

Rapid purification of human plasma lipid transfer proteins

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Abstract Lipid transfer protein (LTP) was isolated from human plasma after lipoproteins were removed by precipitation with dextran sulfate. Three sequential chromatographic procedures were used: butyl-Toyopearl 650, CM-Toyopearl 650, and Toyopearl HW-55. The entire procedure required only a few days and purification was as high as 43,000-fold from the lipoprotein-depleted plasma with the yield of 30%. The final preparation contained two bands on sodium dodecylsulfate electrophoresis; the major and minor components had apparent molecular weights of 69,000 and 66,000, respectively. Both bands catalyzed the transfer of cholesteryl ester with the same specific activity, and had the same N-terminal amino acid sequence. Stabilization of the lipid emulsions with apolipoprotein A-I enhanced the LTP-catalyzed transfer of cholesteryl ester from low density lipoprotein with the reciprocal transfer of triglyceride in a manner similar to that previously observed with partially purified LTP (Nishikawa, O., S. Yokoyama, H. Okabe, and A. Yamamoto. 1988. *J. Biochem.* 103: 188-194). — Ohnishi, T., S. Yokoyama, and A. Yamamoto. Rapid purification of human plasma lipid transfer protein. *J. Lipid Res.* 1990. 31: 397-406.

Supplementary key words cholesteryl ester transfer protein • cholesteryl ester • triglyceride • lipid exchange

Reverse cholesterol transport may play an important role in prevention and/or regression of atheromatous vascular lesions by removing cholesterol accumulated in the cells in arterial walls. Glomset (1) hypothesized the function of lecithin:cholesterol acyltransferase (LCAT) in relation to reverse cholesterol transport many years ago: LCAT catalyzes esterification of cholesterol on high density lipoprotein (HDL) creating a gradient of free cholesterol between the surface of HDL and plasma membrane of the cells. This gradient thereby generates the efflux of cholesterol from the membrane to HDL (1). However, Glomset had already shown that esterified cholesterol on HDL was readily incorporated into low density and very low density lipoproteins in human plasma (2). The same phenomenon was also demonstrated as a change in lipid composition of each lipopro-

tein fraction during the incubation of human plasma in vitro (3). This reaction was characterized more specifically as cholesteryl ester transfer reaction several years later (4, 5). Since the transfer of cholesteryl ester among lipoproteins is nondirectional (6-9) and the rate is much faster than the rate of cholesteryl ester generation in human plasma (6, 9), LCAT-esterified cholesterol on HDL should promptly be distributed to the other lipoprotein fractions. Thus, cholesteryl ester in the core of lipoprotein particles may well be considered as a single kinetic pool in human plasma under the normal kinetic equilibrium of lipoprotein metabolism. Eventually, cholesteryl ester is carried mainly by LDL and is delivered to the liver through the LDL receptor pathway (10). Thus, both lipid transfer reaction and LDL play important roles in reverse cholesterol transport.

The lipid transfer reaction is catalyzed by the lipid transfer protein (LTP) (11-14). LTP(s) was (were) reportedly isolated from human plasma with apparent molecular weights of 58,000 to 74,000 (11, 12, 14-17) and its complete sequence of 476 amino acid residues was predicted according to the base sequence of its cloned cDNA (18). However, it is not clear whether the cloned protein represents every protein claimed to be isolated. There are a few reports that demonstrate at least two electrophoretic bands even in highly purified LTP preparations (11, 12, 14, 15).

The protein accepts cholesteryl ester and triglyceride as substrate as if they were both in a single pool (19, 20). The direction of the transfer is also random among lipoproteins as mentioned above (6-9, 19, 20). The protein

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LTP, lipid transfer protein; LCAT, lecithin:cholesterol acyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; LPDP, lipoprotein-deficient plasma.

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has an affinity for hydrophobic surfaces (11–17), and has a higher affinity for HDL than LDL (21, 22, 23). In order to express full activity, the protein requires stabilization of the surface of the substrate lipid microemulsions with apolipoproteins (20). The protein binds cholesteryl ester. The binding seems reversible so that cholesteryl esters migrate either way between the protein and the substrate lipoproteins (24).

Based on these results, a few hypotheses have been proposed for the mechanism of the reaction. Some kinetic data may support the hypothesis that the protein enhances direct exchange of nonpolar lipids between lipoproteins through their surface (9, 25). Requirement of surface stabilization of the substrate lipid particles (20) may support this as well. The other hypothesis proposes the function of the protein as a carrier of lipids between substrate particles (7). The reversible binding of the lipid to the protein and its exchange with the lipid particles strongly support this thought (24).

Since this is one of the key reactions of reverse cholesterol transport, it is important to know details of the mechanism of this reaction in order to understand the entire pathway. A simple and convenient technique for isolation of LTP is, therefore, crucial for further investigation of the reaction of this protein. Many procedures for the isolation of LTP from human plasma have been reported (11, 12, 14–17). Most of the methods previously reported for the purification of LTP, however, included many complicated steps and were time-consuming. In the present study, we report a relatively simple and rapid procedure for the purification of LTP from human plasma.

