The isolation and characterization of high-density-lipoprotein subfractions containing apolipoprotein E from human plasma

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1. Plasma high-density lipoprotein (HDL) was separated by heparin–Sephacrose affinity chromatography into a non-bound, apolipoprotein E-poor, and a bound, apolipoprotein E-rich, fraction through the binding effect of Mn^{2+} in the column buffer. 2. The application of a series of elution buffers in which the concentration of Mn^{2+} was progressively replaced by Mg^{2+} resulted in the separation of the bound HDL into five subfractions. 3. Each subfraction migrated a different distance on gradient-gel electrophoresis. Three of the subfractions had R_F (relative migration compared with BSA) values within the range of HDL_{2b}. One subfraction contained largely HDL_{2a}, with some material in the regions of HDL_{2b} and HDL_{3a}, and one subfraction spanned the R_F regions of HDL_{2a}, HDL_{3a} and HDL_{3b}. 4. The number of molecules, per HDL particle, of cholesteryl ester, non-esterified cholesterol and phospholipid increased with particle size, whereas triacylglycerol passed through a maximum and the number of amino acid residues remained approximately the same. 5. Apolipoprotein (apo) A-I was the major apoprotein in all five subfractions, but the latter differed appreciably in their contents of apo A-II and apo E. 6. The major fatty acid component of each subfraction was linoleic acid, with moderate amounts of C_{16:0} and C_{18:1} fatty acids and a smaller content of C_{18:0}, C_{20:4, n-6} and C_{22:6, n-3}, with no significant difference in composition between the subfractions. 7. This paper provides the first description of a method for the isolation of three subfractions of HDL_{2b} together with other subfractions in quantities that are sufficient for further analytical or metabolic studies.

INTRODUCTION

High-density lipoproteins (HDLs) play a central role in lipoprotein metabolism. They are involved in the transfer of lipid and apoprotein components between different plasma lipoprotein fractions and between lipoproteins and cell membranes (Eisenberg, 1984). HDL is also implicated in reverse cholesterol transport whereby cholesterol is removed from peripheral tissues and transported through the bloodstream to the liver for excretion as bile salts (Reichl & Miller, 1989; Skinner & Wilson, 1990). It is currently considered that this system provides a basis for the protective effect of high concentrations of plasma HDL against coronary heart disease, which has been proposed on the basis of extensive epidemiological investigations (Castelli, 1984; Gordon & Rifkind, 1989). Alternatively, this effect may arise as a result of the protective action of HDL against the oxidation of low-density lipoprotein (LDL) (Klimov *et al.*, 1989), the inhibitory effect of HDL on platelet aggregation (Spector *et al.*, 1985) or by the reduction of postprandial lipaemia (Patsch *et al.*, 1983).

HDL is highly heterogeneous, and many of the above processes require the presence of HDL particles of particular composition and size in order to fulfil their specific functions. The mechanisms that underly these functions are poorly understood at the present time, largely because methods are not available for the separation of the individual subspecies of which HDL is composed. This present paper describes an affinity-chromatographic procedure for the isolation of HDL subfractions using heparin–Sephacrose, which binds apolipoprotein (apo) E-containing HDL particles that are known to be involved in reverse cholesterol transport and may be derived from the lipolysis of triacylglycerol-rich lipoproteins (Gavish *et al.*, 1987). The separated subfractions were characterized with respect to particle size and lipid and

apoprotein composition. The separation of subfractions of HDL is of considerable current interest, since a change in the concentration of specific subfractions (HDL_{2b}) has recently been shown to be associated with high coronary risk (Wilson *et al.*, 1990; Johansson *et al.*, 1991; Cheung *et al.*, 1991) and in response to exercise (Griffin *et al.*, 1988a).

A preliminary account of some of the results has been presented elsewhere (Wilson *et al.*, 1989).

MATERIALS AND METHODS

Chemicals and materials

Heparin from porcine intestinal mucosa (sodium salt; grade 1) was purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents and solvents were of analytical grade.

Subjects

Healthy male volunteer subjects, aged between 23 and 45 years, who were free from any lipid disorder and had no family history of any condition that was likely to affect the plasma lipoprotein profile, were used in the study. Blood samples (50–100 ml) were drawn from an antecubital vein in the forearm of the non-fasted subjects into bottles containing Na₂EDTA (1 mg/ml of blood). After gentle mixing, the samples were placed on ice and the plasma separated by centrifugation for 10 min at 1500 *g* and 4 °C. Lipoprotein separation was initiated without delay.

