ISSN: 1343-4292
Volume 141, Issue 07, July, 2023

Isolation, purification, and the role of Aryl esterase in the serum of patients with atherosclerosis.

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ABSTRACT— The research included a study about Aryl esterase enzyme in (60) serum samples of patients with atherosclerosis whose ages ranged between 25 to 75 years and compared it with (60) serum samples of the control group whose ages ranged between 25 to 60 years in Tikrit City from November 2022 to February 2023. The results showed a highly significant decrease ($P \leq 0.005$) in the serum levels of aryl esterase of patients with atherosclerosis (71.2 ± 3.7 U/L) compared to the control group (135.36 ± 1.7 U/L). The result also demonstrated no variation due to sex but a decline with age progress. A significant decrease was observed in the activity of aryl esterase and the level of high-density lipoprotein (HDL), and a significant increase in the level of troponin T(TnT), total cholesterol(TCH), triglycerides (TG), very low-density lipoprotein (vLDL), and low-density lipoprotein (LDL) in the serum of patients compared to the control group. The relationship between the activity of the enzyme and many clinical variables was studied in both groups by finding the linear correlation coefficient, as it was found that HDL was associated with the activity of the enzyme with a direct relationship in the group of patients.

KEYWORDS: Aryl Esterase, Atherosclerosis, Troponin, Lipoproteins.

1. INTRODUCTION

PON1 is an enzyme with three activities: paraoxonase, arylesterase and dyazoxonase, the first two of which decrease hypercholesterolemia, diabetes and CVD, Aryl esterase (EC 3.1.1.2) is a hydrolytic enzyme associated with (HDL) that can hydrolyze lipid peroxides. It is considered an antioxidant and anti-atherosclerotic enzyme due to its protective effect on LDL oxidation and the occurrence of atherosclerosis [1], [2], which is mainly due to studies by Mackness and researchers et al., which described the role of HDL-binding PON1 in reducing the accumulation of lipid peroxide on LDL and cardiovascular disease Where the content is surrounded by external protein, as is the case in LDL, and it has an essential role in many diseases, the most important of which are cardiovascular diseases, including atherosclerosis [3]. The researchers also showed that the enzyme aryl esterase is a glycoprotein composed of (453) amino acids, while the researcher Ozols mentioned that it is a polypeptide consisting of (53) amino acids [4]. The enzyme has a molecular weight that varies according to the organism, tissue, and type of nutrition. The researchers, Aviram and Davies, showed that the crystalline structure of the enzyme is in the form of a fan with six blades, with an effective site important for binding with HDL [5]. The enzyme contains only one free (-SH) sulfhydryl group at the amino acid. Cysteine at site (482), which is essential for its work in protecting lipid proteins from lipid peroxides. It was found that replacing cysteine with alanine or serine makes it unable to protect LDL from oxidation and influence the content of lipid peroxides [6]. The aryl esterase enzyme contains two distinct binding sites with Ca^{2+}, which maintains enzyme stability and activity and is necessary for enzyme catalysis, as Ca^{2+} acts as an electrophilic catalyst for the enzyme [7].

Atherosclerosis is the accumulation of fatty acids/lipids and oxidized lipids in the arteries' innermost layer,

the intima. The term atherosclerosis consists of two parts; atherosis (accumulation of fat accompanied by several macrophages) and sclerosis (fibrosis layer comprising smooth muscle cells [SMC], leukocytes, and connective tissue) [8]. Atherosclerotic cardiovascular disease (CVD) is a leading cause of worldwide vascular disease [9]. Epidemiological studies have demonstrated that (LDL) and (HDL) are independent causes of CVD. Clinically, lipoprotein disorders, such as increased LDL-cholesterol or decreased HDL-cholesterol levels, are common characteristics of CVD [10].

2. Materials and methods

The blood was drawn from the vein using a (5 ml medical syringe) and placed in new, sterile plain tubes free of additives. The blood was left for (10) minutes, after which the blood serum was separated from the coagulated part with a centrifuge. Centrifuge at a speed of (4000 rpm) revolutions per minute for (10) minutes, then the clear blood serum was withdrawn and placed in small tubes (Epindroff), as the activity of the aryl esterase enzyme was measured directly, and the residue was kept at a temperature (4ºC) to perform protein determination measurements.

