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Expression and characterization of recombinant human lecithin:cholesterol acyltransferase

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Abstract We have established a baby hamster kidney (BHK) cell line that constitutively expresses significant quantities of human recombinant lecithin:cholesterol acyltransferase (rLCAT). LCAT cDNA was cloned into a mammalian expression vector containing the metallothionein promoter and the dihydrofolate reductase gene. After transfection, the BHK cells were treated with 500 μM methotrexate for 2 weeks to select the successfully transfected cells. Surviving colonies were subcloned and high level secretors were identified by measurement of LCAT activity and mass in the culture medium. The attachment of transfected cells to microcarrier beads enabled the efficient production of large quantities of rLCAT in a serum-free medium. After a single-step chromatography procedure, the rLCAT was purified to homogeneity with yields exceeding 1 mg of rLCAT per 100 ml of culture medium. The molecular weight of rLCAT ($\approx$ 66,000) was identical to that of purified human plasma LCAT on SDS polyacrylamide electrophoresis. The rLCAT was activated by apolipoprotein A-I and had an average specific activity that was similar to purified plasma LCAT. After selective deglycosylation with either neuraminidase or N-glycanase, rLCAT and plasma LCAT had identical molecular weights. The simplification of the production and purification of rLCAT reported here will enable a more in depth analysis of the structure and function of this enzyme.—**Hill, J. S., K. O, X. Wang, S. Paranjape, D. Dimitrijevich, A. G. Lacko, and P. H. Pritchard.** Expression and characterization of recombinant human lecithin:cholesterol acyltransferase. *J. Lipid Res.* 1993. **34:** 1245–1251.

Supplementary key words DNA transfection • expression • recombinant protein • enzymatic deglycosylation

Lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) catalyzes the transfer of an acyl group from the *sn*-2 position of phosphatidylcholine to the 3-hydroxyl group of cholesterol, resulting in the formation of cholesteryl ester and lysophosphatidylcholine. LCAT is synthesized in the liver and secreted into plasma where it associates with high density lipoproteins (HDL) (1, 2). These spherical and nascent discoidal particles contain apolipoprotein A-I which is necessary for maximum LCAT activity (3). By catalyzing the production of the majority of cholesteryl esters in human plasma, LCAT creates an unesterified cholesterol gradient between plasma membranes and circulating lipoproteins, thus facilitating the transport of cholesterol from peripheral tissues to the liver.

The mature LCAT protein is a single polypeptide of 416 amino acids with a relative molecular mass of 65,000–69,000 (4) containing 24% carbohydrate of which up to 7% is sialic acid (5). The primary structure of human LCAT has been determined by the cloning and sequencing of LCAT cDNA (6) and by direct amino acid sequencing (7). The analysis of specific chemical modifications of purified human plasma LCAT (8–10) has provided some insight into the topology of the active site of LCAT. More recently, the catalytic mechanism has been investigated by site-directed mutagenesis (11, 12). Studies with inhibitors of N-linked glycosylation (13) in Chinese hamster ovary (CHO) cells secreting recombinant human LCAT (rLCAT) have also been performed. An in depth analysis of the structure and function of LCAT has often been limited by either the labor-intensive procedures required for the purification of the plasma enzyme or the inability to produce large quantities of rLCAT from cell culture.

To further these investigations, we have developed a baby hamster kidney (BHK) cell line that constitutively expresses significant quantities of rLCAT. In addition, we describe the efficient production and purification of rLCAT including comparative data characterizing both plasma and rLCAT enzyme species.

Abbreviations: rLCAT, (recombinant) lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; CHO, Chinese hamster ovary; BHK, baby hamster kidney; DMDM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TBS, Tris-buffered saline; HBS, HEPES-buffered saline.