Separation of HDL

The density of the plasma was adjusted to 1.063 g/ml by the addition of solid NaBr, and portions (10.4 ml) of the adjusted plasma were centrifuged for 18 h at 105 400 *g* (40 000 rev./min) and 16 °C in polyallomer tubes (7.62 cm × 1.58 cm) in the

Abbreviations used: HDL, high-density lipoprotein; apo, apolipoprotein; LDL, low-density lipoprotein.

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Beckman model L2-65B preparative ultracentrifuge with the type 50Ti rotor. The top layer, containing chylomicrons, very-low-density lipoprotein and LDL, was removed in a volume of 4 ml from each tube by slicing at a point 4 cm from the top of the tube with the aid of a Beckman tube slicer. The bottom fraction was mixed with an equal volume of NaBr of density 1.357 g/ml containing 1 mM- $\text{Na}_2\text{-EDTA}$ to give a final density of 1.25 g/ml and centrifuged for 40 h under the same conditions. The top layer containing the HDL was removed (in a volume of 2 ml) by slicing at a distance of 2 cm from the top of the tube (Skinner, 1992). Densities were routinely measured at 20 °C with an Anton Paar DMA 40 digital density meter.

Heparin–Sephacrose affinity chromatography

Heparin–Sephacrose was prepared by covalent coupling of heparin to Sepharose 4B activated by CNBr (March *et al.*, 1974; Klör *et al.*, 1976) as previously described (Hay *et al.*, 1978; Griffin *et al.*, 1988b) and equilibrated in a column (1 cm $\times$ 12.5 cm) with 50 mM-Tris/HCl/50 mM-NaCl, pH 7.4. Immediately before applying the sample to the column, approx. 15 ml of the above buffer, containing 25 mM- MnCl_2 , was passed through the column. Samples of HDL, prepared by ultracentrifugation, were dialysed exhaustively against 50 mM-Tris/HCl/50 mM-NaCl, pH 7.4, and solid MnCl_2 was added just before applying to the column to give a final Mn^{2+} concentration of 25 mM (Weisgraber & Mahley, 1980). A volume of 1.5 ml containing between 2.5 and 3.5 mg of HDL protein was applied to the column and allowed to equilibrate for 16 h before the eluting buffers were applied. (See the Results section for details of elution buffers.)

Gradient-gel electrophoresis

Gradient-gel electrophoresis was performed on 4–30% polyacrylamide linear gradient gels (Pharmacia PAA 4/30) using the conditions for pre-equilibration and electrophoresis described by Blanche *et al.* (1981). Samples (20 μl) containing 15–20 μg of HDL protein were applied directly after ultracentrifugation without dialysis. Pooled column fractions were concentrated using an Amicon stirred-cell concentrator. A reference protein mixture (HMW Calibration Kit; Pharmacia) was included on each gel.

After electrophoresis, the gels were fixed in 10% (w/v) sulphosalicylic acid for 60 min and stained with Coomassie Brilliant Blue R (0.1%, w/v) in methanol/acetic acid/water (5:1:4, by vol.) for 16 h and destained by washing in methanol/acetic acid/water (2:3:35, by vol.). The stained gels were scanned with the Bio-Rad model 620 video densitometer, and the scan data was programmed on an IBM/XT personal computer. The R_f value of individual bands on each gel was calculated using the ratio of the migration distance of the band relative to the migration distance of BSA in the standard lane of the same gel.

HDL subfractions were defined as described by Blanche *et al.* (1981) as HDL_{2b}, R_f 0.445–0.627 (mean particle diameter 10.57 nm); HDL_{2a}, 0.627–0.711 (9.16 nm); HDL_{3a}, 0.711–0.781 (8.44 nm); HDL_{3b}, 0.781–0.841 (7.97 nm); HDL_{3c}, 0.841–0.962 (7.62 nm). The percentage composition was calculated from the peak areas within the above R_f (coefficient of variation < 7.5%; $n = 4$). The values obtained by this procedure are not affected by the non-linear relationship that has been shown to exist between pore diameter and migration distance (Williams *et al.*, 1990).

