papers and notes on methodology

Enzyme-linked immunosorbent assay for human proapolipoprotein A-I using specific antibodies against synthetic peptide

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Abstract Apolipoprotein A-I (apoA-I), the major protein component of human high density lipoprotein, appears intracellularly as an intermediate precursor (proapoA-I) with a hexapeptide extension (Arg-His-Phe-Trp-Gln-Gln) at its amino terminus. To investigate the regulation of processes that regulate plasma apoA-I levels, a sensitive and simple assay for proapoA-I is required. We describe a specific enzyme-linked immunosorbent assay (ELISA) for quantification of proapoA-I using monospecific rabbit antibodies raised against the peptide : Arg-His-Phe-Trp-Gln-Gln-Asp-Glu-Pro. The monospecificity of antibodies to propeptide has been checked and no cross-reaction with mature apoA-I has been found although three first mature apoA-I amino acids (Asp-Glu-Pro) were included in the immunizing peptide. The assay is a non-competitive sandwich ELISA in which polystyrene microtiter plates were used with antibodies to propeptide adsorbed on the wells. After incubation with plasma samples, the bound proapoA-I was revealed by labeled rabbit polyclonal antibodies directed against mature apoA-I. The working range was 10 to 100 ng/ml, recovery of proapoA-I added to plasma was 94.6 to 106.5%, and the intra- and interassay coefficients of variation were 3.8% and 7.9%, respectively. A delipidation step using diisopropylether–n-butanol was necessary to expose antigen sites of proapoA-I in native lipoproteins. Mean level of proapoA-I in normal subjects was 87 ± 15 μg/ml. It represented 7.1% of total apoA-I while in Tangier serum it represented 29%. — **Barkia, A., C. Martin, P. Puchois, J. C. Gesquiere, C. Cachera, A. Tartar, and J. C. Fruchart.** Enzyme-linked immunosorbent assay for human proapolipoprotein A-I using specific antibodies against synthetic peptide. *J. Lipid Res.* 1988. **29:** 77–84.

Supplementary key words high density lipoproteins • isoelectric focusing • immunoblotting • Tangier disease

Plasma apolipoprotein A-I (apoA-I), the major protein of HDL, is a single polypeptide of 243 amino acids (1) with a molecular weight of about 28,000. It occurs in several isoforms. It is secreted by human intestine and liver (2) as a proprotein which is two charge units more basic than the major plasma apoA-I and contains a six amino acid N-terminal extension with the sequence Arg-His-Phe-Trp-Gln-Gln (3–5) (**Fig. 1**). In human plasma, this proapoA-I represents only 2 to 5% of the total apoA-I, since it is rapidly converted to the "mature" form by proteolytic removal of the hexapeptide prosequence (6). Some studies (7) have reported that patients with Tangier disease (a pathologic state characterized by markedly low levels of plasma HDL) have an abnormal plasma apoA-I isoprotein profile, in which the percentage of proapoA-I is markedly increased. Usually, plasma proapoA-I is measured by two-dimensional gel electrophoresis of radiolabeled apoprotein A-I. To get a better understanding of proapoA-I synthesis and conversion, a more simple assay for determination of protein is required.

The fact that synthetic peptides copying a part of a protein sequence can elicit antibodies capable of reacting with the whole protein has received considerable attention in the recent years (8, 9). Although this approach is suitable for any protein, even those with sequences deduced only from recombinant DNA studies, it may be used in a particular way as a means of differentiating closely

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoproteins; proapoA-I[1–9], Arg-His-Phe-Trp-Gln-Gln-Asp-Glu-Pro; PBS, phosphate-buffered saline.

