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A Simple Purification of Apolipoproteins A-I and B and Their Application to Cholesteryl Ester Transfer Assay

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Abstract

This study describes a stable and simple method for the measurement of cholesteryl ester transfer protein (CETP) activities using reconstituted HDL and LDL as substrates. Apolipoproteins(apo) A-I and -B were purified from hog plasma by a new strategy without ultracentrifugation and delipidation. A simple two-step column chromatography was administered. In the first step of phenyl-sepharose CL-4B column chromatography, hydrophobic plasma proteins were isolated. The most hydrophobic proteins bound to the column appeared to be apo A-I and apo-B. Contaminant proteins were efficiently eliminated from the sample by washing the column with 0.3M NaCl containing buffer after loading the plasma on the column. Two pure proteins showing each single band on SDS-PAGE of apo A-I and apo-B were individually obtained by a subsequent gel filtration column chromatography(Sephadex G-200). This two-step purification was simple and inexpensive compared to the ultracentrifugation and/or delipidation method that are most commonly used. Reconstituted high-density lipoproteins(HDL) and low-density lipoproteins(LDL) were prepared using the purified apo A-I and -B, respectively. When these artificially prepared HDL and LDL were used in the assays for CETP as the cholesteryl ester(CE) donor and acceptor respectively, the specific transfer of CE increased up to two fold compared to that used the native HDL and LDL.

Key words: LDL, HDL, CETP, CE

INTRODUCTION

A plasma CETP functions a key role in the reverse cholesterol transport system which transports cholesterol from peripheral tissues to the liver(1,2). It is one of the plasma components that plays an important role in the regulation of plasma HDL concentration (3,4). Its primary reaction *in vitro* is a transfer of CE from HDL to very low density lipoprotein(VLDL), with a concomitant transfer of TG from VLDL to HDL(5). In this process, the protein-mediated transfer of cholesteryl esters(CE) between lipoproteins has been shown to be closely coupled to the CETP and the cholesterol esterification by lecithin : cholesterol acyltransferase (LCAT)(6,7). The consequences of these transfer reactions are a decrease in HDL cholesterol and an increase in LDL cholesterol levels. It has been well known that the HDL is anti-atherogenic, while the LDL is an atherogenic component(8). Amounts of the two proteins in the plasma could be used as parameters for the early prediction of coronary heart diseases(9,10).

Apolipoproteins also play essential roles in transport and distribution of neutral lipids among different tissues through the blood(6). They are structural components of lipoprotein particles : apo A-I and apo-B are major proteins in HDL and LDL, respectively.

There has been increasing evidence that the CETP may retard the clearance of cholesterol from the blood, resulting in recycling of the cholesterol species between the blood and peripheral tissues(1,11). As the CETP becomes recognized as an atherogenic factor(12), many investigators have attempted to search its inhibitors from various sources(13-15). For an efficient screening the CETP inhibitor, the most important subject would be to establish an easy, effective and inexpensive assay method. Though several laboratories have published methods for CETP assay(11-14,16), most of them have been limited by high cost and low sensitivity in monitoring the CE transfer from the HDL to the LDL. Here, we report an easy way to purify the apo A-I and apo-B

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required to reconstitute HDL and LDL, respectively, without ultracentrifugation(17) and delipidation(18,19). We have reconstituted the proteoliposomes as substrates of CETP using the purified apo A-I and apo-B, and developed a simple and reliable assay system for measuring CETP activities.

MATERIALS AND METHODS

Plasma and chemicals

Hog blood was obtained from local slaughter house in tubes containing Na_2-EDTA(final concentration=1mg/ml) and a fresh hog plasma was obtained by centrifugation(5,000×g) at 4°C for 15min.

1α,2α-[^{3}H]-n-cholesteryl oleate was purchased from Amersham Life Science Co.(Amersham, U.K.). Egg-yolk lecithin(100mg/ml, ethanol solution) and cholesteryl oleate were purchased from Sigma Co.(St Louis, USA). Phenyl Sepharose CL-4B, Sephadex G-200 column media and CNBr-activated Sepharose-4B were purchased from Pharmacia Co.(Uppsala, Sweden).

