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Characterization of Polypeptides From Human Serum Very Low Density Lipoproteins by Isoelectric Focusing Fractionation

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等電點焦點 맞추기 劃分法에 依한 極低密度 血淸 脂蛋白質 Polypeptide의 特性

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SUMMARY

The very low density apolipoproteins were separated by a newly developed method of isoelectric focusing in a narrow pH gradient. Four polypeptides were isolated that differed from the major proteins of the high density or low density lipoproteins. Three of these proteins had indistinguishable amino acid compositions, but different isoelectric points, COOH-terminal alanine, no isoleucine, cysteine or cystine. Two of these polypeptides had NH_2-terminal serine. The polymorphism of apolipoprotein-Ala, so designated from the COOH-terminal residue, was related to sialic acid content; one form contained 2 moles of sialic acid per mole of protein, the second, 1 mole of protein, and the third, no sialic acid. The fourth polypeptide had an amino acid composition different from the first three polypeptides and from other polyptides obtained from very low density lipoprotein. This polypeptide had NH_2-terminal threonine, COOH-terminal resistant to carboxypeptidase A, no histidine, cysteine, cystine or sialic acid. These four polypeptides constituted approx. 40% of the total protein in very low density lipoprotein.

INTRODUCTION

The very low density apolipoproteins have been isolated by Sephadex and DEAE chromatography[1]. A newly developed method of isoelectric focusing in a narrow pH gradient has been applied to the separation of Tris-soluble very low density lipoprotein apoproteins. The technique offers the advantages of high resolving power, excellent reproducibility and direct determination of isoelectric points. Two major very low density apoprotein were described in this investigation. While this work was in progress, a paper appeared in which a third major apoprotein was

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described and characterized[2]. The present investigation describes two polypeptides obtained from Tris-soluble very low density apoprotein. One of these apoprotein Ala, designated from the COOH-terminal residue, was found to exist in three different forms. These forms contained 2 moles, 1 mole, and no mole of sialic acid per mole of protein, respectively. Two forms of apolipoprotein-Ala, with differences in sialic acid content, have been recently reported[3].

MATERIAL AND METHODS

Preparation of Very Low Density Apolipoprotein

Very low density lipoprotein was isolated from a pool of two blood donors. Two experiments were performed with very low density lipoprotein from single donors. The sera were centrifuged at 10,000 rev./min (12,100xg) for 30 min in an ss34 rotor (Sorvall) to remove the chylomicrons. Subsequently, the chylomicron free sera containing 0.05% EDTA were centrifuges in the L2-65 B ultracentrifuge at 30,000 rev./min for 24 h in a 30.2 rotor (Spinco). The top very low density lipoprotein fraction was removed and resuspended in 0.85% NaCl 0.05% EDTA solution (pH 7.4). The purified very low density lipoprotein was delipidated by extraction with ethanol diethyl ether (3 : 1, v/v) for 16 h at −10°. The solvent was decanted and the protein re-extracted with ethanol-dethyl ether (3 : 2, v/v) for 8 h. The insoluble protein residue was washed three times with ether and dried under nitrogen. The very low density apoprotein was partially solubilized in 0.1M Tris-HCl (pH 8.8). The insoluble residue was removed by centrifugation at 4,000 rev./min. The soluble protein remaining (approx. 45% of the total protein) will be referred to as Tris-soluble very low density apoprotein.