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MATERIALS AND METHODS

Plasmid construction and DNA transfection

Full length LCAT cDNA (6) contained within pUC19 was kindly supplied by John McLean, Genentech Inc., San Francisco. The LCAT cDNA was released from this vector by digesting with EcoRI and BamHI. After purification by agarose electrophoresis, the LCAT cDNA fragment was blunt-ended with Klenow polymerase and subsequently ligated into the SmaI restriction site of the pNUT expression vector (14, 15). The newly formed pNUTLCAT plasmid, which places the LCAT cDNA under the control of the mouse metallothionein promoter, was used to establish stable cell lines of baby hamster kidney (BHK) cells, which constitutively synthesize the native LCAT protein. In addition, the pNUT vector contains a mutant form of the dihydrofolate reductase (DHFR) gene permitting the selection of cells stably transfected with the plasmid DNA by their survival in high concentrations of methotrexate.

BHK cells were maintained in Dulbecco's modified Eagle's medium (DMEM;Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS). To mediate the transfection of BHK cells, coprecipitates of plasmid DNA (pNUT or pNUTLCAT) and $CaPO_4$ were prepared (16). Plasmid DNA (20–30 μg) in 0.50 ml of 0.25 M $CaCl_2$ was mixed with 2× HEPES-buffered saline (2× HBS) containing 40 mM HBS (pH 6.96), 280 nM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, and 10 mM glucose. The calcium phosphate–DNA mixture was incubated at room temperature for 30 min before it was added dropwise to a 100-mm culture dish containing a 50% confluent BHK monolayer. After an overnight incubation at 37°C under 5% CO_2, the transfection medium was replaced with DMEM/10% FBS for 24 h before transfected cells were selected over a period of 10–14 days in DMEM/10% FBS containing 500 μM methotrexate (15). Surviving colonies were transferred to 20-mm culture wells and grown to confluency under selected conditions. Clones expressing maximal quantities of LCAT were identified by LCAT enzyme activity and solid-phase LCAT immunoassay. One cell line, termed BHK-LCAT1, was selected for further analysis.

Endogenous radiolabeling and immunoadsorption of rLCAT

BHK-LCAT1 cells were incubated in methionine-free DMEM (DMEM-Met) for 20 min at 37°C to deplete the methionine pool. The endogenous methionine pool was then labeled for 30 min in DMEM-Met supplemented with 100–200 μCi/ml [^{35}]methionine (700 Ci/mmol, New England Nuclear). Subsequently, the labeling medium was removed and the cells were incubated with DMEM/10% FBS. After a specified incubation period, the medium was collected and the cellular protein was harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 1% Nonidet P40, 0.4% sodium deoxycholate, and 1 mM phenylmethylsulfonylfluoride) for subsequent analysis.

Solid phase immunoadsorption was used to detect the presence of LCAT in the culture medium as described previously (17). Polyclonal goat anti-human LCAT antibodies were pre-adsorbed onto agarose-immobilized protein G (GammaBind G Sepharose, Pharmacia LKB) for 30 min at 4°C. Subsequently, a sample volume of medium was added to the suspension and the mixture was rotated end-over-end overnight at 4°C. After centrifugation and washing, the adsorbed material was then eluted from the agarose beads by heating at 90°C in the presence of 2× sodium dodecyl sulfate (SDS) sample buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 40% glycerol). The beads were removed by centrifugation and the supernatant was recovered for electrophoretic analysis. [^{14}C]methylated protein molecular weight markers (Amersham Canada Ltd., Oakville, Ontario) were used as standards. Before autoradiography, the fixed gels were equilibrated with Amplify (Amersham Canada Ltd.) and dried onto Whatman 3MM chromatography paper. Autoradiograms were exposed on X-Omat AR film (Eastman-Kodak) for 16–24 h.

Culture conditions for the optimal secretion of rLCAT by BHK-LCAT1 cells

To maximize the surface area available for cell growth, porous microcarrier beads (Cultisphere-G, Hyclone Laboratories, Inc. Logan Utah) were used. BHK-LCAT1 cells (2×10^6) and 350 mg of sterile microcarrier beads were suspended in DMEM for 24 h permitting the attachment of cells to the beads. Subsequently, the cells were incubated in DMEM/10% FBS while the culture suspension was stirred at a rate of 15 rev/min for 1 minute every hour. After a 4-day growth period, the medium was changed to serum-free Opti-MEM (Gibco-BRL). After 72 h of incubation the culture medium was collected for purification.

