Apolipoprotein H: a two-step isolation method

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Summary A new method for the purification of apolipoprotein H by affinity chromatography followed by continuous-elution electrophoresis is described. It is both simpler and less complicated than the chromatographic and electrophoretic methods usually used. In addition, apolipoprotein H is isolated in a pure, structurally uncleaved form. This is of importance, as impairment has been detected in commercial preparations. The separation and purification of apolipoprotein H is a necessary prelude to its quantitative determination and phenotyping, and hence the clarification of its physiopathological mechanisms in lipid metabolism.—**Gambino, R., G. Ruiu, M. Cassader, and G. Pagano.** Apolipoprotein H: a two-step isolation method. *J. Lipid Res.* 1996. **37:** 902–904.

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Apolipoprotein H (apoH), also known as β2-glycoprotein I, is a 50 kDa plasma protein that has recently attracted considerable interest on account of its many functions, especially those associated with lipoprotein metabolism (1, 2).

Investigated because of its involvement in coagulation (3), apoH has recently been identified as an obligate cofactor for the formation of anti-phospholipid antibodies (4). It is present in plasma and is found in the two major lipoprotein fractions (VLDL and HDL): about 35% is lipoprotein-associated, and the rest is free plasma protein (5). Its ability to activate lipoprotein lipase suggests that it plays a part in triglyceride (Tg) metabolism (6). The rationale for this role seems to lie in apoH's structural polymorphism: three alleles at a single locus code for three isoforms whose phenotypes appear to be correlated with Tg and Chol-HDL levels (1, 2). It has long been known, in fact, that the phenotype of polymorphous apolipoproteins and their plasma level influence lipoprotein levels in various ways (7).

Separation and purification of apoH is a necessary prelude to its quantitative determination and the investigation of its phenotype, and hence the clarification of its physiopathological mechanisms in lipoprotein metabolism. As an alternative to the somewhat complicated chromatographic and electrophoretic methods usually used (4, 8), this report describes a two-step procedure for the isolation of high-purity apoH from normal sera by means of affinity chromatography followed by continuous-elution electrophoresis.

METHODS AND RESULTS

Rabbit anti-apoH antibodies (22 mg) (Behring, Scoppito, Italy) were immobilized on cyanogen–bromide-activated Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) in 5 ml total volume and packed into a 0.7 × 13.5 cm column equilibrated with 0.1 M phosphate buffer, 0.3 M NaCl, 0.05% NaN_3, pH 7.6 (buffer A) at a flow rate of 0.3 ml/min. Absorbance was monitored at 280 nm. Normal serum samples (5 ml per run) were diluted into 40 ml buffer A and applied to the column at 0.17 ml/min. The column was washed with buffer A at 0.3 ml/min until the absorbance reached the baseline, then equilibrated at 0.3 ml/min for 1 h with a lower ionic strength buffer composed of 0.01 M phosphate buffer, 0.15 M NaCl, 0.05% NaN_3, pH 6.8. The apoH bound to the column was eluted with 0.1 M glycine, 0.05% NaN_3, pH 2.5. Sample fractions were collected every minute and the pH was immediately adjusted with 60 µl 0.5 M phosphate buffer, pH 7.6.

Eluates from four column loadings were pooled and concentrated with Centriprep-10 concentrators (Amicon Inc., Beverly, MA) at 3,000 rpm and 20°C in a Beckman-J6B centrifuge (Beckman Instruments, Palo Alto, CA) to a final volume of about 1 ml.

The purity of the sample thus obtained was assessed by electrophoresis in 12% SDS-PAGE. The gel was then silver-stained. As can be seen in **Fig. 1** (lane 1), the approximately 50 kDa band corresponding to apoH was accompanied by higher-weight bands. The presence of these contaminating proteins showed that further purification was required.

Continuous-elution electrophoresis of the proteins (about 2.5 mg) was then performed with a Prep-Cell (Bio-Rad Laboratories, Milan, Italy) through a cylindrical gel (gel tube inside diameter 37 mm; gel length 50 mm) composed of 8.5% acrylamide, 2.7% N,N′-methylene-bis-acrylamide, and 0.1% SDS. The buffers were 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.4. Separation was achieved at 40 mA in about 8 h. Samples from the fractions collected every minute with a tube collector were dot-blotted with specific anti-apoH antibody (data not shown). The samples containing apoH, spanning the 34th to the 52nd fraction, were

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Abbreviations: Tg, triglycerides; Chol, cholesterol; apoH, apolipoprotein H; HDL, high density lipoproteins; VLDL, very low density lipoproteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; HRP, horseradish peroxidase; OPD, *o*-phenylenediamine dihydrochloride; HPLC, high pressure liquid chromatography.

