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Biochemical and Kinetic Study for the partially purified Lecithin: Cholesterol acyltransferase from serum cardiovascular disease

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ABSTRACT

The study includes partial purification of Lecithin: Cholesterol acyltransferase (LCAT) from the blood serum of a person suffering from atherosclerosis. Several techniques were applied including ammonium sulphate precipitation, dialysis, ion exchange chromatography and electrophoresis. It was found that LCAT has one isoenzyme and the highest activity was (1073.46 × 10-3) unit/ml and a molecular weight of 62 KDa. The study also deals with characterizing of LCAT. It was found that the optimum pH and temp. of the enzyme were 7 and 35ºC.

Introduction

Lecithin: Cholesterol acyltransferase (LCAT) (E.C.2.3.1.43) is the enzyme responsible for the free cholesterol uptake on the surface of the lipids and primarily the HDL surfaces[1,2].LCAT is created primarily in the liver and asmall amounts in both testes and stellate cells in the brain[3]. The concentration of LCAT enzyme in the plasma is about 5-6 mg/L, and its concentration influenced by several factors such as age, Food and smoking[4,1].The gene responsible for the synthesis of the LCAT was located in the area of q22.1 in the chromosome (16), which was 4.2 kilo nitrogen base, and consists of six exons[5]. This gene encodes a polypeptide synthesis consisting of (416) amino acids retarded and contains four sites to interact with a sugar group by the N-glycosylation group (Asn 20, 84, 272 and 384). It also contains two sites to interact with a sugar group by O-glycosylation (Thr407 and Ser 409) [6,7]. It was observed that when the enzyme-related diabetes was removed(60 %)[8]. The active site of the enzyme contains three amino acids (Ser and Aspartic acid and histidine) at site 181, 345 and 377, respectively[9].It was found that the amino acid residues from (53) to (71) containing the two-sulfur bridge between Cys50 and Cys74 form the area that binds to the fat and partially covers the active site of the enzyme. The LCAT molecule Free cysteines at site 31 and location 184 These retrograde agents are responsible for enzyme sensitivity to inhibition of active sulfhydryl reactive agents [10].When replacing amino acid elsewhere in the enzyme molecule (through the development of the genetic mutation), the enzyme's activity is affected. The replacement of the amino acid Thr with the amino acid Asn increases the activity of the enzyme, while LCAT activity decreases when replacing Asn 84 or Asn272 with glutamine (82%) and (62%), respectively, and is highly activity when replacing Asn384 with Glu [11]. Genetically, LCAT synthesis is relatively unaffected by changing conditions, but some drugs affect the activity of the enzyme. For example, fibrates reduce LCAT in plasma by 20% while Torcetrapid and Atorvastatin contribute to increase plasma enzyme activity [12,13,14,15,16]. Several studies have indicated that the relationship between high-density lipoprotein (HDL) and cardiovascular disease is inverse [17]. It was found that 30-55% of the changes in HDL concentration are determined by genetic factors [18]. The genetic mutation that occurs in the combination of apoAI and LCAT produces low levels of HDL-C concentration because the LCAT enzyme has an important and specific role in the formation and maturity of the HDL molecule [19] and in the reverse conversion of cholesterol in Blood vessels,

and the LCAT enzyme, have a significant effect on the removal of LDL-C oxidation products [20].

Materials and methods

blood sample (250 ml) was extracted from a 65-year-old with a heart attack (10 years) and was selected as the study model according to the guidelines of the field supervisor and the cardiologist., And after separation of blood serum from the sample subjected the sample to a series of separation and purification necessary to separate and study the characteristics of the enzyme LCAT, as follows:

1.Protein Precipitation and separation using ammonium sulphate:

The protein saturation of the serum was determined by the degree of saturation of the solution [21]. It used 50 ml of the serum and added solid ammonium sulphate and saturated to (70%) gradually, stirring the mixture with the magnetic stirrove at (4 °C) for one hour and leave the mixture for 24 hours in the refrigerator, after which the precipitate was separated from the leachate using a cooled centrifuge for 20 minutes at a speed of 6000 g. After that the precipitate was obtained and the minimum amount of distilled water, the amount of protein and enzyme activity was estimated in the protein precipitate solution before the purification steps were performed And then save the protein precipitate solution at atemperature of (-20° C) until it is used in subsequent steps.

