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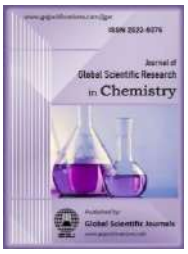

Kinetic Biochemical Study of Partially Purified Lecithin Cholesterol Acyltransferase (LCAT) from Patients with Type I Diabetes

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ABSTRACT

A group of people with type I diabetes are giving blood samples for a study on the Lecithin Cholesterol Acyltransferase (LCAT). Several methods have been used, such as the deposition of ammonium sulfate, dialysis, ion exchange chromatography, and electrical migration. LCAT was found to have one symmetrical molecule with a molecular weight of 68 kDa and a maximum activity of (327.8×10–3) units/ml. The study also looks at the factors that affect the enzyme. It was found that the best temperature for the enzyme was (30 °C), the best pH was (5.5), and the best amount of time for the enzyme to be incubated was (20) minutes.

1. Introduction

Lecithin Cholesterol Acyltransferase (LCAT) rated E.C.2.3.1.43, is the only one that can keep the balance of free cholesterol between peripheral tissues and high-density lipoprotein (HDL) by converting free cholesterol into cholesterol ester and then binding it into HDL particles and sending it through the bloodstream. LCAT activity is needed to reshape lipoprotein, especially for HDL growth and maturation[1][2] Norum and Gjone were the first to write about LCAT deficiency in 1967 in Norway [3]. So far, 117 mutations have been found along the LCAT gene. Most of the genetic defects that have been described led to the familial LCAT deficiency phenotype (77 mutations), 12 mutations led to the Fish Eye Disease (FED) phenotype, and 28 mutations have yet to be categorized [4]. The reverse cholesterol transport rate affects the LCAT ester cholesterol step. The cholesterol esters made by LCAT are stored in the center of the HDL particle. This causes the HDL particle to grow in size and mature, which is used in the protein transfer reaction of the cholesterol ester transfer protein (CETP) to send cholesterol to the liver. So, LCAT activity not only raises levels of good cholesterol (HDL-C), but it also makes HDL particles work better[5].

Epidemiological studies have consistently shown that HDL-C is inversely linked to cardiovascular disease. However, genetic studies and human drugs suggest that HDL-C levels may not always reflect HDL function[6]. Because of this, recent efforts have been focused on making new therapies that can target HDL function selectively, especially reverse cholesterol transfer (RCT)[7]. Several studies on both animals and people have looked into the role of LCAT in atherosclerosis[8,

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9]. Activity of LCAT has also been linked to kidney disease, breast cancer, and liver cancer [10].

The human LCAT gene is found in the q22.1 region of chromosome 16. It is (4.5) kb long, has six exons and five introns, and has a total of 4.2 kg of bases [11, 12]. This gene codes for polypeptide synthesis, which is made up of (416) amino acid residues. The N-glycosylation group has four sites of synergy with a glycosylation group (Asn 20, 84, 272, and 384), and the O-glycosylation carboxylation group has two sites of synergy with a sugar group (Thr 407 and Ser 409)[13]. It has been found that the enzyme is 60% more effective when the glycemic part is associated with the enzyme[14].

In sites 181, 345, and 377, respectively, there are tyrosine, serine, aspartic acid, and histodine acid in the active site of the enzyme[12]. LCAT has six cysteine residues. Four of them are at the protein's active site and are used to make two disulfide bridges (Cys50-Cys74 and Cys313-Cys356). (Cys50-Cys74) are part of the LCAT lid region disulfide, which covers the enzyme's active site and opens when it binds to lipoprotein surfaces [8]. Mature LCAT is a glycoprotein, and it is more Hydrophobe than the lipoproteins in plasma which are Hydrophilic of membrane proteins that are already built into the cell. In its buildup of lipoproteins or fats in the pulp, lipoprotein-associated phospholipase A2 is an enzyme that is 49% like LCAT (LPLA2) (Lipoprotein-associated phospholipase A2)[16]. In plasma, LCAT can combine HDL, LDL, and other protein proteins to make cholesterol ester, but 70–80% of its cholesterol ester goes to the HDL particle[17]. LCAT binds to the lipoproteins in the plasma in the wrong way and makes the free cholesterol ester more active. This happens in a two-step process that involves the hydrolysis of phospholipid alkyl chains and their transfer to free cholesterol. First, the lecithin's site 2 is cut, and the stearic acid is moved to Ser181. After that, the fatty acids are changed into a group of $3-\beta$ hydroxyls in cholesterol, which makes cholesterol ester.

Cholesterol ester is less hydrophilic than free cholesterol, so it goes to the water-loving center of lipoprotein to help it mature[1] [11]. Almost all of the cholesterol ester in the blood comes from the reaction of LCAT [18]. This reaction is helped by an enzyme called α-LCAT, which is activated by the Apolipoprotein AI. It should be noted that LCAT, which is the same as β -LCAT, also works with ApoB to esterify cholesterol to fatty proteins that contain ApoE [19].