MATERIALS AND METHODS

Egg phosphatidylcholine was purchased from Avanti Polar-Lipids, Inc., and [4-¹⁴C]cholesteryl oleate, 60 mCi/mmol, was from Amersham. Other lipids were from Sigma. Butyl-Toyopearl 650(M), CM-Toyopearl 650(M), Toyopearl HW-55(S), and a gel permeation column for high performance liquid chromatography (HPLC) (TSK G3000SW, 0.75 × 60 cm) were obtained from TOSO (TOSHAAS in North America). A TSK G3000SW preparative column (2.5 × 60 cm) was generously provided by the TOSO Corp. Dextran sulfate-cellulose beads were kindly supplied by Kanegafuchi Chem. Ind. Corp. Cholesterol oxidase, cholesterol esterase, and peroxidase were purchased from Toyobo Co. Ltd., and sialidase was from Sigma. Aprotinin was purchased from Miles. The reagents for a gas-phase sequencer were purchased from Applied Bioscience. Other chemicals and reagents used in the study were of the highest quality commercially available.

Preparation of human plasma lipoproteins, apolipoproteins, and triolein/phosphatidylcholine microemulsions

Lipoproteins, LDL, and HDL, were prepared from fresh human plasma at densities between 1.006 and 1.063 g/ml, and 1.091 and 1.21 g/ml, respectively, by sequential ultracentrifugal flotation in NaBr (26). Apolipoprotein A-I was further purified from HDL by means of delipidation, gel permeation chromatography, and DEAE-cellulose ion exchange chromatography in 6 M urea as described elsewhere (27). [¹⁴C]cholesteryl oleate was incorporated into LDL without changing its chemical and protein composition according to a method described previously (28). The specific radioactivity in LDL was 400 cpm/μg cholesteryl oleate. Microemulsions of triglyceride and phosphatidylcholine were prepared according to a procedure described previously using sonication, ultracentrifugal flotation, and gel permeation, with a diameter 26 ± 2 nm (27). The final chemical composition of the emulsions used was 1.15:1 triolein:phosphatidylcholine (w/w).

Assay of lipid transfer reaction

LDL containing [¹⁴C]cholesteryl oleate was used for the assay in order to monitor the lipid transfer reaction during the purification. An appropriate aliquot of each fraction was incubated with HDL and the labeled LDL, corresponding to 80 and 95 μg of cholesterol, respectively, 10 mM Na-phosphate buffer (pH 7.4) containing 0.15 M NaCl and 6% bovine serum albumin in the final volume of 475 μl. The incubation was carried out at 37°C for 60 or 90 min. At the end of the incubation, 25 μl of a solution containing 5% Na-heparin and 1 M MnCl₂ was added to the mixture which was kept at 0°C for 30 min. The LDL was precipitated by centrifugation at 10,000 rpm for 20 min and the radioactivity left in the supernatant was determined.

Lipid transfer was also observed between LDL and microemulsions according to a previously described procedure (20). Lipid microemulsion (10 μg as phosphatidylcholine) was preincubated with apolipoprotein A-I (30 μg) for 60 min at room temperature in 10 mM Na-phosphate buffer containing 0.15 M NaCl and 0.1 mM EDTA-Na. Appropriate amounts of LTP and LDL (corresponding to 100 μg of phosphatidylcholine) were added to give a final volume of 300 μl. After incubation for various periods of time at 37°C, 500 μl of a suspension of dextran sulfate-cellulose beads in the same buffer was added to adsorb LDL at 4°C (29). The suspension contained the adsorbent beads in 83% volume measured by hematocrit and both LDL and lipid microemulsion diffused into the porous beads up to 90% of the suspension volume (29). The concentrations of lipids were

measured in the supernatant; total and unesterified cholesterol, choline containing-phospholipid, and triglyceride were measured by the enzymatic methods as described below.

Purification of lipid transfer protein from human plasma

Concentrated fresh human plasma was obtained as a drainage waste from the retained plasma by the second filter in LDL-apheresis treatment of the patients with familial hypercholesterolemia using a double membrane filtration technique (30). To 2 liters of the plasma, 75 g of MnCl_2 (2 M as final concentration) and 270 ml of 10% sodium dextran sulfate were added. The plasma was then centrifuged at 7,000 *g* for 20 min to remove precipitated lipoproteins. BaCl_2 , 23.5 g, was added to the supernatant to remove the remaining excess dextran sulfate by centrifugation at 7,000 *g* for 20 min. Lipoprotein-deficient plasma (LPDP) was thus obtained within 3 h.

Butyl-Toyopearl 650(M) was packed in a 4.4×11.5 cm column and equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 2 M NaCl and 1 mM EDTA-Na. NaCl was added to LPDP to give a final concentration of 2 M. The supernatant of the centrifugation at 7,000 *g* for 30 min, 1.75 liters, was applied to the column at a flow rate of 10 ml/min. After the column was washed with 500 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA-Na, the bound proteins were eluted with 1 liter of a linear decreasing gradient of the Tris-HCl buffer from 50 mM to 3 mM at a flow rate of 10 ml/min. The elution was contained with 500 ml of 3 mM Tris-HCl buffer and subsequently with distilled water after the elution with the gradient had finished. The eluate was monitored by absorbance at 280 nm and each 15-ml fraction was collected. The fractions containing the lipid transfer activity were collected and concentrated with ammonium sulfate at 80% saturation.

CM-Toyopearl 650(S) was packed in a 2.2×21.5 cm column and equilibrated with 10 mM Na-citrate buffer (pH 5.5). The active fraction from butyl-Toyopearl chromatography was dialyzed against the same buffer and applied to the column at a flow rate of 4 ml/min. After the column was thoroughly washed with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 0 to 1 M for 200 ml at a flow rate of 2 ml/min and each 3-ml fraction was collected. After elution with the NaCl concentration gradient, proteins were further eluted with the citrate buffer containing 1 M NaCl. The active fractions were collected and concentrated with ammonium sulfate.