Purification of apolipoprotein A-I and -B

Ammonium sulfate(final concentration 15%, w/v) was added to hog plasma(200ml) to precipitate highly hydrophobic component in plasma. The supernatant was applied to a phenyl-sepharose CL-4B column(2.5×15cm) without dialysis, which was previously eqilibrated with 5mM sodium phosphate/0.3M NaCl, pH 7.4. After loading a sample on the column, the column was washed with the equilibration buffer until the absorbance of the effluent at 280nm negligible(A_{280}≦0.2). This column was operated under gravity throughout the chromatography. After washing the column, the proteins in the column were eluted with distilled water with a flow rate of 40ml/hr and fractions(20ml) were collected until A_{280}≦0.2. The second elution was conducted with 50%(v/v) ethylene glycol(EG) in 10mM sodium phosphate buffer, pH 7.4 with a flow rate of 20ml/hr until A_{280}≦0.2. The column was finally washed with 75%(v/v) EG in the same buffer with a flow rate of 20ml/hr(fraction size, 3ml) until A_{280}≦0.1.

All fractions of phenyl-sepharose CL-4B were subjected to apolipoprotein analyses by SDS-PAGE and double immunodiffusion, according to the methods described by Laemmli(20) and Ouchterlony(21), respectively. For the double immunodiffusion, polyclonal antisera against apo A-I or -B were placed in a center-well in order to examine presence of the antigens in the samples placed on the surrounding-wells. The samples in the wells were allowed to diffuse for 16hr in a humid chamber at 37°C. The agarose gel was sufficiently washed with PBS-azide solution and dried for 2hr at 37°C in the humid chamber. Then, the immunoprecipitin lines were visualized by staining the gel with coomassie brilliant blue R-250 followed by destaining in the acetic acid-containing solution.

Sephadex G-200 was packed into a column(1.5×90 cm) and the column was equilibrated with 5mM sodium phosphate, pH 7.4 containing 50mM NaCl. Ethylene glycol(75%, v/v) fractions obtained from phenyl-sepharose CL-4B column were dialyzed against 5mM sodium phosphate, pH 7.4 for at least 16hr and applied to the Sephadex G-200 column with a flow rate of 3ml/hr. Absorbance at 280nm of the eluate was monitored and the purity of apo A-I or apo-B was examined by the SDS-PAGE. Purified apo A-I and apo-B were quantitated according to the method described by Bradford(22) and lyophillized for storage.

Reconstitution of HDL and LDL

Proteoliposomes used as substrates of cholesteryl ester transfer assays were prepared by the sodium cholate dialysis technique basically as decribed previously(23). A brief outline of the method and its slightly modified version made in our laboratory are as follows :

In a typical preparation of reconstituted HDL(HDL_R) or LDL(LDL_R), the molar ratio of apo A-I(or apo-B) : phosphatidyl choline : cholesteryl oleate was 0.8 : 250 : 9. A trace amount of [^{3}H]-cholesteryl oleate was added in the HDL_R mixture. The phosphatidylcholine and cholesteryl oleate in chloroform was carefully evaporated to dryness under nitrogen stream at room temperature. To the dried lipid mixture, apo A-I(or apo-B) and sodium cholate solution in Tris-HCl, pH 7.4 were added. The dissolved mixture was gently sonicated in a bath sonicator for 10min at room temperature and incubated in a shaking waterbath at 24°C for 20min. Then, the mixture was dialyzed extensively against 10mM Tris-HCl buffer, pH 7.4, for 24hr at 4°C to remove sodium cholate.

Immobilization of labelled HDLR

Radiolabelled HDL_R was covalently linked to CNBr-

activated Sepharose-4B by the manufacturer's suggested procedures(Pharmacia, Sweden). After coupling, any unreacted groups remained were blocked by incubation of the resin with 0.1M Tris-HCl, pH 8.0, for 24hr at 4°C. The [^{3}H]-HDL_R-Sepharose beads were washed several times with the 0.1M Tris containing 0.5M NaCl, pH 8.0, on the sintered glass funnel. The beads were stored in 0.1M Tris containing 0.5M NaCl buffer at 4°C until use.

Cholesteryl ester transfer assay

To microcentrifuge tubes(1.5ml), [^{3}H]-HDL_R-Sepharose bead solution(50μl) containing apo A-I(approx. 53μg) as a CE-donor, the LDL_R solution containing apo-B (approx. 246μg) as a CE-acceptor and 50μl of human plasma as a CETP source were sequentially added and mixed gently. The reaction mixture was incubated at 37°C with shaking for 2hr. To compare with native lipoprotein system, we incubated the reaction mixture for 10hr with native LDL or HDL which synthesized according to the previously described method(13). After the incubation, samples were centrifuged for 2min at 3,000×g to sediment the [^{3}H]-HDL_R-bound beads. The radioactivity of an aliquot taken out from supernatant was measured by a liquid scintillation counter (Packard Tri-carb 1600TR) to estimate total CE transferred from HDL_R to the LDL_R. Pig plasma was used for determination of nonspecific CE transfer in this assay system, since there has been a minium CETP activity in the rat or pig plasma among experimental animals.