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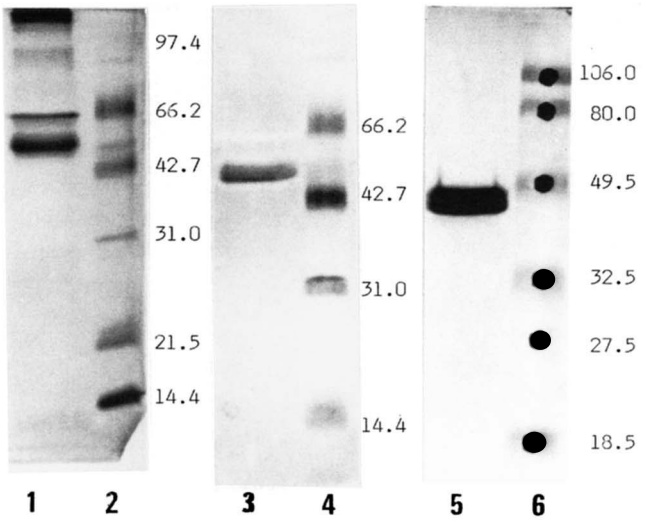

Fig. 1. SDS-PAGE gel electrophoresis of apoH in the two purification steps. Lane 1: silver-stained 12% SDS-PAGE gel of eluted apoH fractions from affinity chromatography column. Lane 3: silver-stained 12% SDS-PAGE gel of purified apoH by continuous elution electrophoresis. Lane 5: Western blot of duplicate gel of lane 3 stained with specific apoH antiserum, using a two-antibody method. In lane 2, molecular mass markers are (from top to bottom): 97.4, 66.2, 42.7, 31.0, 21.5, 14.4 kDa. In lane 4, molecular mass markers are (from top to bottom): 66.2, 42.7, 31.0, 14.4 kDa. Prestained molecular mass marked with black points (lane 6) are (from top to bottom): 106.0, 80.0, 49.5, 32.5, 27.5, 18.5 kDa.

pooled, dialyzed in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4, and subjected in duplicate to 12% SDS-PAGE. One gel was silver-stained (Fig. 1, lane 3), the other was immunoblotted with the specific antibodies (Fig. 1, lane 5). These lanes show a single, approximately 50 kDa band corresponding to the pure protein. Purity as judged by SDS-PAGE was 98–100%. The isolated protein was also subjected to 12% SDS-PAGE after reduction with DTT (data not shown). There was an apparent increase in the molecular weight of the reduced form as reported by others (8, 9). The recovery of pure apoH from the protein mixture eluted from the column in the first purification step was 52%. The final yield from 20 ml starting plasma was about 34%, as in other studies (8).

The apoH thus separated was examined by reverse-phase chromatography on a C_{18} IP Beckman column connected to a Beckman HPLC system. Ten µg was dissolved in 0.1 M H_3PO_4, 0.02 M triethylamine, 0.05 M $NaClO_4$, pH 3 (buffer B). Buffer C was acetonitrile.

The pure protein eluted as a single peak with a retention time of 33.7 min, using a 20–70% acetonitrile gradient for 35 min at 1 ml/min. Absorbance was measured at 214 nm.

Analysis of the N-terminal region with a Model 475A Gas-Phase Sequencer (Applied Biosystems, Foster City, CA) showed that its 10 amino acids were those published for apoH by Steinkasserer et al. (10), namely Gly-Arg-Thr-X-Pro-Lys-Pro-Asp-Asp-Leu.