Toyopearl HW-55(S) was packed in a 2.2×137 cm column and equilibrated with Na-phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1 mM EDTA-Na. The concentrated active fraction from CM-Toyopearl chroma-

tography, 1.5 ml, was applied to the column and chromatography was carried out at a flow rate of 1 ml/min. The eluate was monitored by measuring absorbance at 280 nm and each 10-ml fraction was collected. The active fractions were pooled and stored at -80°C . Some preparations were carried out in the presence of aprotinin, 20 unit/ml, throughout the procedure.

Characterization of isolated lipid transfer protein(s)

The last quarter of the active peak eluted from the Toyopearl HW-55 column, in which two bands were demonstrated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), was further analyzed by HPLC using a gel permeation column. Either analytical or preparative G3000SW columns were connected with a Gilson HPLC system and operated with 10 mM Na-phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1 mM EDTA-Na at a flow rate of 0.4 ml/min. An elution profile was monitored with absorbance at 280 nm and each 0.8-ml fraction was collected. For each fraction containing protein(s), protein concentration was determined, the lipid transfer activity was assayed, the protein(s) were analyzed by SDS-PAGE.

The N-terminal amino acid sequence of each of the two bands separated by HPLC was determined in a gas-phase sequencer (Applied Biosystems 470A Protein Sequencer equipped with a 120A PTH Analyzer). Each isolated protein 3–5 μg dissolved in 30 μl of 1% trifluoroacetate, was applied to the polyblende-coated sheet and then analyzed (31). Degraded PTH-amino acids were analyzed by a built-in HPLC on-line system. The average recovery at each cycle was approximately 95% for the first nine residues.

Each protein, 5 μg , was incubated with sialidase (0.05 unit, 0.005 unit, and 0.0005 unit) in 100 mM ammonium acetate buffer at 37°C for 5 h, and then analyzed by SDS-PAGE.

Other analytical methods

Concentrations of lipids were determined enzymatically. Triglyceride (32) and choline-containing phospholipids (33) were assayed by a colorimetric method using commercial assay kits, Triglyceride-G Test from Wako Pure Chemicals and PL Kit-K from Nihon Shoji, respectively. The microassay of total and unesterified cholesterol was according to the procedure previously described (20, 34, 35) with the following modification. The sample was incubated in 50 mM sodium phosphate buffer containing 0.1% Na-cholate, 0.05% Triton X-100, 8 mg *p*-hydroxyphenylacetic acid, 0.05 IU cholesterol oxidase, 0.5 IU peroxidase, and with or without 0.05 IU cholesterol esterase for total and unesterified cholesterol assay, respectively, in a final incubation volume of 0.5 ml at 37°C for 60 min. Protein concentration was determined by the

Pierce BCA protein Assay Kit using bovine serum albumin as a standard. SDS-PAGE was carried out according to the method described by Laemmli (36) in an 8% gel, with samples heated at 90°C for 10 min in the presence of 1% SDS and 40 mM dithiothreitol prior to application. Proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250.

RESULTS

Purification of LTP

The elution profiles from the butyl-Toyopearl column are shown in Fig. 1. The concentration of Tris-HCl buffer was calculated from the conductivity of each fraction; apparent concentrations were higher than expected probably because of a trace of NaCl from the highly concentrated NaCl used for application of the sample. The active fractions for the lipid transfer reaction were eluted at an apparent Tris-HCl concentration between 20 and 5 mM as a sharp symmetric peak separated from other major protein peaks. Fig. 2 shows an elution profile in the second chromatography using CM-Toyopearl. The active fractions were eluted after a main protein peak. Duplex bands with apparent molecular masses of 66 k and 69 k dalton as demonstrated by SDS-PAGE accounted for 70% of total protein in the peak. Other proteins (mainly below 40 k dalton on SDS-PAGE) were completely removed with Toyopearl HW-55 chromatography (Fig. 3). Although this chromatographic column material is manufactured for gel permeation, the active protein fraction was eluted at 120–150% volume of the total column volume as a single symmetric peak. The preparation from this column contained only duplex

bands described above as demonstrated by SDS-PAGE. The 66 kDa protein seemed to be less than 10% of the total protein included in the entire peak. By judging from staining density of protein bands, however, the later portion of the peak seemed to contain relatively more 66 kDa protein (Fig. 4). The presence of aprotinin throughout the purification procedure did not affect these results.

Table 1 summarizes typical results of the purification procedure. Starting from LPDP, the final-fold purification was about 43,000 and the yield of the activity was about 30%. The apparent decrease in the yield at the step of Toyopearl HW-55 chromatography was mainly due to the low recovery of the activity when the protein was concentrated with ammonium sulfate. The protein concentration in the active fractions eluted from a CM-Toyopearl column was about 30 µg/ml, and the recovery of the protein as a precipitate was 60% after the solution was stirred in 80% saturated ammonium sulfate overnight. When a small butyl-Toyopearl column was used for preconcentration of the sample before ammonium sulfate precipitation, the recovery throughout this concentration step was improved to 80%.