Isoelectric Focusing

The protein were electrofocused by the method of VESTERBERG AND SVENSSON[4], using carrier ampholytes (Ampholine, LKB-Produkter AB, Stockholm). Solutions and density gradient were prepared as described by LKB Instruments.[5] A 110 ml capacity electrofocusing column (No. 8101) and gradient mixer (No. 8121) were used in these experiments (LKB Instruments). Initially, an electrofocusing run was performed with sample but containing 6% ampholine (pH range 4-6) in the presence of 6M urea (Mann). The ampholine was electrofocused for 60 h at 15° with a rotential of 900—1000 V. After the run, approx. 55 2-ml portions were collected and those fractions which contained ampholine in the pH range 4.35—4.85 were pooled. (This pH range, 4.35—4.85, was chosen because preliminary experiments with electrofocusing of Tris-soluble very low density apoprotein indicated that approx. 90% of the protein was found within this pH range.) This prefocused 6% ampholine solution (pH range 4.35—4.85) was subsequently used for the electrofocusing run with the sample. All solutions again contained 6M urea. From 3 to 10 mg of Tris-soluble very low density apoprotein was electrofocused for 64—66 h at 15°. The potential was raised gradually over a 16 h period to a final potential of 900 V. After each run, approx. 90 1.2-ml portions were collected and the pH and A_{280nm} were determined. All pH measurements were made at 25° with a Radiometer pH meter (Copenhagen, PHM 26) and the Radiometer electrode (GK 2322C). Prior to measuring the pH, each tube was diluted 1 : 1 with distilled water to give a final concentration of urea of 3M. The desired portions were pooled and passed through a G-50 column equilibrated with 4M urea-0.5M NaCl-0.1M Tris-HCl (pH 8.6). The protein which appeared in the first peak was subsequently used for chemical characterization. All solutions were concentrated using the Diaflo ultrafiltration cell model 52 and UM-2 membrane (Amico Corp.).

Amino Acid Analysis

Protein samples (0.3-1.5mg) were hydrolyzed in constant boiling 6M HCl for 24 h at 110°. 2-h hydrolyses were performed on Fractions A, B, and C. Quantitative amino acid analysis was determined with a Beckman 120C automatic analyzer equipped with a scale expander and

diigtal integrator according to the procedure of SPACKMAN, *et. al.*[6]. Cystine was independently determined as cysteic acid in performic acid oxidized samples[7]. Tryptohan was determined according to GOODWIN AND MORTON[8]. Corrections were made on all fractions for the loss of serine and threonine during hydrolysis by extrapolating data to zero hydrolysis time and assuming first order kinetics for decomposition.

Terminal Amino Acid Analysis

NH_2-terminal residues were identified by the dansyl chloride procedure[9]. The dansyl derivatives were separated on polyamide sheets (Cheng Chin Trading Co., Ltd.) according to WOODS AND WANG[10].

COOH-terminal residues were determined using carboxypeptidase A (COA DFP, Worthington)[11].

Sialic Acid Determination

Sialic acid was determined by the thiobarbituric acid assay of WARREN[12] after its release from the protein by hydrolysis in 0.05M sulfuric acid at 80° or after release by neuraminidase. Sialic acid was removed enzymatically by incubation of the apoprotein in 0.1M sodium acetate buffer (pH 5.5) containing 0.005M $CaCl_2$ at 37° with neuraminidase (25 units/mg protein) from *Vibria Cholera* (Calbiochem.) for 24h.

Results

Isoelectric Focusing

A typical isoelectric focusing profile from one of five experiments of Tris-soluble very low density apoprotein is shown in Fig. 1. Four peaks (A, B, C, and D) were consistently obtained. These four fractions represented at least 90% of the protein applied to the column. The remaining protein (less than 10%) was present as a broad minor peak between Fraction D and the basic lock (Tubes 66-75). This fraction was not investigated in this study.

The isoelectric points and protein distribution of the isoelectric focusing fractions are shown in Table I. The four protein peaks with pI's of 4.53, 4.70, 4.79, and 4.93, respectively, have a protein distribution of 26, 50, 17 and 7%, respectively.

Table I. Isoelectric Points and Protein Distribution of Very Low Density Apolipoprotein Fractions

Fraction	pI	% Protein (A, B, C, D)	% Protein (A, B, D)**
A	4.53±0.02	26(19—32)	31(23—38)
B	4.70±0.02	50(42—56)	61(51—68)
C	4.79±0.01	17(12—22)	
D	4.93±0.03	7(4—10)	8(5—11)

Values represent the average of five determinations obtained from different preparations. The first column shows the standard error of the mean and the last two columns the ranges obtained from the five determinations.