(1 Partial purification of Aryl esterase from the serum of patients with atherosclerosis

1. Precipitation and protein separation using ammonium sulfate:

The process of sedimentation of the proteins in the serum was carried out using graduated concentrations of ammonium sulfate salt until a saturation rate of 80% was reached, where 3.5 g of ammonium sulfate was added during a time of (40-60) minutes, where the serum was placed in an ice bath with continuous stirring, and then it was separated. The filtrate was removed from the residue, and then the precipitate was dissolved in the smallest amount of buffer solution (Tris-HCL) at a concentration of (10Mm) pH = 7.4) and then the filtrate was taken, and ammonium sulfate was added to it to precipitate the enormous possible amount of proteins [11].

2. Dialysis:

One of the most important and oldest purification methods is that it is used to remove the remaining ammonium sulfate added to precipitate the protein by placing the dissolved protein in a dialysis bag and then immersing the bag in Tris-HCL buffer solution (10 mm (pH = 7.2) and the solution is changed regulator from time to time for 18 hours. This process was done at a temperature of (4 ± 1) to maintain the enzyme's activity (PON1). After the membrane separation process was completed, aryl esterase and protein concentration was measured [12].

1- Kinetic Study of Aryl esterase:

The kinetic properties of these enzymes were studied after they were separated and purified from the serum of Patients with atherosclerosis:

1- Study the effect of substrate concentration:

This effect has been studied using different substrate concentrations (phenyl acetate) and its impact on the activity of the partially purified aryl esterase, where different concentrations (0.2, 0.4, 0.6, 0.8, 1, 1.2). mmol/l to find out the effect of the change in the substrate concentration on the action of the enzyme, where the measurement method used to measure the activity of the enzyme was used and by drawing the relationship between the reaction speed and the substrate concentration to determine the ideal substrate concentration for maximum interaction velocity (Vmax).To define the Michaels-Menten constant value (Km), use the Line Weaver- Burk plot, which has reverse values for both velocity and concentration (1/v vs 1/[s]).

2- Determination of Optimum pH:

The effect of a buffer solution (10mM Tris–HCl pH 7.4 and (Sodium

ISSN: 1343-4292
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Carbonate–Bicarbonate) on Aryl esterase velocity was investigated. At 37°C, pH (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) solutions containing (phenyl acetate) (5mmol/L) were used. The optimum pH was determined by plotting the relationship between interaction velocity and pH and measuring enzyme activity.

3- Influence of Optimal Temperature

The activity of the aryl esterase enzyme was measured in the presence of different temperatures (15, 25, 35, 45, 55, 65) and using a buffer solution (Tris-HCL 10Mm) with a pH = 7.4 and a substrate concentration of 5. Then the relationship linking the reaction speed was drawn with temperature to get the optimum temperature.

5. Electrophoresis:

This method is essential to separate biochemical compounds such as different proteins, which have charged groups with positive and negative charges and have specific sizes, where these charged compounds move in an electric field. And because they have a charge, they move to the opposite pole with the charge when placed in this field. And there are many factors affecting the speed of the particles within the field, such as (the size of these particles, their number, the type of charge they possess, and the strength of the electric field), and the speed of these particles varies when placed at a specific starting line. The electric current is passed towards the opposite poles with the charge. And after a particular time has passed, the location of the particles will vary. These molecules are from the starting point [13].

1- Electrophoresis on a polyacrylamide gel in the presence of SDS to measure the molecular weight of the purified enzyme:

Some modifications have been made to the Laemmli method [14] in preparing gel separation and electrophoresis.

a) The solutions used:

1- Acrylamide _biacrylamide stock solution 30% Dissolve 29 gm of acrylamide and 1 gm of acrylamide in 100 ml of distilled water and keep this substance in a dark vial.

2- The buffer solution for the separating gel (1.5 M Tris-HCL pH 8.8) was prepared by dissolving 12.8 g of Tris-base in 80 ml of distilled water. The pH was adjusted at 8.8 using 1 M HCL, and the volume was completed using distilled water to 100 ml.

3- A solution of 10% Sodium dodecyl sulfate (SDS) was prepared by dissolving 10 g of SDS (sodium dodecyl sulfate) in about 100 ml of distilled water.

4- Bromophenol blue solution 0.5%. Dissolve an amount of bromophenol blue, about 0.5 g, in distilled water, about 100 ml.

5- The solution used for storing the sample (Stock sample solution) Mixing the buffer solution in an amount of 1.2 ml, used for the separation gel with pH 8.8, SDS solution about 10% and sucrose.

