg, and H. B. Brewer, Jr.** Separation of apolipoprotein B species by agarose-acrylamide gel electrophoresis. *J. Lipid Res.* 1986. **27:** 457–460.

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Apolipoprotein B is a major protein constituent of chylomicrons, VLDL, IDL, and LDL. In LDL, apoB is the predominant apolipoprotein and is the apolipoprotein ligand for binding to the high affinity LDL receptor (1). Despite the relative ease of isolation of the apolipoprotein, the detailed physico-chemical characteristics of apoB are still relatively unknown due to its large apparent molecular weight and insolubility in aqueous buffers after delipidation. In addition, it is now known that there are multiple forms of apoB with different molecular weights in the rat (2–4) and in man (5). In man there are two major forms, the larger apolipoprotein being designated apoB-100 and the smaller one apoB-48 (5–7). ApoB-100 is of hepatic origin and is present on VLDL, IDL, and LDL, while apoB-48 is of intestinal origin and is found predominately on chylomicrons and on chylomicron remnants within the VLDL and IDL density ranges (5–7).

The analysis of the structure and metabolism of apoB-48 and apoB-100 necessitates the separation of these two B apolipoproteins. ApoB-48 and apoB-100 can be separated by gel permeation chromatography in $NaDodSO_4$ (8); however, this method is time-consuming and it is difficult to isolate and quantitate small samples of protein. An alternative approach for separation of the B apolipoproteins is by 3% acrylamide $NaDodSO_4$-PAGE (5, 9). However, at the low concentration of acrylamide used, the gel matrix is very weak and handling becomes difficult. In addition, because of the weakness of the gel matrix, vertical slab gel electrophoresis is extremely difficult to perform and can be performed only by utilizing gradient polyacrylamide gels or higher percentage polyacrylamide gels, which results in poorer separation of the protein bands (10). Peacock and Dingman (11) described a method utilizing agarose-acrylamide gel electrophoresis for separation of ribonucleic acids. In this report, we describe a modification of this method for the separation of the multiple forms of apoB. The resulting gel has the characteristic of being able to separate large molecular weight proteins while being of sufficient strength to be utilized in a 3-mm vertical slab gel apparatus, and the gel can be used for immunoblotting or radioautography.

METHODS

Materials and reagents

Acrylamide, bisacrylamide, TEMED, ammonium persulfate, $NaDodSO_4$, electrophoresis purity agarose (low gel temperature), bromphenol blue, and Coomassie brilliant blue R-250 were purchased from Bio-Rad Laboratories. DTT was obtained from Schwarz-Mann, ultrapure Tris from Bethesda Research Laboratories, Inc., boric acid from Mallinckrodt, and certified ACS potassium bromide and EDTA from Fisher Scientific. A buffer containing 0.89 M Tris (pH 8.3) and 0.89 M boric acid (ten times the working concentration) was prepared, filtered, and stored in a glass bottle at room temperature. A 1% $NaDodSO_4$ solution (ten times the working concentration) was kept as a stock solution at room temperature. Ten percent ammonium persulfate was kept at 4°C and made fresh weekly.

Sample preparation

Blood was collected in EDTA (1 mg/ml) and the plasma was quickly separated by centrifugation (2000 rpm) for 30 min at 4°C. Sodium azide (0.05%) and aprotinin (1000 KIU/ml) (Boehringer-Mannheim) were added to the plasma. Lipoprotein fractions were obtained by ultra-

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bisacrylamide, N,N-methylenebisacrylamide; TEMED, N,N,N',N'-tetramethylethylenediamine; DTT, 1,4-dithiothreitol; Tris, tris-hydroxymethyl aminomethane; apo, apolipoprotein.

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centrifugation in a Beckman 40.3 rotor at 39,000 rpm and 4°C (12). Lipoproteins were isolated by tube slicing, dialyzed against 0.01 M EDTA containing 0.05% NaN_3 (pH 8.0), lyophilized, and delipidated with chloroform–methanol 2:1 (v/v) (13). Due to the insolubility of apoB-100 and apoB-48 in aqueous solutions, a technique was developed for dissolving the protein pellets after delipidation in the sample buffer. A small quantity of chloroform–methanol (200–300 μl) was left in the bottom of the tube after delipidation. Tubes were warmed briefly (approximately 5 sec) in a 100°C oil bath. Fifty to 200 μl (depending on the thickness of the gel being used) of 1× Tris-borate buffer containing 3% $NaDodSO_4$ and 1.2% DTT was added while vortexing. The remaining chloroform–methanol was immediately volatilized with a stream of N_2 while still vortexing. To increase the denaturation of the apolipoproteins, samples were left at room temperature for 1 hr and then heated to 100°C for 3 min. One or two drops of 0.1% bromophenol blue in glycerol–water 1:1 (v/v) were added to each sample.