Purification of rLCAT

Approximately 300 ml of culture medium containing rLCAT was loaded onto a phenyl-Sepharose column (10 × 15 cm) that was previously equilibrated with 0.005 M sodium phosphate, 0.3 M sodium chloride, pH 7.4. The column was washed with the same buffer until the absorbance (280 nm) fell below 0.01. Subsequently, the rLCAT was eluted with deionized water.

Antibody preparation

Human plasma LCAT was purified to homogeneity using a modification of a previously described method (18). The dodecylamine-agarose and HDL agarose columns were replaced with three columns connected in sequence: phenyl-Sepharose, Affigel-Blue, and heparin-Sepharose.

The final purified LCAT fraction was used to immunize a goat.

LCAT mass assay

The immunoassay of LCAT was carried out as described previously (17) by using nitrocellulose membranes as a solid phase support. Samples containing either culture medium or purified recombinant LCAT standard were bound to the membrane in a Bio-Rad slot-blot apparatus. Polyclonal goat anti-human LCAT antibodies and Protein G conjugated to horseradish peroxidase (Protein G-HRP, Bio-Rad) were used to visualize the protein. The blot was scanned using a Bio-Rad Video Densitometer (Model 620). Total protein determinations were carried out according to Markwell et al. (19) using bovine serum albumin as the standard.

Electrophoresis and immunoblotting

Samples containing LCAT were mixed 1:1 with 2× SDS sample buffer containing 10% β-mercaptoethanol and 0.1% bromphenol blue. The mixture was boiled for 5 min prior to loading onto a 10% polyacrylamide gel run at a constant current of 15 mA/gel for 45 min. Gels were electroblotted onto nitrocellulose paper (0.45 μM) as described by Towbin, Staehelin, and Gordon (20). Membranes were incubated with polyclonal goat anti-human LCAT antibodies and Protein G-HRP as described earlier to detect the LCAT protein.

LCAT activity assay

LCAT activity was measured using single bilayer vesicles prepared by the method of Batzri and Korn (21). Each assay contained 4.66 nmol of unesterified [^{3}H]cholesterol and 10 μg of apolipoprotein A-I. The molar ratio of cholesterol to egg yolk phosphatidylcholine was 1:4. The substrate (0.03 ml) was pre-incubated with apolipoprotein A-I (apoA-I) in 0.15 ml of 0.1 M Tris and 0.15 M NaCl at 37°C for 30 min. Esterification rates were measured over a period of 30 min at 37°C using 0.015 ml of plasma or culture medium containing rLCAT. The reaction was stopped by adding chloroform–methanol 2:1 and incubating for 2 h at room temperature to extract lipids. Labeled cholesterol and cholesteryl ester were separated by thin-layer chromatography on silica gel layers incubated in petroleum ether–diethyl ether–acetic acid 70:12:1 and radioactivity was determined by liquid scintillation spectrometry.

LCAT activities based on the method of Manabe et al. (22) measured during the production of rLCAT were carried out using the exogenous substrate containing apoA-I.

Enzymatic deglycosylation of LCAT

Neuraminidase (*Clostridium perfringens,* type V, Sigma Chemical Co.) was used to remove the sialic acid residues from LCAT by adding 3 μl of neuraminidase (1 U/ml) to 7 μl of culture medium or purified human LCAT. Samples were incubated at 37°C for 1 h. N-glycanase digestion was performed by adding 10 μl of 0.9 M sodium phosphate, pH 8.7, and 4 μl of 10% Nonidet P-40 to 15 μl of sample. The mixture was boiled for 10 min, cooled, and 1 μl of N-glycanase (250 U/ml) was added before incubating at 37°C overnight. Endoglycosidase H digestion was carried out by adding 10 μl of 0.15 M sodium acetate buffer, pH 5.8, and 4 μl of 10% Nonidet P-40 to 15 μl of sample. The mixture was boiled for 10 min, cooled, and 2 μl of endoglycosidase H (1 U/ml) was added before incubating at 37°C for 16 h.