RESULTS AND DISCUSSION

Purification of apo A-I and apo-B

Fig. 1 shows the elution profile of phenyl-sepharose column chromatography. Hydrophilic proteins in the hog plasma were removed in the early stage of the chromatography. Fractions with hydrophobic proteins including the apo A-I and -B were co-eluted when the column was washed with 50% EG and subsequently with 75% EG, as identified on the SDS-PAGE(Fig. 2) and the double immunodiffusion analyses(Fig. 3). Lane 7 in Fig. 2 shows that fractions eluted with 75% EG contain only apo A-I and apo-B. These two proteins were subsequently separated by Sephadex G-200 gel filtration chromatography(Fig. 4) as confirmed by 10% SDS-PAGE analysis(Fig. 5). From 200 ml of hog plasma, about 37mg of apo A-I and 25mg of apo-B were recovered from each batch. Purified apo A-I was iden-

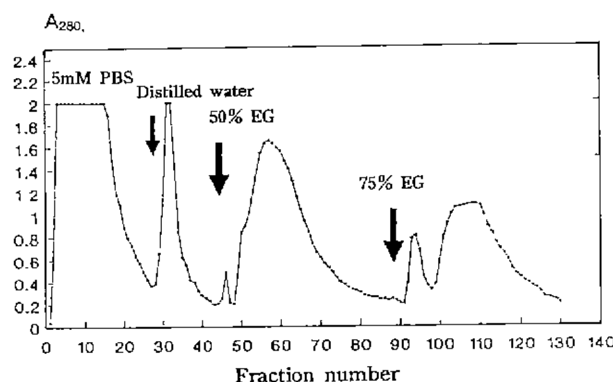

Fig. 1. Elution profile of phenyl-sepharose CL-4B column chromatography.

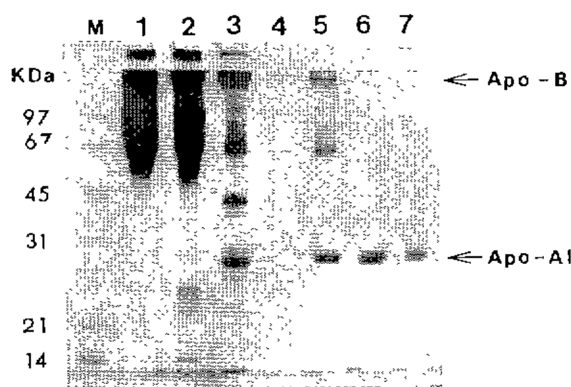

Fig. 2. Electrophoretic patterns of phenyl-sepharose CL-4B fractions obtained from 10% SDS-PAGE.
Stained with coomassie blue R-250.
Lane 1: Fraction number(F #) 7, Lane 2: F# 15, Lane 3: F # 32, Lane 4: F # 46, Lane 5: F # 59, Lane 6: F # 93, Lane 7: F # 110

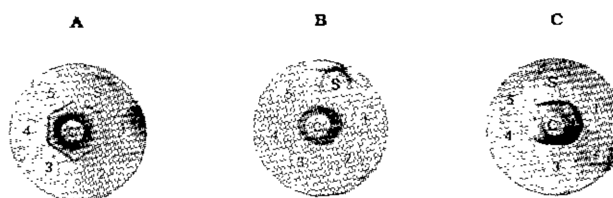

Fig. 3. Immunodiffusion of phenyl-sepharose CL-4B fractions against anti-immunoprecipitin bands visualized by coomassie blue R-250.
Well 1: F# 15, Well 2: F# 32, Well 3: F# 49, Well 4: F# 93, Well 5: F# 110, Well S: positive control (plate A: human apo-B, plate B: human apo A-I, plate C: bovine serum albumin), Well C of plate A: anti-pig apo B antibody, Well C of plate B: anti-pig apo A-I antibody, Well C of plate C: anti- human serum albumin antibody.

tified by N-terminal amino acid sequencing analysis (Protein Sequenator 476A, Applied Biosystem Institute). This method for isolating apo A-I and apo-B simultaneously was more simple and inexpensive than any other methods which include ultracentrifugation and delipidation.