Agarose-acrylamide gel electrophoresis

Agarose (1%) was dissolved in double-distilled H_2O in a reflux apparatus (round-bottom flask and condenser) which was heated just to boiling with a heating mantle. Crystalline acrylamide and bisacrylamide were dissolved in 2× Tris-borate buffer containing 0.2% $NaDodSO_4$ to give final concentrations of 4% and 0.21%, respectively, and stirred in a water bath at 40°C. The agarose solution was then cooled to 40°C (with stirring) under a stream of tap water. The agarose and acrylamide solutions were mixed 1:1 and stirred at 40°C. The final concentrations were 2% acrylamide, 0.5% agarose, 0.105% bisacrylamide, and 0.1% $NaDodSO_4$ in 1× Tris-borate buffer (0.089 M). Ammonium persulfate (0.5 ml of a 10% solution) and TEMED (33 μl) were added per 100 ml of agarose-acrylamide solution just prior to pouring. The solution was then quickly poured into 12 × 14 cm gels in a vertical slab gel apparatus (Hoefer SE 500, San Francisco, CA) which were cooled with circulating water at 20°C. Well-formers cooled to 4°C were inserted and the gels were left to polymerize overnight at 20°C. A 1× Tris-borate buffer (pH 8.3) containing 0.1% $NaDodSO_4$ was utilized as a running buffer in both the upper and lower buffer chambers. Before electrophoresis, the gels were cooled to 4°C and the electrophoresis was performed at a constant voltage of 200 V at 4°C until the tracking dye reached the bottom of the gel (approximately 1.5 hr). Following electrophoresis, the gels were fixed and stained for 30 min in methanol–water–acetic acid 10:10:1 (v/v) containing 0.1% Coomassie brilliant blue R-250. The gels were destained overnight with gentle rocking in 5% methanol–7.5% acetic acid. Immunoblotting was performed with a rabbit anti-B-100 antiserum as previously reported (14, 15). Briefly, the B apolipoproteins were transferred to the nitrocellulose paper for 1 hr at 90 volts. The nitrocellulose paper was blocked with gelatin, followed by incubation with the anti-B-100 antiserum overnight with gentle rocking at room temperature. Goat anti-rabbit IgG antibody conjugated with horseradish peroxidase was then incubated with the nitrocellulose paper and the color was developed on the immunoblot with the addition of 4-chloro-1-naphthol.

The LDL utilized in the recovery experiments were iodinated by the modified iodine monochloride method of Bilheimer, Eisenberg, and Levy (16).

RESULTS AND DISCUSSION

The polymorphic forms of apoB have previously been separated by 3% acrylamide $NaDodSO_4$-PAGE (5), but a difficulty with this system is the fragility of the polymerized gel. The tube gels break easily and the gel matrix is not of sufficient strength to be utilized in vertical slab gel electrophoresis. To overcome this problem, we have adapted the agarose-acrylamide gel electrophoresis system of Peacock and Dingman (11) to separate the different forms of apoB. **Fig. 1** illustrates the separation of apoB forms from VLDL of a normal subject with predominately apoB-100 and of an apoE-deficient subject with both apoB-100 and apoB-48. There is good separation between apoB-100 and apoB-48 even in the lanes in which the gel was markedly overloaded with protein (40–50 μg), and the