6- The electrode buffer solution, about 10 g of SDS, 144 g of chlorine, and 30.3 g of Tris-base were dissolved together, and after completing the process, the volume was completed to a litre of distilled water, and the pH was adjusted to 8.3. To reach the use stage, it is diluted to (1:10) with distilled water.

7- Fixation solution: This solution consists of 10% trichloroacetic acid (TCA) and 40% methanol.

8- 10% (APS) Ammonium persulfate solution: This solution is prepared immediately before use, as it is prepared from 10 g of APS in 100 ml of distilled water.

9- Expansion solution (TEMED) (N, N, N, N-tetramethyl ethylene diamine)

10- Staining solution: Coumas blue R250 (0.1%) CBB R250 Cammasie brilliant blue was used with 10% acetic acid and 40% methanol. The solution is filtered and kept in an opaque vial.

11- Standard Protein: Solution The standard solubilization process for the proteins (BSA, ovalbumin, glycogen phosphorylate, carbonic anhydrase, α-Lactalbumin, β-galactosidase, and trypsin inhibitor) was carried out at a concentration of 5 mg/ml in the sample solution.

12- A destaining solution consisting of 10% acetic acid and 40% methanol.

b) Procedure:

1- Preparation of the 10% separation gel: The 10% separation gel was prepared by mixing 4.6 ml of distilled water with 2.5 ml of buffer solution for the separation gel with pH 8.8, 100 µl of 10% SDS, 100 µl of SPS 10%, and 6 µl of TEMED. These components are mixed well and then transferred to the plate for the migration process and allowed to reach a solid consistency (hardening of the gel).

2- Preparation of sample and protein standard solutions: 25 microliters of β-mercaptoethanol were added to 0.5 ml of sample and protein standard solutions. After the addition process, it was heated at 100 m for 5 minutes and left to cool.

3- The process of adding 15 microliters of standard samples and protein solutions to each hole in the gel was done after placing the buffer solution for the electrode with a pH of 8.3 to cover the plate's surface. When the electric current was passed, the separation process began and continued until the dye was almost finished, after which the gel was extracted from the plate and placed in the fixing solution for two hours. Then it was immersed in a solution of formula CBB R250 for four hours. After the completion of the staining process, the gel was immersed in the dye removal solution, and the switching process continued several times until the blue bands appeared clearly. Gel endorsement device for visualizing the packages and determining the weight, compared with the standard protein packages staged with it.

3. Results and discussion

3.1 Partial Purification of Aryl Esterase from the serum of patients with atherosclerosis:

Proteins are generally concentrated in the first steps of the enzyme purification process to obtain a partially pure enzyme and remove water. Usually several salts are used for this step, including ammonium sulfate $(NH_4)_2\ SO_4$) because it is characterized by good solubility and in order to work equalize the protein charge and its cheapness, which leads to a reduction in The solubility of the protein and its precipitation, and this is called salting out [15], [16] and it is one of the most essential steps in the purification process, which was adopted by most studies for enzymatic purification, the process of purification of aryl esterase from the serum of patients with atherosclerosis takes place in several steps, in the first step the enzyme was precipitated by ammonium sulfate at a concentration of 80% to concentrate the enzyme to obtain it with high purity, which is one of the most essential stages through it, we get a reduction in the volume of the enzyme extract and an increase in the efficiency of the purification process, as well as ridding the enzymatic extract of any other unwanted substances such as proteins, water and sulfates, as they interfere with the effectiveness of enzyme and then get rid of the excess salt during the process of Dialysis by Tris-HCL with pH = 7.4, as the degree of purification of this enzyme at this stage was 3.12 times, with an enzyme yield of 74.4%, and a specific activity (0.00050 U / mg) as shown in Table (1), then the process of purifying aryl esterase was completed by ion exchange chromatography using DEAE-Cellulose resin as filling material for the column and graded solutions of sodium chloride salt, where a single peak was obtained as shown in figure (1), where the purification degree was (4.25 times the enzyme yield). It reached ((44.29) and a specific activity (0.00068 U/mg), as shown in Table (1). To increase the purification after that, the gel filtration method was applied using the gel (Sephadex G-100), as a single peak was also obtained for the aryl esterase. The purified aryl esterase was as shown in figure (2), with a degree of purification of (6.26) times, an enzyme yield of (43.14%), and a specific effectiveness (U/mg 0.001002), as shown in Table (1) below, as the different methods used for purification were combined Aryl esterase and from various sources.