2. Materials and Methods

A blood sample of (110) ml was taken from a group of donors with type I diabetes, whose ages ranged from (20 to 51) years. These people were chosen as the study subjects based on the instructions of the supervisor and a doctor who specializes in diabetes. After separating the blood serum from the sample, the sample went through a series of separation and purification steps that were needed to separate and study the properties of the LCAT enzyme and as follows:

Precipitation and separation of protein using ammonium Sulphate:

The amount of protein in the blood serum was deposited based on how saturated the solution was [20]. (45) ml of blood serum, solid ammonium sulfate with an amount of (60%) was added, and the mixture was stirred by a magnetic stirrer at (4^{o}C) for one hour. The mixture was then put in the fridge for 24 hours, after which the precipitate was separated from the leachate using a cooled centrifuge for 20 minutes at a speed of 20 rpm (1500 ×g). Then, get the precipitate and dissolve it in the least amount of ion-free distilled water. Measure the amount of protein and the enzyme activity in the protein precipitate solution before moving on to the next steps of purification. Store the protein precipitate solution at a temperature of (-20^{o}C) until it is used in the next steps.

Dialysis

This step was done to get rid of the ammonium sulfate added during the sedimentation process and materials with small molecular weights. Cellophane tubes were used to do this. The protein solution made in the last step was put in a cellophane bag and tied tightly from the bottom and then from the top. The tube was then put in a volumetric container with (1) liter of potassium biphthalate solution at a concentration of (50) mmol/L and at (4°C) temperature. The whole process took (24) hours because the dialysis solution had to be changed every two hours[21].

After the dialysis process was done, the final size of the solution, the amount of protein in the solution, and the enzyme activity of the resulted solution. The solution was then kept at a temperature of (-20°C) until it was used in the next steps.

Ion exchange chromatography

The diethylamino-acetylocellulose (DEAE-Cellulose 52) ion exchanger was made according to the instructions from the supplied company (Dibo Biotechnology). A glass separation shaft with dimensions of (40 x 2.5) cm was used, and the column was filled with activated ion exchange resin by quietly pouring it on the walls of the shaft to prevent air bubbles from forming. After preparing the separation shaft, pass the result of the dialysis process by quietly injecting it into the top of the shaft. Then, pass the solution that has been regulated as shown above. A peristaltic pump was used to collect the filtered parts from the end of the separation column in the separation tubes, with (5) ml in each tube and the speed of filtration of the solution from the column (5 ml in 5 minutes), and the filtered protein parts were followed up by measuring the absorption at a wavelength of (280) nm using the spectrophotometer. Also, the activity of the LCAT enzyme in each part of the separated solution was followed up and then collecting the protein parts which show the enzyme activity was followed up.

Protein concentration

The Thermofisher / U.S.A. Spin column is an ultrafiltration centrifuge that has a polyethersulfone membrane (PES). 10 ml of the sample was put into the capacitor, and then the capacitor was put into a cooled centrifuge for 15 minutes at a speed of (1500 ×g). This process was repeated several times, and the regulated solution (potassium diphthalate at (6.1 pH and a concentration of 50 mmol/L) was used for washing and sample concentration[22]. The enzyme is then kept at a temperature of (-20 °C) until it is used to estimate the molecular weight and figure out the best conditions for the enzyme.

Sodium dodecyl Sulphate poly acrylamide gel electrophoresis (SDS-PAGE)

The sulfate compound sodium dodecyl sulfate can break down proteins into chains of different sizes surrounded by negatively charged SDS molecules. These chains move by electrical migration based on the molecular weights of the peptide chains only, and the protein bound to SDS goes to the positive electrode based on the ratio of charge to mass. When electrical migration is done on a polycarylamide gel using SDS, it all moves in the same direction. Each protein is given a certain distance in centimeters by the gel of the electrical migration device (cm). At the same time, standard solutions with proteins of known molecular weight are passed. By plotting the distances traveled by known protein compounds against the logarithm of their molecular weights, the molecular weight of the unknown enzyme can be found by projecting the distance it travels on the standard curve. This method was found around 1969, and many people still use it today because it is easy[23], [24].

Determination of lecithin - cholesterol acyltransferase activity in blood serum:

Enzyme of lecithin-cholesterol acyltransferase activity in blood serum was measured using Manabe, et al.,[25] which includes esterifying cholesterol substrate into cholesterol ester by LCAT, it depends on measuring the colorful complex absorption resulted from free cholesterol when interacting at (545) nm.

3. Results and Discussion

Isolation and purification of LCAT and study of properties

In order to get the LCAT enzyme out of the blood serum of people with insulin-dependent type I diabetes, different separation and purification methods have been used. For example, it has been deposited by saline displacement and then purified by dialysis and ion exchange chromatography, which will be talked about later.

1. Separation by salting out

By adding more ammonium sulfate salt to the serum [20], the protein was deposited by salt displacement. The most protein was deposited when the saturation rate was used (60%), and the

precipitate was re-dissolved in the smallest amount of the regulated solution at 6.1 PH.