Chemical analysis

The concentrations of total cholesterol and non-esterified cholesterol in the isolated HDL subfractions were determined by

means of the Boehringer CHOD-PAP diagnostic kit, and cholesterol ester was taken as the difference. Triacylglycerol and phospholipid estimations were made with the triacylglycerol fully enzymatic kit and the total phosphorus phospholipid kit (Boehringer) respectively. Precinorm standard plasmas (Boehringer) were included in all the above assays. Total protein was determined by the modification of the method of Lowry *et al.* (1951) described by Hartree (1972), BSA being used as standard. The composition of the HDL subfractions in terms of molecules of components per particle was calculated by the method of Shen *et al.* (1977).

Apo analysis

Apo composition was determined by means of non-competitive e.l.i.s.a., using horseradish peroxidase-conjugated goat anti-(mouse immunoglobulin) serum with 2,2'-azinobis-(3-ethylbenz-thiazolinesulphonic acid) as hydrogen donor (Campbell, 1984). Assays for apo A-I were performed by using a mixture of three different monoclonal antibodies to human apo A-I (1:1:1, by vol.), with an inter-assay coefficient of variation of 7.5% and an intra-assay coefficient of 3.4%. Monoclonal antibodies from single clones were used for assay of apo A-II (Boehringer) and apo E (generously provided by Dr. R. W. James, Hôpital Cantonal, Geneva, Switzerland) which gave inter-assay and intra-assay coefficient of 6.4% and 6.1% respectively for apo A-II, and 7.3% and 9.6% for apo E. Standard curves were prepared by using standard plasma (Boehringer) for apo A-I and apo A-II and reference standard apoprotein (Immuno Ltd., Dunton Green, Sevenoaks, Kent, U.K.) for apo E.

Fatty acid analysis

Total lipids were extracted by a modification (Hanson & Olley, 1963) of the Bligh & Dyer (1959) method and saponified, and the fatty acids were methylated by heating under reflux with methanol in the presence of 2,6-di-*t*-butyl-4-methylphenol.

The composition of methyl esters of fatty acids was determined by g.l.c. using an on-column injector fitted in a Hewlett–Packard 5880A gas chromatograph equipped with a 30 m $\times$ 0.253 mm (inner diameter) fused silica column coated with DB-wax, with N_2 as carrier gas. Identification of fatty acid methyl esters was made by comparison with authentic standards and by low-resolution electron impact or chemical-ionization m.s.

RESULTS

Heparin–Sephacrose affinity chromatography

When HDL, separated by ultracentrifugation in the density range 1.063–1.25 g/ml, was applied to a heparin–Sephacrose column in 5 mM-Tris/HCl/50 mM-NaCl/25 mM- MnCl_2 , pH 7.4, as described in the Materials and methods section, a non-bound, apo E-poor, fraction of HDL was eluted. Stepwise addition of 5 mM-Tris HCl/70 mM-NaCl, followed by 5 mM-Tris/HCl/0.6 M-NaCl, resulted in the elution of a bound, apo E-rich, HDL fraction and a small fraction containing lipoprotein (a) [Lp(a)] and some LDL. These results are in agreement with those previously reported by Weisgraber & Mahley (1980). Analysis by gradient-gel electrophoresis, however, revealed that the bound fraction of HDL was heterogeneous, as judged by the inflexions on the scanned curve, and contained subpopulations of HDL that spanned the R_f region of HDL_{2b}, HDL_{2a} and HDL_{3a}, with at least three components in the HDL_{2b} region (Fig. 1).

In an attempt to separate these components, the effect of applying a series of eluting buffers containing different concentrations of NaCl, MnCl_2 and MgCl_2 was investigated empirically. Optimal resolution of the subfractions, as determined by

Fig. 2. Separation of HDL subfractions by heparin-Sepharose affinity chromatography

