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Note

Purification of human apolipoprotein A-IV by fast protein liquid chromatography

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Apolipoprotein A-IV (apo A-IV) is a major constituent of rat high-density lipoprotein (HDL) [1], but occurs in human plasma mainly unassociated with lipoproteins [2,3]. As a chylomicron component, human apo A-IV is difficult to obtain in larger amounts from normal human plasma, and in the past pathological conditions such as hypertriglyceridemia [3], chylous ascites, pleural effusions [4,5] or chyluria [6] had to be used to isolate this apoprotein.

As the bulk of apo A-IV in human plasma is recovered in the fraction with a density (d) > 1.21 g/ml after ultracentrifugation, isolation procedures were developed to use this fraction as a source of apo A-IV. Therefore, the $d > 1.25$ g/ml fraction was incubated with a triglyceride–phospholipid emulsion, and the lipid-associated proteins including apo A-IV were recovered by flotation of these lipid protein particles [7,8]. Further purification of apo A-IV was then carried out by preparative sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

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(PAGE) [8], ion-exchange chromatography [9] or a combination of both [7,10]. Even then it was tedious to obtain milligram amounts of this apoprotein.

This paper describes a new and rapid purification of apo A-IV from the mixture of proteins isolated from the $d > 1.25$ g/ml fraction of human serum after adsorption on a triglyceride–phospholipid emulsion with a fast protein liquid chromatographic (FPLC) system (Pharmacia) using the Mono Q HR 10/10 preparative anion-exchange column. On a preparative scale, several milligrams of apolipoprotein A-IV can thus be obtained quickly and easily.

EXPERIMENTAL

Isolation of apo A-IV from lipoprotein-depleted serum

Apo A-IV from lipoprotein-depleted serum was recovered by absorption on a triglyceride-rich phospholipid emulsion, as described by Weinberg and Scanu [8]. Briefly, fasting human serum was adjusted to a density of 1.25 g/ml by adding solid potassium bromide and centrifuged in a 60 Ti Beckman rotor at 330 000 *g* for 48 h at 10°C. After removal of floating lipoproteins, the infranate was extensively dialysed against 0.15 *M* sodium chloride, 0.05 *M* potassium phosphate buffer (pH 7.4) and 0.05% EDTA (pH 7.4) and incubated in 4 *M* sodium chloride (pH 7.4) for 1 h at 37°C with a triglyceride–phospholipid emulsion (Intralipide, Kabi Vitrum, Noisy le Grand, France). Triglyceride–phospholipid emulsion particles were then separated from lipoprotein-depleted serum by centrifugation in an SW 27 type rotor at 132 000 *g* for 35 min at 4°C. The creamy layer was recovered, resuspended in distilled water and lyophilized.

Delipidation and solubilization of intralipid-associated proteins

The lyophilized proteins were stirred overnight at +4°C in diethyl ether–ethanol (1:1, v/v, 5 ml per mg of protein) and centrifuged. The delipidation procedure was repeated three times at 1-h intervals of stirring at +4°C. A final extraction with diethyl ether was made at −20°C. After centrifugation the proteins were solubilized in 10 m*M* Tris–HCl buffer (pH 8.2) containing 7 *M* urea and stirred overnight to evaporate the remaining diethyl ether. Prior to chromatography, the protein solution was filtered through a 0.22-μm Millipore membrane filter.

Anion-exchange FPLC

The FPLC system (Pharmacia, Uppsala, Sweden) was used to purify apo A-IV from other intralipid-associated proteins. The system employed two P-500 high-pressure pumps and a GP-250 gradient programmer to form the gradient. The elution was controlled by a single-path UV monitor. A chart recorder with two channels documented the UV absorbance, the programmed gradient and the collected fractions (FRAC-100 fraction collector).

All buffers were freshly prepared with deionized urea solution (Amberlite resin MB 13, Prolabo, Paris, France), filtered through 0.22-μm Millipore membrane filters and degassed before use. The Mono Q HR 10/10 (10 cm × 1 cm I.D.) column was equilibrated with 10 m*M* Tris–HCl buffer (pH 8.2) and 7 *M* urea at a

flow-rate of 2.0 ml/min, and 10–20 mg of intralipid-associated proteins were applied. The column was operated at room temperature.