The results are shown in Table (1) shows the specific activity of the LCAT enzyme after the deposition process increased to (44.5×10^{-3}) units per mg of protein, which is (1.84) times higher than before purification, and the total activity of the enzyme was recovered by 104.7% compared to the crude enzyme.

2. Dialysis

The next step in separating and purifying the LCAT enzyme was dialysis. During this step, the enzyme's specific activity went up to (99.9×10^{-3}) units/mg of protein, which is (4.14) times higher than it was before purification. The enzyme's total activity was also recovered by (212.1%) compared to the total activity of the raw enzyme, as shown in Table (1). This shows that the dialysis process is a key part of cleaning up the enzyme molecule and making it more active.

Table (1) Stages of purification of the enzyme lecithin cholesterol acyltransferase in the blood serum of patients with type I diabetes mellitus

Purification stages	Vol. (ml)	Protein concentration (mg/ml)	Total protein (mg)	Enzyme activity (unit/ml)×10^{-3}	Total activity (unit×10^{-3})	Quality activity (unit/mg)× 10^{-3}	Number of purification	Sample of retrieved amount
Blood serum	45	3.60	162.1	87.0	3914.4	24.1	1.00	100.00
Sedimentation using ammonium sulfate	40	2.31	92.2	102.5	4099.3	44.5	1.84	104.72
Dialysis	35	2.37	83.1	237.3	8303.8	99.9	4.14	212.13
Ion exchange chromatography	60	0.67	45.7	327.8	19668.9	430.8	17.84	502.47

Purification by ion exchange chromatography

The technique of ion exchange chromatography was used on the protein solution from the dialysis. By measuring the concentration of the protein in the Rogan solution, the model found two distinct peaks (A,B) for the presence of the protein, as shown in Figure (1), where the volume of the Rogan for the two peaks was (130) ml and (345) ml, respectively. By keeping track of how well the LCAT enzyme worked in each band, it was found that the enzyme's activity is centered in the Rogan solutions of the protein band (A), and that it worked best at the volume of Rogan (130) ml (band I),where its activity was about (327.8×10^{-3}) units/ml (Figure 1). The specific activity was (430.8×10^{-3}) units/mg of protein, which is (17.84) times more than it was before purification, and the total activity of the enzyme was recovered to (502.47%) of what it was before purification. Since the LCAT enzyme wasn't very effective except at (band I), it has only one symmetrical form. This is in line with what other researchers found when they used ion exchange

chromatography to separate and purify the LCAT enzyme in pig blood serum and found that it only has one symmetrical form [26] and when they separated the LCAT enzyme from the plasma of healthy people and partially purified it by using deposition with ammonium sulfate [27]. Then, with the help of DEAE-cellulose and ion exchange chromatography, it was found that the enzyme has one identical During the arrival of a single band and the separation of it from the serum of a patient with heart disease, it was found to be the same as one. This was done by using a technique called ion exchange chromatography [28].

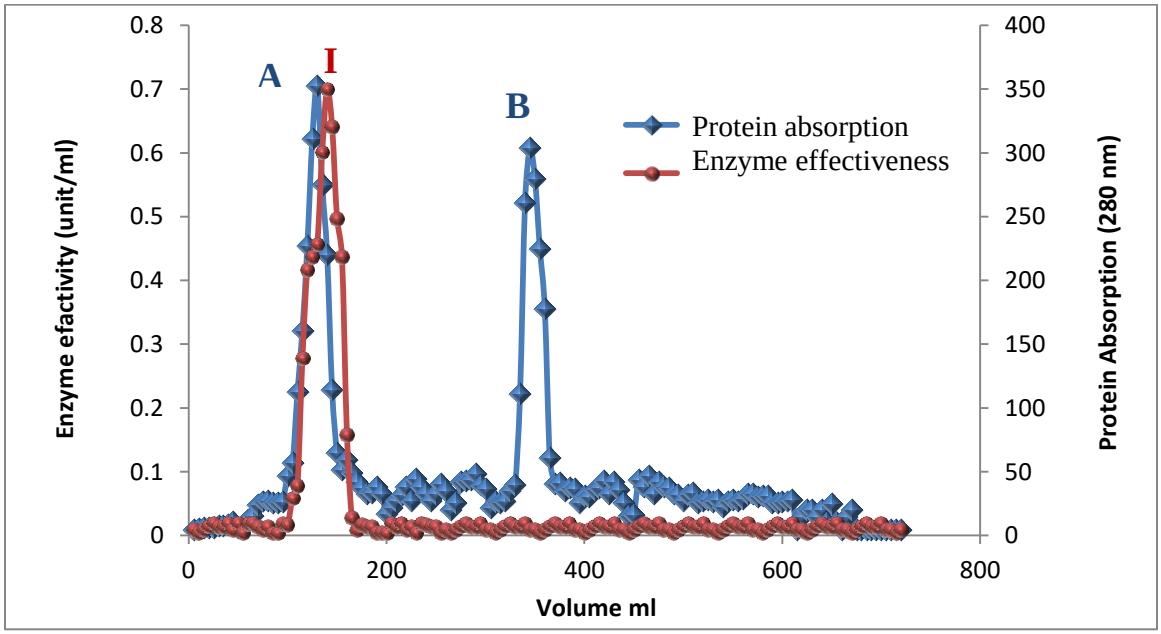

Figure (1): Profile of Rogan Protein Bands of LCAT Enzyme Produced by Ionic Exchange Separation Shaft Using DEAE-Cellulose and Yield to Serum of Type I Diabetes