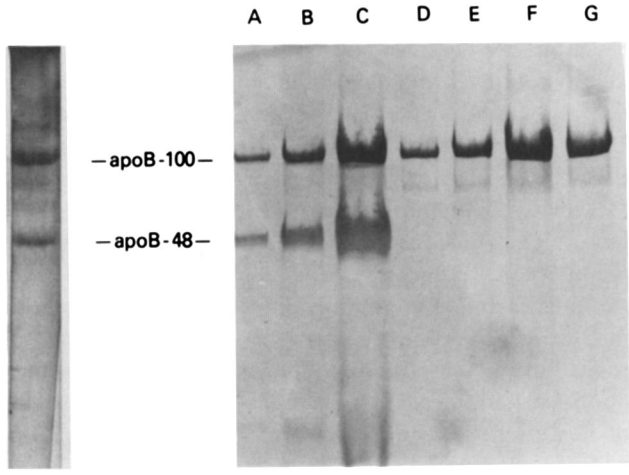

Fig. 1. Electrophoresis of apoB-100 and apoB-48 by acrylamide $NaDodSO_4$ tube gel electrophoresis and agarose-acrylamide slab gel electrophoresis. Left: 70 μg of VLDL protein from an apoE-deficient patient separated by acrylamide $NaDodSO_4$ gel electrophoresis in a tube gel (7 × 100 mm). Right: lanes A, B, and C contain 10 μg, 25 μg, and 50 μg of VLDL protein, respectively, isolated from a patient with apoE deficiency; lanes D to G contain 10 μg, 25 μg, 50 μg, and 40 μg of normal VLDL protein, respectively, separated by agarose-acrylamide slab gel electrophoresis.

separation is comparable to that obtained with 3% acrylamide tube gel electrophoresis (Fig. 1). In the tube gel and in lanes D–G, one can also visualize very small amounts of apoB-74. In other samples that had more apoB-74, one could also visualize apoB-26 but it was always much less apparent than apoB-74. In the tube gel, trace amounts of presumably Lp(a) were present above the apoB-100 band; these were not seen after more extensive reduction. In Fig. 1, as well as in Figs. 2 and 3, the top of the gel is included in the picture demonstrating that there was no aggregated apoB at the top of the gel and that the apoB was completely resolubilized by the method that was developed for this purpose.

The separation of delipidated VLDL apolipoproteins on a preparative scale utilizing agarose-acrylamide slab gel electrophoresis is illustrated in **Fig. 2.** There was an effective separation of apoB-100 and apoB-48 at a protein load of 200 to 700 μg of protein per lane. In three separate experiments, the recovery in the apoB-100 band of ^{125}I-labeled LDL radioactivity added to LDL (200 μg) was 77 ± 4% (± SD), which compares favorably with the tetramethyl urea and isopropanol precipitation of LDL apoB (73% and 86% precipitation of apoB, respectively) (17). Of the 23% of the counts lost, 12% was lost during the delipidation procedure, while 9% was lost in the preparation and transfer of the delipidated sample to the gel electrophoresis apparatus. Virtually all of the applied radioactivity (98%) was recovered in the apoB-100 band. The coefficient of variation was quantitated in separate experiments in which 200 μg of protein from LDL in the presence of trace quantities of ^{125}I-labeled LDL was separated 9 times in a 3-mm slab gel apparatus. The coefficient of variation of apoB isolated by the gel was 13% with an average recovery of 75% of the initial radioactivity.

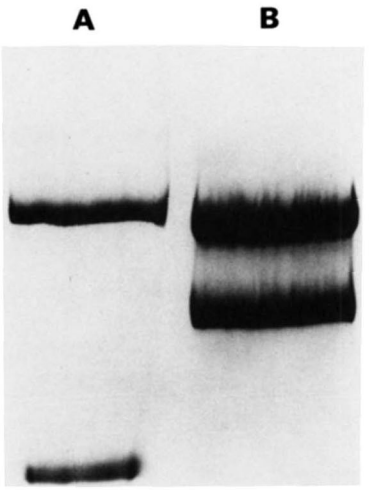

Fig. 2. Preparative separation of apoB-100 and apoB-48 agarose-acrylamide slab gel electrophoresis (3 × 120 × 140 mm slab gel). Two hundred μg of normal VLDL and 700 μg of VLDL protein from an apoE-deficient patient were electrophoresed in lanes A and B, respectively. In lane A, the upper band is apoB-100, while the lower band consists of lower molecular weight proteins which migrate as a single band in this gel electrophoresis system. In lane B, the upper band is apoB-100 while the band below it is apoB-48.