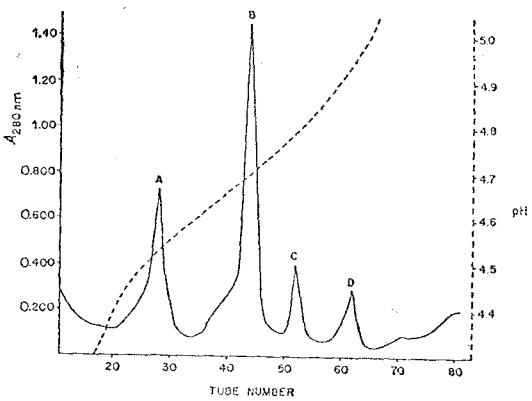

Fig. 1. Isoelectric focusing profile of Tris-soluble very low density apoprotein.
—— A_{280nm} ·····, pH gradient.

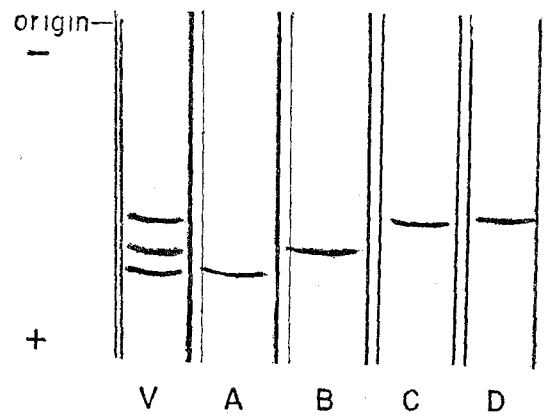

Fig. 2. Polyacrylamide gel electrophoresis of isoelectric focusing fractions (A, B, C, D) performed in 8 M urea (pH 8.9) and 7.5% acrylamide. Tris-soluble very low density apoprotein is shown for comparison(V).

Polyacrylamide Gel Electrophoresis

Each fraction obtained from isoelectric focusing (A, B, C, and D) gave a single band on polyacrylamide electrophoresis (Fig. 2). Fractions C and D had nearly identical electrophoretic mobilities. An equal mixture of Fractions A, B, and D that had previously been treated with neuraminidase for 24 h at 37° gave a single band on polyacrylamide gel electrophoresis (not shown).

Table II. Amino Acid Composition of the Isoelectric Focusing Fractions

Values shown in the columns are the means and ranges of four analyses each of A and B, three analyses each of C and D. Values are expressed as moles of amino acid per 100,000g of protein. n.d. stands for not determined.

Amino Acid	A	B	C	D
Lysine	71 (69—74)	70 (68—72)	70 (67—73)	71 (68—75)
Histidine	11 (10—12)	11 (9—12)	0	10 (9—11)
Arginine	23 (19—25)	24 (21—27)	13 (12—13)	24 (20—26)
Aspartic Acid	85 (82—88)	85 (81—89)	64 (62—67)	86 (80—90)
Threonine	58 (53—62)	58 (53—61)	101 (95—105)	58 (54—62)
Serine	117(111—122)	119(114—123)	99 (93—104)	119(113—123)
Glutamic Acid	120(119—122)	119(117—122)	164(160—168)	118(116—121)
Proline	26 (24—27)	26 (24—28)	42 (41—43)	26 (24—27)
Glycine	46 (43—49)	44 (42—48)	28 (26—29)	45 (42—47)
Alanine	118(115—121)	117(114—122)	75 (71—80)	117(113—121)
Cysteine and Cystine*	0	0	0	0
Valine	69 (63—72)	72 (65—74)	45 (43—46)	70 (68—72)
Methionine	19 (18—19)	19 (18—20)	21 (21—22)	19 (18—19)
Isoleucine	0	0	10 (10—11)	0
Leucine	58 (57—60)	60 (58—61)	92 (88—98)	59 (56—61)
Tyrosine	21 (19—22)	20 (17—22)	53 (50—57)	21 (19—21)
Phenylalanine	44 (42—46)	46 (43—48)	24 (22—26)	45 (42—47)
Tryptophan**	27	27	n.d.	28

* No cysteic acid was present following performic acid oxidation.
** Determined spectroscopically on a single preparation.

Amino Acid Analysis

The amino acid composion of Fractions A,B, and D were indistinguishable with in experimental error (Table II). A,B, and D contained no isolecine, cysteine, or cystine. The amino acid composition of C differed from A,B, and D, and from all previously reported fractions obtained from low density lipoprotein and high density lipoprotein. Fraction C contained no histidine, cysteine, or cystine.