ISSN: 1343-4292
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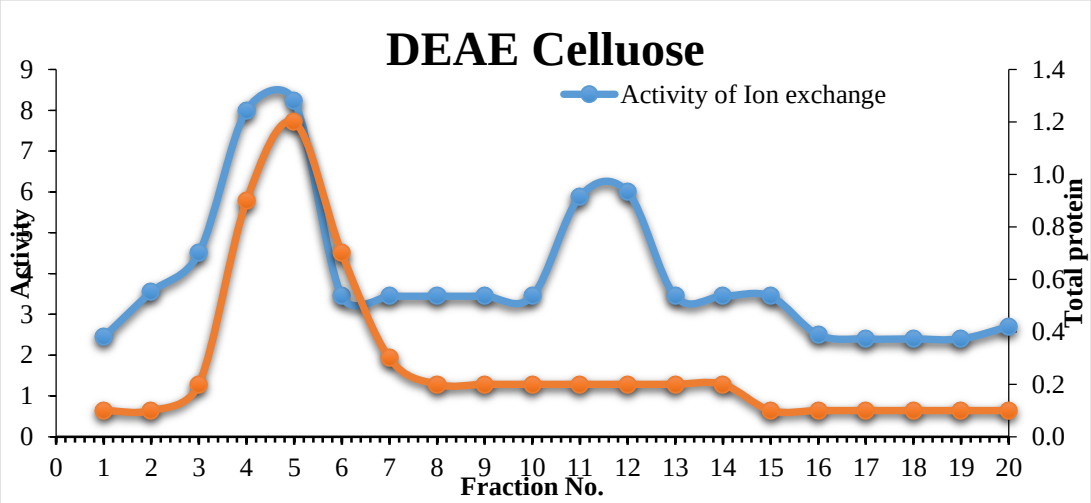

Figure (1): Purification of aryl esterase by ion exchange chromatography

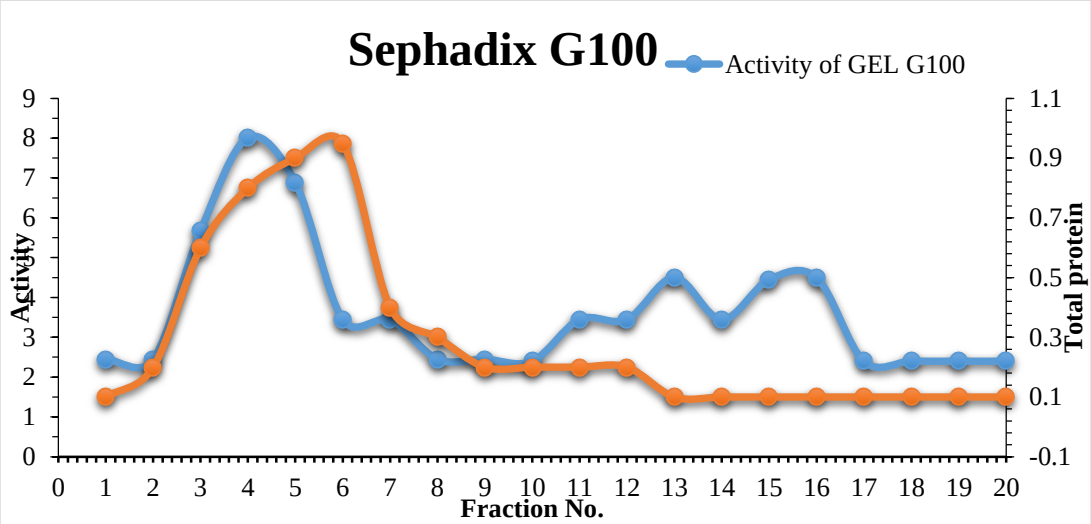

Figure (2): Purification of aryl esterase by gel filtration chromatography

Table (1): Stages of purification of aryl esterase from the serum of patients with atherosclerosis

Purification Steps	Volume Ml	ACTIVITY IU/ml	Total Activity	Protein Concentration mg/ml	Specific Activity U/mg	Recovery Yield %	Fold of purification	Total protein
Crude	9	8.81833	8.8929	62	8.88816	188	1	558
Precipitation	8	8.88789	8.86312	57	8.888138	67.9	8.86	456
Dialysis	8	8.88865	8.8692	17	8.88858	74.4	3.12	136
Ion exchange (DAEA-Cellulose)	5	8.88823	8.84115	12	8.88868	44.29	4.25	68
Gel Filtration	5	8.888816	8.84888	8	8.881882	43.14	6.26	48