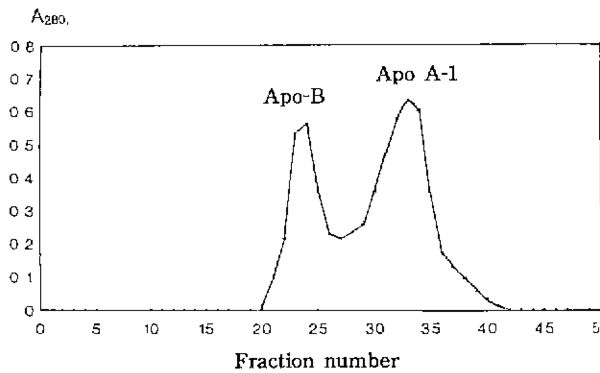

Fig. 4. Elution profile of Sephadex G-200 column chromatography.

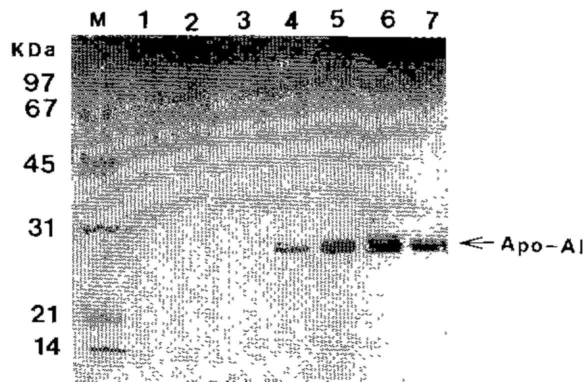

Fig. 5. Electrophoretic patterns of Sephadex G-200 fractions obtained from 10% SDS-PAGE.
Stained with coomassie blue R-250.
Lane 1: fraction number(F #) 23, Lane 2: F# 25, Lane 3: F # 26, Lane 4: F # 32, Lane 5: F # 34, Lane 6: F # 35, Lane 7: F # 37

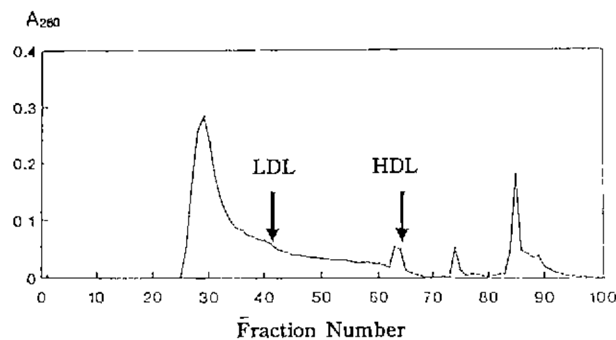

Fig. 6. Bio-Gel A-5m column chromatography of artificial LDL.

Reconstitution of lipoproteins and immobilization of HDLR

HDL_R(apo A-I containing proteoliposome) was composed of phosphatidylcholine single bilayer, cholesteryl oleate and apo A-I. About 97% of total protein was bound to the beads through immobilization process, 50~60% of the total radioactivity was removed through washing procedures. LDL_R was composed of only apo-B intercalated phosphatidylcholine single bilayer. This apo-B containing proteoliposome(LDL_R) was slightly increased in its size over the native LDL as shown in Bio-Gel A-5m column chromatography(Fig 6).

Cholesteryl ester transfer assay

The amount of [^{3}H]-cholesteryl ester transferred from [^{3}H]-HDL_R-sepharose to LDL_R increased with the duration of incubation and appeared to be saturated within 1.5hr at 37°C and 30~40% of the total radioactivity in CE-donor(HDL_R) had been transferred to the CE-acceptor(Fig. 7). Throughout this CE-transfer assay, HDL_R containing radiolabelled CEs were incubated with LDL_R at 37°C in the absence(as blank control) or presence of CETP source. As illustrated in Fig. 7, human plasma and lipoprotein deficient plasma showed similar CE-transfer activity. After 2hr incubation, about 30% of the total radioactivity was recovered in LDL_R which is a CE-acceptor. [^{3}H]-cholesteryl ester transferred was dependent on the amount of plasma added as a CETP source in incubation mixtures

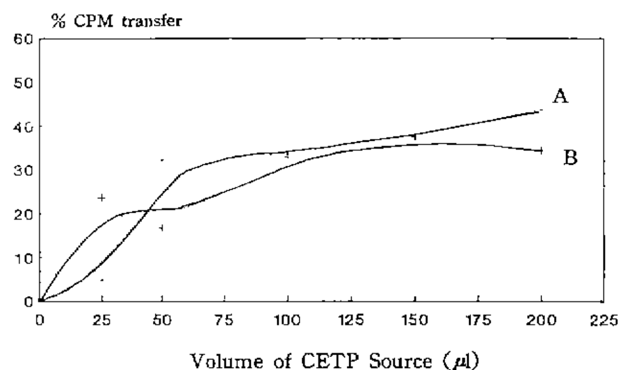

Fig. 7. Changes in CETP activity with increasing concentration of CETP using artificial substrate system.
[^{3}H]-CE-HDL_R(53μg of apo A-I) was used as a CE-donor and LDL_R(246μg of apo-B) used as a CE-acceptor. Human plasma(A) and lipoprotein deficient plasma(B) were used as CETP sources, respectively.