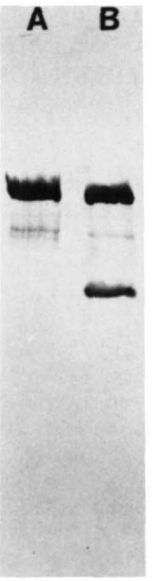

Fig. 3. Immunoblot of VLDL protein (2 μg) from a normal subject (A) and an apoE-deficient patient (B) following separation by agarose-acrylamide gel electrophoresis. The protein was transferred to nitrocellulose paper by electrophoresis, the paper was blocked with gelatin followed by incubation with a rabbit polyclonal IgG anti-human apoB, and then horseradish peroxidase-conjugated goat anti-rabbit IgG was added followed by color development. The major upper band in lanes A and B is apoB-100 while the major lower band in lane B is apoB-48. There is a very small amount of apoB-74 present between apoB-100 and apoB-48 in both lanes A and B.

The increased stability of the agarose-acrylamide gel permits immunoblotting of electrophoretically separated B apolipoproteins. VLDL from a normal subject and from an apoE-deficient patient were separated by agarose-acrylamide gel electrophoresis followed by immunoblotting with a rabbit polyclonal apoB antiserum (**Fig. 3**). Effective transfer and blotting were obtained despite the large molecular weight of the B apolipoproteins.

With the agarose-acrylamide gel method, it has been our experience that the temperature and timing of the different steps in the formation of the slab gel are important. In addition, during the sample preparation it is also very important not to completely dry the apolipoproteins after delipidation, since this induces aggregation and a significant fraction of the apolipoproteins may not enter into the gel matrix.

In conclusion, we have found that an agarose-acrylamide gel method for apoB separation is fast, has the advantage of being able to be performed on an analytical or preparative scale in a vertical slab gel apparatus, and the polymerized gel is of sufficient strength to be used in immunoblotting and/or radioautography. This method should be useful in the isolation and characterization of

the different apoB forms and should facilitate the investigation of the in vivo metabolism of apoB in both triglyceride- and cholesterol-rich lipoproteins.

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REFERENCES

1. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212:** 628–635.
2. Krishnaiah, K. V., L. F. Walker, J. Borensztajn, G. Schonfeld, and G. S. Getz. 1980. Apolipoprotein B variant derived from rat intestine. *Proc. Natl. Acad. Sci. USA.* **77:** 3806–3810.
3. Elovson, J., Y. O. Huang, N. Baker, and R. Kannan. 1981. Apolipoprotein B is structurally and metabolically heterogenous in the rat. *Proc. Natl. Acad. Sci. USA.* **78:** 157–161.
4. Sparks, C. E., and J. B. Marsh. 1981. Metabolic heterogeneity of apolipoprotein B in the rat. *J. Lipid Res.* **22:** 519–527.
5. Kane, J. P., D. A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* **77:** 2465–2469.
6. Meng, M. S., R. E. Gregg, E. J. Schaefer, J. M. Hoeg, and H. B. Brewer, Jr. 1983. Presence of two forms of apolipoprotein B in patients with dyslipoproteinemia. *J. Lipid Res.* **24:** 803–809.
7. Kane, J. P., G. C. Chen, R. L. Hamilton, D. A. Hardman, M. J. Malloy, and R. J. Havel. 1983. Remnants of lipoproteins of intestinal and hepatic origin in familial dysbetalipoproteinemia. *Arteriosclerosis.* **3:** 47–56.
8. Sparks, C. E., and J. B. Marsh. 1981. Analysis of lipoprotein apoproteins by SDS-gel filtration column chromatography. *J. Lipid Res.* **22:** 514–518.
9. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
10. Stalenheof, A. F. H., M. J. Malloy, J. P. Kane, R. J. Havel. 1984. Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. *Proc. Natl. Acad. Sci. USA.* **81:** 1839–1843.
11. Peacock, A. C., and C. W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry.* **7:** 668–674.
12. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
13. Folch, R. J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
14. Sprecher, D. L., L. Taam, and H. B. Brewer, Jr. 1984. Two-dimensional electrophoresis of human plasma apolipoproteins. *Clin. Chem.* **30:** 2084–2092.
15. Gabelli, C., R. E. Gregg, and H. B. Brewer, Jr. 1986. Immunoblot analysis of different apolipoprotein B species. *In* Apolipoprotein Mutants. C. Sirtori and G. Franceschini, editors. Plenum Press, New York. In press.
16. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. *Biochim. Biophys. Acta.* **260:** 212–221.
17. Egusa, G., D. W. Brady, S. M. Grundy, and B. V. Howard. 1983. Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein apolipoprotein B. *J. Lipid Res.* **24:** 1261–1267.