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Lecithin: cholesterol acyltransferase (LCAT) is a key enzyme in mammalian lipoprotein metabolism. Associated with the surface of high-density lipoproteins (HDL), LCAT contributes to the homeostasis of circulating free and esterified cholesterol via the reverse cholesterol transport pathway. The purpose of these studies was to characterize a recombinant form of LCAT, secreted by a human lung cell line (Beta gene 1069/111) and to evaluate a new expression system for LCAT using transformed *Pichia pastoris* cells. A human lung cell line (Beta gene 1069/111), transfected with *pBIISK* (Stratagene)+ vector was used as the source of recombinant (rLCAT) for the first stage of characterization studies. Human lung cells were expanded in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum for the expression of the recombinant LCAT. At 80 – 90 % confluency, the medium was changed to a serum free preparation and the flasks were incubated for 48 hrs at 37°C to facilitate the secretion of the enzyme. Beta gene (1069/111) LCAT was purified from the conditioned medium using phenyl sepharose chromatography. The purified enzyme was characterized according to: carbohydrate composition, and enzyme kinetic parameters. The enzymatic characteristics, of the human lung cell line LCAT had similar *Km* and *Vmax* values to other LCAT preparations, isolated from other expression systems and human plasma. Deglycosylation reduced the molecular weight of the enzyme from about 67,000 to about

43,000 suggesting a carbohydrate component of 25-32% of the enzyme's total mass. Detailed analysis of the carbohydrate structures revealed N-glycan structures in a complex pattern of sialylated and fucosylated tri and tetra-antennary glycosides (8).

In addition to the Beta gene expression system, a *Pichia pastoris* yeast expression system was also developed consisting of human LCAT cDNA cloned into *pPICZαA* vector along with a removable amino-terminal polyhistidine tag. The *Pichia pastoris* cells were transformed with a vector containing the LCAT gene cDNA and transformants were selected on agar plates containing zeocine (100μg/ml). Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) were used to confirm the correct integration of the LCAT gene cDNA into the *pPICZαA* vector. The recombinant LCAT produced by the yeast cultures was purified by Talon affinity chromatography, taking advantage of the removable histidine tag. The enzymatic activity was determined using proteoliposome vesicles. The Yeast expression system yielded ~18 mg of enzyme protein/500 ml and thus may provide an appropriate enzyme source for characterization studies via NMR analysis and x-ray crystallography.

CHARACTERIZATION OF RECOMBINANT

LECITHIN: CHOLESTEROL ACYLTRANSFERASE,

SECRETED BY A HUMAN LUNG CELL LINE (1069-111)

AND BY *PICHIA PASTORIS* YEAST CELLS.

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DISSERTATION

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INTRODUCTION

Lecithin: cholesterol acyltransferase (LCAT; EC 2.3.1.43) is a key enzyme in lipoprotein metabolism, because it catalyzes the formation of plasma cholesteryl esters. Circulating plasma LCAT is produced in the liver and is primarily associated with HDL. LCAT is synthesized in the brain, but at a very low concentration. The role of LCAT in the brain is not yet understood. In the process of cholesteryl ester formation, LCAT forms high-density lipoproteins (HDL) from nascent HDL precursors, and small HDL (1). The gene and cDNA of LCAT, was first cloned in 1986 by McLean and colleagues (2) and sequenced in 1987 by Yang et al. (3). LCAT is composed of a single polypeptide chain of 416 amino acids (diagram 1) with a molecular mass of about 62,000 (4). The degree of glycosylation of LCAT differs depending on the expression system used (5, 6). N-linked carbohydrates analysis of recombinant LCAT also revealed that the level of glycosylation differs from one expression system to another (7). The amount of carbohydrates is approximately 32%. This was revealed by the digestion assay using N-Glycosidase, which reduced LCAT molecular weight from 64,000 to 44,000. Carbohydrate structure analysis of recombinant LCAT (beta gene 1069/111 rLCAT) by GC-MS and MALDI-MS has shown that N-linked carbohydrates structures are the largest in a complex pattern of sialylated and fucosylated tri and tetra-antennary N-linked glycosides (Figure 1 &Table 1). Further enzymatic cleavage and mass spectrometric analysis of LCAT (9) have revealed four N-glycosylation sites (Asn_{20}, Asn_{84}, Asn_{272} and Asn_{384}) with a similar

general carbohydrate chain composition of Hex6 $HexNAc_{4,5}$ $NeuAc_{1,3}$ as reported by Lacko et al (8). These sites are utilized for glycan chain attachment. In addition, to the N-glycosylation sites, plasma LCAT has two O-glycosylation sites at Thr407 and Ser409 occupied by carbohydrates chains with a general composition of Hex HexNAc NeuAc (9). The precise function of the LCAT glycan chains is not known. They likely contribute to the solubility properties of LCAT, and removal mechanism of the desialylated enzyme from plasma via the asialo-glycoprotein receptors of the liver. The carbohydrates in LCAT might also be required for the correct folding and efficient secretion of LCAT from cultured cells (10 -12). Data from the analysis of the linear sequence of LCAT and circular dichroism have shown that LCAT has a relatively low (~22%) alpha helix content and a higher (27-47%) Beta-sheet content. LCAT shares the Ser/Asp-Glu/His triad with lipases, and esterases. However, because of the low sequence homology between LCAT and lipases, esterases, the LCAT fold is still to be determined.

Esterification of cholesterol in plasma is mediated by LCAT. Under physiological conditions, LCAT converts cholesterol and phosphatidylcholine (PC) (lecithin) to cholesteryl ester and lysophosphatidylcholine (lysoPC) (Figure 2). In the mechanism of that reaction, LCAT transfers fatty acids from the C-2 position of lecithin to C-3-OH of cholesterol. The LCAT reaction utilizes highly water insoluble substrates (lipoproteins) that possess an outer monolayer similar to the surface of membranes. Lipoproteins are spherical macromolecules consisting of core of triglycerides and cholesteryl esters

surrounded by an outer shell of cholesterol and phospholipids and apolipoproteins. The reaction takes place on the surface of high-density lipoprotein (HDL) in multiple steps: Binding and activation of the enzyme LCAT, phosphatidylcholine binding, acyl enzyme formation, release of lysoPC, cholesterol binding and cholesterol ester and enzyme release (13). Fielding and co-workers showed that in the deacylation of phospholipid substrates an Asp-His-Ser triad is involved (12). Dolphin, and co-workers also suggested that Ser and His residues are involved in the phospholipase A_2 step of the LCAT reaction (14, 16). A number of research studies have focused on the acyl chain specificity of lecithin: cholesterol acyltransferase. By using dipalmitoylphosphatidylcholine (DPPC) and arachidonyl CoA, Dolphin and coworkers again demonstrated the formation of acyl-enzyme intermediates during LCAT activity. In a different study, Dolphin and colleagues also showed the existence of a tetrahedral adduct by using aromatic boronic acids (17) and sn-2 difluoroketene phosphatidylcholine analogs (18). LCAT has a variety of substrates, which, in the physiological state are present in aggregated form either in active lipoproteins or in synthetic lipid vesicles or reconstituted HDL (rHDL) (21). LCAT binds not only to HDL, but also to LDL and phosphatidylcholine (PC) (33). Recent studies have shown that the interaction on-rate constants of LCAT with rHDL, low-density lipoproteins (LDL), and egg phospatidylcholine (eggPC) vesicles are equal ($1.4 - 2.5X10^5\ s^{-1}\ M^{-1}$) and are not affected by lipid nor the apolipoproteins composition or content of the surface. (20,21). However, the off-rate constants depend on the

apolipoproteins and the nature of the lipids surface (20). These experiments indicate that LCAT binds first to the surface of lipids, and second is activated by interacting specifically with Apolipoprotein A-I (ApoAI). Fielding and co-workers have reported similar results on the specificity of lecithin: cholesterol acyltransferase with respect to phospholipid classes (22). Fielding reported that N, N-dimethylamino PE, egg PC, and dioleoyl PC were suitable substrates for lecithin: cholesterol acyltransferase (33). These researchers have demonstrated that LCAT reaction on phosphatidylcholine vesicles is activated by ApoA-I.

Site directed mutagenesis studies have shown that the activation of LCAT reaction requires the helices between residues 143 – 187 (23) on the LCAT gene. However, the exact region of LCAT that is involved in the activation by ApoA-I is not known. LCAT is involved in the transformation of lipoproteins by converting discoidal small spherical HDL into mature spherical HDL species. This role of LCAT has been studied by analyzing the effect of LCAT addition on the plasma lipoproteins of LCAT-deficient patients (25-26). Esterification of unesterified plasma lipoprotein cholesterol is catalyzed by Lecithin: cholesterol acyltransferase (LCAT), a plasma enzyme that plays a central role in maintaining reverse cholesterol transport through action with high density lipoprotein (HDL) and the subsequent action of Cholesterol ester transfer protein (CETP). HDL is considered protective against atherosclerosis, at least in part due to its role in reverse cholesterol transport, whereby excess free cholesterol (FC) is removed

from cells in peripheral tissues, such as macrophages within the arterial wall, and returned to the liver for excretion, primarily as bile acids. Free cholesterol (FC) is generated in part by the hydrolysis of intracellular cholesteryl ester (CE) stores. Several key molecules play a role in reverse cholesterol transport, including ATP-binding cassette protein A1 (ABCA1), a membrane proteins, lecithin: cholesterol acyltransferase (LCAT), and scavenger receptor class-B, type I (SR-BI). Promotion of this pathway (Figure 4) could also reduce cardiovascular diseases risks via preventing or reversing atherosclerosis. LCAT activity on the surface of HDL and LDL are designated as LCAT α-activity and the LCAT β-activity. While the α-activity requires the presence of ApoA-I or ApoAIV as cofactors, the β -activity does not require these cofactors (28). Mutations in the LCAT gene lead to Familial LCAT Deficiencies (FLD) or Fish-Eye Disease (FED) (34). There is a close relationship between specific mutations and the severity of clinical and biochemical abnormalities in patients with FLD and FED. Markedly reduced HDL cholesterol (< 10 mg/dL), ApoA-I levels, and corneal opacity are characteristic of both types of LCAT deficiencies. However, Familial LCAT Deficiencies (FLD) is uniquely characterized by a total loss of LCAT activity, progressive proteinuria and renal insufficiency whereas the ,-activity is retained in FED (25). Therefore, LCAT reactivity is essential for normal lipoprotein metabolism and for a proper equilibrium between tissue and plasma cholesterol. The role of LCAT in the prevention of atherosclerosis development has been controversial. Transgenic overexpression of human LCAT in mice results in no

significant difference in atherosclerosis development compared with non-transgenic controls (35) or greater atherosclerosis (36). Transgenic overexpression of human LCAT in rabbits results in decrease of atherosclerosis (37). The conflicting results in mice may be due to the impact of LCAT overexpression on HDL concentration and sub fraction size. Very high levels of LCAT over expression (50-100-fold) result in a 2-fold increase in plasma HDL concentrations and the appearance of large HDL_1 particles and ultimately in greater atherosclerotic lesion development in mice (36). However, more modest levels of LCAT over expression (10-20-fold) result in a smaller increase in plasma HDL cholesterol, no change in HDL particle size, and no effect on atherosclerotic lesion size compared with non-transgenic mice (37). An apparent explanation for the increased atherosclerosis with high levels of LCAT over expression is that the HDL_1 particles that result are unable to support reverse cholesterol transport.

Even though purified LCAT has been available over 25 years, and the primary structures of LCAT as well as the essential features of the catalytic site have been determined (8, 28), the three-dimensional structure of the enzyme remains to be elucidated due to the lack of diffraction–grade crystals for X-ray analysis. The first part of this research project focuses on the characterization of recombinant LCAT (rLCAT) expressed by human lung cell line by establishing the basic kinetic parameters using water soluble and macromolecular substrates and analyzing carbohydrate composition using N-Glycosidase digestion assay. LCAT activity was measured using proteoliposome vesicles. Whereas

LCAT esterase (phospholipase) activity was measured using a water soluble fluorescent substrate (phosphatidylcholine substrate, 1,2-bis [4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC)) by continuous fluorescence assay which represents the first step in the LCAT mechanism (32). The second part of this research project also consists of the characterization of the recombinant LCAT, utilizing a *Pichia pastoris* yeast expression system. The production of a functional protein is intimately related to the cellular machinery of the organism producing the protein. Many proteins have been expressed using *E. coli* as the expression system. *E. Coli* is an attractive system because its genome has been fully mapped and it requires a simple, inexpensive procedure to maintain growth and the secretion of recombinant proteins into the medium. However, *E. coli* is a prokaryote and lacks intracellular organelles, such as the endoplasmic reticulum and the Golgi apparatus that are responsible for the post-translational modifications of proteins, including glycosylation. Many eukaryotic proteins, produced in *E. coli,* are non-functional because the appropriate post-translational modifications do not occur. Therefore, researchers have recently started looking for eukaryotic yeast and mammalian expression systems for the large-scale production of mammalian proteins. *Pichia pastoris* expression system has been developed as an outstanding host for the production of foreign proteins because of the presence of its alcohol oxidase promoter (31). Compared to other eukaryotic expression systems, *Pichia* offers many advantages, because it does not secrete endotoxins or present viral contamination compared to animal cell cultures.

Furthermore, *P. pastoris* can utilize methanol as a carbon source in the absence of glucose, and produces proteins with short glycan chains. The *Pichia pastoris* expression system uses the methanol-induced alcohol oxidase (AOX1) promoter, which controls the gene that codes for the expression of alcohol oxidase. This promoter has been characterized and incorporated into a series of *Pichia pastoris* expression vectors. Since the proteins produced in *Pichia pastoris* are typically folded correctly and secreted into the medium, the *Pichia. pastoris* expression system provides an excellent alternative to *E. coli* expression systems. So far a number of proteins have been produced using this system, including tetanus toxin fragment, human serum albumin and lysozyme (28,30). Therefore, because the production of large amounts of enzyme is the major goal of this study, the *Pichia pastoris* expression system is ideally suited. The expression of recombinant human LCAT in a human lung cell line for this project thus, should allow the acquisition of basic kinetic parameters including specific activity and turnover number, using water soluble and macromolecular substrates. In order to elucidate the three-dimensional structure of LCAT through x-ray crystallography and multi-dimensional NMR analysis, the pichia pastoris expression system is proposed to yield the adequate amounts of proteins for these projects.