RESULTS

Expression of rLCAT in BHK cells

The autoradiogram in **Fig. 1** indicates that BHK cells transfected with pNUTLCAT secreted a predominant protein that was recognized by antibodies specific for human plasma LCAT. This protein migrated as a broad band typical of glycosylated proteins in SDS-polyacrylamide gels and spanned a molecular weight of 60,000–67,000. This is consistent with previous determinations for purified human LCAT (4, 5). No LCAT mass was detected in the medium from those cells transfected with the pNUT vector containing no LCAT cDNA insert.

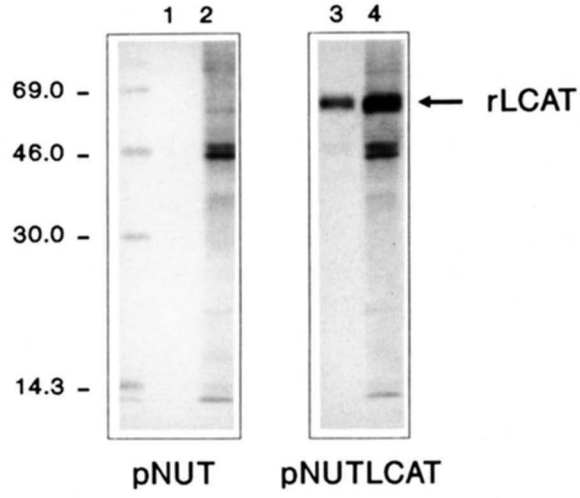

Fig. 1. Expression of rLCAT. BHK cells transfected with pNUT or pNUTLCAT plasmids were subsequently labeled with [^{35}S]methionine for a 30-min pulse. After 6 h (lanes 1 and 3) and 24 h (lanes 2 and 4) of incubation, medium was collected and rLCAT was immunoadsorbed as described in Materials and Methods. The immunoadsorbed protein was reduced and electrophoresed in 10% SDS-polyacrylamide gels. After gel drying, radioactivity was detected by autoradiography. Lanes 3 and 4 show labelled rLCAT (60,000–67,000) secreted by BHK cells transfected with pNUTLCAT. Lanes 1 and 2 represent cells transfected with the pNUT vector containing no LCAT CDNA insert. Molecular weight standards are indicated in kilodaltons.

Properties of rLCAT

Aliquots of serum-free medium were collected at several time intervals from 35-mm culture dishes containing BHK-LCAT1 cells and assayed for the presence of LCAT mass and activity. As shown in **Table 1,** the levels of both LCAT mass and activity continued to increase during a 48-h incubation period. The average specific activity of the LCAT enzyme calculated from these values of mass and activity was 3.75 ± 0.39 nmol/h per μg. The specific activity of rLCAT was similar to plasma LCAT (5.64 ± 0.32 nmol/h per μg) (23) and LCAT purified from plasma (0.75 ± 0.08 nmol/h per μg) using the same substrate. In addition, the ability of apoA-I to activate both rLCAT and human plasma LCAT was compared. Both enzymes demonstrated a typical saturation curve with a maximal activation between 5 and 7.5 μg of apoA-I/assay (**Fig. 2**). After numerous cell passages, both the activity and mass of rLCAT secreted from this stable BHK cell line remained consistent.

Production and purification of rLCAT

As mentioned previously, the LCAT cDNA is under the control of the mouse metallothionein promoter which can be stimulated by the presence of divalent cations (24). Due to its lower cell toxicity, the effect of Zn^{2+} ion concentration on the secretion of rLCAT from the BHK-LCAT1 cells was investigated. As shown in **Fig. 3,** a Zn^{2+} ion concentration of 20 μM corresponded to the highest secretion rate of rLCAT.