In order to evaluate the ability of apoH to bind negatively charged phospholipid, we incubated apoH at different dilutions with a constant concentration of cardiolipin. For binding experiments, apoH was labeled with horseradish peroxidase (HRP) by the two-step glutaraldehyde method. Briefly, glutaraldehyde was diluted in 0.1 M phosphate buffer, pH 6.8, to a final concentration of 1.25%. Five mg HRP was dissolved in 0.1 ml glutaraldehyde solution and allowed to incubate overnight at room temperature. The HRP-glutaraldehyde mix was added to an apoH solution (400 µg in 200 µl 0.1 M carbonate/bicarbonate buffer, pH 9.5) and incubated overnight at room temperature. The remaining sites were blocked with 0.2 M ethanolamine, pH 7, for 2 h at 4°C. Labeled apoH was dialyzed in 0.05 M Tris, 0.15 M NaCl, pH 7.4, overnight at 4°C. The unconjugated HRP molecules were removed by gel filtration.

Microtiter wells (Sigma Chemical, St. Louis, MO) were coated with 100 µl of cardyolipin (Sigma Chemical) at 30 µg/ml in ethanol, dried under vacuum, and blocked with 350 µl of 1% milk powder/0.3% gelatine in phosphate buffer–saline, pH 7.6. HRP-apoH was diluted 100- to 204,800-fold in washing buffer (0.1 M bicarbonate, 0.5 M NaCl, 0.1% milk powder, 0.1% Tween-20, pH 8.1). One hundred µl from each dilution was added to each well in duplicate, incubated for 2 h at 37°C, and then washed four times with washing buffer.

The plate was washed six times with assay buffer solution. One hundred µl of a freshly prepared enzyme substrate solution made by dissolving an OPD tablet in 0.05 M phosphate-citrated buffer, pH 5.0, to a final concentration of 1 mg/ml was added to the wells. The plate was briefly agitated, covered, and left in the dark at room temperature for 20 min. The reaction was then stopped by adding 50 µl 3 M HCl to each well, and the plate was agitated to ensure thorough mixing. Well absorbance was measured with a Bio-Rad 3550 reader at 490 nm. HRP-apoH was able to bind to wells coated with cardiolipin in a dose-dependent manner. Binding of apoH was found for 1:100, 1:200, and 1:400 dilutions, corresponding to 840, 420, and 210 ng/well, respectively.

DISCUSSION

Apolipoproteins play a key role in lipid metabolism as the structural or coenzymatic units of the lipoproteins. The levels of the main plasma lipids, too, are influenced by changes in the quantity or phenotype of some apolipoproteins (7).

Recent work (2) has shown that apoH's genic polymorphism influences plasma lipid levels, though their relation to its own plasma level has not been established. Isolation of apoH in a pure, structurally uncleaved form is thus a matter of importance, as impairment has been detected in commercial preparations (11).

The technique currently used for this purpose requires three or more chromatographic steps. Our procedure is much simpler and avoids all precipitating steps that could lead to protein loss. We found that pure apoH could not be obtained with a single affinity chromatography step. Higher molecular weight proteins, in fact, co-eluted with apoH (Fig. 1, lane 1). They were also recognized, albeit with a weaker signal, by the anti-apoH antibodies in a Western blot analysis (data not shown), probably because of the polyclonality of the antibody binding apoH in the column, or because apoH formed indirect bonds with other proteins due to its strong electric charge, as reported by other authors (8). Subsequent purification by means of continuous-elution electrophoresis, on the other hand, gave 98–100% pure apoH (Fig. 1, lanes 3 and 5). The purified material displayed only one band reacting with the antisera, suggesting that our procedure included conditions for dissociation of the apoH complexes in plasma or, better still, was selective for free apoH. The final yield after the two purification steps was in agreement with that reported by McNally et al. (8), who observed binding of apoH to many biological substances by hydrophilic and other interactions.

The ability to bind a negatively charged phospholipid was evaluated by incubating apoH at different dilutions with a constant concentration of cardiolipin. Only native apoH and hence the active form is suitable for experiments designed to test whether purified apoH has retained lipid-binding activity. When clipped between Lys-317 and Thr-318, apoH lost its ability to bind negatively charged phospholipid. It is possible that such a clip could result in a conformational change in lipid-binding sites and alter the ability of apoH to bind lipid (11). Our apoH preparation satisfied all these criteria and bound successfully to cardiolipin in microplate wells. Our purification technique allowed the isolation of an uncleaved protein without other contaminants, such as a protease, which may be copurified with apoH as frequently reported.

In conclusion, this combination of two relatively simple methods was found to be an effective way of separating useful amounts of high-purity apoH for study of its role in lipid metabolism.

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