Terminal Amino Acid Analysis

A and B contained NH_2-terminal serine. NH_2-terminal threonine was identified in C. No NH_2-terminal analysis was performed on D. The kinetic data on the amino acids released by carboxypeptidase from Fractions A,B, and D were similar to the results previously published for apolipoprotein-Ala[2]. Carboxypeptidase released 2 moles of alanine per mole of valine from Fractions A, B, and D. No amino acid was released from Fraction C by 24 h digestion with carboxypeptidase A at pH 8.5.

Sialic Acid Determination

Fraction A yielded 2 moles of sialic acid per mole of protein (assuming a mol. wt. of 10,000). Fraction B yielded 1 mole of sialic acid per mole of protein. Fractions C and D contained no sialic acid.

Molecular Weight Determination

The approximate molecular weight of the

Fractions A, B, C, and D as determined by polycrylamide gel electrophoresis in sodium dodecyl sulphate was 10.000±1,000 for A,B, and 9000±900 for C.

DISCUSSION

In the present study the very low density apolipoproteins have been separated by a newly developed technique of preparative isoelectric focusing in a narrow pH gradient. Fraction A appears to be essentially identical to Fraction D_4 and Fraction B essentially identical to Fraction D_3 on the basis of amino acid composition, NH_2-and COOH-terminal analysis, sialic acid content, and position on polyacrylamide gel electrophoresis. Apolipoprotein-Glu from purified D_2 appears to be identical to Fraction C since it contains 18 mole % glutamic acid, no histidine, cysteine or cystine; it has NH_2-terminal threonine, COOH-terminal resistant to carboxypeptidase; and it has the same relative mobility on disc electrophoresis as Fraction C.

Fraction D, which was identical in amino acid composition to Fractions A and B and has COOH-terminal alanine but not sialic acid, was not obtained in the previous investigations. Three forms of apolipoprotein-Ala were present in all the preparations studied in the approximate ratio of 31 : 61 : 8 (Table I). We shall designate these forms as apolipoprotein-Ala_2, apolipoprotein-Ala_1 and apolipoprotein-Ala_0 with the subscript to denote the moles of sialic acid per mole of protein. The possibility that apolipoprotein-Ala_0 was formed during the isoelectric focusing procedure by acid hydrolysis is unlikely since less than 0.5% of the total protein would be expected to be in contact with the acid lock solution. Furthermore, the polyacrylamide electrophoretic pattern of Tris-soluble very low density apoprotein that had been previously incubated with an equal volume of the acid lock solution at 15° for 6 h appeared identical to untreated very low density apoprotein. The fact that neuraminidase treated apolipoprotein-Ala_2 and apolipoprotein-Ala_1 gave a single band in the position of apolipoprotein-Ala_0 supports the concept that the charge differences of the polymorphic forms of apolipoprotein-Ala are due to differences in the sialic acid residues. The possibility that other differences exist between the three forms of apolipoprotein-Ala, *e.g.* differences in the carbohydrate prosthetic group, must be considered.

The method of isoelectric focusing in a narrow pH gradient as developed for Tris-soluble very low density apolipoprotein offers some definite advantages over other fractionation procedures. The separation of Fraction B from Fraction C with a pI difference of only 0.09 pH units indicates a high resolving power. Secondly, excellent reproducibility and direct determination of isoelectric points are two additional features of the method. However, since the pH was taken in the presence of 3M urea, the isoelectric point obtained does not necessarily refer to the pI at zero ionic strength. The isoelectric focusing method allows essentially complete recoveries of the protein applied to the column. Other methods, such as DEAE-cellulose chromatography permit only partial recoveries. Shore and Shore obtained only 50-60% of the total very low density apoprotein in the eluate. The yield of very low density apoprotein obtained after DEAE chromatography were not reported by Brown, *et. al.*[1,2]; thus, no comparison can be made. Since all the protein is recovered from the isoelectric focusing column, an accurate estimate of the relative distribution of polypeptides applied to the column can be obtained. DEAE-cellulose chromatography does not usually permit such an estimation since losses are generally obtained.

The isoelectric focusing method in narrow pH gradients should be useful for the separation of other apolipoproteins which have proved difficult to separate by the more conventional chromatographic methods.

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