3.2 Determination of molecular weight by electrophoresis using SDS:

Determination of the molecular weight of aryl esterase by relay method on a polyacrylamide gel) in the presence of sodium dodecyl sulfate (SDS-PAGE). This method is widely used in biochemistry to separate large biological molecules such as proteins, nucleic acids and enzymes. As the enzyme is treated with SDS to disintegrate the protein, chains of variable sizes are surrounded by negatively charged SDS particles, as it removes the original charge of the protein, and these chains move by electrical migration depending on the molecular weights of the molecule. The protein surrounded by the SDS molecules moves towards the positive pole depending on the charge-to-mass ratio. When migration occurs on a polyacrylamide gel using SDS, it also moves depending on its size,195. As previously mentioned, during electrical migration, the movement of proteins or enzymes depends on several factors. These factors include molecular weight, charge, and electric current. Therefore, proteins will move at different distances depending on the molecular weight. figure (2) shows the shape of aryl esterase when transported with standard solutions of known molecular weight.

As shown in the figure below (2) [17] and by comparison between the enzyme package and the standard compound packages, it was found that the molecular weight of aryl esterase purified from the serums of patients with atherosclerosis is equal to (80 kD), as shown in the following figure:

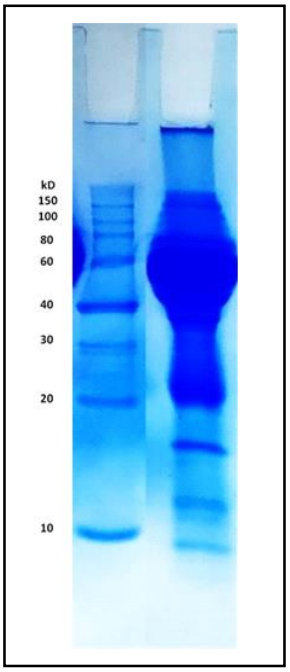

Figure (2): shows the electrophoresis of purified aryl esterase from the serum of patients with atherosclerosis.

3.3 Kinetic Study of optimum condition for aryl esterase partially purified from serum of patients with atherosclerosis:

1- Effect of Different Substrate Concentrations on Enzyme Activity with Determination of Michaelis-Menten's Constant (Km) and Maximum Velocity (Vmax):

The effect of the substrate concentration (phenyl acetate) on the speed of the enzymatic reaction of the aryl esterase partially purified from the serum of patients with atherosclerosis and separated from the column (Sephadex G-100) was studied, where it was found that there was an increase in the speed of the enzymatic reaction with an increase in the substrate concentration (phenyl acetate) and the reaction speed continued to

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increase until the maximum speed is reached at a concentration of (1M) (as in figure 3), which shows an increase in the speed of the enzymatic reaction by increasing the substrate concentration, as the figure itself shows the aryl esterase enzyme purified from the serum is subject to the Michaelis-Menten equation, as the resulting form is a hyperbola. There are several ways to calculate the value of the Michaelis-Menten constant Km, which is defined as the affinity between the enzyme and the substrate. Familiarity increases between them, and through it can determine the efficiency of enzyme analogues to catalyze vital reactions and to know the stability of enzymes and the effect of inhibitors and activators on enzymatic activity [18] or the substrate concentration when the rate of speed is half the value of the maximum speed (V max), when the enzyme is subject to the Michaelis-Menten equation, Km can be calculated for it, as the Lineweaver-Bark method is the most accurate and best in practice due to its ease of use and the lack of mathematical operations in it and its efficiency Indicating the accuracy of the experiment.

To calculate the values of the constant Km for the enzyme purified by gel filtration, the above method was followed as in figure (4), and the value of Km was equal to (16.66 mM), as well as its maximum velocity at the base material itself was equal to (0.909 ng/ml). These results contrasted with some previous studies, which were conducted to determine the kinetic constants of the enzyme, and the differences between all these studies were clear and almost natural due to the different sources from which the enzyme was purified and the various methods used in purification.