Characterization of isolated protein(s)

The peak of the lipid transfer activity eluted from a Toyopearl HW-55 column still contained two protein bands of 69 kDa and 66 kDa as demonstrated by SDS-PAGE. Further separation of these two bands was attempted in order to examine whether or not both proteins had lipid transfer activity. Although only 10% of the protein was accounted for by the 66 kDa protein in the entire peak, about 30% was due to the 66 kDa protein in the last quarter of the peak (Fig. 4). This part of the peak was collected and concentrated with ammonium

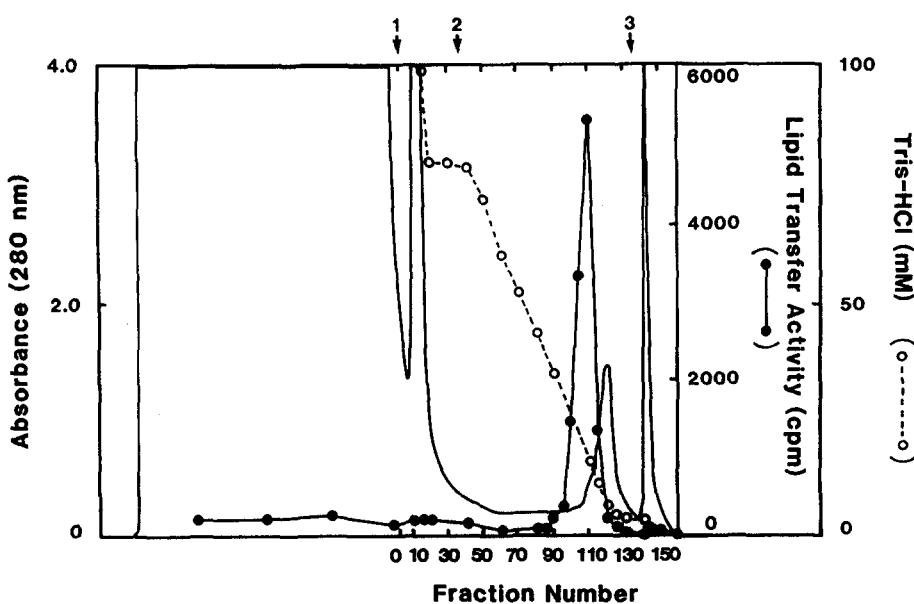
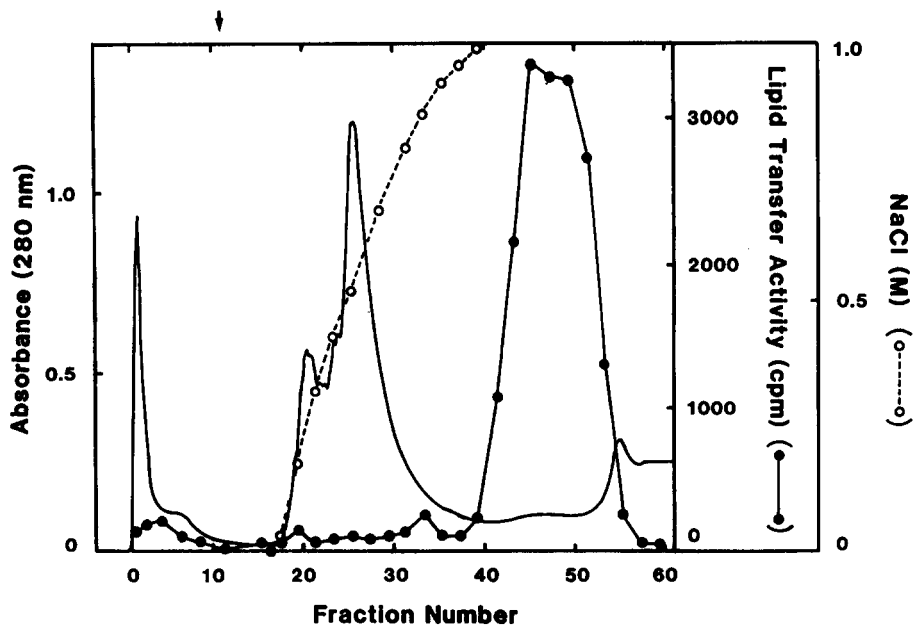


Fig. 1. Butyl-Toyopearl column chromatography. LPDP was applied to a Butyl-Toyopearl (M) column (4.4 × 11.5 cm) with 2 M NaCl. The column was washed with 500 ml of 50 mM Tris-HCl containing 1 mM EDTA-Na (arrow 1). Chromatography was conducted with a linear gradient of 50 mM to 3 mM of Tris-HCl (arrow 2) at a flow rate of 10 ml/min. Elution was continued with 500 ml of 3 mM Tris-HCl and then the column was washed with distilled water (arrow 3). Protein was monitored at 280 nm (—) and 15-ml fractions were collected. Fifty microliters of each fraction was used for the assay of lipid transfer activity. The lipid transfer activity was represented as cpm of a supernatant of an assay mixture (●—●). Conductivity of each fraction was measured and shown in the figure as the concentration of Tris-HCl (○---○).

Fig. 2. CM-Toyopearl column chromatography. The active fractions from a Butyl-Toyopearl column were applied to a CM-Toyopearl column (2.2 × 21.5 cm) equilibrated with 10 mM Na-citrate buffer (pH 5.5) at a flow rate of 4 ml/min, and then the column was washed with the same buffer. A linear gradient elution from 0 to 1 M of NaCl was performed at a flow rate of 2 ml/min for 100 min (arrow). The elution was monitored at 280 nm (—) and the NaCl concentration was calculated from conductivity measurements (○—○). Fifty microliters of each fraction was used for the assay of the lipid transfer activity (●—●).



sulfate, and then analyzed with a TSK G3000SW gel permeation column, and the eluate was monitored by absorbance at 280 nm (Fig. 5).