MATERIALS AND METHODS

Two types of expression systems are used in this project. The *Pichia pastoris* yeast expression system and Human lung cell line (Beta Gene) transfected with *pBIISK* (Stratagene)+ vector LCAT were the source of recombinant rLCAT.

I – EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN LCAT

Human lung cell line (Beta gene) was first plated in a 75 cm^2 and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (complete DMEM) until the flask is 100% confluent. Remove the medium from the flask, rinse with phosphate buffered saline (PBS) buffer. Then 1ml of 0.05% trypsin-EDTA was added to the flask and incubated at room temperature for 1 minutes. 10 ml of DMEM supplemented with 10% FBS was added to the flask to inhibit trypsin digestion. The medium was then transferred into 15 ml centrifuge tube and spin for 5 minutes. The supernatant was removed, and the cells were resuspended in complete DMEM. The homogeneous suspensions of the cells were transferred to four 75 cm^2 flasks already containing 15 ml of complete DMEM. The flask were then incubated for 48 hours at 37°C in a 5% CO_2 incubator to allow the flasks to be 100% confluent. Once the 100% confluency is reached, split the cells as described above into twelve 500 cm^2 triple layer flasks (Nunc) containing 150 ml of complete DMEM. The flasks were incubated at 37°C in a 5% CO_2 incubator until 60 % confluent. The medium was replaced with 150 ml of fresh OPTI-MEM then flasks were incubated for 30 minutes. Replaced the medium with fresh OPTI-MEM, then incubated flasks at

37°C in a 5% CO_2 incubator to allow the secretion of the enzyme. The 24 hrs and 48 hours conditioned medium were collected from the culture of beta gene cell line 1069/111, centrifuged to remove cellular debris and subjected to phenyl-sepharose chromatography. Approximately 800 ml to 900 ml of adjusted 3 Ohms collected medium containing the r-LCAT was loaded on a phenyl sepharose column (2.5x18 cm), which had previously been equilibrated with high buffer (0.005 M PO_4, 0.3 M NaCl, pH 7.4, conductivity 3 Ohms). The column was washed with the same buffer until the $absorbance_{280}$ decreased below 0.01. Then the LCAT was eluted with deionized water subsequently.

II – CHARACTERIZATION OF LCAT

A – SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

1ml of purified LCAT and conditioned media was harvested and frozen. Samples were later thawed and run on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Samples for each lane were 30 µl purified LCAT and 10 µl 4X loading dye. Samples were boiled for 5 minutes and centrifuged at 1000xg before loading. The gel was then run at 130V for 90 minutes and stained with coomassie blue G250.

B – WESTERN BLOT ANALYSIS OF THE RECOMBINANT LCAT

1ml of conditioned media and purified LCAT from phenyl sepharose chromatography were harvested and frozen. Samples were later thawed and run on a 10% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Samples for each lane were 30 µl media (~ 3 µg of protein) and 10 µl 1x loading dye. Samples were boiled for 5 minutes and centrifuged at 1000xg before loading. The gel was run at 130V for 90 minutes and electroblotted to a PVDF membrane (Biorad) at 30V for 1 hour. The membrane was blocked 1 hr at 4°C in 3% nonfat dry milk, 1x PBS. The human rLCAT antibody was diluted 1:5000 into the blocking solution. The membrane was incubated with the antibody solution overnight at room temperature. After rinsing with phosphate buffered saline containing tween 20 (1X PBST), a mouse anti-goat antibody conjugated to alkaline phosphatase (Sigma, diluted 1:500) was incubated with the blot for 1 hour at room temperature. The blot was rinsed three times in 1xPBST and visualized with nitro blue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP).

C - ENZYME ACTIVITY

LCAT esterase (phospholipase) activity was determined first by using water soluble substrate for continuous fluorescence assay, which represents the first step in the LCAT reaction. Second by using macromolecular (proteoliposome) substrate to characterize the enzymatic properties of LCAT secreted by the human lung cell line (Beta gene 1069/111).

1– FLUORESCENCE

Phosphatidylcholine,1,2-bis[4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC; Molecular Probes) was used for the continuous fluorescence assay of lecithin: cholesterol acyltransferase. The principle of the fluorescence test is based on the increase in fluorescence intensity, which is due to the separation of the two pyrene groups that form the DpybPC used as substrate (Figure 3). A stock solution of 1.25 mM of DPybPC was prepared. Then from that stock solution a series of DPybPC solutions with concentrations of 3×10^{-8} M , 6×10^{-8} M , 8×10^{-8} M , 2×10^{-7} M ,5×10^{-7} M , 10^{-6} M was prepared to conduct the assays. The LCAT fluorescence activity assay was carried out on a digital phase-modulation spectrofluorometer ISS K2 (ISS, Champagne, IL) at 25°C. The excitation wavelength was 332 nm and the emission 398. The experimental measurements were carried out for 180 seconds. The fluorescence units (FU) were then converted to molar concentrations of DpybPC using the formula: [DpybPC] $x10^{-7}$M = FU_{380} (0.88).

2 - RADIOASSAY

LCAT activity in this part of the experiment was determined using proteoliposome vesicles containing Apolipoprotein A-I (ApoA-I), lecithin, and cholesterol in a molar ratio of 0.8/250/12.5 labeled with 20μci/50 ml of [^{3}H]cholesterol (46ci/mMole). The assay components (50 μl of enzyme and 100 μl of proteoliposome vesicles) in duplicate, was shaken on a vortex mixer and incubated at 37°C for 4 hours. At the end of the incubation

period, isopropanol was used to stop the reaction. The supernatant was decanted into another tube and evaporated to dryness in a boiling water bath. Then after, the residues of each sample were taken up in about 30 µl of chloroform and spotted on a thin-layer chromatography plate. The chromatograms were developed in a solvent mixture of petroleum ether: ethyl ether: acetic acid in a ratio of 90:10:1 to separate the free and esterified cholesterol components (FC and CE). Following the run, the chromatogram was air-dried and then exposed to iodine vapor in a closed chamber to visualize the lipid containing spots. The appropriate zones for cholesterol and for cholesteryl esters were marked, the iodine was allowed to sublimate, and then the marked zones of free cholesterol (FC) and cholesterol ester (CE) were cut out for the scintillation counting. The radioactive counts for free and total cholesterol were utilized to calculate the percentage of free cholesterol esterified during the incubation period.

[CPM CE / (CPM CE + CPM FC)] X 100% = % CHOLESTEROL ESTERS

The rate of cholesterol Esterification (LCAT units) is calculated by multiplying the fractional rate (% of cholesterol Esterification/time) by the free cholesterol concentration present in the macromolecular (proteoliposome) substrates.

3. CARBOHYDRATES COMPOSITION

Purified LCAT produced by Beta gene 1069/111 cell lines cultures was subjected to digestion by N-Glycosidase (PNGaseF, New England Biolabs Inc. Beverly, MA) as follows. A stock solution of PNGaseF containing 500,00 units/ ml was used. The LCAT

sample was denatured in a solution (135 µl) containing 0.1% SDS and 0.5% mercaptoethanol by heating at 100°C for 10 minutes. Subsequent to cooling to 25°C, 20 µl of 0.5 M sodium phosphate , pH 7.5, 20 µl of 10%NP-40 were added and incubated with increasing amount of PNGaseF for 30 minutes.

II- *PICHIA PASTORIS* YEAST EXPRESSION SYSTEM

A- DEVELOPMENT OF THE *PICHIA PASTORIS* YEAST EXPRESSION SYSTEM

Pichia pastoris yeast expression system consists of human LCAT cDNA cloned into pPICZαA vector along with a removable polyhistidine tag at the amino-terminal of LCAT (Fig 7.) LCAT yeast expression system was constructed and cloned into *pPICZαA* by Dr. Min Li at the University of British Columbia, Vancouver, British Columbia, Canada).

1- TRANSFORMATION OF *PICHIA PASTORIS*

The EasyComp™ (Invitrogen) transformation method was used to transform the *Pichia pastoris* yeast GS115 (*his4, AOX1, AOX2*) strains. The EasyComp™ (Invitrogen) transformation method uses the EasySelect™ Pichia expression kit, which is composed of three solutions. Solution I (sorbitol solution containing ethylene glycol and dimethylsulfoxide (DMSO)), for the preparation of competent cells, solution II (Polyethylene glycol (PEG)) for the transformation, and solution III (salt solution), for washing and plating transformed cells. Transformants were selected on agar plate containing Zeocine (100µg/ml) as antibiotic and screened by replica plating. Polymerase

chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) were used to confirm the correct integration of the LCAT gene cDNA + *pPICZαA* vector into Pichia genome.

2 – POLYMERASE CHAIN REACTION (PCR) ANALYSIS OF PICHIA INTEGRANTS

The polymerase chain reaction (PCR) was used to determine whether or not the LCAT construct has integrated into *pichia* genome. Total DNA was isolated from *Pichia pastoris*. recombinants strains were grown at 30 °C in a shaking incubator, in 10 ml minimal dextrose medium + Histidine (MDH) to an OD_{600} of 5 to 10. Then the cells were collected by centrifugation at 1500 x g for 10 minutes. The cells were washed with sterile water by centrifugation and resuspended in 2 ml of SCED buffer (1 M sorbitol, 10 mm sodium citrate pH 7.5, 10 mm EDTA, 10 mm dithiothreitol). 0.2 mg of lyticase (sigma) was added. The sample tube was then incubated at 37 °C for 50 minutes in order to achieve about 80% spheroplasting or less. After incubation, 2 ml of 1 % SDS was added to the tube, mixed gently and set on ice for 5 minutes. After, 1.5 ml of 5 M potassium acetate, pH 8.9 was added to the tube. Was then centrifuged at 10,000 x g for 10 minutes at 4 °C. The supernatant was transferred in a new microcentrifuge tube. To the supernatant 2 volumes of ethanol was added. The tube was then incubated for 15 minutes. After the tube was centrifuged at 10,000 x g for 20 minutes at 4 °C. The pellet was resuspended in 0.7 ml of Tris-EDTA (TE) buffer, pH 7.4 and transferred into a new

microcentrifuge tube. The extraction was achieved with equal volume of phenol:chloroform (1:1 v/v) followed by equal volume of chloroform:isoamyl alcohol (24:1). The aqueous layer was then split into two microcentrifuge tubes. A 1/2 volume of 7.5 M ammonium acetate. pH 7.5 and a 2 volumes of ethanol were added to the tubes. The tubes were then placed on ice for 10 minutes. After incubation on ice, the tubes were centrifuged at 10,000 x g for 20 minutes at 4 °C. The pellets were washed once with 1 ml of 70 % ethanol, and air dried. The pellets were then resuspended in 50 µl of Tris-EDTA (TE) buffer, pH 7.5. The concentrations of the DNA samples were (.935µg/µl - 2.94 µg/µl).

Nova Taq™ polymerase chain reaction (PCR) kit was used to amplify the DNA. the reaction mix consisted of 5 µl of 10X PCR buffer, 4 µl of 25 mM of Mg^{2+} , 1 µl of dNTP mix (10 mM each), 2 µl of 10 mM 5'AOX1 primer (5'-GAC TGG TTC CAA TTG ACA AGC-3'), 2 µl of 10 mM 3'AOX1 primer (5'-GCA AAT GGC ATT CTG ACA TCC-3'), 5 µl of DNA extracted from yeast recombinants, 30 µl of PCR graded water, and 1 µl of 1.25 U Nova*Taq* DNA polymerase. DNA from non transformed yeast (GS115 wild type) was used as control. The reactions were layered with 50 µl of mineral oil. Then the genetic Thermal Cycler was loaded and run with the program shown on table 2. After running the PCR, 10 µl polymerase chain reaction products were analyzed on a 1X Tris Acetate EDTA (TAE), 0.8 % agarose gel. Loading samples were prepared by

mixing 2 µl of the DNA standard molecular weight, 2 µl of the DNA loading dye (1 %), and 8 µl of 1X TE buffer for the molecular standard. The cDNA loading samples were prepared by mixing 2 µl of the loading dye with 10 µl of the PCR products. The electrophoresis was run at 80 volts for 1 hour. After the gel was analyzed under the UV transilluminator (UVP, Inc.) equipped with Polaroid camera. Polaroid black & white instant sheet films were used.

3 – REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

The reverse transcription polymerase chain reaction (RT-PCR) was performed to determine how much full-length of mRNA is induced. Total RNA was isolated from Pichia recombinant strains. Pichia cells were grown in 100 ml buffered glycerol-complex medium (BMGY). Cells were grown at 30 °C in a shaking incubator at 250 rpm until culture reaches an OD_{600} between 2 and 6. Cells were harvested by centrifugation and resuspended in buffered methanol-complex medium (BMMY) in order to induce the expression. After 24 hours cells were collected by centrifugation. The pellet was washed with diethyl pyrocarbonate (DEPC) treated water. Then the pellet was resuspended in 20µl of DEPC-treated water and transferred into a microcentrifuge tube. To the resuspended pellet, 1ml of triazol reagent was added and the tube was kept on ice for 15 minutes. After 0.2 ml of chloroform was added to the tube, and the tube was again kept on ice for 15 minutes. At the end of the incubation time, the tube was centrifuged at

40,000 x g for 10 minutes at 4°C, then the top aqueous layer was pipetted out into a new 1.5 ml tube. 600 µl of isopropanol was added to that aqueous layer. The tube was then mixed and incubated on ice for 15 minutes. After the incubation time, the tube was centrifuge at 14,000 rpm for 10 minutes at 4°C, then the supernatant was discarded. 1 ml of 70% ethanol was added to the pellet. Then the tube was again centrifuged at 14,000 xg and the supernatant discarded. Excess of 70% ethanol was removed without dislodging the pellet. The pellet was air dry for 15 minutes, and resuspended into 70 µl of autoclaved water. The tube was then incubated at 65°C for 5 minutes then kept on ice. Immediately after the tube was stored at -80°C until ready to use. The concentration of the isolated RNA was 1.88 µg/ml

The Retroscript ™ first strand synthesis kit for RT-PCR (Ambion) was used for the cDNA synthesis, then Nova Taq™ polymerase chain reaction (PCR) kit was used to amplify the cDNA. The reverse transcription reaction mix consisted of 5 µl of total RNA (1.88 µg/ml), 2 µl of random decamers, 2 µl of 10X Reverse transcriptase buffer, 4 µl of deoxyribonucleotide tri-phosphate (dNTP) mix , 1 µl of placental Rnase inhibitor, 1 µl of reverse transcriptase, and 5 µl of Nuclease-free water. The tube content was then mixed, spins gently and incubated at 55 °C for 1 hour. After the tube was incubated at 92 °C for 10 minutes to inactivate the reverse transcriptase. The PCR was proceeded immediately as described above (see Polymerase chain reaction (PCR) analysis of Pichia integrants).