Approx. 3.0 mg of HDL protein in 1.5 ml of 50 mM-Tris HCl/50 mM-NaCl/25 mM-MnCl₂ was applied to a column (1 cm × 12.5 cm) and eluted with the following buffers, all of which contained 5 mM-Tris/HCl, pH 7.4: (1) 25 mM-NaCl/80 mM-MnCl₂; (2) 50 mM-NaCl/25 mM-MnCl₂; (3) 75 mM-NaCl/25 mM-MnCl₂; (4) 50 mM-NaCl/12.5 mM-MnCl₂/12.5 mM-MgCl₂; (5) 50 mM-NaCl/6.2 mM-MnCl₂/18.6 mM-MgCl₂; (6) 0.1 M-NaCl; (7) 0.6 M-NaCl. The fractions indicated by the bar lines were pooled for further investigation. The proportion of the total HDL contributed by the individual separated fractions was I, 64.2 ± 13.9 (s.d.)%; II, 9.1 ± 3.3%; III, 7.8 ± 3.9%; IV, 6.4 ± 3.5%; V, 5.2 ± 2.4%; VI, 7.3 ± 3.3% (n = 7).

analysis on gradient gels, was obtained by stepwise elution with the seven buffers listed in the legend to Fig. 2. Gel scans of the subfractions obtained by elution with these buffers (Fig. 3) demonstrated that the non-bound material (Fraction I) contained

Fig. 3. Densitometric scans of 4-30%-polyacrylamide gradient gels containing subfractions (SF) of HDL isolated by heparin-Sepharose chromatography

Fractions I-VI are those indicated in Fig. 2. The broken lines represent scans of a single unfractionated sample of HDL as a reference, analysed on the same gel as each isolated subfraction.

the major portion of the HDL₃ subclass, with most of the remainder of the HDL₃ being eluted in Fraction II. Fraction III was partially enriched with HDL_{2a} (*R_F* 0.627-0.711), but also contained some HDL_{2b} and HDL_{3a}. Fractions IV, V and VI appeared as almost symmetrical peaks within the *R_F* range

Table 1. Compositions of HDL subfractions isolated by heparin-Sepharose affinity chromatography (n = 7)

Values represent the mean numbers of molecules per HDL particle (± s.d.). Abbreviations: CE, cholesteryl ester; NC, non-esterified cholesterol; PL, phospholipid, TG, triacylglycerol

Fraction	CE	NC	PL	TG	Amino acid residues
I	56.2 ± 6.1	21.7 ± 6.2	65.7 ± 3.1	9.6 ± 6.3	1327 ± 118
II	62.3 ± 6.3	22.3 ± 5.4	67.2 ± 16.2	9.9 ± 6.2	1303 ± 97
III	69.9 ± 12.3	34.3 ± 5.3	82.3 ± 18.4	13.2 ± 3.3	1263 ± 124
IV	87.0 ± 15.2	36.7 ± 6.1	83.4 ± 16.6	15.9 ± 4.0	1389 ± 107
V	97.1 ± 16.2	38.2 ± 4.7	92.1 ± 24.1	20.1 ± 6.3	1329 ± 134
VI	115.4 ± 15.0	50.2 ± 10.3	125.2 ± 23.4	12.6 ± 4.2	1335 ± 144

Table 2. Apoprotein composition of HDL subfractions isolated by heparin-Sepharose affinity chromatography ($n = 7$)

Values represent mean percentages of apoproteins $\pm$ S.D. The values given below are the means $\pm$ S.D. for the individual results obtained in seven separate experiments.

Fraction	A-I	E	A-II	A-I/E	A-I/A-II	A-II/E
I	99.24 $\pm$ 0.41	0.15 $\pm$ 0.06	0.44 $\pm$ 0.27	673.5 $\pm$ 52.1	202.01 $\pm$ 96.21	5.39 $\pm$ 3.44
II	99.41 $\pm$ 0.42	0.27 $\pm$ 0.07	0.30 $\pm$ 0.19	312.9 $\pm$ 95.3	342.00 $\pm$ 61.21	3.91 $\pm$ 2.14
III	99.33 $\pm$ 0.37	0.24 $\pm$ 0.13	0.34 $\pm$ 0.22	475.55 $\pm$ 97.10	322.55 $\pm$ 47.53	1.94 $\pm$ 0.86
IV	98.65 $\pm$ 0.87	0.77 $\pm$ 0.21	0.57 $\pm$ 0.17	357.35 $\pm$ 86.21	169.63 $\pm$ 35.90	1.84 $\pm$ 0.97
V	98.15 $\pm$ 0.83	0.67 $\pm$ 0.19	0.84 $\pm$ 0.23	197.47 $\pm$ 61.21	427.57 $\pm$ 71.51	1.51 $\pm$ 0.58
VI	97.17 $\pm$ 0.97	2.13 $\pm$ 0.89	0.66 $\pm$ 0.22	53.12 $\pm$ 22.34	423.53 $\pm$ 47.40	0.36 $\pm$ 0.11