The protein concentration and the transfer activity in each fraction of the peak are summarized in Table 2. Fractions 2 and 3 contained mainly the 69 kDa protein and fractions 6 and 7 mainly the 66 kDa protein as demonstrated by SDS-PAGE, although the separation was incomplete (Fig. 5). The specific lipid transfer activity was similar throughout these fractions as described in Table 2. The lipid transfer activity for both proteins (fraction 3 and fraction 6) were related to the mass of the protein assayed as shown in Fig. 6. Although the N-terminal amino acid of both proteins could not be de-

tected, the sequence of the next nine amino acid residues did not show any secondary sequence. The amino acids were coincident with each other and with the sequence deduced from the cDNA base sequence previously reported (18) (Table 3). No change was observed in either of the two proteins after incubation with various amount of sialidase as judged by SDS-PAGE analysis (data not shown).

Consequently, both protein bands coisolated in the present procedure were demonstrated to have very similar properties as far as they were characterized. The entire peak fraction from Toyopearl HW-55 chromatography was, therefore, used for further characterization of the transfer reaction catalyzed by LTP.

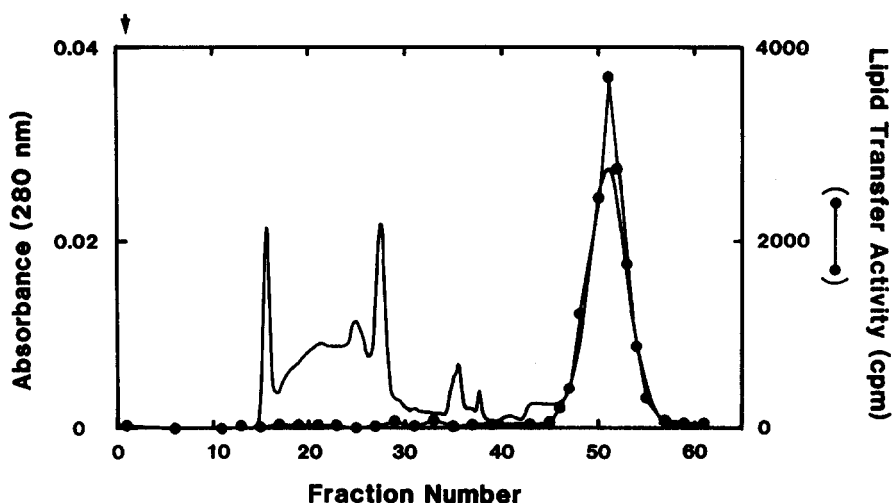
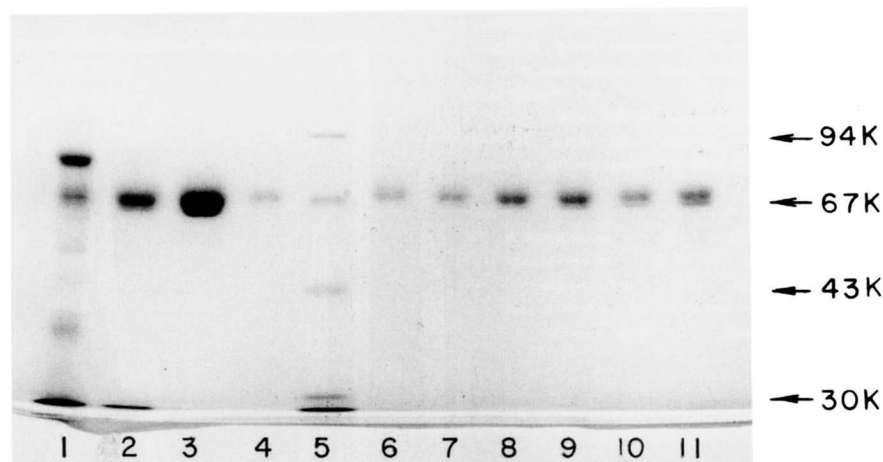


Fig. 3. Toyopearl HW-55 column chromatography. LTP fractions from CM-Toyopearl chromatography were concentrated with ammonium sulfate and applied on a Toyopearl HW-55 column (2.2 × 137 cm) (arrow). Chromatography was conducted with sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1 mM EDTA-Na, and the column was monitored at 280 nm (—). Twenty-five microliters of each 3-ml fraction was assayed for lipid transfer activity (●—●).

Fig. 4. SDS-PAGE of purified LTP. LTP fractions from CM-Toyopearl and Toyopearl HW-55 chromatography were analyzed by SDS-PAGE (8% polyacrylamide gel). Molecular weight marker proteins (phosphorylase b; 94,000, bovine serum albumin; 67,000, ovalbumin; 43,000 and carbonic anhydrase; 30,000) are in lane 5. Lane 1, 4 μ g of eluate from a Butyl-Toyopearl column; lane 2, 1.5 μ g of eluate from a CM-Toyopearl column; lanes 3 and 4, 3.5 and 0.3 μ g of eluate from a Toyopearl HW-55 column, respectively; lanes 6 and 11, 70 μ l of fraction 48 and 53 from a Toyopearl HW-55 column, respectively; and lanes 7–10, 30 μ l of fractions 49–52.