1. Determination of molecular weight by electrophoresis

The molecular weight of the LCAT enzyme separated from the blood serum of type I diabetics was estimated by applying SDS-PAGE electrophoresis. Molecular weight markers consisting of molecular weight materials with molecular weights ranging from (24,000-100,000 daltons) were used for this purpose. Through this process, a protein band was characterized at a distance of (4.2) cm from the starting point as shown in Figure (2), and this band was adopted in estimating the molecular weight of the enzyme, which was found to be equal to (68) kDa through the use of the standard curve (Fig. 3)[29]. They had concluded that the molecular weight of the LCAT enzyme purified from human plasma was about (65) kDa, They also[30] came to the conclusion that the molecular weight of the LCAT enzyme secreted from human hepatocytes into plasma ranges from (63-67) kDa and that about 40% of it is made up of carbohydrate components. This means that the LCAT enzyme is a glycol protein. On the other hand, they have separated and purified the LCAT enzyme from pig plasma and studied its amino acid content. They found that its amino acids are similar to the amino acids of the enzyme separated from human plasma and that its molecular weight is about (69) kDa [31].

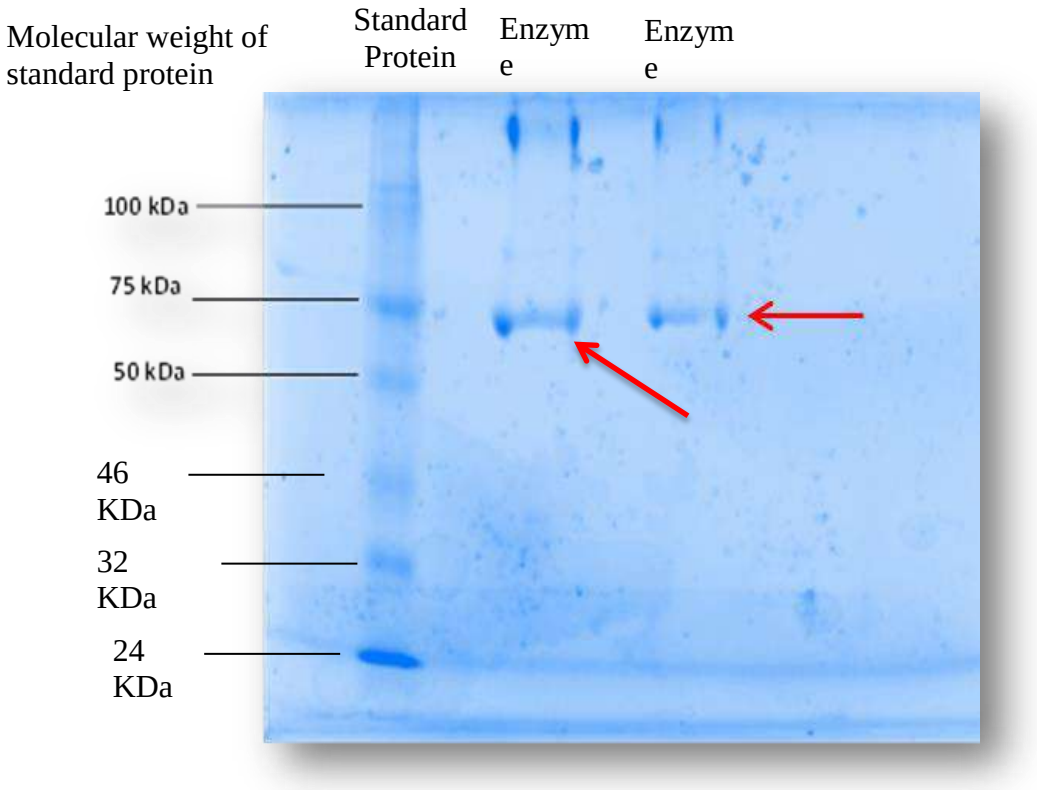

Figure (2): Separated Protein Bands On The Gel After Applying SDS-PAGE electrophoresis