of HDL_{2b} (0.445–0.627) with mean R_f values ($\pm$ S.D.) of 0.542 ± 0.005 , 0.529 ± 0.004 and 0.506 ± 0.009 respectively. The validity of the differences in the distance migrated by the material in these fractions was substantiated by comparison with a standard HDL sample from a single subject analysed on the same gel. Thus the R_f maximum of Fraction IV was close to the peak maximum of HDL_{2b} of the standard HDL, whereas Fractions V and VI migrated in positions corresponding to the upper and lower ends respectively of the high-molecular-mass side of the standard HDL_{2b} curve. Similar results were obtained in seven experiments using HDL from different subjects, demonstrating the reproducibility and general applicability of the separation procedure. Fractions IV and V appeared as essentially single peaks, whereas Fraction VI contained an additional component of larger particle size.

Composition of HDL fractions

The overall composition, per particle of HDL, of the isolated fractions (Table 1) is in general agreement with that previously reported for the total HDL₂ subclass of human serum (Keddy, 1977). Increasing particle size was accompanied by an increase in the number of molecules per particle of cholesteryl ester, non-esterified cholesterol and phospholipid, whereas triacylglycerol passed through a maximum at Fraction V and the number of amino acid residues per HDL particle remained approximately the same. The apoprotein composition of the HDL subfractions, as determined by e.l.i.s.a., is given in Table 2. These values provide reliable estimates of relative apoprotein concentration (see the Materials and methods section), but the absolute concentrations of apo A-I were overestimated in relation to apo A-II and apo E. It was therefore not possible to estimate the molar distribution of apoproteins per HDL particle.

The fatty acid composition of the seven HDL subfractions was similar and displayed no discernable trend of changes in the various subfractions. The major fatty acid components were C_{18:0} [which ranged between 23.3 ± 3.2 (S.D.) and $26.3 \pm 2.6\%$ of the total fatty acid composition] and C_{18:2, n-6} (39.2 ± 3.0 to $43.6 \pm 2.8\%$), with appreciable quantities of C_{18:0} (9.4 ± 1.2 to $10.6 \pm 2.2\%$), C_{18:1} (13.7 ± 1.6 to $16.9 \pm 2.0\%$) and C_{20:4, n-6} (6.4 ± 1.8 to $9.7 \pm 2.6\%$) and a small content of C_{22:6, n-3} (2.1 ± 0.8 to $2.9 \pm 0.63\%$).

DISCUSSION

Although it has long been known that HDL contains two major subclasses, HDL₂ and HDL₃, and methods have been available for their separation (Gofman *et al.*, 1954; Havel *et al.*, 1955), the isolation of further subfractions of HDL has since eluded the attempts of many investigators because of the close similarity of the different subspecies, both in their particle size and composition. Analysis of HDL patterns obtained from a

large group of human subjects by gradient-gel electrophoresis (Blanche *et al.*, 1981) showed that the frequency distribution of the relative migration distances (R_f values) of the subpopulation peaks showed five apparent maxima, corresponding to HDL_{2b} to HDL_{3c}, as defined in the Materials and methods section, whose characteristics were confirmed by analysis in the analytical ultracentrifuge and by electron microscopy (Anderson *et al.*, 1977). These observations and the detection of up to ten subfractions in both HDL₂ and HDL₃ by a combination of electrophoretic and isoelectric focusing techniques (Marcel *et al.*, 1984) have provided a stimulus for the development of new isolation procedures. Cheung & Albers (1982), for example, using immunoaffinity methods, isolated two populations of HDL, one containing both apo A-I and apo A-II and the other containing apo A-I without apo A-II, and Clifton *et al.* (1987), using gel-permeation chromatography, separated fairly pure preparations of subpopulations HDL_{2a} and HDL_{3a}.