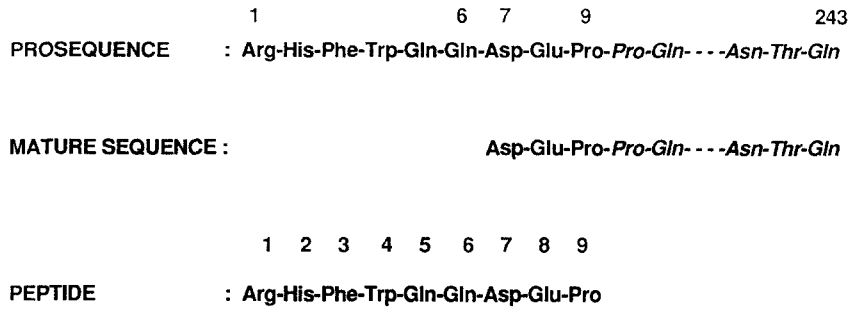

Fig. 1. Human apolipoprotein A-I (prosequence and mature sequence) and synthetic peptide sequences.

related proteins that vary from each other in only part of their primary sequence. In such cases, immunization with the complete protein will elicit cross-reactive antibodies, while the use of synthetic peptides that mimic the most divergent domains will allow the production of specific antibodies. The determination of proproteins is particularly relevant to this method. Indeed, except for the propeptide, the remainder of the sequence is common to both pro- and mature forms.

Using antibodies raised against a synthetic peptide copying the NH_2-terminus of proapoA-I, we have developed an enzyme-linked differential antibody immunosorbent assay for the evaluation of human proapoA-I, which offers significant advantages over two-dimensional gel electrophoresis. Our assay is simple, rapid, sensitive, needs only small amounts of plasma, and avoids the use of radioisotopes.

METHODS

Plasma samples

Human plasma samples were obtained by venipuncture from asymptomatic normolipidemic male and female donors who had fasted overnight. The blood was collected into 10-ml Vacutainer tubes containing 0.5 ml of 5 mmol/l EDTA and centrifuged for 15 min at 2,500 rpm and 4°C to separate cells from plasma. Delipidization was performed with diisopropylether–n-butanol 60:40 (v/v) according to Cham and Knowles (10).

Lipoproteins and apoHDL preparation

Plasma lipoproteins were isolated by sequential preparative ultracentrifugation (11) which was carried out in a Beckman 50.Ti rotor at 4°C at 40,000 rpm for 24 hr. All fractions were dialyzed against 0.9% NaCl containing 0.1 g/l of NaN_3. ApoHDL were prepared by delipidization of HDL with diethylether–ethanol according to Scanu and Edelstein (12).

Peptide synthesis

The peptide Arg-His-Phe-Trp-Gln-Gln-Asp-Glu-Pro, proapoA-I [1–9] was prepared by a solid phase method, according to Merrifield (13). The C-terminal amino acid (proline) was bound to the 1% cross-linked styrene-divinylbenzene resin (100–200 mesh beads) as its cesium salt. N-α Boc protection was used and trifunctional amino acids were protected as follows: Glu (O-benzyl); Asp (O-benzyl); His ($N_{imidazole}$-tosyl); Arg (tosyl).

After deprotection and cleavage from the resin by hydrogen fluoride treatment, the crude peptide was extracted into 5% (vol/vol) acetic acid and purified by gel filtration (GF 05 Trisacryl) and preparative reversed phase HPLC (Whatman Magnum 9, ODS 2). The purity of the peptide was checked by reversed phase HPLC, amino acid analysis, and thin-layer chromatography performed on pre-coated TLC aluminium sheets containing silica gel 60 (Merck, ref 5953) developed in ethyl acetate–pyridine–acetic acid–water 5:5:1:3 (v/v/v/v) for 80 min (R_f = 0.6).