After a 72-h incubation in serum-free Opti-MEM, BHK cells attached to microcarrier beads secreted rLCAT at levels exceeding 10 μg/ml. The secreted rLCAT was purified by phenyl-Sepharose chromatography with the elution profile depicted in **Fig. 4.** After elution with deionized water, 97% of the original activity was recovered resulting in a 29-fold purification (**Table 2**). A homogeneous LCAT protein was eluted from the column as observed by a single band in SDS polyacrylamide electrophoresis.

TABLE 1. Secretion of rLCAT mass and activity into serum-free medium

Hours of Incubation	Mass	Activity	Specific activity
	μg/ml	*nmol/h/ml*	*nmol/h/μg*
24	4.36	17.90	4.11
30	6.91	26.39	3.82
48	10.30	34.30	3.33

Confluent BHK-LCAT1 cells were incubated in serum-free Opti-MEM for the indicated time intervals. LCAT activity and mass were determined as described in Materials and Methods. Activity units are expressed as nmol of cholesteryl ester formed per hour per ml. The values depicted are the mean of two separate experiments, each done in duplicate.

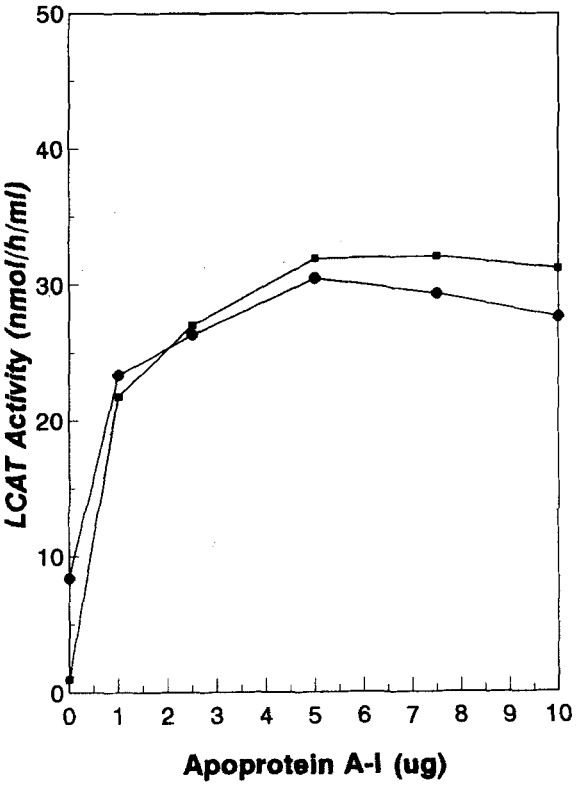

Fig. 2. Activation of plasma LCAT and rLCAT as a function of apolipoprotein A-I concentration. Assays were performed by using single bilayer cholesterol-lecithin 1:4 vesicles. LCAT activity was measured using 0.015 ml of plasma (●) or culture medium containing rLCAT (■) and expressed as nmol of cholesteryl ester formed per hour per ml. Data points are means of duplicate assays.

Enzymatic deglycosylation of plasma LCAT and rLCAT

To compare the carbohydrate structure of purified human LCAT and rLCAT, each was selectively deglycosylated with either neuraminidase to remove sialic acid residues or N-glycanase to digest N-linked carbohydrate chains. **Fig. 5** shows a Western blot of an SDS polyacrylamide gel which demonstrates that both enzymes have equivalent molecular weights before and after enzymatic deglycosylation. However, it is apparent that rLCAT migrates as a broader band compared to plasma LCAT. Digestion with neuraminidase was accompanied by a reduction in molecular weight which was consistent with previous data (5, 12) indicating that sialic acid makes up a significant portion of the total carbohydrate mass. In addition, reaction with N-glycanase reduced the apparent molecular weight of LCAT to about 48,000, which was comparable to the calculated molecular weight for the mature protein of 47,090 (6). In addition, the removal of the N-linked carbohydrate significantly decreased the size heterogeneity of rLCAT. Both plasma LCAT and rLCAT were resistant to digestion with endoglycosidase H (data not shown) in-