The RT-PCR products were then analyzed on a 1X Tris Acetate EDTA (TAE), 0.8 % agarose gel.

4 – EXPRESSION OF RECOMBINANT LCAT BY *PICHIA PASTORIS* INTEGRANTS

After characterizing the selected strains, by PCR and RT-PCR, Pichia recombinant strains (GS115, Mut^+) were grown in a small scale to test the effectiveness of the expression.

10 ml of Buffered Minimal Glycerol / Buffered Minimal Methanol (BMGY/BMMY) media were used. BMGY media is composed of (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base media, $4x10^{-5}$%, 1% glycerol in 700ml of water). While BMMY is composed of: (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base media, $4x10^{-5}$ %, 0.5% methanol in 700 ml of water). A single colony of GS115 Mut^+ was inoculated to 25 ml of BMGY in a 250 ml baffled flask. The flask was covered with 2 layers of sterile gauze. Then the cells were grown at 30°C in a shaking incubator (250 rpm) until the culture reaches an OD_{600} between 2-6. Then the cells were harvested by centrifugation at 1500 x g for 5 minutes at room temperature. The cell pellet was then resuspended in 200 ml of BMMY medium to induce the expression of the recombinant protein LCAT. To maintain the induction, 100% of methanol was added to a final concentration of 0.5% every 24 hours. Then at each times points (hours): 0, 6, 12, 24, 36, 48, 60 and 72, 1 ml of the expression culture was transferred to a 1.5 ml microcentrifuge tube and frozen until ready to use for the

analysis of the expression levels by InVision™ His-tag In-gel stain (invitrogen) protocol followed by detection on the Alpha Imager 3400 with a 302 nm light source and Ethidium Bromide Filter. Following InVision staining, a SimplyBlue stain was performed on the gel to show total protein load. After establishing the highest expression level time (6 hours), the expression was scaled up in order to produce more enzyme. The cultured media was then subjected to Talon™ resins (cobalt-based immobilized metal affinity chromatography resins (IMAC) for the purification of the recombinant LCAT.

5 – FRACTIONATION OF THE *PICHIA PASTORIS*

Transformed *Pichia pastoris* cells were broken by standard mechanical method using glass beads. 10 ml of the culture was collected for the mechanical lysis. The harvested medium was centrifuge at 1500 x g for 5 minutes at room temperature. The supernatant was decanted and the pellet resuspended in the breaking buffer: 50 mM sodium phosphate, pH 7.4, 1mM phenylmethylsulfonyl fluoride (PMSF, protein inhibitor), 1mM EDTA, and 5% of glycerol in 900 ml of deionized water; (100 µl of breaking buffer for 1 ml of sample each). Then to the cell suspension, an equal volume (equal volume estimated by displacement) of acid-washed glass beads size 0.5 mm (Sigma). The tubes were then vortex for 30 seconds, then incubated on ice for 30 seconds. Vortex/incubation on ice were repeated for a total of 8 cycles. Then the tubes were centrifuged at a maximum speed for 10 minutes at 4°C. The supernatants were transferred to fresh microcentrifuge and frozen until ready to use.

6 – INVISION ™ HIS-TAG IN-GEL STAIN

The samples were prepared by adding the following to a micro centrifuge tube: 10ÌL NuPAGE LDS Sample Buffer (4X), 4 ÌL NuPAGE Sample Reducing Reagent (10X), 26 ÌL Vortexed samples (culture supernatants or cells extracts previously prepared). The total volume of prepared samples was 40 ÌL in each microcentrifuge tube. Samples were then heated at 70ÆCfor 10 minutes then cooled to room temperature. One, 4-12% Bis Tris NuPAGE gel (1.0 mm thick, 10 well) was loaded and electrophoresed in MES SDS Buffer at 200V for 35 minutes. After electrophoresis, the gel was fixed for 1 hour in the fixing solution (methanol 40 ÌL, acetic acid 10 ml, ultra pure water completed to 100 ml). After the fixation, the gel was washed twice for 10 minutes each with ultra pure water to remove the fixative. Then the gel was stained with the ready-to-use solution of InVisionTM His-tag In-gel stain (invitrogen) for 1 hour at room temperature. After the gel was washed twice for 10 minutes each with 20 mM phosphate buffer (sodium phosphate monobasic 1.3 g, ultra pure water 500 ml, pH adjusted to 7.8 with 3M NaOH). Immediately after the gel was visualized on the Alpha Imager 3400 with a 302 nm light source and Ethidium Bromide Filter. Following InVision staining, a SimplyBlue stain was performed on the gel to show total protein load.

7 – TALON™ CELL THRU IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

Talon affinity chromatography (BD Biosciences), taking advantage of the removable histidine tag, was used for the purification of the recombinant rLCAT. Talon™ resins (BD Bioscience), which is cobalt-based immobilized metal affinity chromatography resins (IMAC)) was used for the purification of the recombinant LCAT expressed by Yeast cells (Figure 8). The purification of fusion protein by Talon™ Cell Thru immobilized metal affinity chromatography (IMAC) can be done in native or denaturing conditions. In this research project native purification was used (figure 9). 15 ml BD Talon resin was transferred to a sterile tube and centrifuged to remove the supernatant. Then 10 bed volumes of 1X extraction buffer (50mM sodium phosphate, 300 mM NaCl, pH 8.0) were added to the resin to pre-equilibrate it. The resin was then centrifuged at 700 x g for 2 minutes and the supernatant discarded. After equilibration, the resin was packed into a 2 ml gravity-flow column with end-cap in place. The resin was allowed to settle out of suspension. The end cap was then removed after to allow the buffer to drain until it reaches the top of the resin bed. After draining the buffer 500 ml of the culture supernatant or 50ml of the cell extract was run through the column. The column was then washed once with 5 bed volumes of 1 X extraction buffer. Then, the polyhistidine-tagged protein was eluted by 5 bed volumes of the elution buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM Imidazole, pH 7.0). The eluate was collected in 9 ml fractions.

Fractions were analyzed by reading the UV absorbance at 280 nm. Figure 13, shows the fraction numbers versus UV absorbance at 280 nm plot.

8 – CHAEACTERIZATION OF LCAT SECRETED BY *PICHIA PASTORIS*

A – SIMPLYBLUE STAINING (Invitrogen).

1 ml of purified LCAT, conditioned media, and cell extract were harvested and frozen. Samples were later thawed and run on a 4-12% Bis Tris NuPAGE gel (1.0 mm thick, 10 wells) in MES SDS Buffer according to a SimplyBlue procedure (Invitrogen). Samples for each lane were 26 µl of the purified LCAT, the conditioned media, and the cell extract; 10 µl 4X NuPAGE LDS Sample Buffer (Invitrogen) and 4 µl of 10X NuPAGE sample reducing reagent (invitrogen). Samples were boiled for 10 minutes and cooled to room temperature. The gel was then run at 200 V for 35 minutes and then SimplyBlue stained.

B – WESTERN BLOT ANALYSIS OF THE RECOMBINANT LCAT SECRETED BY *PICHIA PASTORIS* INTEGRANTS

1ml of conditioned media and purified LCAT from Talon™ Cell Thru immobilized metal affinity chromatography (IMAC), were harvested and frozen. Samples were later thawed and run on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Samples for each lane were 30 µl (~ 9µg) media and 10 µl 1X loading dye. Samples were boiled for 5 minutes and centrifuged at 1000xg before loading. The gel was run at 130 V for 90 minutes and subsequently electroblotted to a PVDF membrane (Biorad) at 30 V for 1 hour. The membrane was blocked 1 hour at 4°C in 3%

nonfat dry milk, 1X PBS. The human rLCAT antibody was diluted 1:5000 into the blocking solution. The membrane was incubated with the antibody solution overnight at room temperature. After rinsing with phosphate buffered saline containing tween 20 (1X PBST), a mouse anti-goat antibody conjugated to alkaline phosphatase (Sigma, diluted 1:500) was incubated with the blot for 1 hour at room temperature. The blot was rinsed three times in 1xPBST and visualized with nitro blue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP).

C - ENZYMATIC ACTIVITY OF THE RECOMBINANT LCAT EXPRESSED BY *PICHIA PASTORIS*

1 – RADIOASSAY

The recombinant yeast LCAT activity was determined using proteoliposome vesicles as previously described. Proteoliposome vesicles containing Apolipoprotein A-I (ApoA-I), lecithin, and cholesterol in a molar ratio of 0.8/250/12.5 labeled with 20μci/50 ml of [^{3}H]-cholesterol (46ci/mMole) were used as substrate. The assay components (50 μl of enzyme purified from recombinant cells and 100 μl of proteoliposome vesicles) in duplicate, was shaken on a vortex mixer and incubated at 37°C for 4 hours. At the end of the incubation period, isopropanol was used to stop the reaction. The supernatant was transferred into a new tube and evaporated to dryness in a boiling water bath. Then after, the residues of each sample were taken up in about 30 μl of chloroform and spotted on a thin-layer chromatography plate. The chromatograms were developed in a solvent mixture

of petroleum ether: ethyl ether: acetic acid in a ratio of 90:10:1 to separate the free and esterified cholesterol components (FC and CE). Following the run, the chromatogram was air-dried and then exposed to iodine vapor in a closed chamber to visualize the lipid containing spots. The appropriate zones for cholesterol and for cholesteryl esters were marked, the iodine was allowed to sublimate, and then the marked zones of free cholesterol (FC) and cholesterol ester (CE) were cut out for the scintillation counting. The radioactive counts for free and total cholesterol were utilized to calculate the percentage of free cholesterol esterified during the incubation period.

[CPM CE / (CPM CE + CPM FC)] X 100% = % CHOLESTEROL ESTERS

The rate of cholesterol Esterification (LCAT units) is calculated by multiplying the fractional rate (% of cholesterol Esterification/time) by the free cholesterol concentration present in the macromolecular (proteoliposome) substrates.

2 – FLUORESCENCE ASSY

The lipase activity of the recombinant yeast LCAT was determined by using Phosphatidylcholine, 1,2-bis [4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC; Molecular Probes) as substrate for the continuous fluorescence assay. A stock solution of 1.25 mM of DPybPC was prepared. Then from that stock solution, a series of DPybPC solutions with concentrations of 3×10^{-8} M , 6×10^{-8} M , 8×10^{-8} M, 2×10^{-7} M, 5×10^{-7} M, 10^{-6} M was prepared to conduct the assays. The LCAT fluorescence activity assay was carried out on a digital phase-modulation

spectrofluorometer ISS K2 (ISS, Champagne, IL) at 25°C. The excitation wavelength was 332 nm and the emission 398. The measurement the experimental measurements were carried out for 180 seconds. The fluorescence units (FU) were then converted to molar concentrations of DpybPC using the formula:

[DpybPC] $x10^{-7}M = FU_{380}(0.88)$.

RESULTS

A - EXPRESSION OF RECOMBINANT LCAT IN BETA GENE CELLS 1069/111

Beta Gene cells transfected with the *pBIISK* (Stratagene) , expressed and secreted LCAT into the cell culture medium. Subsequent gel electrophoresis and Western blotting of the clones with the anti-rhLCAT antibody revealed a single band with a molecular weight of ~67kd (Figure 5), the expected size for the native glycosylated, plasma LCAT. The recombinant LCAT expressed by the beta gene cells line, 1069/111, was then purified by phenyl sepharose chromatography, and characterized by further studies

B – PRODUCTION AND PURIFICATION OF LCAT FROM BETA GENE 1069/111

The beta gene cells, 1069/111 were expanded from 75 cm^2 flask to 500 cm^2 triple layer tissue culture flasks in DMEM medium supplemented with 10% fetal bovine serum. When the cell layer was near confluence, the cells were washed and incubated with serum free DMEM medium. The serum free DMEM medium media was aspirated and replaced with 150 ml of serum-free DMEM. The flasks were then incubated at 37°C in a 5% CO_2 incubator to allow the secretion of the enzyme. The conditioned medium were collected 24 and 48 hours from the cultures of Beta gene cells line, 1069/111, centrifuged to remove cellular debris and subjected to phenyl-sepharose chromatography. Approximately 900 ml of adjusted 3 Ohms collected medium containing the r-LCAT was loaded on a phenyl sepharose column (2.5x18 cm), which had previously been equilibrated with high buffer (0.005 M PO_4, 0.3 M NaCl, pH 7.4, conductivity 3 Ohms). The column was washed

with the same buffer until the absorbance$_{280}$ decreased below 0.01. Then the LCAT was eluted with deionized water subsequently. The purity of the LCAT preparation, following phenyl–sepharose chromatography is shown in Figure 5, indicating an essentially homogeneous band upon SDS-PAGE.

C – CHARACTERIZATION OF THE PURIFIED HUMAN RECOMBINANT LCAT

a - ENZYMATIC ACTIVITY

Both water soluble and macromolecular (proteoliposome) substrates were used to characterize the enzymatic properties of LCAT secreted by the human lung cell line (1069/111). The purpose of these dual measurements was to establish a comparative steady state kinetic parameters for the human lung cell enzyme in addition to providing a primary standard for LCAT specific activity measurements. Table 1 and

Table 2 show respectively the percentage of rLCAT activity recovery and the kinetic data obtained with both water soluble and macromolecular substrates. The *Km* and V_{max} values were comparable with both types of substrates and for LCAT isolated from the conditioned media collected from human lung cell line culture (beta gene 1069/111).

b) CARBOHYDRATES AMOUNT IN EXPRESSED LCAT

The recombinant LCAT produced by 1069/111 cultures and purified by phenyl–sepharose chromatography was subjected to digestion by N-Glycosidase (PNGaseF, New England Biolabs Inc. Beverly, MA) as follows. A stock solution of PNGaseF containing

500,00 units/ ml was used for the deglycosylation of rLCAT. About 20 μg of the rLCAT was denatured in 1X glycoprotein denaturing buffer (0.1% SDS and 0.5% mercaptoethanol) by heating at 100°C for 10 minutes. Subsequent to cooling to 25°C, 20 μl of 10X G7 buffer (0.5 M sodium phosphate , pH 7.5), 20 μl of 10%NP-40 were added and incubated with increasing amount of PNGaseF for 30 minutes. The digestion of Beta gene LCAT (1069/111) with PNGaseF resulted in a reduction of molecular weight from approximately 64,000 to 44,000 (Figure 6) representing a carbohydrate component of about 32%.