Conjugation of synthetic peptide with tetanus toxoid

Fifteen mg of proapoA-I [1–9] (12 μmol) was dissolved in 2.0 ml of phosphate-buffered saline (PBS), pH 5.6, and then mixed with 12.8 mg of tetanus toxoid. After 30 min, 20 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (HCl salt) in 500 μl of PBS was added and the mixture was stirred for 1 hr at room temperature in the dark. The resulting mixture was then dialyzed exhaustively against 0.01 M sodium phosphate–0.15 *M* NaCl, pH 7.2. Rabbits were immunized as described previously (14). Five hundred μl of (1 mg/ml) conjugate (tetanus toxoid with synthetic peptide) emulsified with an equal volume of Freund's complete adjuvant (FCA) was injected subcutaneously and boosted with 200 μl of the same mixture (conjugate + FCA) every 15 days.

Purification of anti-proapoA-I antibodies by affinity chromatography

A column was prepared by covalently linking the synthetic human pro-segment to CNBr-activated Sepharose

4B (15). Immunoglobulins obtained from immune sera by Na_2SO_4 precipitation and protein A affinity chromatography (to remove proteases) were loaded on this synthetic peptide–Sepharose column and the flow was stopped. After 2 hr at room temperature, the column was then washed with 0.1 M phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.01% NaN_3. The specific anti-proapoA-I immunoglobulins retained on the column were eluted with 1.0 M acetic acid and collected into 1 M K_2HPO_4 buffer. The solution was dialyzed against 25 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.01% NaN_3) and concentrated by ultrafiltration. The affinity-purified antibodies were stored as 1-ml aliquots (0.5 mg) at −30°C.

ApoA-I antibodies–polyclonal peroxidase conjugate

Affinity antibodies to apoA-I were prepared as described elsewhere (15). Labeling of antibodies with horseradish peroxidase was accomplished following a previously described procedure (16). The conjugate was stored at −20°C in small aliquots.

Two-dimensional gel electrophoresis

An LKB vertical electrophoresis unit was used for isoelectric focusing. The gel was prepared as follows: 2 g of acrylamide and 80 mg of N,N'-methylene bisacrylamide were dissolved in 30 ml of freshly deionized 8 M urea. Eight ml of 10% (w/v) ampholine (pH 4.2–6.2, Sebia) was added and the volume was adjusted to 40 ml with deionized water. The solution was de-aerated, and then 120 μl of 10% N,N,N',N'-tetramethylene diamine and 280 μl of 10% ammonium persulfate solution were added prior to casting the gel.

Eighty μg of apoHDL in 0.2 M Tris-HCL (20 μl) was applied to the gel following the addition of 50 μl of ampholine (pH 4.2–6.2) and 15 μl of 50% glycerol.

The anode solution was 0.01 M phosphoric acid and the cathode solution was 0.02 M NaoH. After prefocusing for 30 min at 100 v, the cathode solution was replaced and isoelectrofocusing was allowed to proceed at 4°C for 1 hr at 200 V and finally for 18 hr at 400 V. Immediately after the focusing, the gel was fixed and stained for 1 hr in 0.5% Coomassie Blue R250–methanol–acetic acid–water 12.5:40:10:37.5 (v/v/v/v) and destained in methanol–water–acetic acid–glycerol 20:68:10:2 (v/v/v/v).

After focusing, one of the sample tracks was sliced and equilibrated for 10 min in 0.06 M Tris-HCl buffer, pH 6.8, containing 2.3% SDS and 5% β-mercaptoethanol for electrophoresis in the second dimension. This focused gel was placed on the top edge of a 10% polyacrylamide slab gel prepared according to Laemmli (17).

Electrophoresis was conducted at 40 mA per gel at 18°C for 4 hr.

After staining of the SDS-polyacrylamide gel, apoproteins were identified by comparison of apparent molecular weights with those of known standards.

Immunoblotting

After isoelectric focusing, proteins were directly electro-transferred to nitrocellulose sheets as previously described (18). These sheets were then soaked for 2 hr at room temperature in TNT buffer (15 mM Tris-HCl, 140 mM NaCl, 0.05% Tween 20). They were then incubated with gentle agitation overnight at 4°C in PBS containing 0.05% Tween 20 (buffer A) and rabbit anti-human proapoA-I specific antibodies (4 μg/ml or immunserum diluted to 1/100) or rabbit anti-human apoA-I polyclonal antibodies (4 μg/ml).