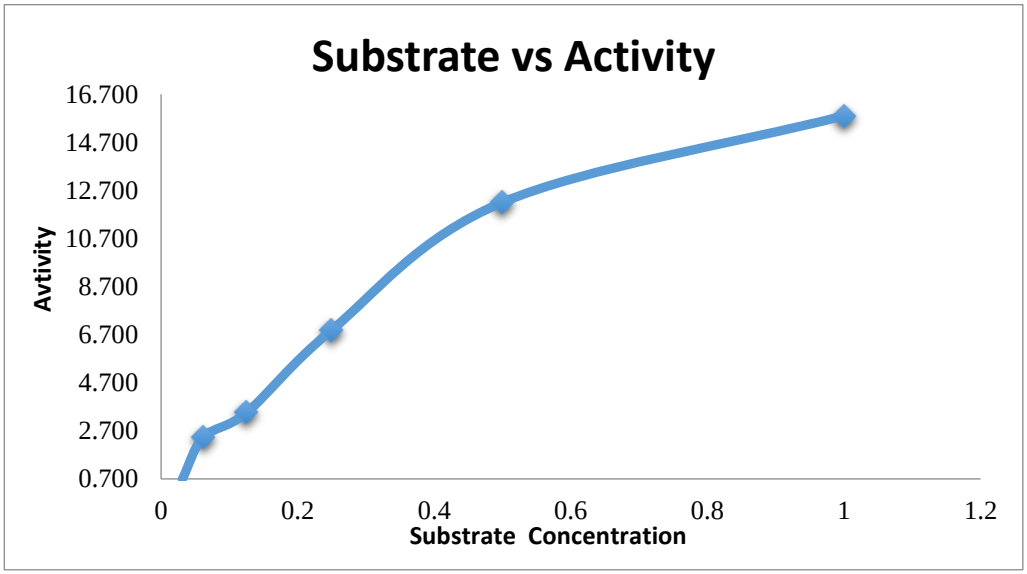

Figure (3): Michaelis-Menten's plot showed the effect of Substrate Concentration on Aryl esterase activity.

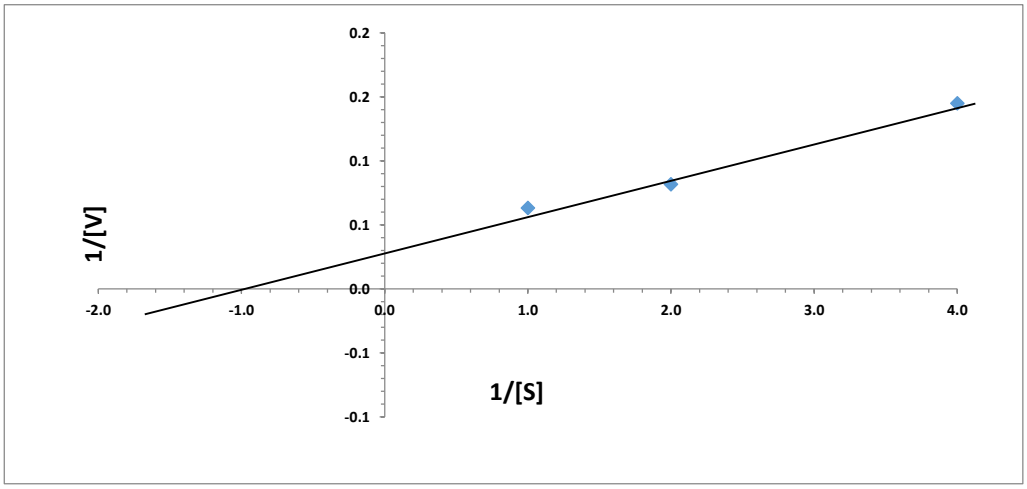

Figure (4): Line Weaver–Burk plot for partially purified.

2- Effect of pH on Enzyme Activity:

The degree of pH affects the activity of the enzyme as a result of the difference in the nature of the enzyme and its chemical composition as well as the multiple ionic groups carried by the enzyme, as each enzyme has an optimum pH because the enzymes are very sensitive to the change in the concentration of the hydrogen ion H + [19] and an increase in the speed of the enzymatic reaction was observed with increasing pH until reaching the maximum speed at pH = 7.5, after which a decrease in the speed of the enzymatic reaction was observed, and it may be due to the occurrence of internal electrical repulsion or due to the loss of electrical charge on the side of the amino acid chains resulting from the dissociation of the protein constituent of the enzyme (leading to protein denaturation) and this What prevents the enzyme from forming a complex - enzyme precursor (ES) [20] and figure (5) shows the relationship between the rate of the enzymatic reaction and the altered pH values of the purified aryl esterase enzyme from patients with atherosclerosis.