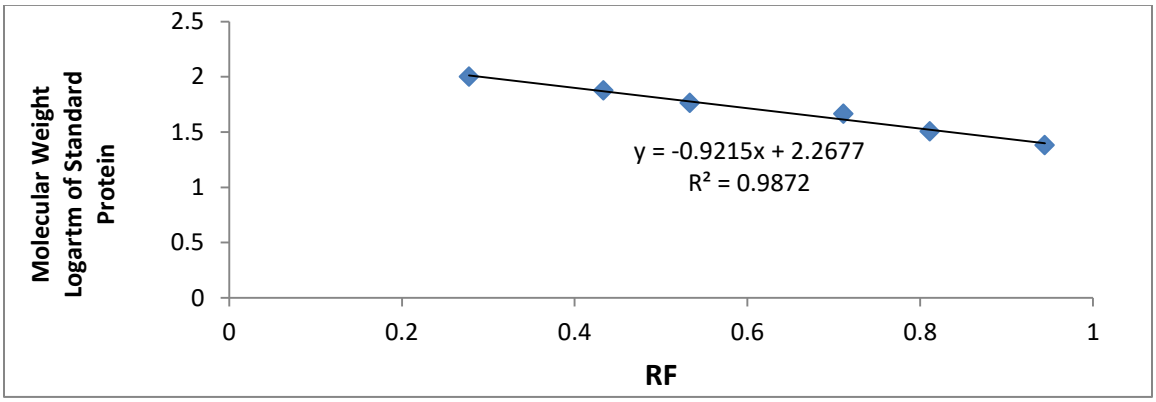

Figure (3): Standard Curve For Estimating The Molecular Weight Of The Enzyme Lecithin Cholesterol Acyltransferase Using SDS-Polyacrylamide Type Electrcophoresis

1. Factors affecting the enzyme activity of lecithin-cholesterol acyl transferase

Some factors that affect the LCAT enzyme activity (or how fast the enzymatic reaction goes) have been studied, as have the best conditions for it to work. The protein band (A) has been used as a source of the enzyme.

Effect of enzyme volume

In the presence of different sizes of the enzyme purified from the blood serum of patients, the LCAT enzyme's activity was measured. The volumes ranged from (50-450) μl, and figure (4) shows the linear relationship between the enzyme activity and how much of it there is. It was noticed that the speed of the enzyme reaction increases with the size of the enzyme when there is a lot of the base substance nearby. This is because there

are more active sites that are bound to the base material, which makes the reaction happen faster[32].

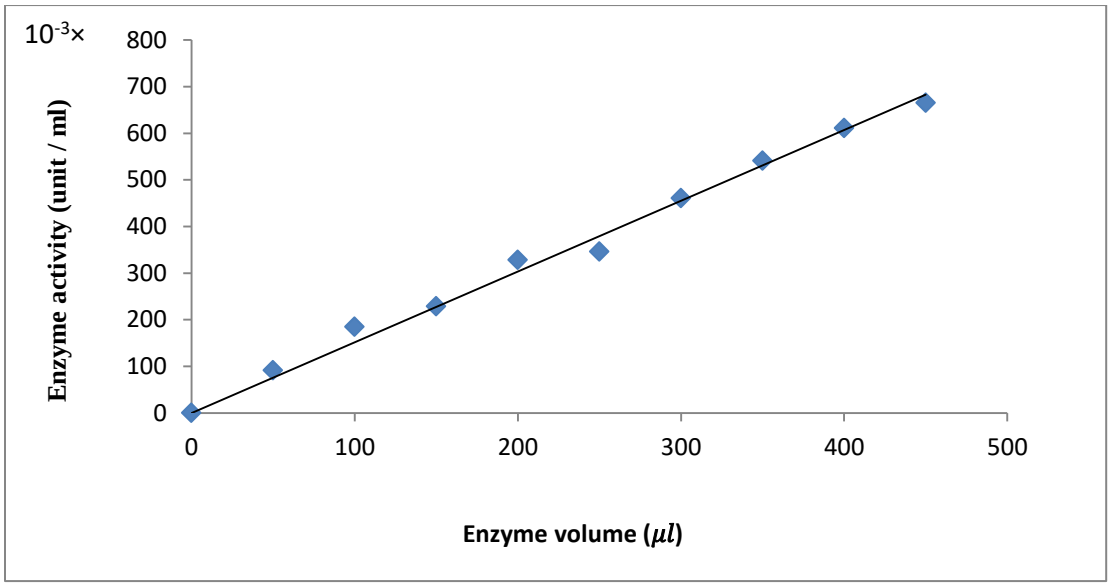

Figure (4): Effect of Enzyme Volume on Enzyme Activity

Effect of temperature

When figuring out the LCAT enzyme activity at different temperatures (ranging from 5-50°C), it was found that the enzyme's activity goes up slowly as the temperature goes up to a certain point (30°C), and then it goes down clearly after that point, figure (5). The increase in reaction rate or enzyme activity caused by collisions and then fusions between the molecules of the enzyme and the molecules of the substrate, which speeds up the reaction or makes the enzyme more effective. The decrease in enzyme activity after the optimum temperature of (30 °C) may be due to a mutation in the enzyme's synthesis, which has a negative effect on the enzyme's vital activity[32] [20].