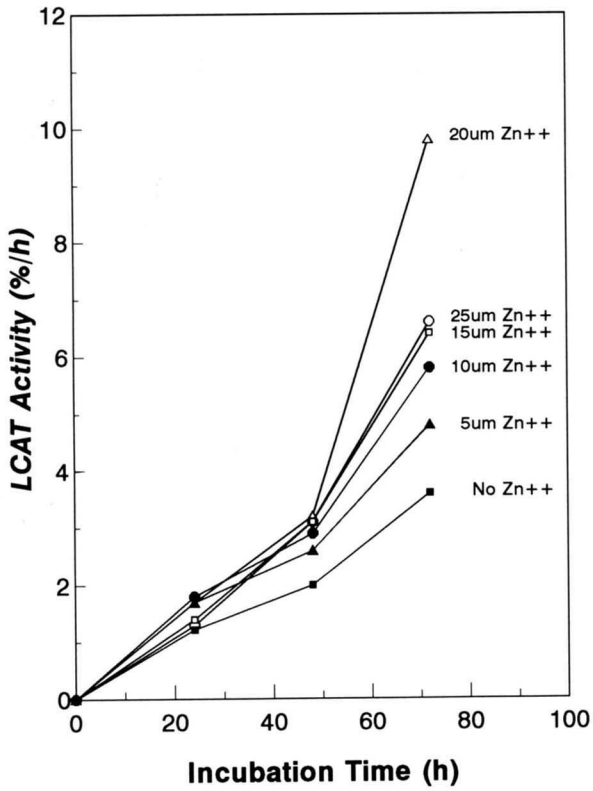

Fig. 3. Effect of Zn^{2+} ion concentration on the secretion of rLCAT. Different concentrations of $ZnSO_4$ were added to a suspended culture of BHK-LCAT1 cells after the initiation of incubation in serum-free medium. Culture medium was analyzed for LCAT activity expressed as a percentage of cholesterol esterified per hour.

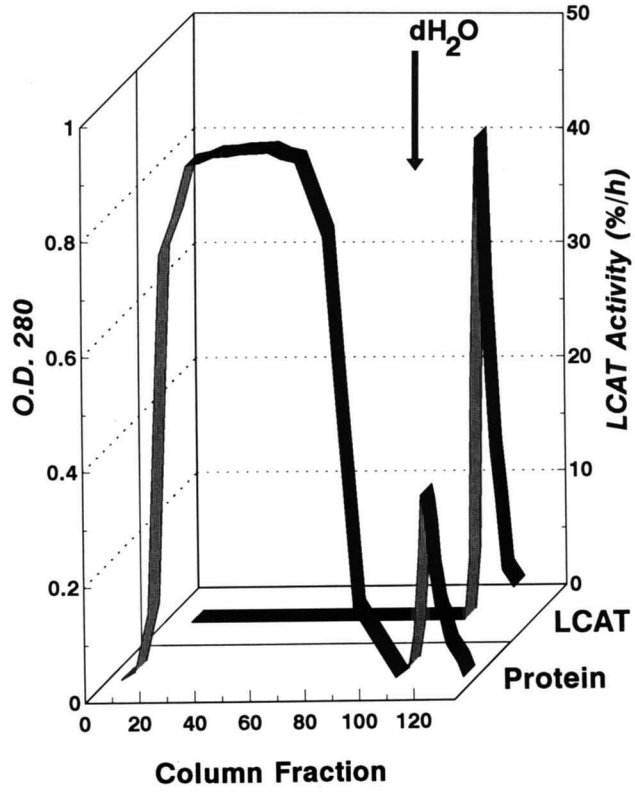

Fig. 4. Elution profile of phenyl-Sepharose CL-4B chromatography. An aliquot (300 ml) of culture medium containing rLCAT secreted from BHK cells was applied to a phenyl-Sepharose CL-4B column (10 × 15 cm) equilibrated with 0.005 M potassium phosphate buffer (pH 7.4) containing 0.3 M sodium chloride. The column was washed with the same buffer until the absorbance (280 nm) fell below 0.01 at which point the rLCAT was eluted with deionized water (arrow). The protein absorbance and LCAT activity expressed as a percentage of cholesterol esterified per hour were determined in each fraction.

dicating the assembly of either complex or hybrid oligosaccharide chains in the Golgi apparatus prior to the secretion of LCAT from the cell (25).