The sheets were then washed three times in buffer A and incubated at room temperature for 3–4 hr under gentle agitation, with labeled peroxidase–anti-rabbit immunoglobulin diluted 1000 times with buffer A.

After washing three times with buffer A and twice with distilled water, apoprotein bands were visualized by addition of peroxidase substrate (Immunostaining Kit, Sebia, 92130 Issy Les Moulineaux, France).

Noncompetitive inhibition

The microtiter plate wells were coated by incubation for 14 hr at 25°C with 100 μl of PBS containing 10 μg of peptide/ml.

Different dilutions of synthetic peptide (1 mg/ml) or plasma were mixed with the immune serum (final dilutions 1/2000) in the final volume of 300 μl and incubated overnight at 4°C with gentle agitation.

After three washings of the microtiter plate with the PBS, pH 7.4, 100-μl volumes of pre-incubated dilutions were added to microtiter plate wells and incubated for 2 hr at 37°C.

After washing, 100 μl of diluted peroxidase-labeled rabbit anti-IgG was added and incubated 2 hr at 37°C. The plate was then washed again.

Finally, 100 μl of freshly prepared O-phenylene diamine (OPD) substrate was added. After 30 min in the dark, 100 μl of 1.0 N HCl was added and the enzymatic color reaction was read at 492 nm.

Calibration curve

ApoHDL containing known amounts of proapoA-I was used as primary standard. The proapoA-I content of this apoHDL preparation was determined as follows. ApoHDL was radiolabeled according to Hunter (19) and after isoelectric focusing and autoradiography, the band corresponding to each apoA-I isoprotein was cut out and counted. The distribution of radioactivity permitted assessment of the proportion of proapoA-I in the sample. From this ratio and a knowledge of the total apoA-I content, previously determined by ELISA (16), the exact

amount of proapoA-I in our apoHDL preparation was calculated (0.015 mg of proapoA-I/mg of HDL apoA-I).

Enzyme-linked immunoassay (sandwich)

In this noncompetitive enzyme immunoassay, oligoclonal anti-proapoA-I antibodies were coated to the plate and polyclonal anti-apoA-I antibodies were used as labeled antibodies. The coating and washing buffer was PBS, pH 7.4. The same buffer containing 1% bovine serum albumin was used for the dilution of standard, samples, and labeled antibodies.

Microtiter plates (96-well) were coated by incubation for 16–18 hr at 25°C with 100 μl of PBS containing 25 μg/ml of anti-proapoA-I. The solution was then removed by aspiration and the wells were washed four times. Standards or delipidated samples (dilutions determined by previous titration experiments: 1/1500, 1/2500) were diluted and 100 μl of each dilution was added to microtiter plate wells in duplicate. Incubation was continued for 14–15 hr at 4°C in a humid chamber. After washing, 100 μl of labeled antibody solution (dilutions determined by previous titration experiments) was added to each well. Following a further incubation for 2 hr at 37°C, the plate was washed again. Finally, 100 μl of O-phenylene diamine (OPD) substrate (Sigma), freshly prepared before each use (75 mg of OPD, 25 ml of 0.1 M citrate–phosphate buffer, pH 5.5, and 16 μl of H_2O_2) was added.

The color was developed over a period of 45 min in the dark and the enzymatic reaction was stopped by the addition of 100 μl of 1.0 N HCl to each well. The color was read at 492 nm.