The column was then eluated for 40 min at the same flow-rate with a linear gradient of sodium chloride from 0 to 200 mmol (0 to 20% buffer B: 10 m*M* Tris–HCl, 7 *M* urea, 1 *M* sodium chloride). The sodium chloride concentration was then increased to 1 *M* to remove the remaining proteins. Eluted fractions were stored at +4 °C for rechromatography or immediately dialysed to remove urea. For rechromatography of apo A-IV the fractions containing apo A-IV were diluted with an equal volume of 10 m*M* Tris–HCl buffer and 7 *M* urea, and reapplied to the column with a 50-ml superloop (Pharmacia). The column was then operated as described above.

Ion-exchange chromatography and preparative SDS-PAGE

For comparison apo A-I and apo A-IV were also isolated from intralipid-associated proteins by chromatography on DEAE-Sephacel (Pharmacia) and subsequent preparative SDS-PAGE as described earlier [10].

Characterization of proteins

Proteins were quantified according to Lowry et al. [11] using bovine serum albumin as standard. For isoelectric focusing (4 °C, 1 h), 7% acrylamide gels were used as described earlier [12]. SDS-PAGE was performed in a 5–30% linear acrylamide gradient containing a 3% stacking gel. For immunoblotting analysis, isoelectric focusing was carried out in slab gels as described by Menzel et al. [13]. Proteins were then electrophoretically transferred to nitrocellulose and developed with specific antibodies to apo A-IV as outlined earlier [14].

Amino acid analysis of purified apo A-IV was made in a Beckman 6 300 amino acid analyser after 72 h of acid hydrolysis. The average amount for determination of one amino acid was 182 pmol.

RESULTS

Fast anion-exchange chromatography of lipid-associated proteins

As apolipoprotein A-I is one of the major intralipid-associated proteins [8,10] (see Fig. 1A), we adapted the conditions for FPLC described before for the separation of HDL apoproteins [15]. As apo A-IV eluted at a higher ionic strength than apo A-I, a linear gradient of sodium chloride up to 200 m*M* was introduced, and the pauses in the gradient slope developed initially for separation of apo A-I and A-II were omitted [15]. It was important to develop the linear gradient in a volume of 80 ml (40 min) to get a complete separation of apo A-IV from apo A-I (see Fig. 2A). The position of apo A-I and apo A-IV were the same when isolated apoproteins were added to the mixture or chromatographed as pure proteins. After the first separation apo A-IV still contained protein contaminants. Apo A-IV could be further purified by rechromatography under identical conditions after lowering the sodium chloride concentration in the pooled apo A-IV-containing fractions from a first separation chromatography by dilution with 10 m*M* Tris–HCl buffer and 7 *M* urea without sodium chloride (see Fig. 2B).

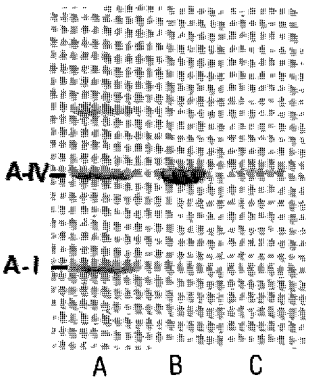

Fig. 1. SDS-PAGE in a 5–30% linear polyacrylamide gradient gel of 35 μg of intralipid-associated proteins (A) and 25 μg (B) and 10 μg (C) of purified apo A-IV. Purified apo A-IV was obtained from two successive purifications on the Mono Q HR 10/10 anion-exchange column. Apo A-IV and A-I were identified with known standards.