2.Dialysis:

The membrane filtration process was performed by placing the protein solution recorded in paragraph (1) in the tight cellophane bag, binding from the bottom, then connecting the top tightly and placing the tube in a volumetric container containing 2.5 liters of potassium biphthalate solution at a concentration of (50) mM / L .The process was continued for 24 hours, taking into account the change of membrane filtration solution every two hours [22]. After the screening process was completed, The final volume of the resulting solution was determined by the amount of protein and enzyme activity of the solution obtained from the screening and then conserve the solution at a temperature of (-20 °C) until it is used in subsequent steps.

3.Ion exchange chromatography technique:

Diethylaminoethyl cellulose (DEAE-Cellulose52) was prepared according to the instructions of the supplied company (Whatman)and supplied with the processing peak. It used a glass separation column with dimensions of 40×2.5cm. The column was filled with the activated ion exchanger Quietly after the completion of the Colum design, the resulting model of the dialysis process was passed through the syringe at the top of the column and then gently passed the regulator solution referred to above and used the alternating pump in the collection of parts Candidate from the end of the seperation column in tube (5 ml / 5 min). The protein samples were followed by absorption measure at a wavelength of (280) nm using optical spectrophotometer. The activity of LCAT was also monitored in Each part of the separated solution, to follow up the activity during the separation process and then collect the protein parts in which the enzyme's activity appears.

4.The technique of lypholyzation:

The resulting high protein LCAT was obtained after obtaining the enzyme in a solid form and preserved at (-20 ° C) until it was used to estimate the molecular weight, determine the optimal conditions of the enzyme and study the effect of inhibitors on its activity.

5.Estimating the Approximate Molecular Weight of LCAT by SDS-PAGE Electrophoresis:

The method of the researchers Roy and Kumar (2014) [23] was used to estimate the approximate molecular weight, which includes the addition of sodium dodecyl sulphate (SDS), which has the ability to separate proteins into secondary units and give them a large number of negative charges thus neutral and make separation depends on the molecular weight.

6.Measure the Activity of the LCAT enzyme:

The activity of the lecithin-cholesterol acyltransferase (LCAT) in the serum was estimated by using the Manabe and its group method (1976) [24]. The method involves the cholesterol substrate esteration to cholesterolester by the LCAT enzyme. The method is based on the measurement of the intensity absorption of the quinone- Of the reaction at a wavelength of 545 nm.

Results and Discussion:

In this study, LCAT was isolated from the serum. The results shown in Table 1 indicate that the specific activity of the LCAT obtained after the precipitation process increased to ($7.33x10^{-3}$) unit/mg protein any they multiplied by (2.08) time fo what it was befor purification, and that the amount of retrieval total activity for enzyme (118.5%) compared to the total activity of the crude enzyme, any that there is a concrete purification of the enzyme in this process. The specific activity of the enzyme increased to (17.24) unit/ mg protein, it was multiplied by (4.91) than it was before the purification using the dialysis process and the amount of retrieval The total activity of the enzyme was (202.6%) compared to the total activity crude enzyme, as well as increased activity after using the ion exchanger type DEAE-cellulose.

Table (1):Stages of lecithin: cholesterol acyl transferase purification in blood serum cardiovascular patients

Purification steps	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Enzyme activity (unit/ml)x10^{-3}	Total activity unit x10^{-3}	Specific activity (unit/mg)x10^{-3}	Purification time number	Recovery rate activity
Blood serum	50	90.54	4527	318.18	15909	3.51	1	----
Precipitation by ammonium sulphate (70%)	35	73.42	2569.7	538.64	18852	7.4	2.09	118.50
Dialysis	38	49.21	1869	848.25	32233	17.24	4.91	202.6
Ion exchange chromatography peak(A)	48	12.75	612	1073.86	51545	84.22	23.99	323.99

Ion exchange chromatography technique:

The ion exchange chromatography technique was applied to the protein solution obtained from the membrane sorting. By tracking the protein concentration in the Elution solution of the sample, two distinct peaks (II, I) were found for the protein presence as shown in Figure (1) The volume of Elution for the two coats (95) ml and (200) ml, respectively.