RESULTS

Specificity of antibodies

Isoelectric focusing and immunoblotting. Isoelectric focusing separated the different HDL apoproteins according to their isoelectric point (pI). The different apoA-I isoforms were identified by two-dimensional electrophoresis and by immunoblotting using polyclonal antibodies directed against apoA-I. Five apoA-I isoproteins were found. The pIs of the two minor isoforms were 5.89 and 5.81, while those of mature isoforms were 5.65, 5.45, and 5.4, respectively, as determined according to Warnick et al. (20).

Immunoblotting using polyclonal anti-apoA-I antibodies (**Fig. 2A**) confirmed the isoelectric pattern of apoprotein A-I but showed in addition one more isoform which was visualized between the two major forms, and which did not appear by direct gel staining. Lanes B and C of Fig. 2 show the immunoblotting patterns of apoHDL using immune serum and purified antibodies directed against peptide [1–9]. Only the band corresponding to the more basic isoform (proapoA-I) was revealed.

Noncompetitive inhibition. **Fig. 3** shows the noncompetitive inhibition of antibody binding to coated synthetic peptide with soluble antigen (the same synthetic peptide or plasma apoprotein). Clearly, the antibodies recognize the

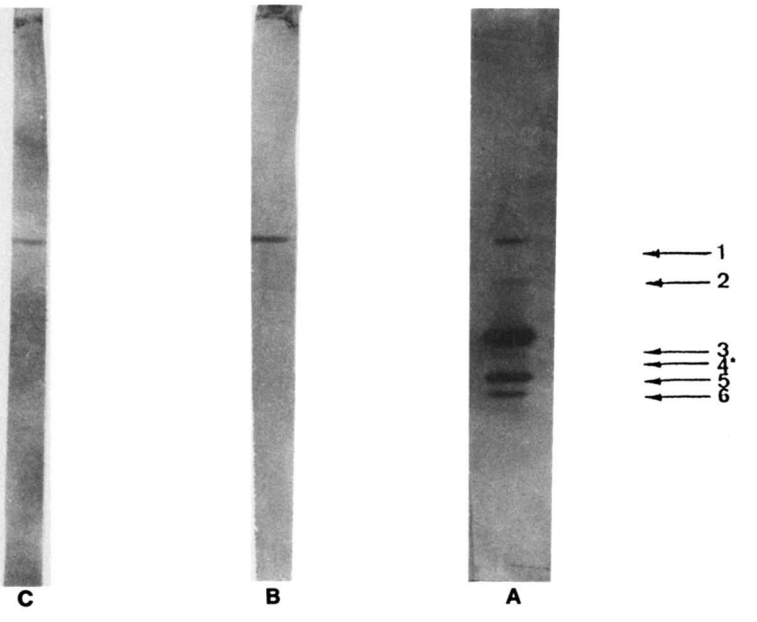

Fig. 2. Immunoblotting (after isoelectric focusing in 2% ampholine, pH 4.2–6.2) of apoHDL with polyclonal anti-apoA-I antibodies (A) (4 μg/ml), oligoclonal anti-proapoA-I antibodies (B) (4 μg/ml), and whole immune serum antipeptide (C) (diluted 1/100). *ApoA-I isoform was not revealed by Coomassie blue staining.