D – EXPRESSION OF LCAT IN *PICHIA PASTORIS* YEAST SYSTEM

1 –TRANSFORMATION AND SCREENING OF *PICHIA PASTORIS* INTEGRANTS

Pichia pastoris cells were transformed using the EasyComp™ (Invitrogen) transformation method. Transformants were selected on agar plate containing Zeocine (100μg/ml) as antibiotic and screened by replica plating. Figure 10, shows the selecting plate of different clones on yeast extract peptone dextrose (YPD) containing 100 μg/ml of the antibiotic Zeocine. The transformants from the selection plate were further screened for the correct integration of the LCAT cDNA gene by PCR and RT-PCR using *5'AOX1* (5'-GAC TGG TTC CAA TTG ACA AGC-3') and 3'AOX1 (5'-GCA AAT GGC ATT CTG ACA TCC-3') as primers, that annealed upstream and downstream region of the plasmid multiple cloning site. Figure 11, shows the results of the genomic PCR analysis. As it can be noticed on the PCR result, the pichia transformant two bands compared to the Pichia

non-transformed cell (wild type). The first band on the PCR result of the transformant corresponds to the size of the *AOX1* gene (approximately 2.2 kb), and the second band to the cDNA of the LCAT gene (1.2Kb). This result shows that the pichia integrants screened were Mut+.

The RT-PCR was used to determine whether or not the LCAT gene was being induced and transcribed. Total RNA was isolated from induced cultures as described previously in material and method. Figure 12, shows the results of the RT-PCR analysis. It can be noticed that the LCAT gene was transcribed at different levels in the different clones screened. All the clone screened showed two bands compared to the wild type, which showed one bands.

2 – EXPRESSION OF RECOMBINANT LCAT FROM TRANSFORMED PICHIA PASTORIS INTEGRANTS

Although rlCAT was not supposed to be expressed intracellularly, by its native yeast host, both secreted and intracellular expression of the rLCAT were attempted in P. pastoris. Transformed P. pastoris were grown in Buffered Minimal Glycerol (BMGY) in a 250 ml baffled flask at 30°C with vigorous shaking, to an OD_{600} between 2-6 OD_{600} between 2-6. Cells were harvested by centrifugation at 1500 x g for 5 minutes at room temperature. The cell pellet was then resuspended in 200 ml of Buffered Minimal Methanol (BMMY) medium to induce the expression of the recombinant protein LCAT. Methanol was replenished to a final concentration of 0.5% every 24 hours from a 100%

stock to maintain the induction. Samples were removed periodically for analysis. Samples were centrifuged at 1500 x g, the supernatants were transferred into news microcentrifuge and frozen until ready to use. The cell extracts were prepared from the cell pellets as described in the material method. Both supernatant and cell extracts were analyzed by Standard InVision™ His-tag In-gel stain (invitrogen) protocol followed by detection on the Alpha Imager 3400 with a 302 nm light source and Ethidium Bromide Filter. Following InVision staining, a SimplyBlue stain was performed on the gel to show total protein load. Figures 14 and 15 show the gels patterns after InVision and SimplyBlue staining. After induction, a histidine tag signal can clearly been seen in the extract.

3 – PURIFICATION OF RECOMBINANT LCAT BY TALON™ AFFINITY CHROMATOGRAPHY

Samples prepared from the cell cultures supernatant and cells extract were analyzed by: Standard InVision staining, SimplyBlue stain, and Western blot. A single colony of GS115 Mut$^+$ was inoculated to 25 ml of BMGY in a 250 ml baffled flask. The flask was covered with 2 layers of sterile gauze. Then the cells were grown at 30°C in a shaking incubator (250 rpm) until the culture reaches an OD_{600} between 2-6. Then the cells were harvested by centrifugation at 1500 x g for 5 minutes at room temperature. The cell pellet was then resuspended in 200 ml of BMMY medium to induce the expression of the recombinant protein LCAT. To maintain the induction, 100% of methanol was added to a final concentration of 0.5% every 24 hours. Then at each times points (hours): 0, 6, 12,

24, 36, 48, 60 and 72, 1 ml of the expression culture was transferred to a 1.5 ml microcentrifuge tube and frozen until ready to use for the analysis. Figures 14 and 15 show the gels patterns after InVision and simplyBlue staining of the media, purified samples from Talon chromatography, and the cell extract preparations. As previously stated, the detection was performed on the Alpha Imager 3400 with a 302 nm light source and Ethidium Bromide Filter. Following InVision staining, a SimplyBlue stain is performed on the gel to show total protein load. Figure 16 shows: The western blot analysis of the purified recombinant LCAT by Talon chromatography using human rLCAT antibody (diluted 1:5000) as primary antibody and a mouse anti-goat antibody conjugated to alkaline phosphatase (Sigma, diluted 1:500) as secondary antibody. The blot was then visualized with nitro blue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP). After elution of LCAT, about 50% of the original activity was recovered resulting in a 6-fold purification (table 4).

4 – EZYMATIC ACTIVITY OF THE *PICHIA PASTORIS* RECOMBINANT LCAT

Macromolecular (proteoliposome) and Phosphatidylcholine, 1,2-bis [4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC; Molecular Probes) were used as substrates to characterize the enzymatic properties of LCAT secreted by yeast transformants. The purpose of these dual measurements was to compare the kinetic parameters for the LCAT expressed in the yeast to the human lung cell enzyme. After many attempts to assay the activity of the recombinants LCAT from yeast, no significant

activity was observed especially with the fluorescence assay, which had always shown a straight line (graph not shown). Table 4 shows a comparison of the percentage of the conversion of the cholesterol to the cholesterol esters after fours hours between beta gene LCAT and recombinant yeast LCAT.

DISCUSSION

The characterization of Lecithin Cholesterol Acyltransferase in this research project started with the purification, the characterization, and the establishment of basic kinetic parameters of LCAT secreted by the human lung cell line (beta gene 1069/111). For the purpose of eventual enzyme replacement therapy, the main goal of this project was to produce LCAT from a human source with maximum immune compatibility. Although the enzyme secreted by the human lung cell line (beta gene 1069/111) reacted strongly with the antibody prepared against human recombinant LCAT, the immune determinants may be represented by specific epitopes, including the oligosaccharide components. Consequently further research will be required to ascertain that the human lung cell line (beta gene 1069/111) LCAT is indeed compatible with the LCAT deficient patients' immune system. The initial velocity of proteoliposome esterification, as determined by the amount of the label in CE per hour and per μg of protein increased linearly with increasing NSL concentration over the range of 0.22 nmoles to 4.32 nmoles. Some previous works (38) have shown that Phosphatidylcholine, 1,2-bis [4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC) can be used to study the LCAT phospholipase reaction by monitoring the hydrolysis of DPybPC in continuous manner (39). The hydrolysis of DPybPC by LCAT could be detected at very low substrate concentration. The initial velocities were proportional to the substrate concentration up to $8x10^{-8}$ M, but decreased at high concentration substrate concentration possibly due to

the saturation of the enzyme. Some previous works (38) have shown that Phosphatidylcholine, 1,2-bis [4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC) can be used to study the LCAT phospholipase reaction by monitoring the hydrolysis of DPybPC in continuous manner (39). The hydrolysis of Phosphatidylcholine,1,2-bis[4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC) by LCAT could be detected at very low substrate concentration. The initial velocities were proportional to the substrate concentration up to $8x10^{-8}$ M, but decreased at high concentration substrate concentration possibly due to the saturation of the enzyme. The enzymatic characteristics of the human lung cell line LCAT (beta gene 1069/111) had similar *Km* and *Vmax* (table 2) values to other LCAT preparations, isolated from several sources (40), including the BHK cell line we reported earlier (8). The inability to compare the specific activities of enzyme preparations that had been prepared in different laboratories has been one of the persistent difficulties in LCAT research. This problem had been primarily due to the numerous procedures developed for the preparation of the macromolecular substrates for LCAT (19, 33, 38) and the lack of uniformity of these procedures. In this research project, we report for the first time, an approach to standardize the specific activity for LCAT based on the enzyme assay developed by Bonelli and Jonas (38), utilizing a fluorescent phosphatidylcholine substrate, 1,2-bis [4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC). Even though accurate enzyme assays require standardization of the fluorometer before

undertaking a set of rate measurements, the data obtained are reliable, reproducible and should be comparable to similar assays conducted in other laboratories. The enzymatic characteristics of the human lung cell line LCAT had similar *Km* and *Vmax* values to other LCAT preparations, isolated from several sources, including the BHK cell line and human lung cell line reported on earlier (7, 8). The carbohydrate analysis of LCAT derived from the human lung cell line (beta gene 1069/111) revealed glycan components similar to human plasma and other recombinant forms of LCAT (40). Deglycosylation substantially reduced the molecular weight of the enzyme from about 67,000 to about 43,000 (Figure 6) suggesting the presence of a carbohydrate component representing 25-30% of the enzyme's total mass. Further carbohydrate analysis done by Lacko and coworkers (8) suggested that the carbohydrate structures appear to be exclusively the N-linked type as no O-linked glycans were detected. However, recently studies have shown the existence of O-linked glycans (48). The most probable carbohydrate structures (Figure 1), based on the mass spectrometry analysis appear to be primarily tetra-antennary glycans compared to the tri-antennary saccharides found in human plasma LCAT and the biantennary structures found in the enzyme secreted by the McArdle 7777 cells (40). The discrepancy in the respective n-acetyl neuraminic (sialic) acid contents of the human lung cell LCAT versus the enzyme secreted by the McArdle 7777 cells (Table 3) may be due to different concentrations of sialidases present in the respective cultures. The presence of the difucosyl structures in the human Lung cells (Beta gene, 1069/111) is

unique and may be characteristic of the complex glycoproteins synthesized in lung tissue. In summary, the LCAT produced by the human lung cell line is very similar in most respects to the circulating human plasma enzyme (47) as well as to other recombinant species secreted by mammalian cells (40). Consequently, the human lung cell LCAT and other forms of LCAT (Table 1) could be appropriate for enzyme replacement therapy, provided that immune compatibility of the preparation is confirmed and that appropriate large scale production can be developed. Yeast expression system was designed to provide a reasonable amount of the LCAT enzyme in order to allow the completion of the important fundamental studies in LCAT enzyme chemistry. As already mentioned in the introduction, when compared to other eukaryotic expression systems, *Pichia pastoris* has many advantages, because it does not have the endotoxin or the viral contamination problems compared to bacteria. In addition, *P. pastoris* produces proteins with short carbohydrates and can utilize methanol as a carbon source in the absence of glucose. Data obtained from PCR, RT-PCR confirmed the correct integration of the vector along with the LCAT cDNA (Figure10, 11). Even though the recombinant LCAT was supposed to be secreted, both the culture media and the cell extract were analyzed. LCAT produced by transfected *Pichia pastoris* cell cultures after elution had about 50% yield resulting in 6 fold purification. The Yeast expression system is expected to yield about 100 mg/ml to support crystallization trials or labeling with stable isotopes. Therefore, with 18 mg of

the protein enzyme /500 ml, obtained from cultures, it may be possible to characterization studies via NMR analysis and x-ray crystallography.

The InVision staining (Invitrogen) of the gel showed a signal on the cell extract, the culture media, and no signal on the purified and enterokinase digested enzyme. However, a unique and clear band can be seen on the SimplyBlue (Invitrogen) staining. These gels patterns showed that the recombinant protein is expressed, but not fully secreted since the LCAT bands are seen on both cultured media and cell extract. Glycosylation can be most likely one of the reasons of this incomplete secretion of the recombinant LCAT (40). In addition, interaction between newly synthesized proteins can lead to the formation of protein aggregates. A. Helenius and collaborators have shown that interaction between newly synthesized proteins can cause them to be retained in the endoplasmic reticulum (ER). From in vitro folding studies, It is well known that formation of large aggregates were due to incompletely folded proteins (41). A unique protein band was observed between 60 – 80 kda on the SimplyBlue staining (Figure 14). The western blot (figure 15) showed a band, which exhibited three non distinct major bands. This western blot pattern can be explained by level of expression of LCAT isoforms. In fact, it has been shown that at least six isoforms of LCAT can be isolated by isoelectric focusing (42; 43, 44). The Carbohydrate residues (sialic acid) determine the polymorphism of all glycoprotein. A glycan on a given amino acid in a glycoprotein always presents a structural heterogeneity, called microheterogeneity, which is characterized by the substitution of sugar residues on

a similar core structure (45). N-linked glycosylation of recombinant protein secreted by Pichia pastoris might have an impact on their subsequent immunoreactivity with the antibody prepared against that protein (46). Consequently, visualization of the expressed protein will depend on several factors, including its expression level, its solubility, and its molecular weight. The activity of the recombinant yeast LCAT was low on the culture supernatant, the cell extract, and the purified enzyme by talon chromatography (table 4). Non digested enzyme, digested enzyme (with the enterokinase enzyme (Sigma) for the removal of the histidine tag at the N-terminal), culture media, and cell extract were assayed for the LCAT activity. All the results were low compared to the LCAT expressed in the human lung cell line activity. 5', 5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) is a compound that reversibly inhibit LCAT. In the presence of beta mercaptoethanol, the inhibitory effect of DTNB, is suppressed. The use of DTNB alone reduced the activity of the enzyme nearly to zero compare to the used of DTNB in the presence of Beta mecaptoethanol. This reduction of the activity is an evidence that the LCAT enzyme was effectively expressed, but was not so active as the LCAT expressed from Beta gene cell line. Since glycosylation is one of the most important modifications of proteins after their synthesis, we can attribute this apparent lack of recombinant enzyme activity to the different post-translational processing. Miller and coworkers have shown that the N-linked oligosaccharides are responsible for the proper folding and the conformational stability of the LCAT, and determine the ability of LCAT to interact with

its substrates (5) It has been shown that glycosylation patterns vary with the cell line, the culture medium, culture supplements, time after infection and the protein expressed (49). Pichia pastoris and mammals have some same initial steps in the glycosylation. The first same step is the transfer of $Glc_3Man_9GlcNAc_2$ from the endoplasmic recticulum (ER) to the newly synthesized protein by an oligosaccharyltransferase complex. The second same step is the trimming by glucosidases II and I. The third same step is the removal of α-1,2-mannose residue by endoplasmic reticulum α-1,2-mannosidase. The $Man_8GlcNAc_2$ resulting from these three steps is the common N-glycan found on most glycoproteins leaving the fungal or mammalian endoplasmic reticulum. After these three steps, the glycosylation pathway diverge (Figure 17) (50). In the golgi, human mannosidase remove from $Man_8GlcNAc^2$ to yield $Man_5GlcNAc_2$, which is a precursor for the complex N-glycan formation. While glycosylation in Pichia pastoris yield hypermannosylated N-glycans with more than 100 mannose residues (51, 52). Therefore, as it can be noticed, LCAT glycosylation patterns vary with its source. Miller coworkers also evaluated the effect of LCAT source on its function, by evaluating the K_m and V_{max} of LCAT from different sources. The results have shown that the difference in glycosylation had an impact on the enzyme kinetics by reducing the apparent V_{max} of the enzyme, with small changes in the apparent K_m (9). Site directed mutagenesis studies have also shown that there is a reduction in the apparent V_{max} but not in the apparent K_m (11, 12). Binding affinity studies have also shown differences between LCAT from SF21 cells infected with

a recombinant baculovirus (bLCAT), HepG2 cell-expressed LCAT, and plasma-derived LCAT. Others studies on α-*N*-Acetylgalactosaminidase (rαNAGAL rαNAGAL) (53) have also shown that N-glycosylation may be critical for proper folding and efficient secretion of rαNAGAL in *P. pastoris.*