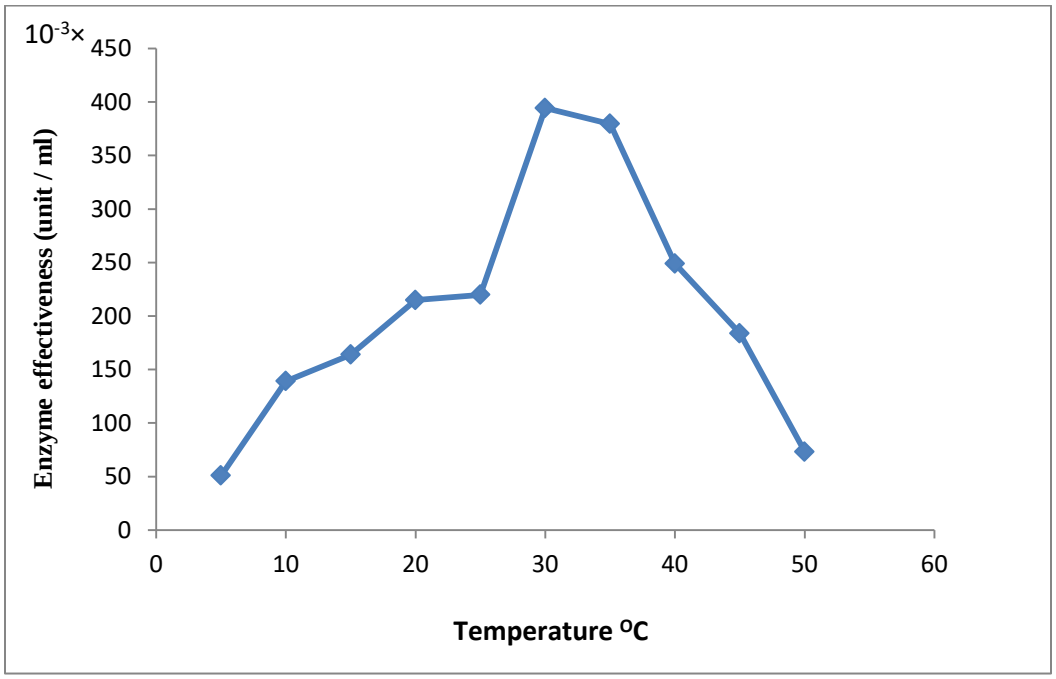

Figure (5): Effect of Temperature on Enzyme Activity

Effect of PH buffer solution

In order to find the optimal pH at which the LCAT enzyme works, the activity of the enzyme was tracked using a regulated solution of potassium biphthalate at a concentration of (50) mmol/L and a range of pH 4 to pH 8.5. Figure (6) shows that the enzyme worked best at pH 5.5, which means that pH (5.5) leads to the best modification in the spatial regulation of the enzyme's active site. When the pH goes above 5.5, the structure of the active site changes in a way that makes it harder for the enzyme to bind to the base material. This makes the enzyme less effective or slows down the rate at which it works[32],[20].

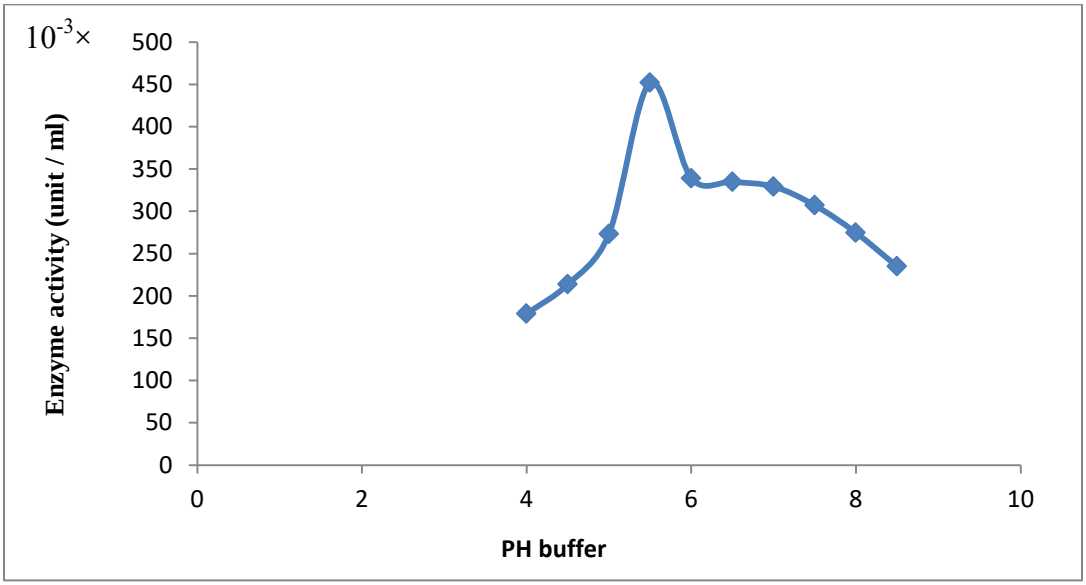

Figure (6): Effect of PH Buffer on Enzyme Activity

Effect of incubation time

The effect of incubation time on the activity of the LCAT enzyme was studied using different incubation times that ranged from (5-45) minutes. Figure (7) shows the relationship between the activity of the enzyme and the incubation time. The best incubation time for the enzyme was found to be (20) minutes.