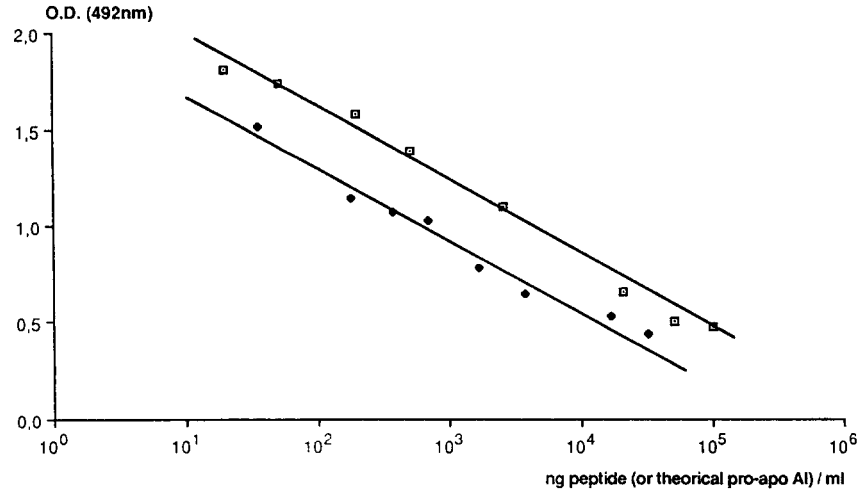

Fig. 3. Non-competitive inhibition of anti-proapoA-I binding to synthetic peptide-coated wells, using soluble synthetic peptide (♦) or delipidated plasma (□). Different dilutions of synthetic peptides (1 mg/ml) or delipidated plasma mixed with the immune serum (final dilution was 1/2000) were preincubated overnight at 4°C. Synthetic peptide [1-9] was coated (1 μg/well, 14 hr at 25°C). One hundred-μl volumes of the preincubated dilutions were added and incubated for 2 hr at 37°C with precoated peptide. After washing, 100 μl of diluted peroxidase-labeled anti-IgG rabbit antibodies was added and incubated for 2 hr at 37°C.

synthetic peptide and are specific for it. Moreover, the amount of peroxidase-anti-rabbit immunoglobulin that binds to the precoated peptide decreases with increasing soluble peptide or plasma antigen concentration, and the inhibition curves obtained with the peptide and the plasma are parallel. However, this study did not show that the antibody specificity to the nonapeptide was determined by the hexapeptide prosegment [1-6] and not by the three last aminoacids [7-9] common with apoA-I (Fig. 1). This was confirmed by immunoblotting after isoelectric focusing (Fig. 2).

Standard curves obtained by enzyme-linked immunoassay

ApoHDL was used as standard. The level of proapoA-I in this preparation was determined previously as described in Methods. The proapoA-I concentration was 58.5 μg/ml. The standard curve obtained using this preparation (**Fig. 4**) showed the characteristic sigmoidal shape of an enzyme immunoassay.

The working range of the assay was from 10 to 100 ng of proapoA-I (Fig. 4). The calibration curves obtained with untreated plasma, delipidated plasma (diisopropyl-ether-n-butanol 60:40 (v/v)) and apoHDL are shown in **Fig. 5.** The curves obtained with apoHDL and delipidated plasma are parallel but untreated plasma did not react with the coated anti-proapoA-I antibodies. This indicated that proapoA-I antigenic sites are not accessible in native plasma.