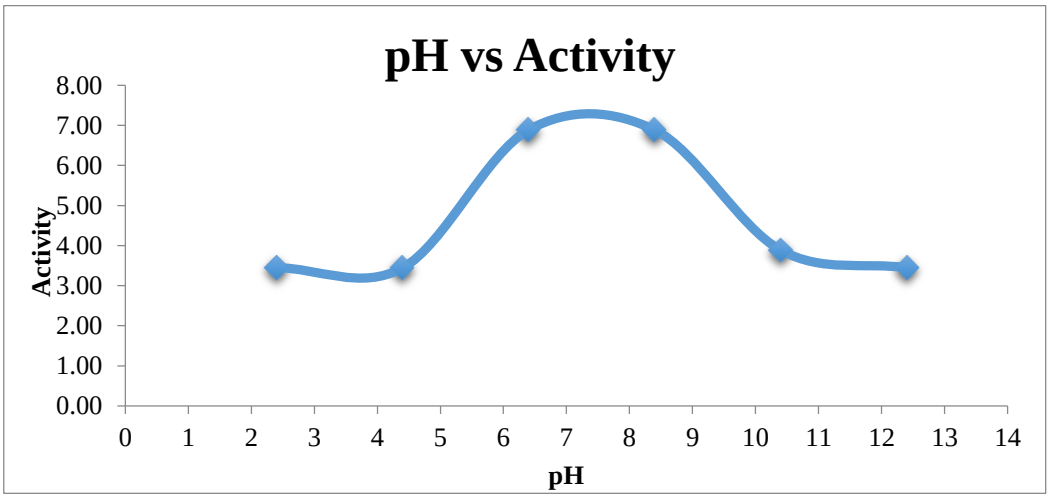

Figure (5): Effect of pH on Aryl esterase activity.

3- Effect of Temperature on Enzyme Activity:

The optimum temperature can be defined as the temperature at which the enzyme is at its maximum speed without affecting the enzyme's effectiveness within a period of no less than the period required to estimate the enzyme's effectiveness. The enzyme thermally and the extent of ionization of the active groups and the substrate [21], where the enzymes have a temperature Optimum (equal or more or less) than that

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temperature of the cell that contains it, and the enzymatic reaction speed begins to increase with an increase in temperature until the enzyme reaches the temperature at which it has the highest activity, and that is the optimal temperature for this enzyme, and after that the activity begins to decrease with an increase in temperature This is due to the occurrence of damage and denaturation of the enzyme, due to the effect of temperature on the ionization energy of the active aggregates present on the surface of the enzyme and the substrate, and due to the increase in the kinetic energy of the molecules that break the bonds between the active amino acids, and this causes the enzyme to lose its effectiveness, and because the enzymes are complex protein molecules whose catalytic activity is affected in Regular triple structural structure, so high temperatures change the geometry and natural shape of the enzyme, causing the enzyme to lose its activity [22]. The results showed an increase in the reaction speed of the aryl esterase enzyme with the temperature rise, and the maximum was at the temperature (37 ° C), which is the optimal temperature for the enzyme to work, as the enzymatic reaction speed reaches its maximum value and the pH is constant. Figure (6) below shows the effect of temperatures Heat affects enzyme activity.