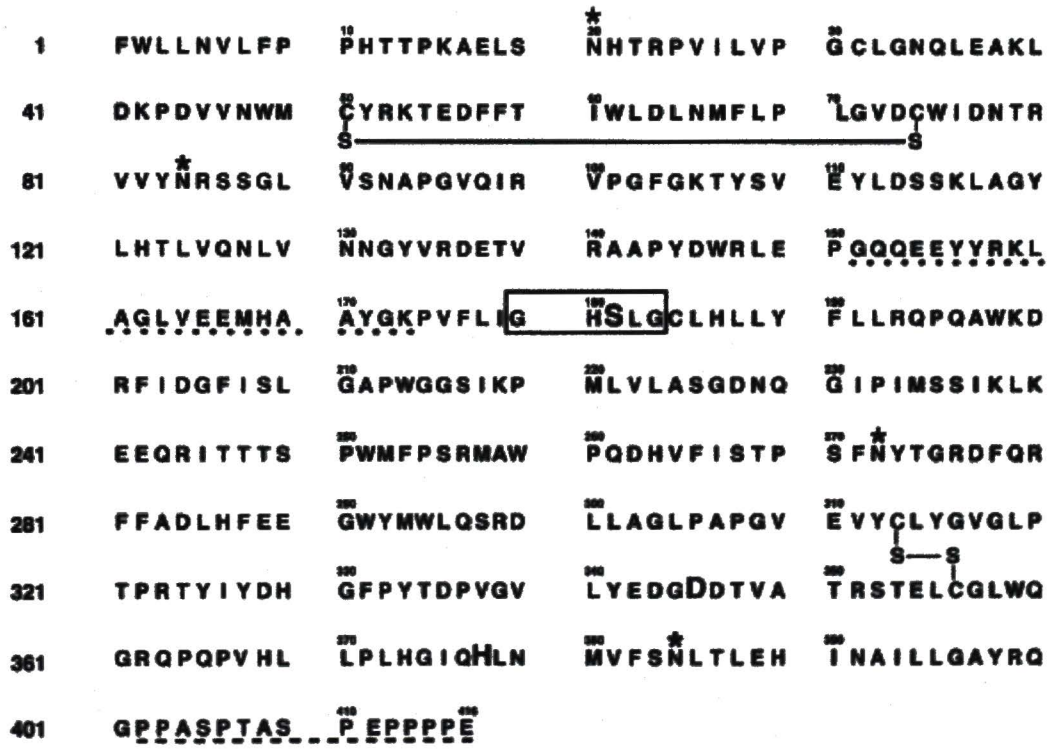
In summary data from this research project show that differences in protein glycosylation did not have an impact on the expression level of the recombinant LCAT (rLCAT) from Pichia pastoris transformant, but on the secretion efficiency and the activity of the recombinant enzyme.

SUMMARY

There are similarities between LCAT secreted by the human lung cell line (beta gene 1069/111) and the circulating human plasma enzyme (47) as well as to other recombinant LCAT secreted by mammalian cells (40). Consequently, the human lung cell line (beta gene 1069/111) and other forms of human lung cell LCAT could be appropriate for enzyme replacement therapy, provided that immune compatibility of the preparation is confirmed and that appropriate large-scale production can be developed. The kinetic parameters of the human lung cell line had similar *Km* and *Vmax* values to other LCAT preparations, isolated from several sources, including the BHK cell line and human lung cell line reported on earlier (7, 8). The carbohydrate analysis of LCAT purified from beta gene 1069/111 revealed glycan components similar to human plasma and other recombinant forms of LCAT (40). Deglycosylation substantially reduced the molecular weight of the enzyme from about 67,000 to about 43,000 (Figure 6) suggesting the presence of a carbohydrate component representing 25-32% of the enzyme's total mass. LCAT produced by transfected *Pichia pastoris* cell cultures after elution had about 50% yield resulting in 6 fold purification. The Yeast expression system is expected to yield about 100 mg/ml to support crystallization trials or labeling with stable isotopes. Therefore, with 18 mg of recombinant LCAT/500 ml, obtained from cultures in this project, it may be possible to characterization studies via NMR analysis and x-ray crystallography. The low activity of the recombinant LCAT expressed from *Pichia*

pastoris might be due to the differences in glycosylation between mammalian cells and *Pichia pastoris* yeast cell line. The structure, composition, and amount of glycosylation most likely have a direct or an indirect effect on the overall structure of LCAT, thus influencing the active site, the interfacial binding site and/or the activation of LCAT. Experiments indicate that LCAT binds first to the surface of lipids. Then second is activated by interacting specifically with Apolipoprotein A-I (ApoAI). Therefore, the recombinant LCAT from Pichia pastoris might be improperly folded due to the N-glycosylation in Pichia pastoris, which yield hypermannosylated N-glycans with more than 100 mannose residues (51, 52). Results from this research project show that a specific expression system has an impact on the expression level and the activity of a given protein.

Diagram. 1. Linear sequence of human LCAT [2 and 3]. The four stars indicate the *N*-glycosylation sites on LCAT. In addition, T_{407} and S_{409} are *O*-glycosylated. The heavy line between C_{50}–C_{74} demarcates the lid region of LCAT. Dots under residues 151–174 indicate an amphipathic helix (helix 4–5) that may be involved in the binding of phospholipid at the active site. The boxed sequence GXSXG is conserved in the active site of various lipases. In LCAT, the S_{181} residue found in this sequence is one of the components of the catalytic triad; the other two members of the triad are D_{345} and H_{377}, indicated in large, bold letters. The Pro rich C-terminal sequence, marked by the dashed line, can be removed without affecting LCAT activity on lipoproteins.



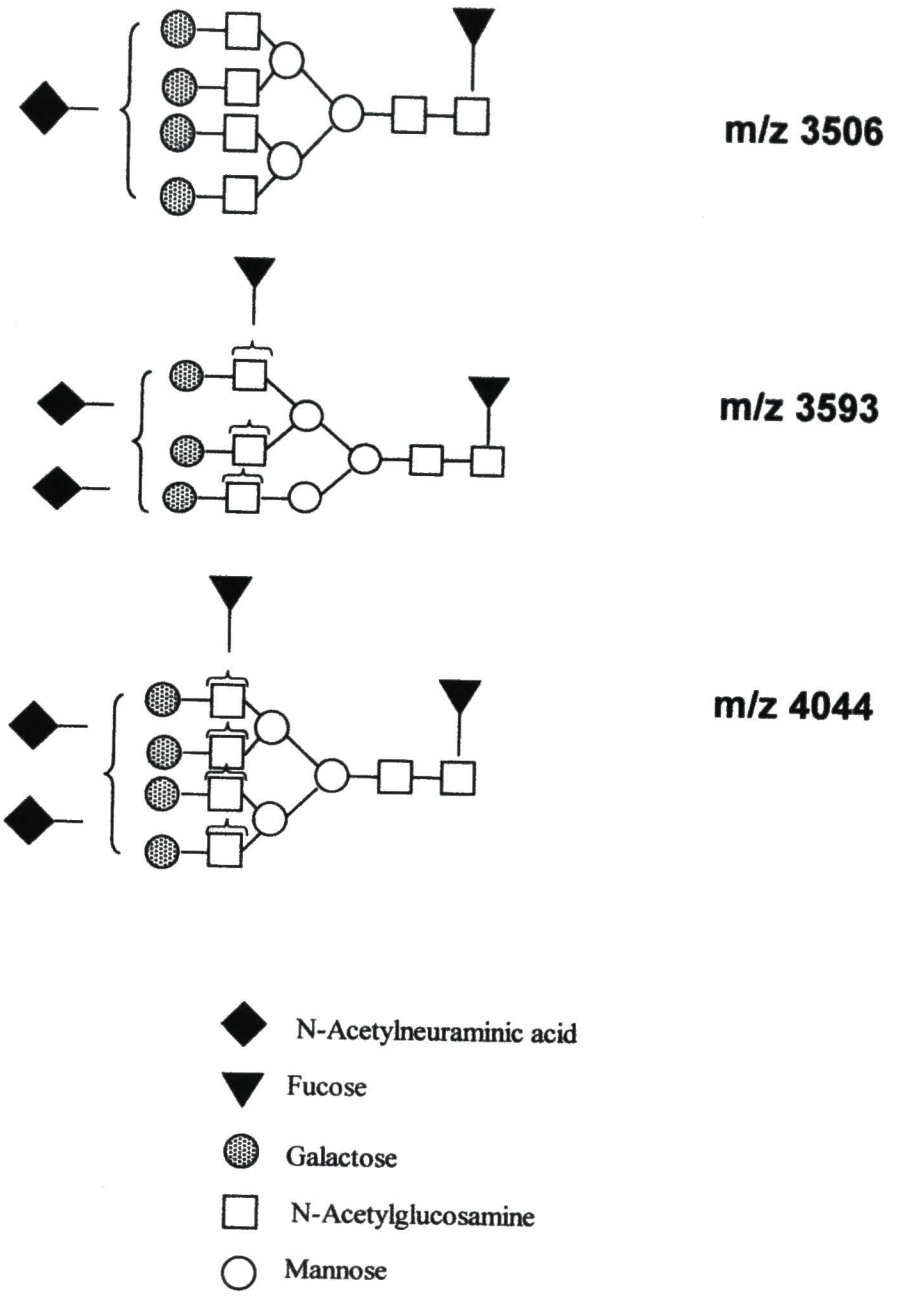


Figure 1. Possible structures of N-linked oligosaccharides released from Human lung cell line (beta gene 1069/111) purified LCAT

Signals m/z	Possible oligosaccharide assignments
2607	$NeuAc(Fuc)Hex_5HexNAc_4OMe^+Na^+$
2664	$(Fuc_2)Hex_5HexNAc_5OMe^+Na^+$
2695	$(Fuc)Hex_6HexNAc_5OMe^+Na^+$
2837	$(Fuc_3)Hex_5HexNAc_5OMe^+Na^+$
2870	$(Fuc_2)Hex_6HexNAc_5OMe^+Na^+$
3044	$(Fuc_3)Hex_7HexNAc_5OMe^+Na^+$
3057	$NeuAc(Fuc)Hex_6HexNAc_5OMe^+Na^+$
3114	$(Fuc_2)Hex_6HexNAc_6OMe^+Na^+$
3146	$(Fuc)Hex_7HexNAc_6OMe^+Na^+$
3233	$NeuAc(Fuc_2)Hex_6HexNAc_5OMe^+Na^+$
3320	$(Fuc_2)Hex_7HexNAc_6OMe^+Na^+$
3494	$(Fuc_3)Hex_7HexNAc_6OMe^+Na^+$
3506	$NeuAc(Fuc)Hex_7HexNAc_6OMe^+Na^+$
3593	$NeuAc_2(Fuc_2)Hex_6HexNAc_5OMe^+Na^+$
4044	$NeuAc_2(Fuc_2)Hex_7HexNAc_6OMe^+Na^+$

Table 1. Molecular weights of molecular ions (above 2500) separated during the MALDI-MS run from the PNGase digest of tryptic fragments of recombinant LCAT secreted by the human lung cell line (1069/111)(Lacko et al., 1998).