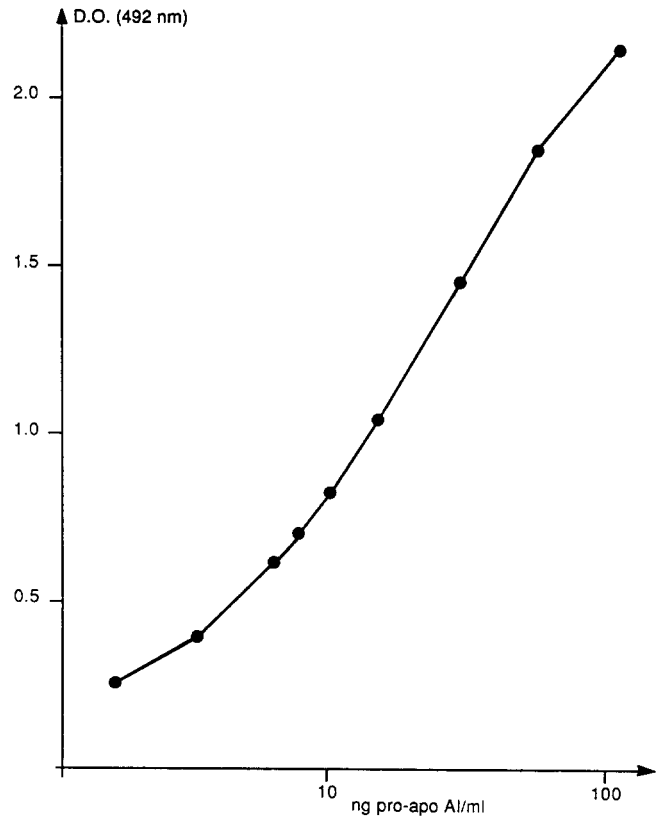

Fig. 4. A representative standard curve of proapoA-I obtained with apoHDL as primary standard. The apoA-I level of apoHDL was determined by ELISA. Radiolabeled apoproteins were isoelectrofocused (2% ampholine, pH 5.2-6.2). Each apoA-I isoform was counted and the ratio proapoA-I/apoA-I was determined. The working range of the assay was from 10 to 100 ng of proapoA-I/ml.

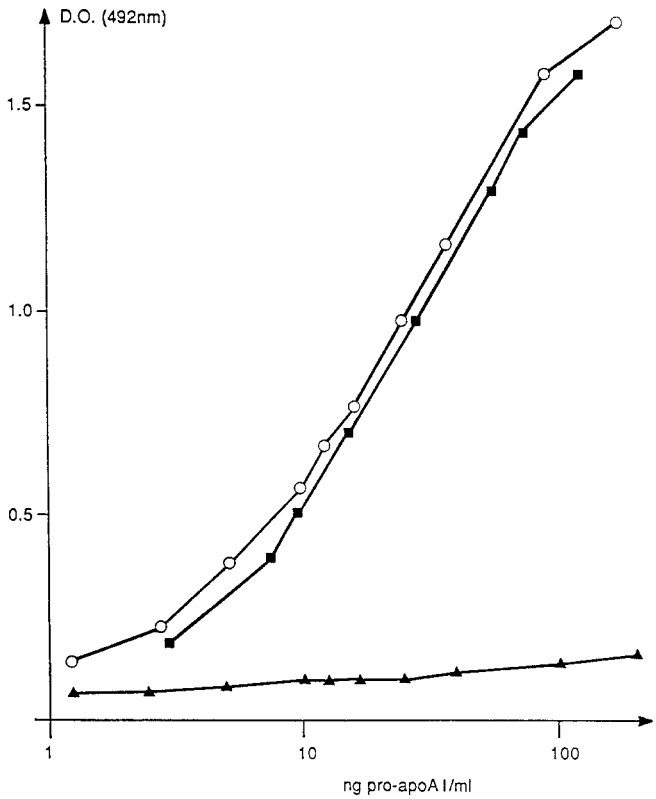

Fig. 5. Standard curves obtained with apoHDL (○), delipidated plasma (■), and untreated plasma (▲).

Recovery and reproducibility

When varying amounts of apoHDL standard were added to delipidated plasma of known proapoA-I content, recoveries ranged from 94.6 to 106.5%. The intra- and interassay coefficients of variation were, respectively, 3.8% and 7.9%.

Concentrations of proapoA-I and the ratio of proapoA-I to apoA-I in plasmas from normolipidemic and Tangier subjects

The average level of proapoA-I in the plasma of normolipidemic subjects determined by ELISA was 88 ± 15 μg/ml. According to Student's *t* test, there was no significant difference between female and male subjects (**Table 1**).

In order to determine the ratio of proapoA-I to apoA-I, levels of apoA-I were also measured by ELISA (15). The mean value for the levels of apoA-I was 1.20 ± 0.21 mg/ml. The mean ratio proapoA-I to apoA-I was 7.1% and ranged from 5.6 to 9%. In female subjects, this ratio was higher than in male subjects but the difference was not significant (Table 1). In the sample from a Tangier patient (not shown), the ratio of proapoA-I to apoA-I was 29%. The levels of apoA-I and proapoA-I were 0.0042 mg/ml and 1.2 μg/ml, respectively.