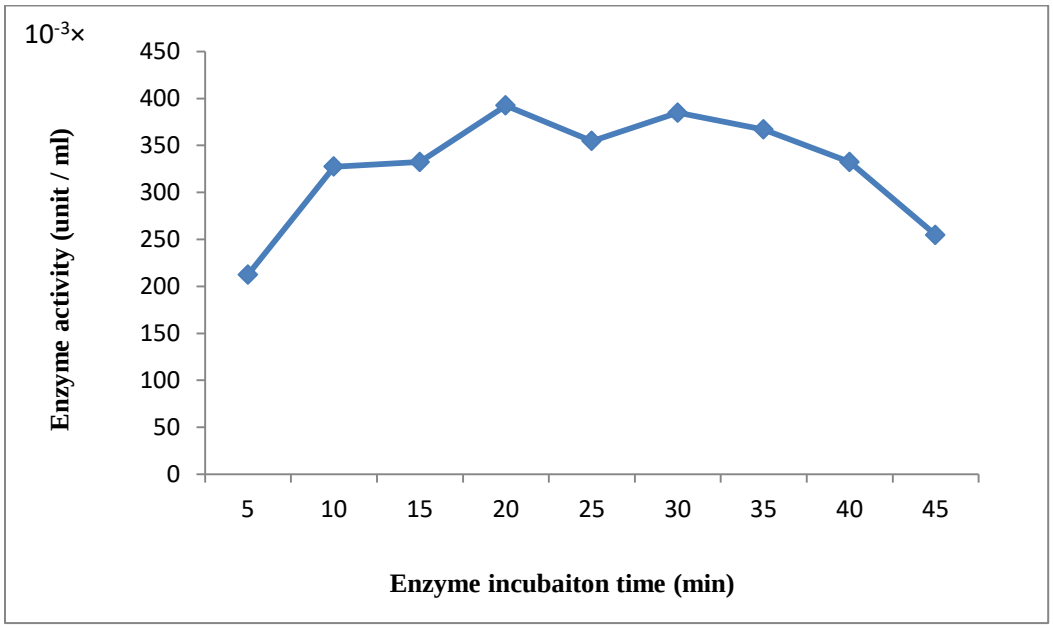

Figure (7): Effect of Incubation Time on Enzyme Activity

Effect of substrate concentration

When the concentration of the enzyme in the reaction mixture stays the same, an increase in the concentration of the base material makes the rate of reaction speed go up quickly at first, but then it slows down and stays the same no matter how much the concentration of the base material goes up and this is called (V_{max}). To find out how the concentration of the substrate (lecithin-cholesterol) affects the LCAT enzyme activity, the activity of the enzyme was measured in the presence of different concentrations of the base substance that ranged from (7–75) μg/ml. Figure (8) shows that as the concentration of the base substance went up, the speed of the enzymatic reaction went up, too, until it reached a certain point where the speed of the enzymatic reaction could not go up any more. At this point, the reaction reached its maximum speed, or Vmax. Michaelis and Minten tried to explain this by saying that when low concentrations of the base material are used, the active sites of the enzyme are not filled up. This means that the speed of the enzyme reaction is not at its fastest. However, when the concentration of the base material goes up, the active sites are filled up. This means that the enzyme is working at its best and the reaction speed is at its fastest. Adding more base material won't change this[33]. For the LCAT enzyme, it was decided that the lecithin-cholesterol concentration was what led to reaching the mid-speed. The most was (5) µg/ml. V_{max} and Km were measured to be (377.4×10^{-3}) units/ml and (5) μg/ml. Figure (9) shows that when the Lineweaver-Burke equation was used, Vmax and Km were found to be (384.6×10^{-3}) units/ml and (5.2) microgram/ml, respectively.