DISCUSSION

In this study, we have stably transfected BHK cells with an expression vector containing an LCAT cDNA. The cells secrete rLCAT whose properties closely resemble those of plasma LCAT with respect to molecular weight, activation by apoA-I, specific activity, and carbohydrate content. However, there was a greater size heterogeneity associated with rLCAT in SDS polyacrylamide electrophoresis when compared to LCAT purified from plasma. Similar observations have recently been reported for recombinant antithrombin secreted from BHK and CHO cells (26). In each case, removal of the N-linked carbohydrate resulted in the migration of a single sharp band with no apparent difference in mobility compared to the plasma protein, suggesting a greater degree of heterogeneity in the glycosylation of the recombinant protein. To determine the basis for these differences, a comparative analysis of the specific composition and structure of the carbohydrate chains for both rLCAT and plasma LCAT is currently under investigation. It is possible that

TABLE 2. Purification of rLCAT secreted by transfected BHK cells

Fraction	Volume	Protein	Specific Activity	Purification	Yield
	ml	*mg*	*units/mg*	*-fold*	*%*
Opti-MEM culture medium	350	149	105		100
Phenyl-Sepharose eluent	110	4.9	3,090	29	97

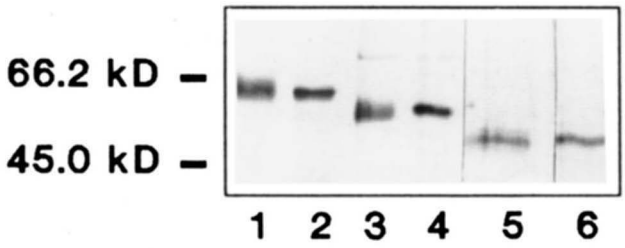

Fig. 5. Comparison of the effects of enzymatic deglycosylation of human and rLCAT. The molecular weights of human plasma LCAT and rLCAT were determined by SDS polyacrylamide gel electrophoresis. LCAT protein was blotted onto nitrocellulose membranes and detected immunologically as described in Materials and Methods. Lane 1, untreated rLCAT; lane 2, untreated human plasma LCAT; lane 3, neuraminidase-treated rLCAT; lane 4, neuraminidase-treated human plasma LCAT; lane 5, N-glycanase-treated rLCAT; lane 6, N-glycanase-treated human LCAT.

differences in the specific activity of LCAT reported for different cell lines such as COS-1 cells, 3–12 nmol/h per μg (17, 27); CHO cells, 1–2 nmol/h per μg (11–13); and human embryonic-293 cells, 46 nmol/h per μg (28) could be attributed in part to differences in the glycosylation patterns present in each mammalian cell type. In addition, the variety of methods employed for substrate preparation and activity analysis are also likely to contribute to this heterogeneity.

The increased surface area of microcarrier beads permitted a greater number of cells to grow per unit volume of culture medium. This form of tissue culture in conjunction with the use of serum-free medium has enabled us to produce greater quantities of rLCAT which can be purified to homogeneity by a single-step chromatography procedure.

The simplification of the production and purification of rLCAT reported here will pave the way for a more in depth analysis of the structure and function of this enzyme. The knowledge of natural mutations causing fish eye disease (23, 29, 30) and LCAT deficiency (26, 27, 31–33) combined with the application of site-directed mutagenesis will enable the expression and purification of mutant recombinant proteins. As a result, this expression system will be an invaluable tool to investigate the physical/chemical properties of these mutant LCAT proteins and their interaction with lipoproteins. Finally, the preparation of large quantities of homogeneous enzyme should allow the crystallization of LCAT and the elucidation of its three-dimensional structure.

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