By monitoring the activity of LCAT in each pack, the activity of the enzyme was found to be concentrated in the Elution solutions of protein (I). The highest enzyme activity was at the size of Elution volume (100) ml (peak A) as shown in Fig.1, About ($1073.86x10^{-3}$) unit / ml. ($84.26x10^{-3}$) unit / mg protein , it was multiplied by (18.68) times more than it was before purification. The total recovery rate of the enzyme was (323.99%) compared to the total activity of the crude enzyme.Since the LCAT enzyme showed activity except in peak A, the enzyme has one symmetry. This is consistent with other researchers. The LCAT enzyme was separated and purified in the pig symmetry and the activity ($1626\ x10^{-3}$) unit/ ml [25], and Kaplan (1969)[26] isolated the LCAT enzyme from plasma of healthy subjects and then partially purified the enzyme by applying ammonium sulphate deposition and ion exchange chromatography using DEAE-cellulose. That the enzyme is the same as one by reaching a single peak and showed activity The total activity of the enzyme was about ($1372x10^{-3}$) units / ml and a recovery rate of (52.6%).

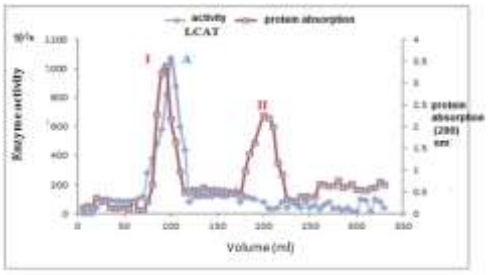

Fig. 1: Elution volume profile Proteins of the LCAT enzyme derived from the ion exchange column using DEAE-Cellulose for blood of a patient with cardiovascular disease

2. Molecular Weight by Electrophorsis Technology:

The molecular weight of the LCAT enzyme isolated from a patient with atherosclerosis was determined by the application of SDS-PAGE electrophoresis. The protein solution obtained from the lyophilize and isolated peak I was injected from the separation process using ion exchange chromatography.

In this process, a protein bundle was identified at a distance of (3.5) cm from the starting point as shown in Fig. 2. This peak was used to estimate the molecular weight of the enzyme and it was found to be approximately (62) kDa, fig(3). Miller and his group (1996) [27] found that the molecular weight of the LCAT synthesized from human plasma was about (65) kDa, and Doi and Nishida (1983) [28] had concluded that the molecular weight of the LCAT, which is produced from human liver cells, Plasma is between (63-67) kDa and about 40% of it is carbohydratea, any that the LCAT is a glycoprotein, while the LCAT is separated from the pig plasma and its amino acid content is found. Amino Acids mimic the amino acids of the enzyme separated from the human plasma and that its molecular weight is about (69) kDa [29].

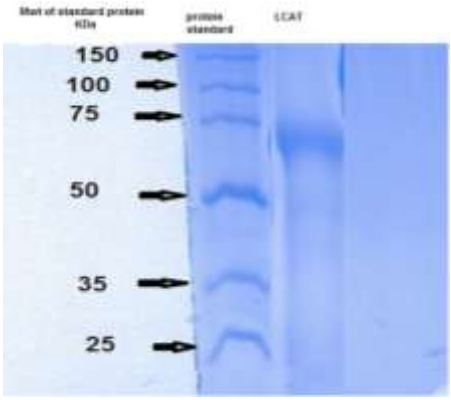

Figure 2: Separated protein bundles after application of SDS-PAGE electrophoresis