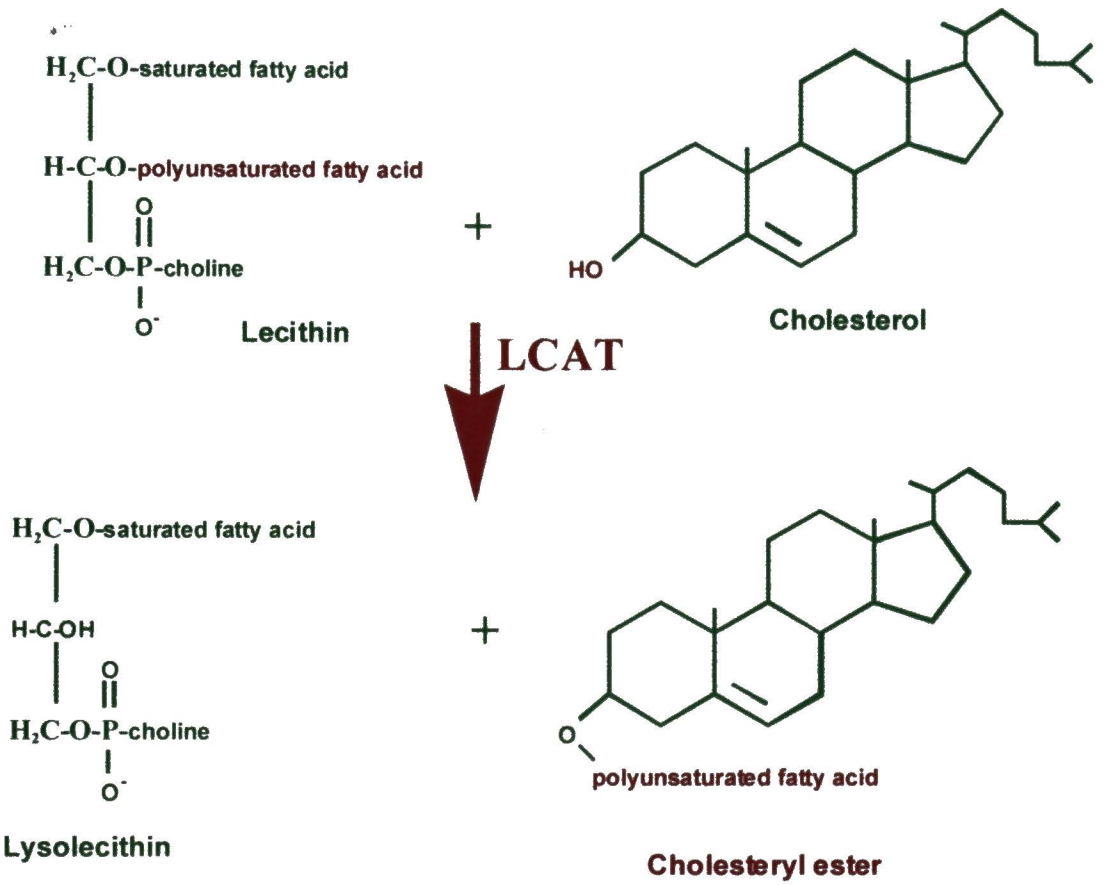


Figure 2. The Catalytic mechanism of LCAT

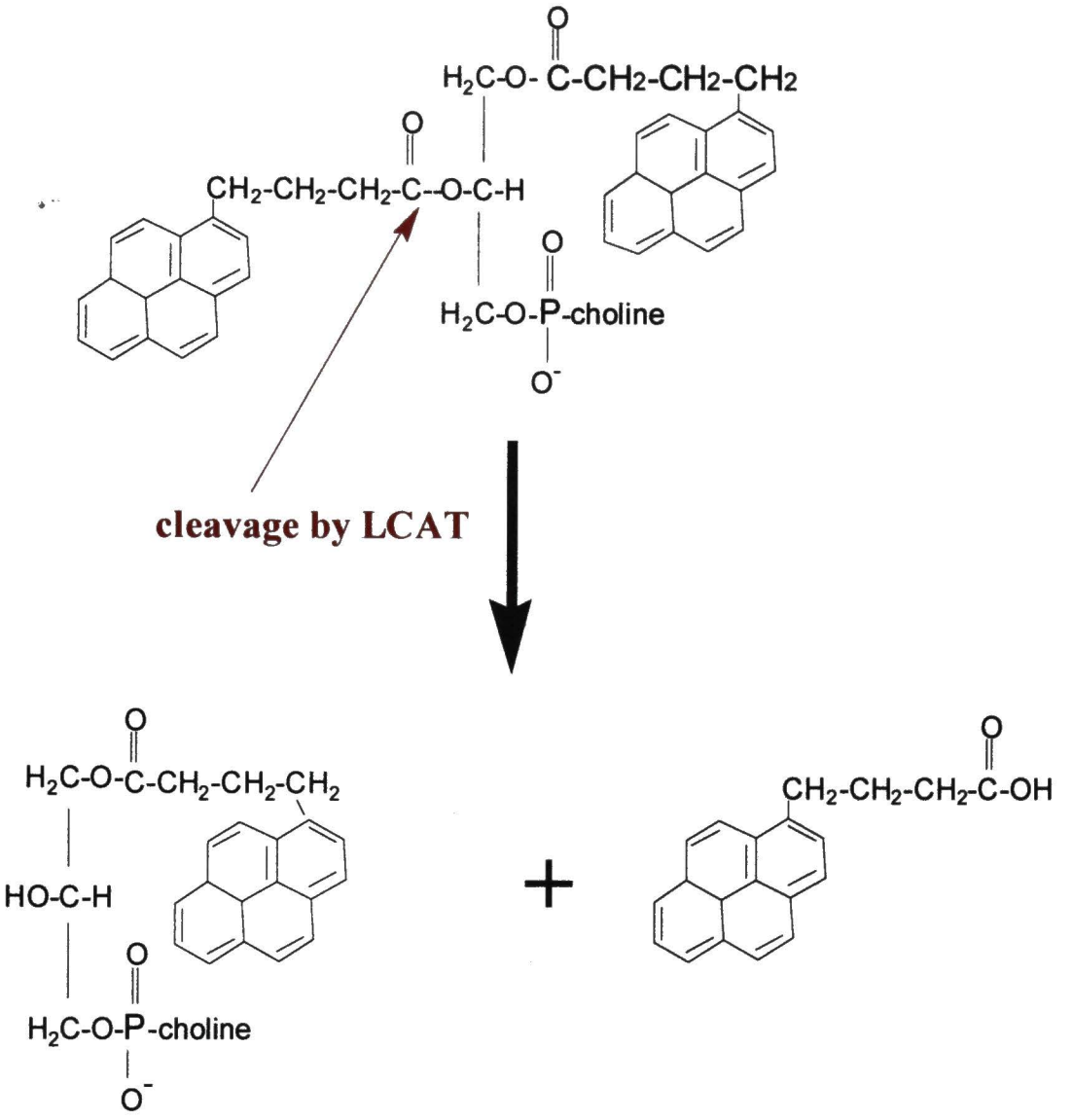


Figure 3. Principle of the LCAT fluorescence assay using Phosphatidylcholine,1,2-bis[4-(1-pyreno-butanoyl]-sn-glycero-3-phosphocholine (DpybPC; Molecular Probes) as substrate.

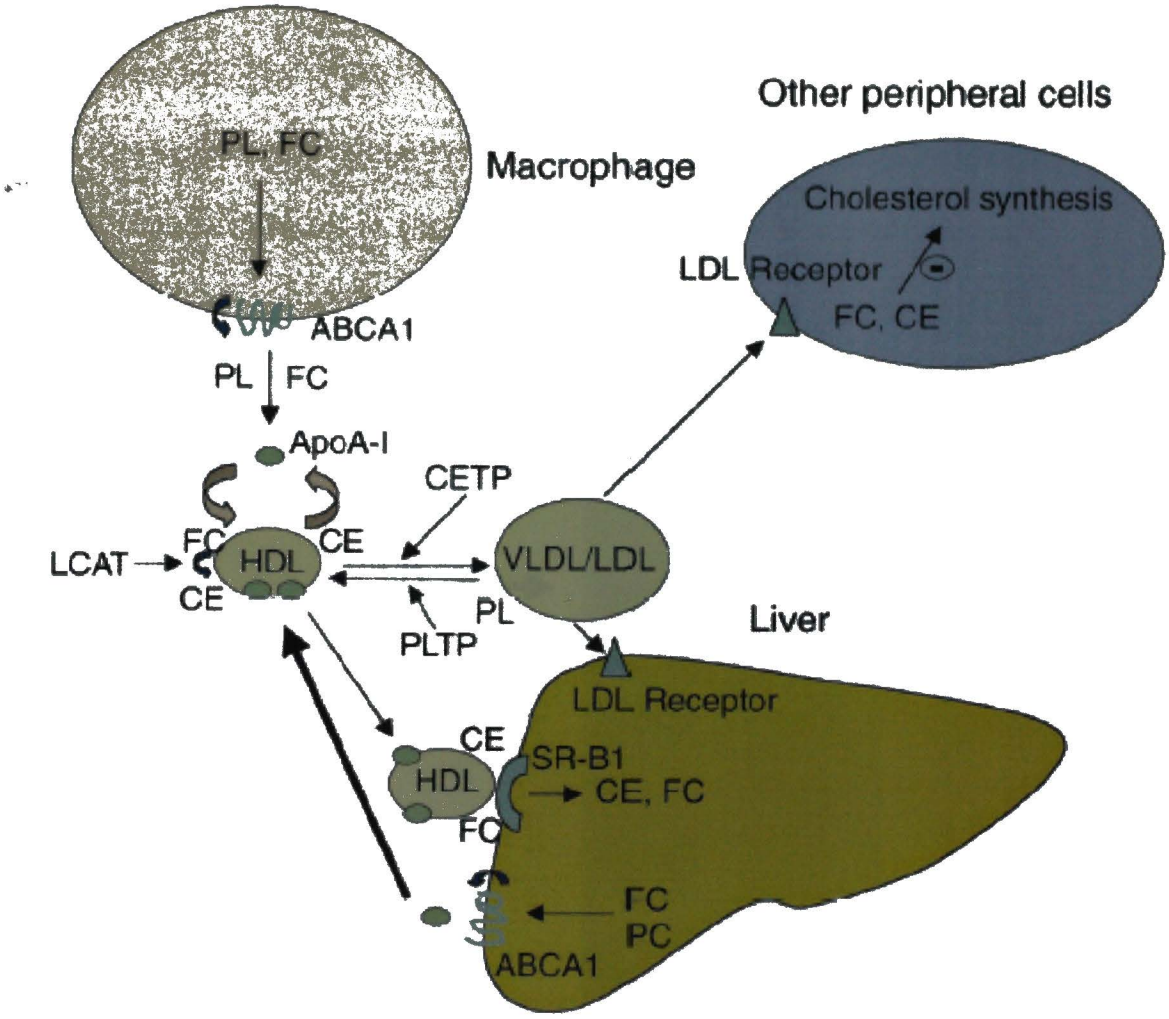


Figure 4. HDL and Reverse cholesterol transport (Alan R. Tall, 2001).
Several key molecules play a role in reverse cholesterol transport, including ATP-binding cassette protein A1 (ABCA1), lecithin: cholesterol acyltransferase (LCAT), and scavenger receptor class-B, type I (SR-BI). Promotion of this pathway could in theory help reduce atherosclerosis

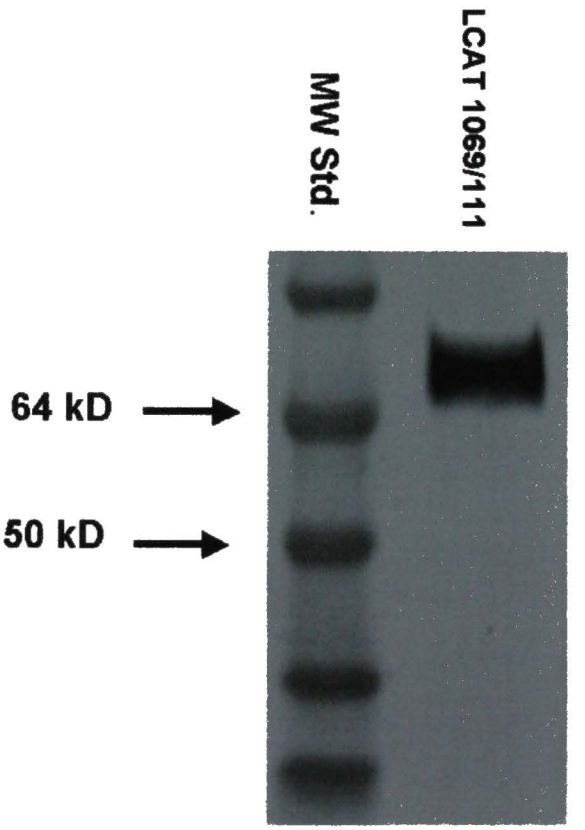


Figure 5. SDS-PAGE pattern of purified LCAT, secreted by the human lung cell line (Beta gene, 1069/111)

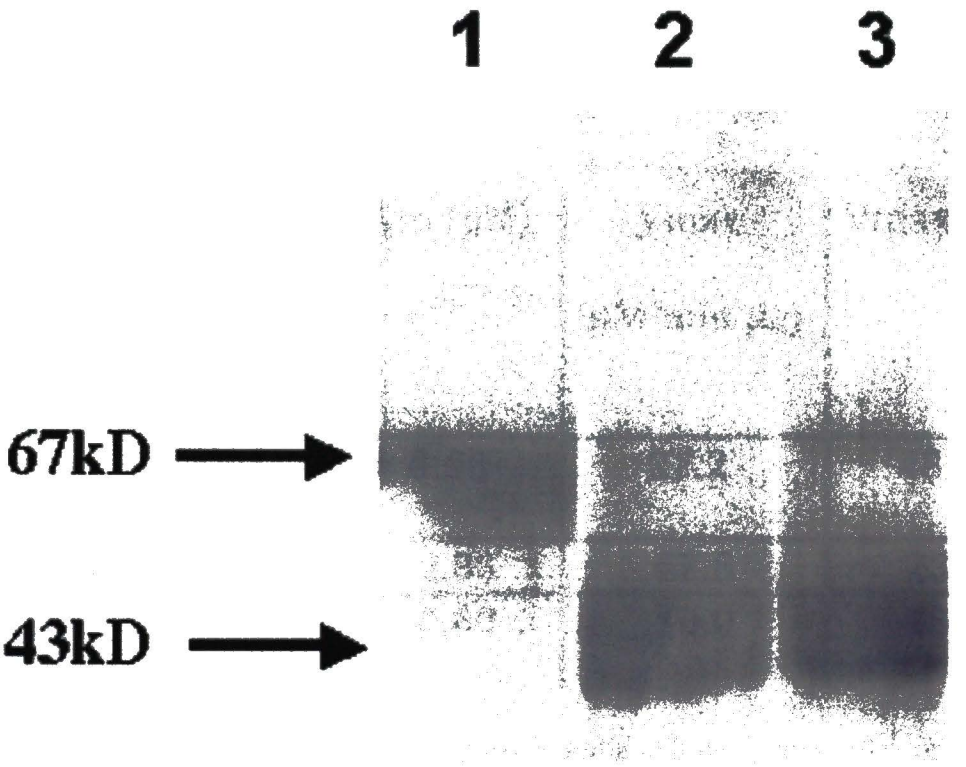


Figure 6. Digestion of LCAT (Beta gene, 1069/111) with PNGaseF.

Lane 1. 0 time, Lane 2. 3hr. 25 μg PNGaseF,
Lane 3. 3hr 50μg PNGaseF

Table 2. Enzyme assays conducted with both water-soluble fluorescent substrate and with radioactively labeled macromolecular substrate.