The separation of HDL into an apo E-rich and an apo E-poor fraction was achieved by the use of heparin-Sepharose affinity chromatography (Weisgraber & Mahley, 1980). The observation that the gradient-gel scan of the apo E-rich fraction contained at least three components suggested that the use of different elution solvents might result in the separation of these subfractions. The precipitation procedures described by Burstein *et al.* (1970) for the separation of lipoprotein fractions by the use of different concentrations of MgCl₂ and polyanions suggested the possibility that progressive replacement of Mn²⁺ by Mg²⁺ ions in the elution buffer might be effective in resolving the subfractions of the bound apo E-rich HDL. In the present study it was shown that, by such a strategy, it was possible to resolve this material into five subfractions. On analysis by gradient-gel electrophoresis the first fraction of the heparin-Sepharose bound HDL (Fraction II) to be eluted contained a mixture of HDL_{2a}, HDL_{3a} and HDL_{3b}, whereas the second fractions (Fraction III) contained largely HDL_{2a}, with some contamination with HDL_{2b} and HDL_{3a}. Of particular interest is the observation that HDL_{2b} was resolved into three components (Fractions IV, V and VI).

The high degree of symmetry of the peaks representing the different subfractions, together with their different R_f values and the large differences in the apoprotein composition, especially in the distribution of apo A-II and apo E, of the individual subfractions, strongly suggests that each of the isolated fractions contains predominantly a different subpopulation of HDL. Although the microheterogeneous nature of the gradient-gel bands has been realized for many years (Anderson *et al.*, 1977), this is the first report of a method capable of achieving the physical separation of HDL_{2a} and HDL_{2b} and of further resolving the latter into three subfractions in quantities that are suitable for analytical or metabolic investigations. The overall lipid and protein compositions of these fractions are in close agreement with those reported by Clifton *et al.* (1987) for HDL_{2b} and

HDL_{3a} isolated in essentially pure form and for preparations containing HDL_{2a} and HDL_{3b} by gel-permeation chromatography on Superose 6B, though the apoprotein compositions of the subfractions was not reported in that study.

The structure and composition of the HDL subfractions are likely to have undergone some alteration from their native state in the circulating plasma owing to the dissociation of apos, especially apo E and apo A-IV, as a result of the high centrifugal force and concentrated salt solutions used in their separation (Hay *et al.*, 1978). The observation that the distribution of HDL subfractions undergoes specific and reproducible changes in certain diseases, such as coronary heart disease (Wilson *et al.*, 1990; Cheung *et al.*, 1991) in response to physiological stimuli such as exercise (Griffin *et al.*, 1988b) and to drugs (Skinner *et al.*, 1989) provides strong evidence for a physiological role for the isolated subfractions.

A knowledge of the distribution and characteristics, including the composition, of the subspecies that comprise plasma HDL is important in understanding the metabolic role of HDL and, in particular, the mechanism by which HDL protects against the development of coronary heart disease, a process that, at least under some conditions, implicates a fraction of HDL rich in apo E. The observation that survivors of myocardial infarction and subjects of high coronary risk have significantly reduced plasma concentrations of HDL_{2b} and apo E-rich HDL (Wilson *et al.*, 1990; Cheung *et al.*, 1991; Johansson *et al.*, 1991) demonstrate the importance of specific HDL subfractions in these processes and suggest that measurement of HDL subfractions may provide a more effective indicator for detecting subjects at risk from coronary heart disease than the use of total plasma HDL cholesterol concentration.

HDL may also play an important role in the delivery of fatty acids to peripheral tissues, either by lipid transfer to, or exchange with, tissue cell membranes or by receptor-mediated uptake (Eisenberg, 1984). This may be particularly important with respect to the essential polyunsaturated fatty acids which are required for eicosanoid synthesis and for the maintenance of membrane fluidity. It may be significant that the major fatty acid associated with each HDL subfraction isolated in the present study is linoleic acid, the principle metabolic precursor of arachidonic acid and prostaglandins. In total HDL this acid is present largely as cholesteryl ester (Scanu, 1972). The similarity of the fatty acid composition of the different HDL subfractions may arise through the rapid exchange of phospholipids and cholesteryl esters between different HDL particles.

In conclusion, a method has been described for the isolation of subfractions of HDL, including three subfractions of HDL_{2b}, by the use of heparin-Sepharose affinity chromatography. The subfractions were distinct with respect to their migration distance on gradient-gel electrophoresis and in composition, and were isolated in quantities sufficient for metabolic studies.

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