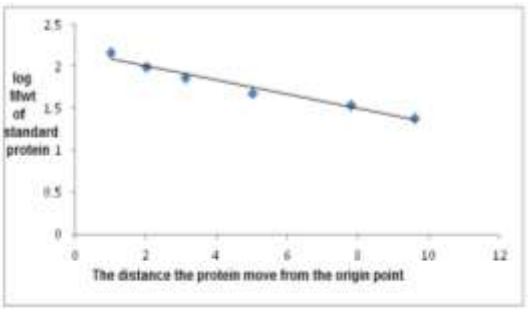

Fig. 3: Standard curve for estimating the molecular weight of the lecithin: cholesterol Acyl ransferase using SDS

3.Factors affecting the Activity of Lecithin-Cholesterol Acyl transferase:

Some factors affecting the activity of LCAT (or the speed of the enzymatic reaction) were studied, as well as optimal conditions in which the protein peak (I) was used as an enzyme source.

✓ **Effect of enzyme quantity**

The activity of LCAT was estimated to have different concentrations of the purified enzyme from patients' plasma. Concentrations between (50-500) µg / ml were shown. Figure 4 shows the linear relationship between the enzyme's activity and its concentration with other factors stability. The enzymatic reaction speed is increased by increasing the concentration of the enzyme with an abundance of the substrate in the reaction environment. This is due to the availability of an increase of active sites that are associated with the base material, due to increasing the speed of the reaction[30,31].

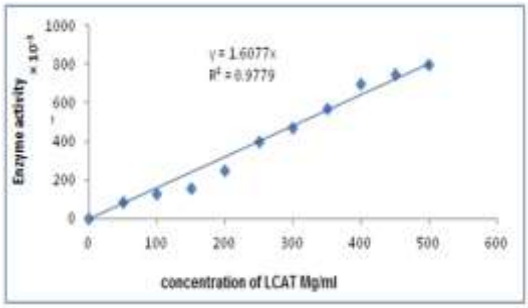

Fig. 4: Effect of different concentrations of LCAT on its activity

✓ **Temperature effect**:

When the LCAT was evaluated at different temperatures (ranging from 5-50 °C), it was observed that the gradual increase in temperature resulted in the enzyme being gradually increased to (35°C). After this degree, decrease in enzyme activity was observed (Figure 5). The reason for the increase in the speed of the reaction or the activity of the enzyme is due to the increased collision and then the docking between the molecules of the enzyme and the particles of the substrate as a result of increased kinetic energy, and the decrease in the activity of the enzyme after this degree of thermal, may be due to a malignant in the synthesis of the enzyme and thus the negative impact on The vital activity of the enzyme's active site[21,30].

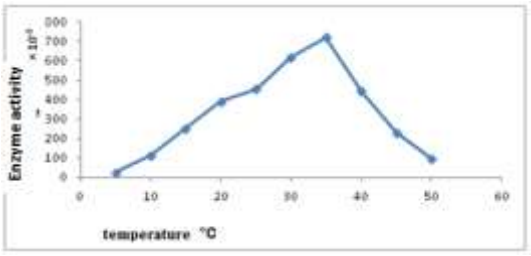

Fig. 5: Effect of temperature on the activity of LCAT enzyme

✓ **Effect of pH:**

In order to reach the optimum pH in which the LCAT enzyme works, the enzyme's activity was followed by the potassium-biphthalate solution at a concentration of 50 mM / L and between pH 4 and pH8.5. pH 7 as shown in Fig. 6. This indicates that pH 7 leads to the best modification of the spatial regulation of the enzyme's active site and thus helps in the best cohesion between the substrate and the active location of the enzyme.

When pH exceeds 7, the spatial structure of the active site will be altered in such a way as to weaken the possibility of binding the enzyme to the base material, which means a decrease in its activity or decrease in the speed of the enzymatic reaction[21,30]

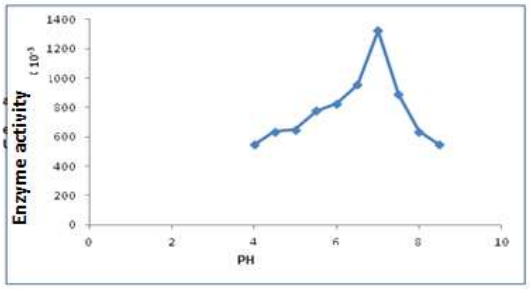

Fig. 6: Effect of pH on the activity of LCAT.