Assay Type	Km (μM)	Vmax (μM/min/μg)	Vmax/Km
Fluorescence assay[1]	**0.56**	**27.2**	**48.6**
Fluorescence assay[2]	**2.86**	**67.6**	**23.6**
Radioassay[1]	**0.60**	**10.0**	**16.7**

Conducted with LCAT, purified from conditioned media of human lung cells[1] or baby hamster kidney[2] (BHK) cells.

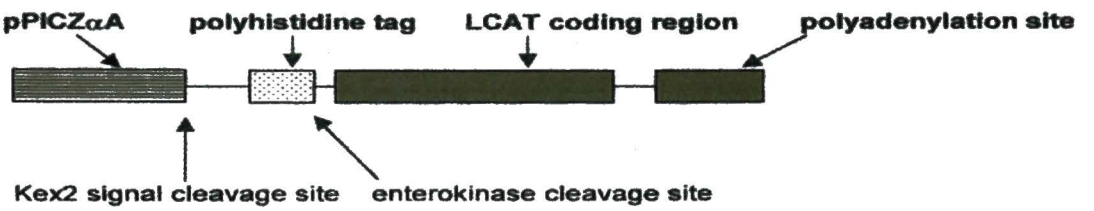


Figure 7: The pPICZαA-LCAT construct

Step	Temperature	Time	Cycle
Heat Soak	94°C	2 minutes	1
Denaturation	94°C	1 minute	25
Annealing	55°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1

Table 3. PCR Thermocycler running program

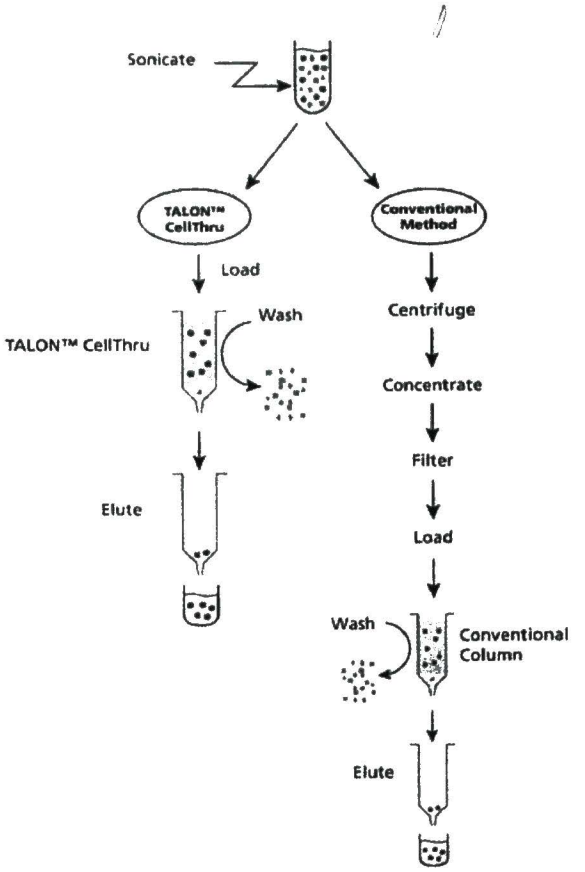


Figure 8. TALON™ Cell thru immobilized metal affinity chromatography (IMAC) procedure.

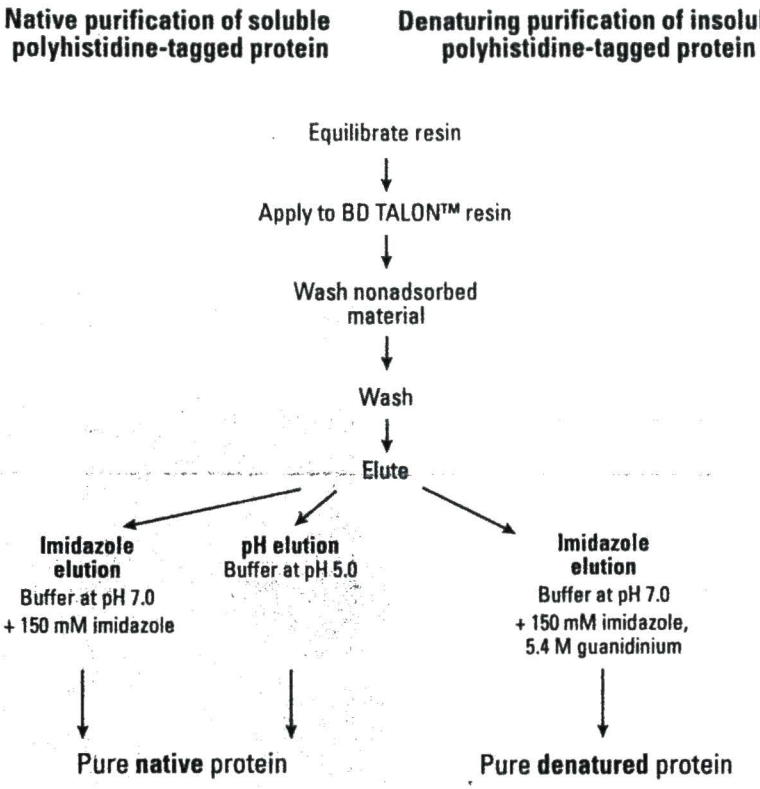


Figure 9. Purification procedure of polyhistidine-tagged proteins using BDTalon™ Resin The TALON system employs a resin with special affinity for His-tagged proteins. The Talon resin allows the elution of the target protein in its native or denatured form at the pH 7. In this research project, LCAT will be eluted in its native form and therefore, will be expected to retain its native conformation and activity. The LCAT expressed by Pichia cells (see construct on (Figure1) contains a removable hexahistidine segment and thus will be an ideal candidate for the highly efficient TALON purification procedure.

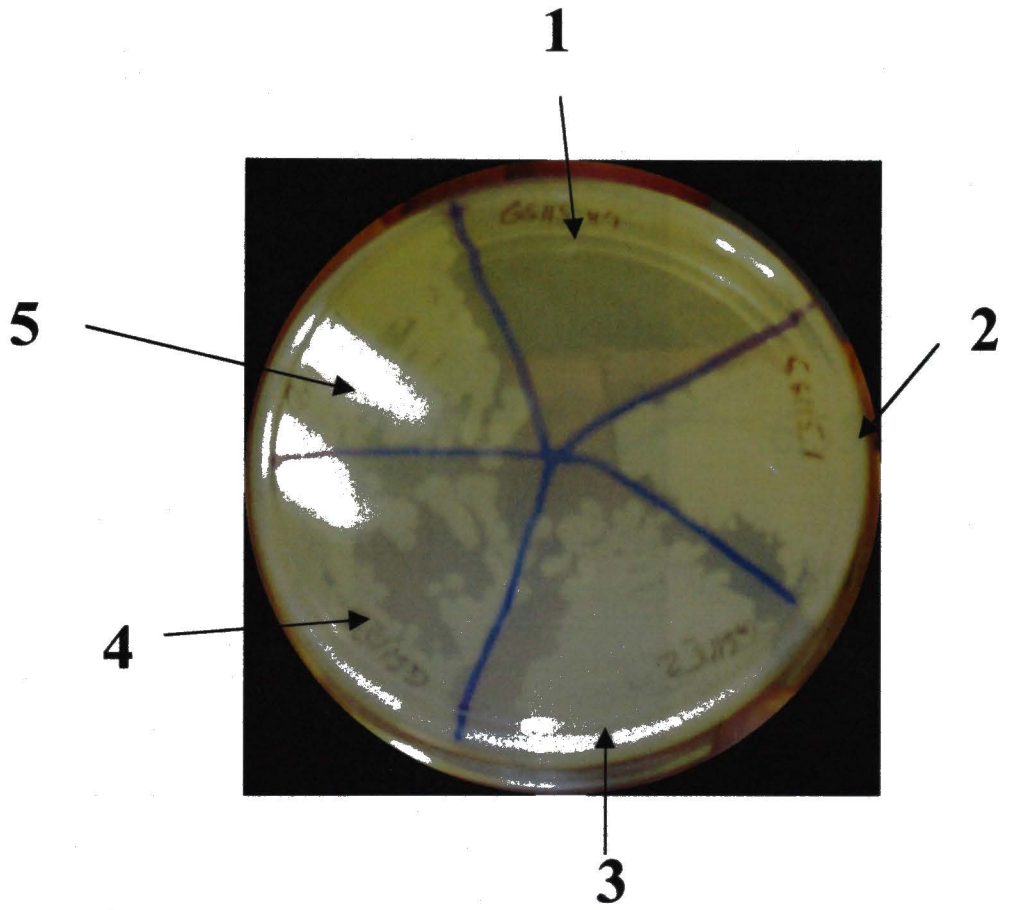


Figure 10. *Pichia Pastoris* Selection Plate (Containing the antibiotic Zeocine 100µg/ml):

1- *GS115* Wild Type (Non transformed), **2-** GS115 Transformant # 1, **3–** GS115 Transformant # 2, **4–** GS115 Transformant # 3, **5–** GS115 Transformant # 4

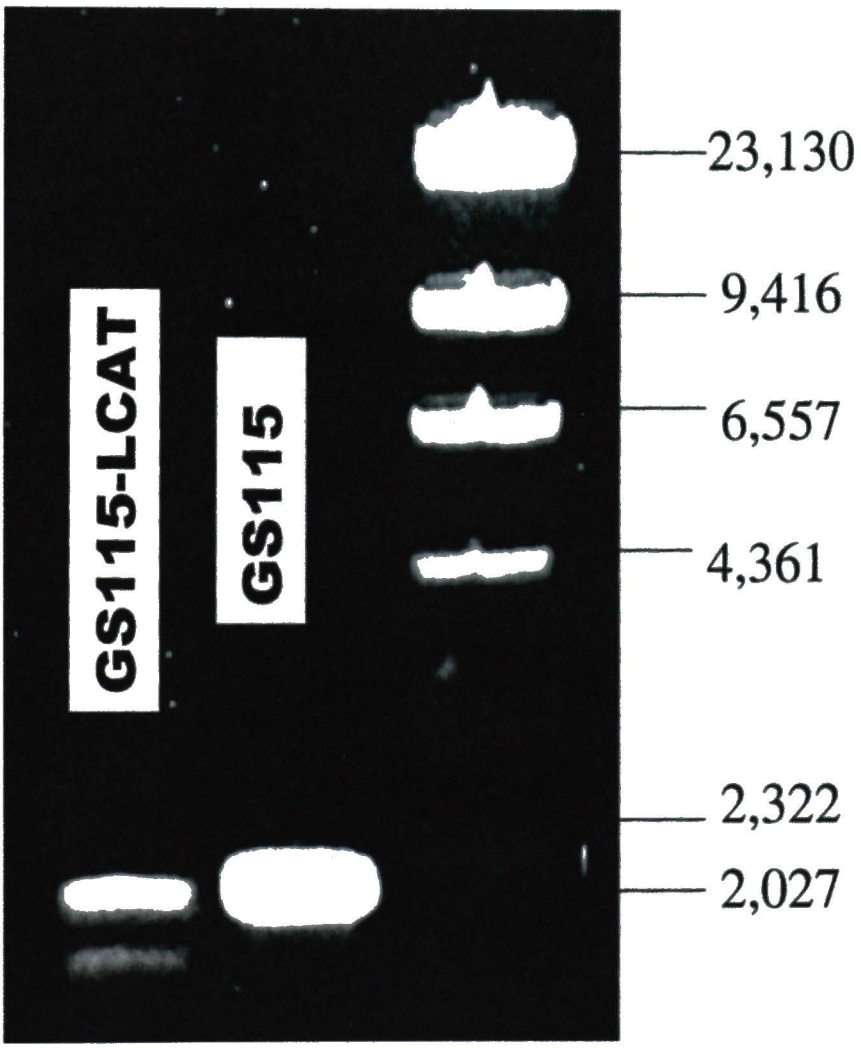


Figure 11. Polymerase Chain Reaction Analysis of Pichia integrants

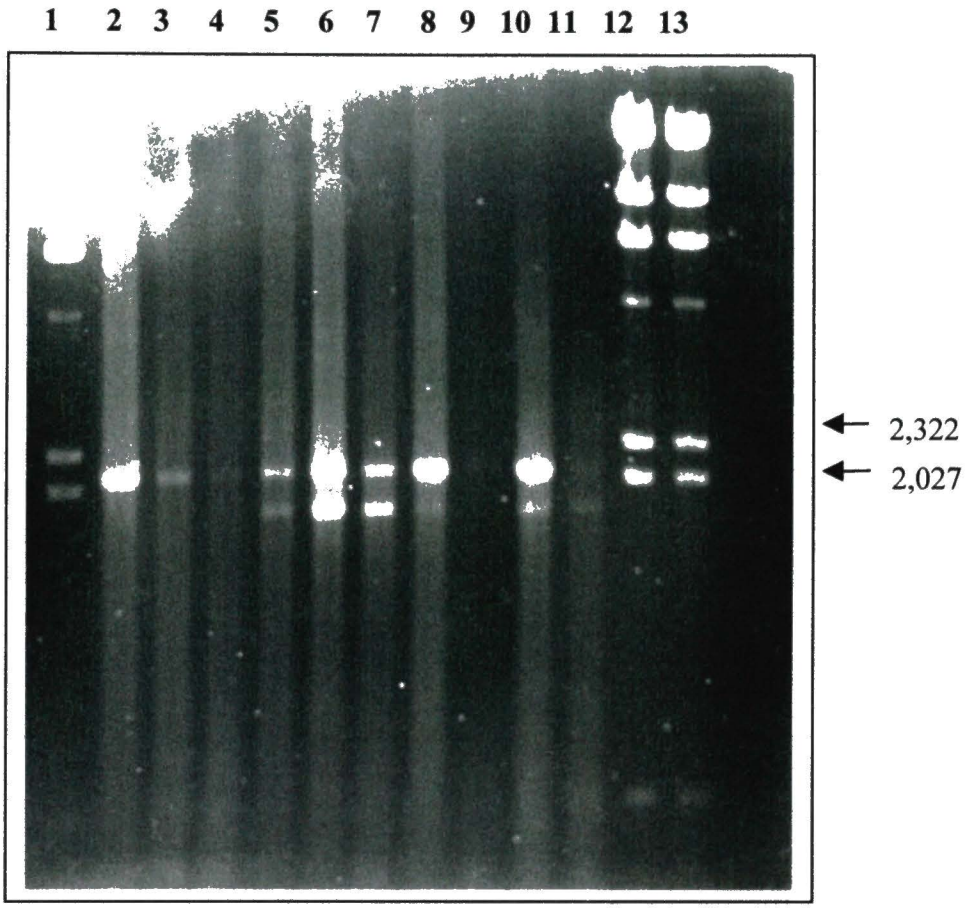


Figure 12: Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of Pichia integrants:

1: Molecular Weight, **2:** GS115 Wild Type, **3:** GS115 Wild Type negative Control, **4:** GS115 transformant #1, **5:** GS115 transformant # 1 negative control , **6:** GS115 transformant # 2, **7:** GS115 transformant # 2 negative control, **8:**GS115 transformant # 3, **9:** GS115 transformant # 3 negative control, **10:** GS115 transformant # 4, **11:** GS115 tansformant # 4 negative control, **12 & 13:** Molecular Weight.