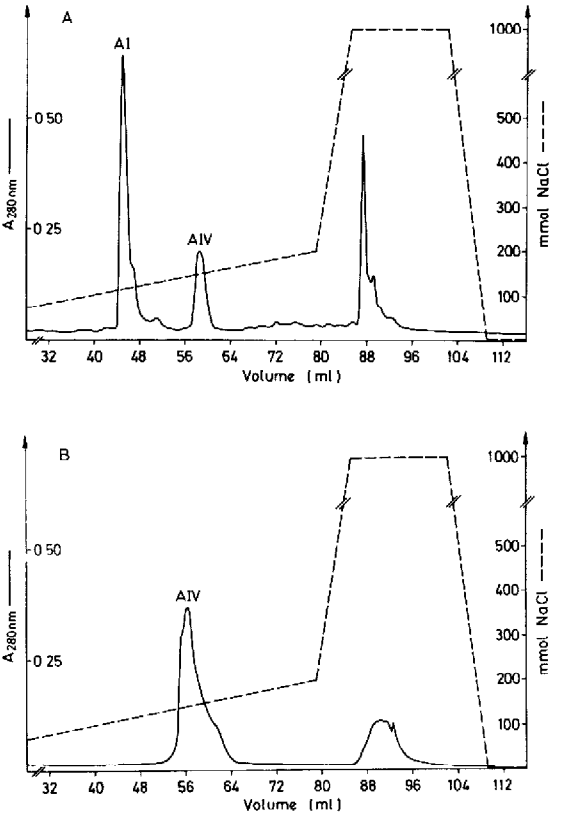

Fig. 2. Elution profiles of apo A-IV on the Mono Q HR 10/10 column, (A) after injection of 10 mg of delipidated intralipid-associated proteins and (B) pooled apo A-IV-containing fractions from four chromatographies of intralipid-associated proteins for rechromatography. Flow-rate, 2.0 ml/min, starting buffer, 10 m*M* Tris-HCl (pH 8.2) and 7 *M* urea. The gradient is developed with the same buffer containing 1 *M* sodium chloride.

Apo A-IV purified by anion-exchange chromatography showed the same apparent molecular mass of 46 000 upon SDS-PAGE as plasma apo A-IV (as judged by immunoblotting after electrophoresis of whole plasma, data not shown) or as recovered by adsorption on the triglyceride–phospholipid emulsion (see Fig. 1). It also showed the major plasma isoforms as demonstrated by isofocusing (see Fig. 3).

The identity of the isolated protein with apo A-IV was shown with monospe-

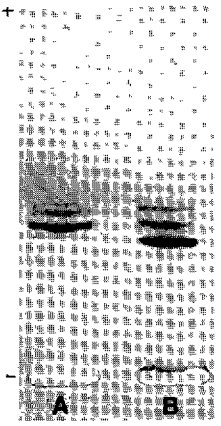

Fig 3. Isoelectric focusing of purified apo A-IV in a pH gradient of 4-6: 10 μg each of apo A-IV (A) and, for comparison, apo A-I (B) were applied. Purified apo A-IV was obtained from two successive purifications on the Mono Q HR 10/10 column and shows the main isoforms.

TABLE I

AMINO ACID COMPOSITION OF APO A-IV ISOLATED BY FPLC

Amino acid	Composition expected [18]	Composition found
Asp(+Asn)	35	35.53
Thr	15	12.54
Ser	18	17.89
Glu(+Gln)	88	91.30
Pro	12	11.82
Gly	15	21.33
Ala	30	29.68
Cys	0	0
Val	19	18.80
Met	5	N.D.*
Ile	9	5.47
Leu	53	55.85
Tyr	7	4.24
Phe	11	13.35
His	9	8 02
Lys	28	25.47
Arg	25	24 70
Trp	1	N.D.*

*N.D = not determined

cific antibodies to human apo A-IV after isofocusing or SDS-PAGE (data not shown). Furthermore, amino acid analysis of the purified apo A-IV protein was compatible with the composition derived from published sequences [16–18]. The results are shown in Table I.

DISCUSSION

Only a small portion of human apo A-IV is associated with lipoproteins and thus it is difficult to isolate in large amounts via isolation of lipoproteins. The isolation of apo A-IV advanced by the use of its ability to associate with a triglyceride–phospholipid emulsion when incubated with lipoprotein-deficient serum [7,8]. Weinberg and Scanu [8] established exact conditions under which, apart from apo A-IV, only a few different proteins were recovered with reisolated phospholipid–triglyceride particles after incubation with lipoprotein-deficient serum. Thereafter a major problem remained with the purification of apo A-IV from the mixture of these proteins, which was initially performed by preparative SDS-PAGE [8]. Also ion-exchange chromatography was introduced [7,9] or a combination of both isolation procedures was employed [7,10].

This study establishes the use of FPLC for the rapid, high-resolution purification of apo A-IV. Usually 10–20 mg of total protein were applied to the column. The apo A-IV recovered showed the major isomorphic forms also found in plasma. The presence of several isoforms may also explain the asymmetrical elution peaks of apo A-IV (see Fig. 2). The overall recovery of apo A-IV was 25% of that present in the initial incubation mixture. This yield resembles that reported by Weinberg and Scanu [8]. Separation can be achieved in 40–50 min, and with several runs large amounts of apo A-IV can be obtained in a short time.

The interest in apo A-IV has recently increased as evidence accumulates that it may play a role in reverse cholesterol transport. It is obviously capable of promoting cellular cholesterol efflux [19] and of binding to hepatocytes and endothelial cells [20,21]. The technique described here makes it possible to obtain large amounts of apo A-IV for investigations of a number of problems associated with this unusual protein.

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