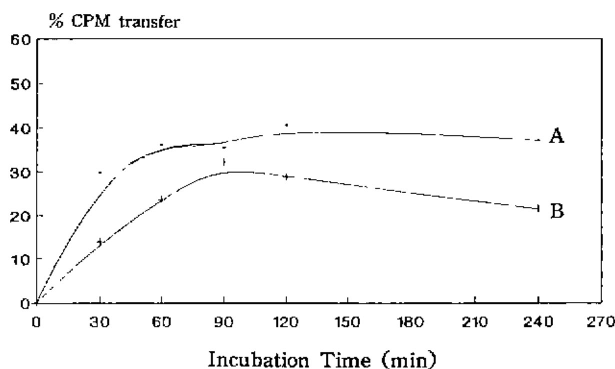

Fig. 8. CETP activities with time course using artificial substrate system.
[^{3}H]-CE-HDL_R(53μg of apo A-I) was used as a CE-donor, LDL_R(246 μg of apo-B) as a CE-acceptor. Human plasma (A) and lipoprotein deficient plasma (B) were used as CETP sources, respectively. Results are expressed as mean of tiplicate samples.

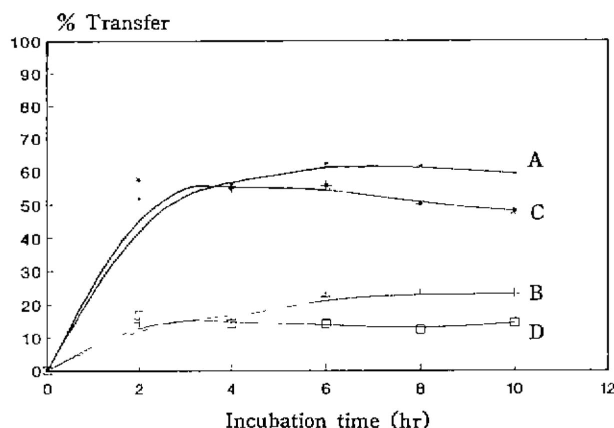

Fig. 9. Comparison of cholesteryl ester transfer activity in artificial and native substrates system.
Human plasma(0.05ml) was used as a CETP source Results are expressed as mean of triplicate samples. A: [^{3}H]-CE-HDL_R (53μg of apo A-I)+LDL_R (246μg of apo-B), B: [^{3}H]-CE-HDL_R (53μg of apo A-I)+LDL (240μg of apoprotein), C: [^{3}H]-CE-HDL(60μg of apoprotein)+LDL_R(246 μg of apo-B), D: [^{3}H]-CE-HDL(60μg of apoprotein)+LDL(240 μg of apoprotein).

up to 0.2ml. The time course of the transfer of radiolabelled CEs from HDL_R to LDL_R is shown in Fig. 8. The time course experiment has shown an increasing trend of CE-transfer activity up to 2hrs. Thus the addition of CETP to the incubation mixture induced a time-dependent redistribution of radiolabelled CEs from HDL_R to LDL_R.

The various CETP activities were expressed when used in artificial and native substrates. HDL_R was incubated LDL_R("A" in Fig. 9) or native LDL("B" in Fig 9) and native HDL was incubated LDL_R("C" in Fig. 9) or native LDL("D" in Fig. 9). CETP activities were highest when HDL_R and LDL_R were used as CE donor and acceptor respectively, and were lowest when native HDL and LDL were used. Native LDL was also a good substrate only when HDL_R was used as a CE-donor.

As described above, two major apolipoproteins were purified from hog plasma using only two steps of column chromatographies. The measurements of CETP activities can be done in a short time without ultracentrifugation which normally is required for reisolation of lipoproteins after incubation of CETP reaction mixtures. This time-consuming procedure can be eliminated by using immobilized HDL_R as a CE donor. This method, which is highly reliable so far, has been used for clinic setup mass-screening system of CETP inhibitors in our laboratory.

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