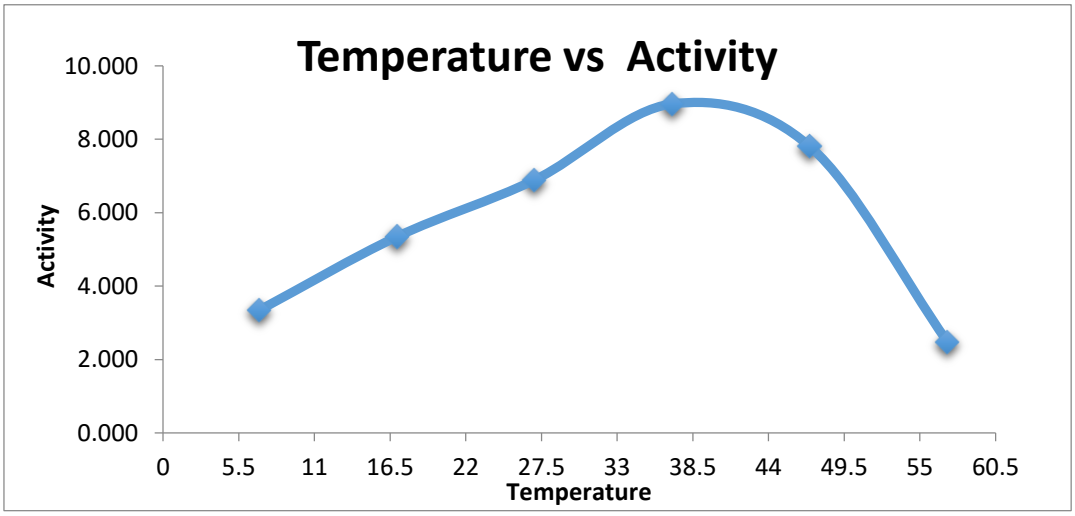

Figure (6): Effect of Temperature on Aryl esterase activity.

4- Determination of the optimal time for the activity of aryl esterase:

The activity of the enzyme was measured at different times (from 1.5 to25) minutes. It was found that the optimal time to measure the activity of the purified aryl esterase enzyme from patients with atherosclerosis is (5) minutes, as the speed of the enzyme reaction increases with increasing time until reaching the highest effectiveness at a time (5) minutes until the enzyme is saturated with the base material during this period, after which the activity begins to decrease gradually, and the reason for this decrease is the thermodynamic properties. For the enzyme, the hydrogen bonds of the enzyme are broken due to its dynamic activity, and thus the stability of the three-dimensional shape of the enzyme decreases. Thus the effectiveness decreases [23].

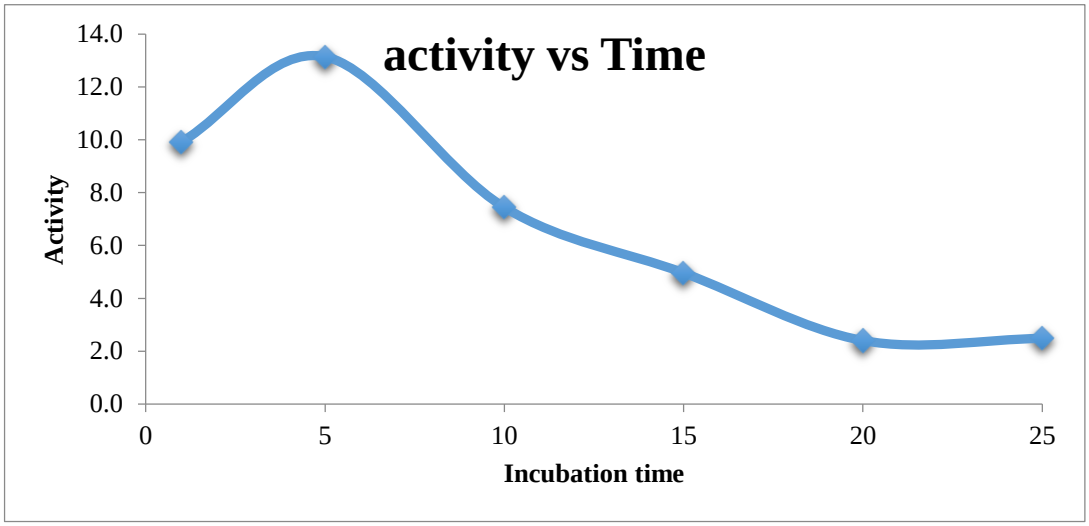

Figure (8): Effect of Time on Aryl esterase activity.

4. Conclusion

1- Low levels of aryl esterase, (HDL) and high levels of (cTnT, TG, VLDL, LDL) in patients with atherosclerosis compared to healthy people.

2- The degree of purification of the partially purified aryl esterase by gel filtration chromatography (Sephadex G-100) was (6.26) times, with an enzyme yield of (43.14%) and a specific activity (U/mg 0.001002).

3- The values of Km and Vmax of the purified enzyme are (Km = 16.66 mM) (Vmax = 0.909 ng/ml), and the optimum temperature for enzyme action was (37 °C), while the optimum pH for the enzyme was (pH = 7.5).

Recommendation:

1- A biochemical and molecular study of aryl esterase analogues in patients with atherosclerosis.

2- Studying aryl esterase levels in other diseases such as cancer and diabetes type 1 and type 2.

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