✓ **Effect of concentration of the Substrate**:

The LCAT was followed by different concentrations of substrate (lecithin-cholesterol), which ranged from (5-75) µg/ml. Figure (7) shows that increasing the concentration of the substrate increased the speed of the enzymatic reaction until it reaches a value that

does not occur after the increase in the speed of the enzymatic reaction, that is, the reaction reaches the maximum speed Vmax, and interpreted the worlds Machiles and Mentin The use of low concentrations of the base material makes the active sites of the enzyme unsaturated. However, when the concentration of the substrate is increased, the active sites will be saturated and the enzyme activity or reaction rate will be at its peak [21] for the LCAT enzyme was reached The concentration of (Lecithin-cholesterol) which led to the maximum speed was (17.5) µg/ml. The value of Vmax and Km (1000.3 x 10^{-3}) units/ml and (17.5) µg/ml, respectively. When the Lineweaver-Burke equation was adopted, the value of Vmax and Km (1000.2 x 10-3) Were found to be (17.56) µg / ml respectively, as shown in Fig. 8.

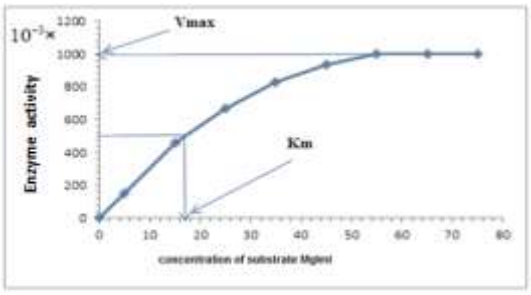

Figure (7): Effect of the concentration of the substrate on the activity of the LCAT enzyme

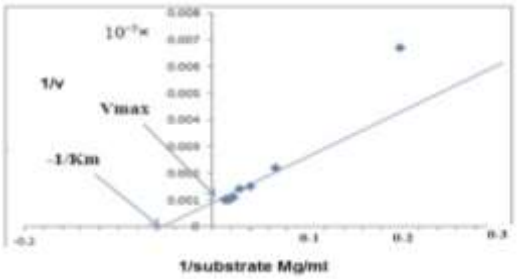

Figure (8): Effect of the concentration of the substrate on LCAT activity by applying the Line weaver_ Burke equation

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دراسة كيموحيوية حركية لانزيم LCAT المنقى جزئيا من مصل مرضى القلب والاوعية الدموية

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الملخص

تضمنت الدراسة تنقية جزئية لإنزيم اللسثين – كوليستيرول اسايل ترانسفريس (LCAT) من مصل دم شخص يعاني من أمراض الأوعية الدموية، إذ سبق أن اصيب بجلطة قلبية، وقد طبقت تقنيات عدة للتنقية ابتداء من الترسيب بكبريتات الامونيوم ثم الفرز الغشائي فكروماتوغرافيا التبادل الايوني واخيرا الهجرة الكهربائية. وتم التوصل الى ان لإنزيم LCAT متماثلا واعلى فعالية كان مقدارها (1073.46×10^{-3}) وحدة انزيمية/ مل، كما تم الحصول على حزمة منفردة عند تطبيق الهجرة الكهربائية على الانزيم المنقى من التبادل الأيوني، وكان بوزن جزيئي تقريبي مقداره (62)كيلو دالتون. اهتمت هذه الدراسة كذلك بتوصيف الانزيم، إذ وجد أن للإنزيم درجة حامضية مثلى مقدارها pH7 عند استخدام محلول البوتاسيوم باي فثاليت كمحلول منظم، وكانت درجة الحرارة المثلى (35°م).