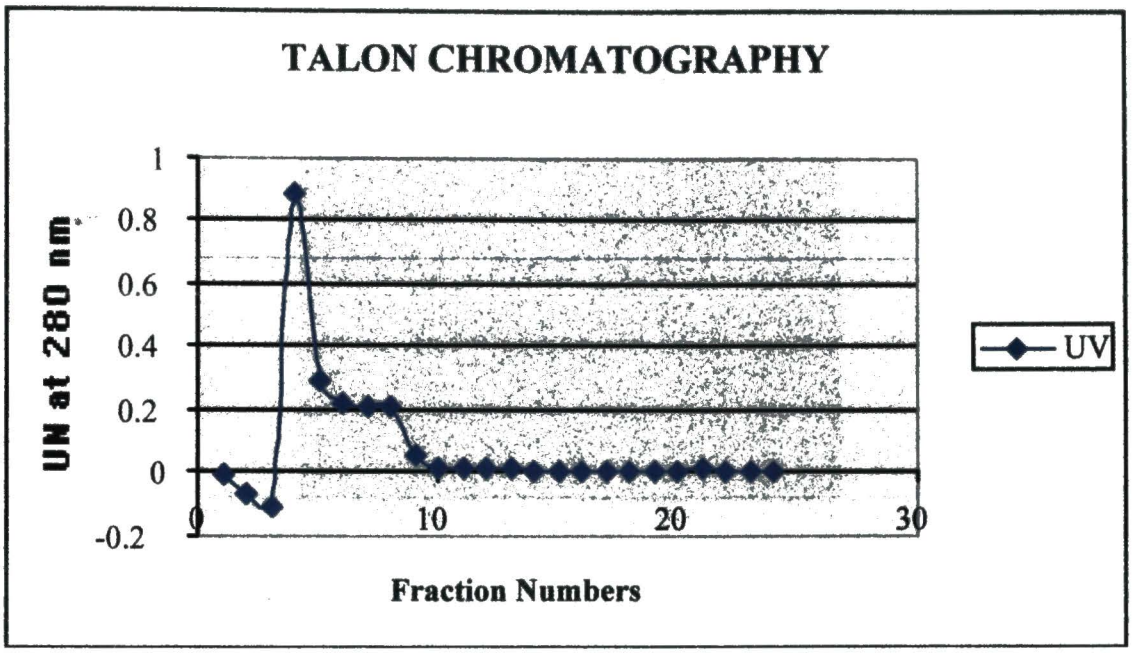


Figure 13. Talon Chromatography of the conditioned media collected from yeast transformant culture elution plot (fraction numbers vs. absorbance at 280 nm).

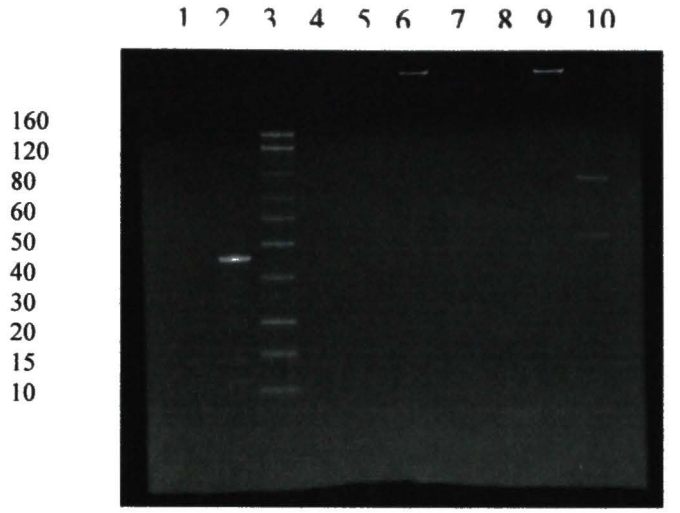


Figure 14: InVision Staining of the NuPAGE gel (1.0 mm thick, 10 well)

1-Pierce Neg. Control, **2** – Pierce Pos. Control, **3** – BenchMark Hist-tagged protein,
4- Talon purified LCAT from(GS115 clone 1) Yeast, **5**-Talon LCAT + Enterokinase,
6- Yeast Media (GS115 clone 1), 7- GS115 clone1 Extract ,
8- LCATx6His (Sample From Canada), **9**- GS115 clone 3 media (BMMY),
10- GS115 clone 3 Extract.

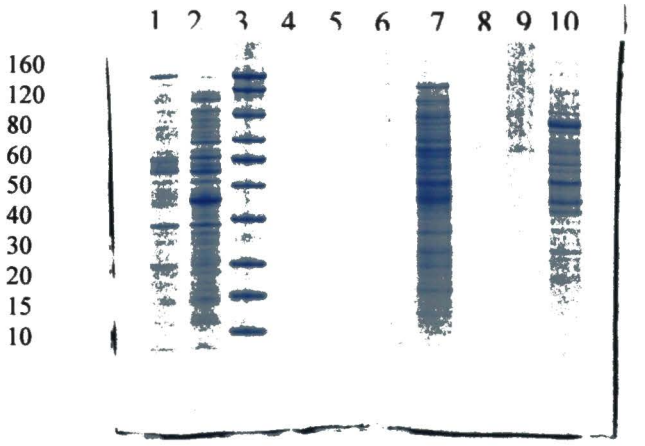


Figure 15: SimplyBlue Stain NuPAGE gel (1.0 mm thick, 10 well)

1-Pierce Neg. Control, **2** – Pierce Pos. Control, **3** – BenchMark Hist-tagged protein,
4- Talon purified LCAT from(GS115 clone 1) Yeast, **5**-Talon LCAT + Enterokinase,
6- Yeast Media (GS115 clone 1), 7- GS115 clone1 Extract ,
8- LCATx6His (Sample From Canada), **9**- GS115 clone 3 media (BMMY),
10- GS115 clone 3 Extract.

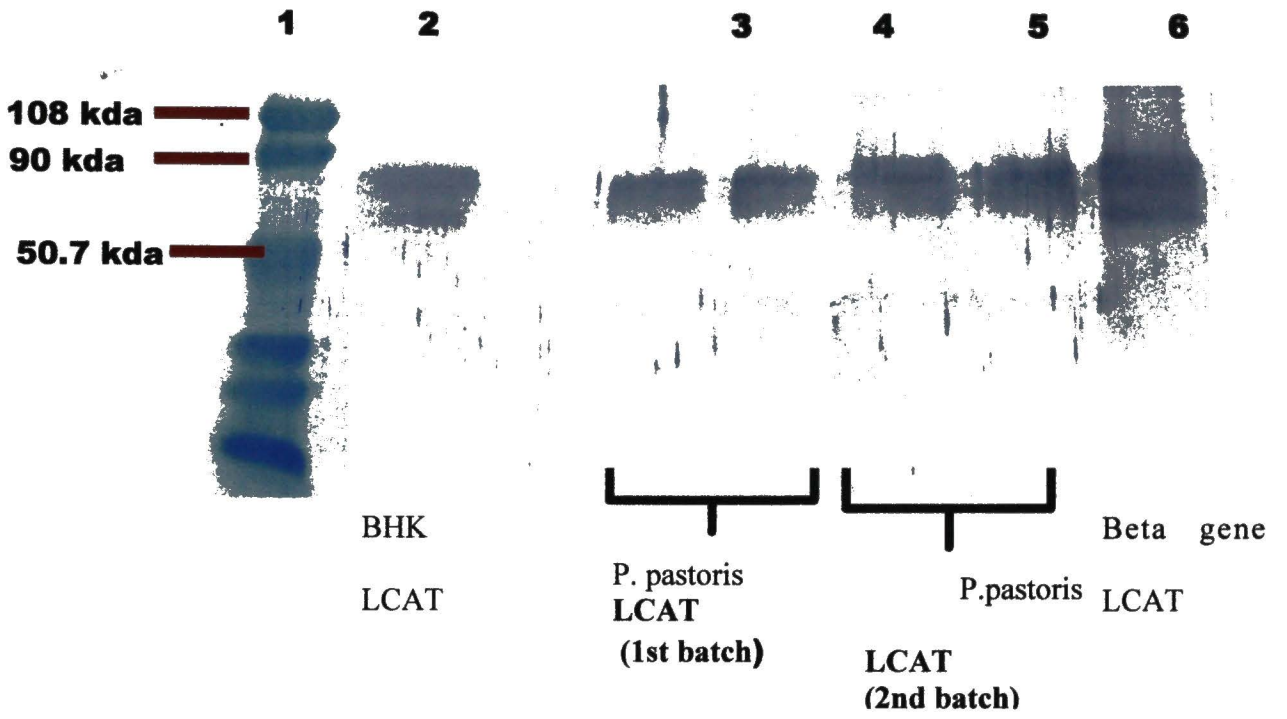


Figure 16. Western blot analysis of the yeast recombinant LCAT.

Samples	Protein (mg)	Activity (Units)	Recovery LCAT Activity (%)	Activity (*nmol/h/l*)	Specific activity (*nmol/h/ug*)
Media (Before Column)	202.5	15137.3	100	0.025	0.12
Purified LCAT (After Column)	18	7389.3	48.8	0.013	0.7

Table 4: Yeast LCAT purification and activity

Samples	Percentage of Count/Minutes (%CPM)	β-Mercapto-ethanol
Beta Gene LCAT	16.52	-
Yeast Recombinant LCAT. Non digested with Enterokinase	4.06	-
Yeast Recombinant LCAT. Digested with Enterokinase	2.45	-
Yeast Recombinant LCAT.digested with enterokinase + DTNB*	0.56	-
Yeast Recombinant LCAT.digested with enterokinase + DTNB	4.27	+
Yeast Extract	2.05	_

Table 5. Yeast recombinant enzyme assay conducted with radioactively labeled macromolecular substrate.

* 5',5'-Dithiobis-(2-Nitrobenzoic Acid)

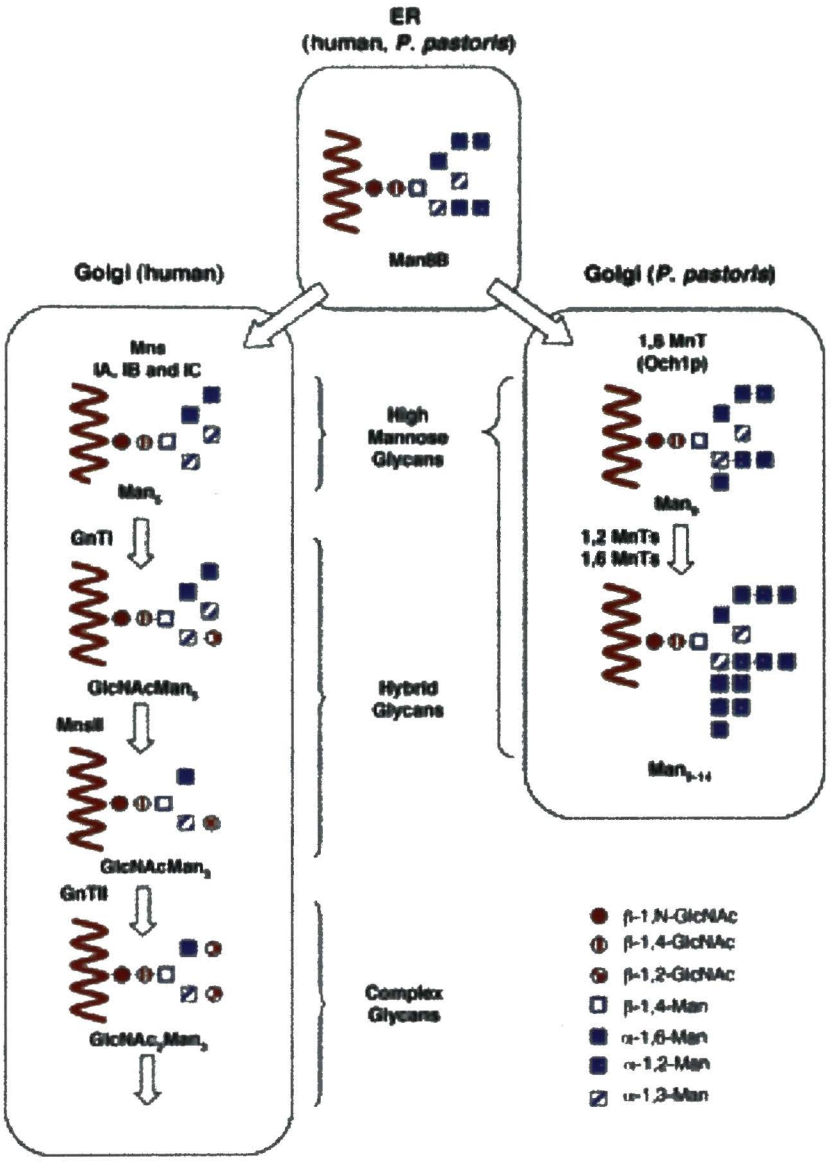


Figure 17. N-linked glycosylation pathway in human and Pichia pastoris (50). Mns,α-1,2-mannosidase; MnsII, mannosidase IIGnTI, β-1,2-N-acetylglucosaminyltransferase I; GnT II, β-1,2-N-acetylglucosaminyltransferase II; MnT, mannosyltransferase.

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