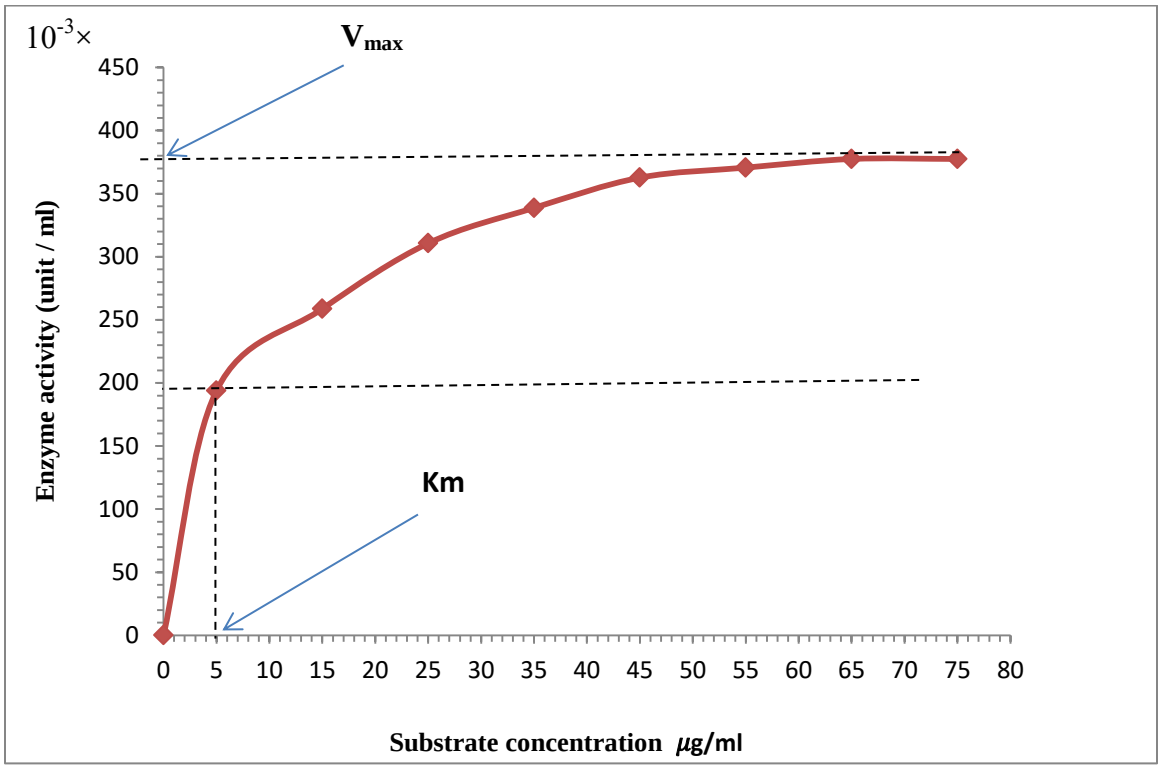

Figure (8): Effect of Substrate Concentration on Enzyme Activity

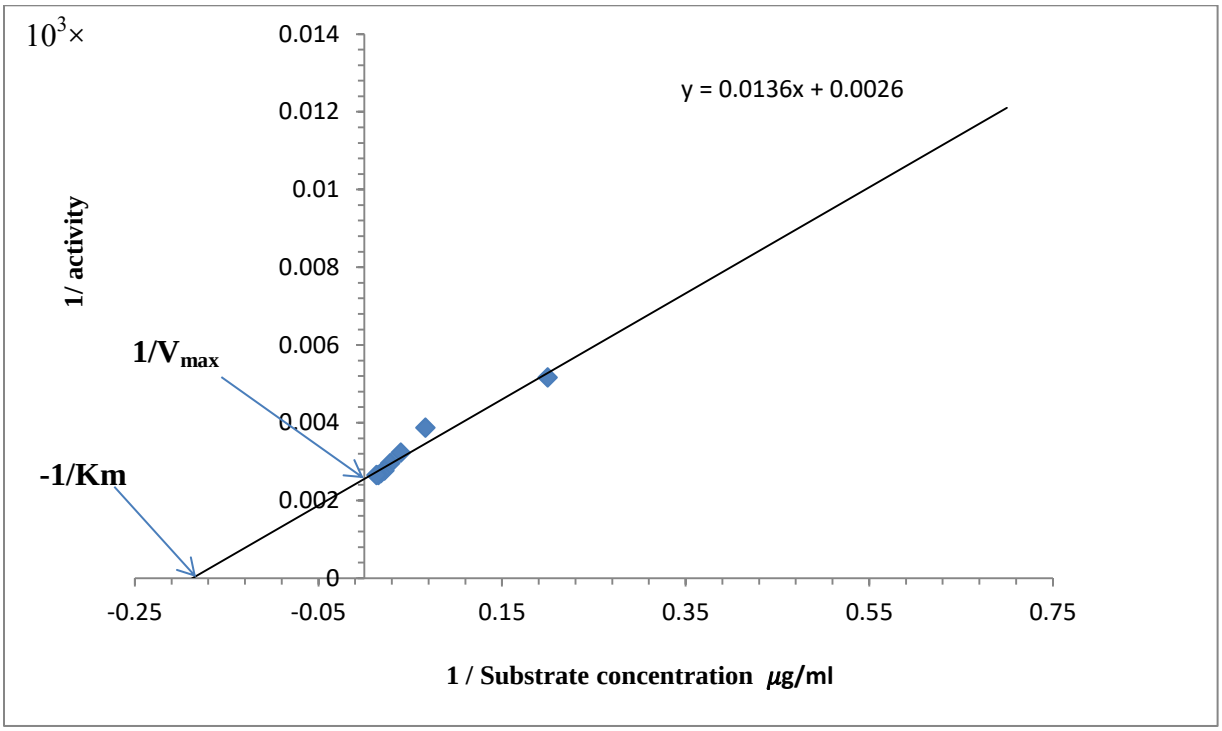

Figure (9): Effect of Substrate Concentration on Enzyme Activity LCAT Applying Lineweaver-Burke equation

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