Lipid transfer reaction between LDL and lipid microemulsion

A phosphatidylcholine/triolein microemulsion was incubated with LDL in the presence or absence of LTP, and the transfer of cholesteryl ester was examined (**Fig. 7** and **Fig. 8**). The microemulsion was stabilized with apolipoprotein A-I or, alternatively, with an excess amount of bovine serum albumin as a control. With bovine serum albumin, both phosphatidylcholine and triolein decreased in a time-dependent mode in the supernatant after LDL was precipitated with dextran sulfate-cellulose (**Fig. 8**). The ratio of the two lipids, however, remained constant, indicating that this process is due mainly to aggregation or fusion of the emulsion to LDL. This reaction was minimized by covering the emulsion with apolipoprotein A-I (**Fig. 8**). Cholesteryl ester transfer was hardly mea-

surable in the supernatant in the absence of LTP. LTP-catalyzed cholesteryl ester transfer between the emulsion and LDL was dependent on the amount of LTP in the incubation mixture when the emulsion was stabilized with apolipoprotein A-I (**Figs. 7, 8**). At 5 h incubation, 10 and 15% of LDL-cholesteryl ester were transferred to the microemulsion with 0.5 and 1.5 μ g LTP, respectively. Reciprocal decrease of triglyceride and increase of cholesteryl ester in the emulsion were observed at the same time on the basis of a 1:1 molar exchange (**Fig. 8**). However, LTP enhanced cholesteryl ester transfer only to a limited extent with the emulsion when bovine serum albumin was used instead of apolipoprotein A-I (**Fig. 7**). The apparent rate of the net transfer of cholesteryl ester facilitated by LTP under these conditions was less than one-third of the rate with the apolipoprotein-stabilized emulsion, even when the decrease of the emulsion in the supernatant was considered (**Figs. 7, 8**).

TABLE 1. Purification table of LTP

Preparation	Volume ^a	Protein ^b	Activity ^c	S.A. ^d	Fold ^e	Yield ^f
	ml	mg	$\times 10^6$ cpm/h	cpm/ μ g/h		%
LPDP	2,000	222,040	6.68	0.031	1	100
Butyl-Toyopearl	404	118.0	4.62	39.2	1,302	69.2
CM-Toyopearl	149	4.23	4.21	995.3	33,066	63.0
Toyopearl HW-55	93	1.56	2.03	1301.3	43,233	30.4

^aTotal volume of each preparation.

^bTotal protein of each preparation.

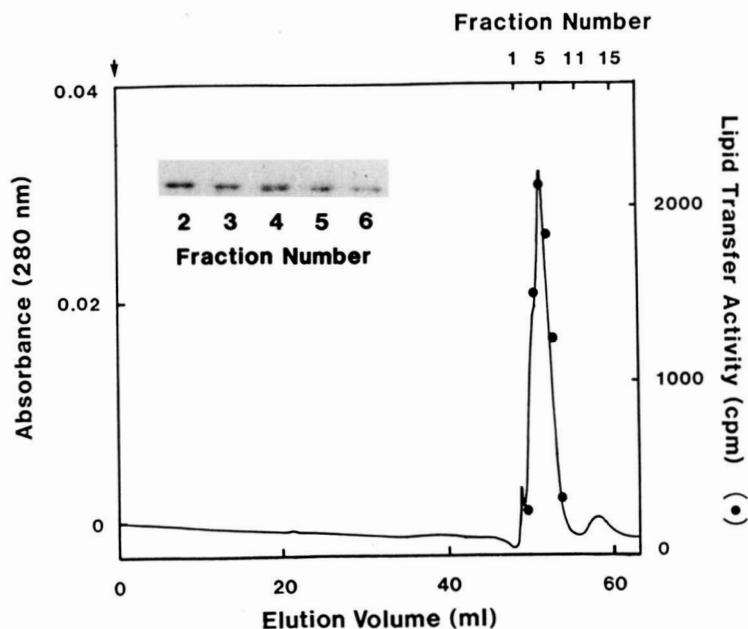
^cTotal activity of each preparation.

^dSpecific activity (S.A.) was calculated on the basis of the lipid transfer activity assayed by the method using radio-labeled LDL. Each preparation (200 mg of LPDP, 15 μ g for Butyl-Toyopearl, 1.4 μ g for CM-Toyopearl, and 0.8 μ g for Toyopearl HW-55) was incubated at 37°C for 60 min for the data presented in the table.

^eThe ratio of the S.A. of each preparation to that of an LPDP preparation.

^fThe percent of the total activity of each preparation compared to an LPDP preparation. The values represent a typical preparation. The average purification fold was $41,061 \pm 14,213$ and the average recovery was $30.2 \pm 10.3\%$ for five preparations (mean \pm SD).

Fig. 5. HPLC on a G3000SW column. The last quarter of LTP fractions from a Toyopearl HW-55 column was applied on a G3000SW column (0.75 × 120 cm) (arrow), and HPLC was conducted with 10 mM sodium phosphate buffer containing 0.15 M NaCl and 0.1 mM EDTA-Na at a flow rate of 0.4 ml/min. The eluent was monitored at 280 nm (—) and each 0.8-ml fraction was collected. Lipid transfer activities (●) were assayed for the peak fractions (2–6, Table 2) using 25 μ l of each fraction. Protein in fractions 2–6 (0.3 μ g each) was analyzed by SDS-PAGE using an 8% gel; the patterns are shown in the inset.



DISCUSSION

In the present report, we described a simple and efficient procedure for the purification of LTP from human plasma. Although LTP was isolated as duplex bands in electrophoresis in this procedure, the two molecules were both active with similar specific activity and had the same N-terminal amino acid sequence which was similar to that already reported.