DISCUSSION

Although anti-protein antibodies have, in some cases, been successfully raised using short peptides of only five or six residues in length (3–5, 21–23), our experience has shown that longer peptides are better candidates. This study, designed to prepare antibodies against proapoA-I, a hexapeptide, illustrates the problem. In order to circumvent this difficulty, some investigators have added a linker sequence, unrelated to the target protein, mainly to allow a better presentation of the peptide when coupled to its carrier protein. We decided, instead, to elongate the [1–6] peptide with three amino acids [7–9] found in mature apoA-I (see Fig. 1). Using this approach, the immunizing peptide then consisted of nine amino acids. Since only three of these were in common with the leader tripeptide of apoA-I, it was considered unlikely that the nonapeptide would elicit antibodies that would cross-react with the mature A-I protein to any significant extent.

The peptide was coupled to tetanus toxoid using carbodiimide activation of the α-carboxyl group of Pro_9 or the side chains of Asp_7 and Glu_8.

In each case, the linkage occurred at the C-terminus of the peptide and the N-terminus of the peptide was thus exposed in a similar manner as in the native protein. Rabbit immunization with this conjugated peptide [1–9] resulted in the production of specific anti-proapoA-I anti-

TABLE 1. Concentrations of proapoA-I and apoA-I in the plasma of normolipidemic subjects

Normolipidemic Subjects	ProapoA-I	ApoA-I	ProapoA-I / ApoA-I
	mg/ml		%
Male (A)	0.084 ± 0.013 (14)	1.18 ± 0.15 (14)	6.9 ± 1.0 (14)
Female (B)	0.089 ± 0.015 (15)	1.18 ± 0.18 (15)	7.4 ± 0.8 (15)
A + B	0.087 ± 0.015 (29)	1.18 ± 0.16 (29)	7.1 ± 0.9 (29)
Comparison A to B:T (Student's *t* test)	0.92	0	1.44

Values are given as mean ± SD; number of samples in parentheses.

bodies as demonstrated by immunoblotting after isoelectric fusing of apoHDL (Fig. 2).

It is known that proapoA-I represents the intracellular form of the protein (2), which is converted in plasma to apoA-I (6), the main protein of HDL, commonly considered as a negative marker of risk of coronary heart disease. Determination of proapoA-I might, therefore, improve our understanding of processes that lead to low plasma levels of HDL. Until now, levels of proapoA-I have been determined by measurement of radioactivity after two-dimensional electrophoresis of labeled delipidated lipoproteins. This technique has disadvantages; it is a complex technique (isolation and delipidization of lipoproteins); it is time consuming, and requires the use of radioisotopes.

The anti-proapoA-I antibody prepared as described in this report has enabled us to develop an enzyme immunoassay of proapoA-I which, in contrast, allows rapid, simple, and sensitive quantitation of proapoA-I on a large scale. This assay requires a delipidation step, indicating that, when localized inside lipoproteins, the NH_2-terminus of proapoA-I is not accessible to antipeptide antibodies.

The mean ratio of proapoA-I to apoA-I was 7.1%. This is higher than the ratio found by others (4, 7, 24). A possible explanation for this discrepancy is the difference in the assay method. Values usually found by others were determined by a technique that may have allowed in vitro proapoA-I conversion and degradation to occur.

The enzyme immunoassay described in this paper could be applied to the determination of proapoA-I in different pathophysiological states where proapoA-I synthesis and conversion are impaired or accelerated. Moreover, the sensitivity of this assay should allow quantitative studies of proapoA-I metabolism in cultured cells. Finally, the results presented here substantiate the usefulness of synthetic peptides as a means of obtaining specific antibodies to small peptides. Such enzyme immunoassays could be applied to other pro-proteins.

Manuscript received 30 January 1987 and in revised form 8 June 1987.

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