Since LTP fractions obtained from CM-Toyopearl chromatography and Toyopearl HW-55 chromatography may be correctly determined simply by the elution profile monitored with absorbance at 280 nm, one might be able to skip the assay of the transfer activity after these two chromatographies and proceed to the next step. Thus, the entire procedure may require 3 days. In comparing the present method with the procedures described in refer-

ences 16 and 17, the yields were 30, 27 and 1.7%, respectively, and purification was 43,000-, 55,000-, and 108,000-fold, respectively. However, because of the different assay systems, estimated specific activities of isolated LTP were 20, 4.6, and 145 μ mol/h per mg, respectively. Thus it is difficult to compare the results on the basis of degree of purification and specific activity. Both of the previous methods included ultracentrifugal flotation of HDL, so that the entire purification process was longer.

TABLE 2. The protein concentration and the transfer activity of fractions from G3000SW

Fraction	Protein	Activity	S.A.
	μ g	cpm/90 min	cpm/ μ g/h
2	0.19	264	937
3	0.62	1501	1614
4	1.11	2122	1274
5	1.03	1834	1187
6	0.43	1240	1922
7	0.16	329	1371

Twenty five μ l of each fraction from a G3000SW column was assayed. Incubation was carried out for 90 min. Specific activity is expressed as cpm/ μ g/h in order to make the values comparable to those in Table 1.

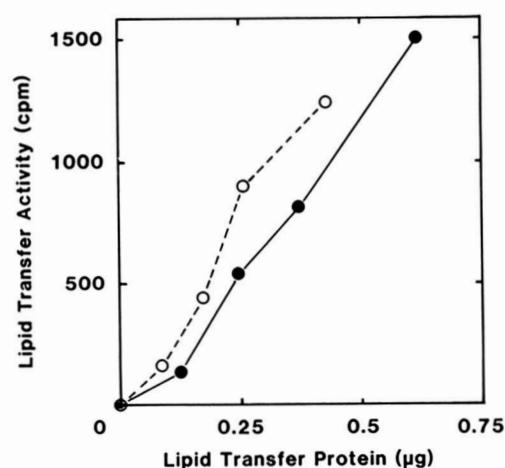


Fig. 6. Relationship of the amount of the two proteins to lipid transfer activity. Various amounts of the 66 k protein (○---○) or the 69 k protein (●—●) were incubated with labeled LDL and HDL at 37°C for 90 min. The transfer activity is represented by the counts in the supernatant after precipitation of LDL. Details are described in Materials and Methods.

TABLE 3. N-Terminal amino acid sequence of two species of LTPs

LTP ^a	Cys-Ser-Lys-Gly-Thr-Ser-His-Glu-Ala-Gly
LTP-69kD	X-Ser-Lys-Gly-Thr-Ser-His-Glu-Ala-Gly
LTP-66kD	X-Ser-Lys-Gly-Thr-Ser-His-Glu-Ala-Gly

^aAmino acid sequence of LTP deduced from cDNA (18). LTP-69kD and LTP-66kD represent fractions 3 and 6 of the HPLC analysis, respectively, shown in Fig. 5 and Table 2.

The important feature in the separation of LTP by column chromatographies used in this procedure seemed to be nonspecific interaction of LTP with a gel matrix of Toyopearl. Toyopearl HW-55, polyvinylalcohol porous beads, is manufactured for gel permeation chromatographic media. It was, however, reported that this type of gel is a good adsorbent for hydrophobic chromatography in the presence of a high concentration of salts (37). With the lower ionic strength of the buffer, LTP was eluted earlier from a Toyopearl HW-55 column as well as from a TSK G3000SW column. This suggested that this interaction was due mainly to hydrophobic interaction. On Toyopearl HW-55 chromatography, LTP interacted strongly with the gel matrix under the conditions described, while most of the other proteins were eluted by the ordinary gel permeation chromatography process. This interaction was not specific for LTP, since other

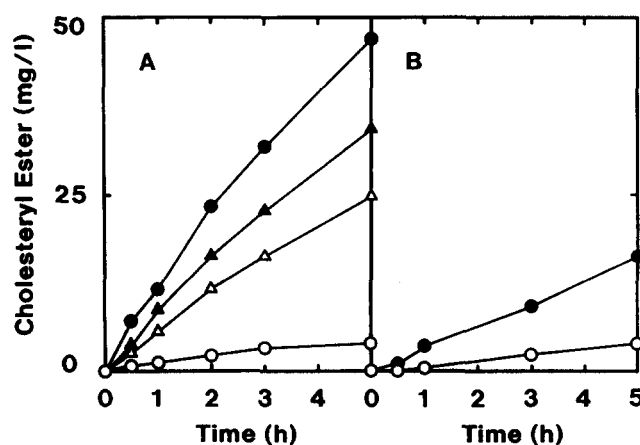


Fig. 7. Time course of cholesteryl ester transfer from LDL to lipid microemulsion. A phosphatidylcholine/triolein (1:1.15) microemulsion (w/w) was prepared as described in Materials and Methods. A lipid microemulsion (150 µg as phospholipid) was preincubated with 30 µg of apoA-I (panel A) or 200 µg of bovine serum albumin (panel B) for 60 min at room temperature in 10 mM sodium phosphate buffer containing 0.15 M NaCl and 0.1 mM EDTA-Na. Then LDL (100 µg of phospholipid and 130 µg of esterified cholesterol) and purified LTP (0 µg, ○—○; 0.5 µg, △—△; 1.5 µg, ▲—▲; and 4 µg, ●—●) were added in a final volume of 200 µl. After incubation for various periods of time at 37°C, 500 µl of the dextran sulfate-cellulose beads suspension was added to adsorb LDL at 4°C. The concentrations of esterified cholesterol were enzymatically measured in the supernatant after the beads were precipitated (6). The concentration of cholesteryl ester is expressed in terms of esterified cholesterol.

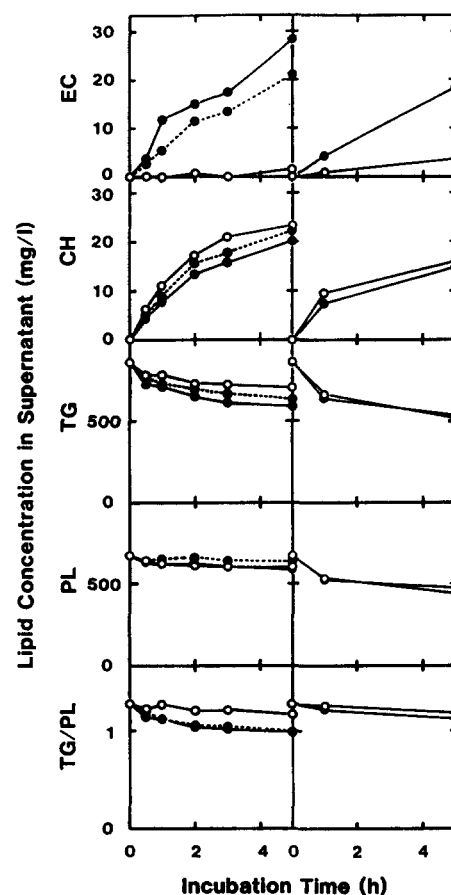


Fig. 8. Lipid transfer reaction between the lipid microemulsion and LDL. Left: phosphatidylcholine/triolein (1:1.15) microemulsion (w/w) was prepared as described in Materials and Methods. A lipid microemulsion (100 µg phospholipid) was preincubated with apoA-I (30 µg) for 60 min at room temperature in 10 mM sodium phosphate buffer containing 0.15 M NaCl and 0.1 mM EDTA-Na. Then LDL (100 µg of phospholipid and 130 µg of esterified cholesterol) and purified LTP (0 µg, ○—○; 0.3 µg, ●—●; and 1 µg, ●—●) were added in a final volume of 300 µl. Right: similar incubations using 200 µg bovine serum albumin instead of apoA-I. The amount of LTP added in the incubation mixture was 0 µg (○—○) and 3 µg (●—●). After incubation for various periods of time at 37°C, 500 µl of the dextran sulfate-cellulose suspension was added to adsorb LDL at 4°C. The lipid concentrations (EC, esterified cholesterol; CH, free cholesterol; TG, triglyceride; and PL, phospholipid) were enzymatically measured in the supernatant (6) and the ratio of triglyceride to phospholipid (TG/PL) is also shown in the bottom of the figure. The percent decrease in triglyceride in the lipid emulsion was statistically significant ($P < 0.05$) between control and 0.3 µg LTP, and between 0.3 and 1 µg LTP, for 2, 3, and 5 h of incubation with apoA-I-stabilized emulsion.

proteins with apparent molecular weights below 40,000 coeluted with LTP from a Toyopearl HW-55 column when CM-Toyopearl chromatography was omitted. The elution profiles of the LTP activity from ion-exchange chromatography also suggest nonspecific interaction of LTP with the gel matrixes. With the cation- and anion-exchange gels of Toyopearl and Sepharose (Pharmacia) that we examined, the chromatographic elution profile of LTP was always broad and overlapped many other sharp protein

peaks. Several other ion-exchange columns for HPLC (from TOSO and Synchropak) and for FPLC (Pharmacia) also gave similar results.

A chromatographic separation with CM-Toyopearl column was affected by various factors. The concentration of NaCl with which the active fraction was eluted appeared to be dependent on the volume of the column. When a small column was used or when a less steep concentration gradient was used, the peak of the activity was eluted earlier than the position described in this report, overlapping with the main protein peak. This also suggests interaction of LTP with the gel matrix.

While the rates of the transfer reaction were linearly proportional to the amount of active fractions in each purification step, there was no relationship to the amount of LPDP. Thus, it was difficult to estimate the amount of LTP present in LPDP from the activity measured. The final fold of purification, therefore, appeared to be between 20,000 and 60,000, being dependent upon the results of the lipid transfer activity assay in LPDP.

The present report demonstrated that the two protein bands were copurified with the LTP activity from human plasma. A few other reports also indicated that at least two bands might be found in the final isolated LTP preparation (11, 12, 14, 15). Both the 66 kDa and the 69 kDa proteins were shown to have similar specific lipid transfer activity and similar resistance against sialidase treatment. The amino acid sequences were identical beyond the undetected N-terminal acid for the following nine residues, suggesting that the N-terminal is cysteine (18) rather than serine (16). Although the results were similar whether or not aprotinin was used throughout the preparation procedure, we cannot yet completely eliminate possibilities of artificial cleavage at the C-terminal end during the course of isolation for the presence of the two bands. It also remains possible that the difference is in glycosylation protected against sialidase.

The experiments using phosphatidylcholine/triolein microemulsion and LDL as substrate particles for LTP revealed that the net increase of cholesteryl ester and the reciprocal decrease in triglyceride in the emulsion were caused by LTP. As was observed previously (20), apolipoprotein A-I stabilized the surface of the emulsion and protected it from aggregation or fusion to LDL. Thus, LTP has been shown to require the lipid particle surface stabilized with apolipoproteins for the maximum expression of its activity. LTP did facilitate the lipid transfer reaction but only to a limited extent when the apolipoprotein did not stabilize the particles. The enhancement by LTP was much greater with the stabilized emulsions than with those less stabilized. These findings were consistent with our previous results observed with the partially purified LTP (20). ■

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