A Kinetic and Thermodynamic Study of The Enzyme Lactate Dehydrogenase Purified from Cardiac Patients' Blood

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Abstract

A kinetic and thermodynamic study was conducted for the enzyme partially purified from heart patients by gel filtration and dialysis. It was found that the optimal concentration of the enzyme was (0.8M), and the optimal concentration of polyphenols to inhibit the purified enzyme was (5M). The values of Km and Vmax for the purified enzyme are (Km=0.243M) and (V_{max}=1.5197 M/min). The optimum temperature for enzyme action was (40°C), while the optimum pH was (7.4). The thermodynamic results of the purified enzyme showed that the activation energy is 74545J.K^{-1}.mol^{-1} and the activation enthalpy for the transition state of the ES complex was 9818 J.mol^{-1}, while the activation entropy for the transition state was 57.557 + KJ.K^{-1}.mol^{-1}, And the value of the free energy of the transition state is 14.568 - Kj.mol^{-1}. The positive value of enthalpy indicates that the enzymatic reaction is energy-absorbing. In contrast, the positive value of entropy suggests that the enzyme complex ES is less regular than the enzyme and the substrate and more random, while the negative value indicates free energy offers that the course of the reaction towards the formation of the enzyme complex is automatic, that is, it does not need to be equipped with energy. The study aimed to study the kinetics of the enzyme lactate dehydrogenase purified from the serum of heart patients, such as temperature, pH, and concentration of the substrate, as well as calculating the Michaelis-stencil constant Km for the enzyme and the maximum speed Vmax and then extracting polyphenol compounds from pomegranate peels and studying their effect on the activity of lactate dehydrogenase enzyme purified from serum of heart patients. And a study of the thermodynamic parameters of the enzyme lactate dehydrogenase purified from the serum of heart patients.

Keywords: Lactate Dehydrogenase (LDH), Inhibition, Polyphenols, Thermodynamic properties.

1. Introduction

Lactate dehydrogenase (LDH) is a vital tetramer enzyme in the anaerobic metabolic pathway and belongs to the oxidoreductase class [1]. Oxidoreductase, whose function is to catalyze the reverse conversion of lactate to pyruvate with the reduction of NAD+ to NADH and vice versa. The enzyme is present in various organisms, including plants and animals [2]. It is present in all tissues and serves as an essential checkpoint in the process of gluconeogenesis and the metabolism of deoxyribonucleic acid (DNA) [3]. Heart, liver, skeletal muscle, kidney, and erythrocytes, while smaller amounts are found in the lung, smooth muscle [4], and brain because of its diffuse activities in many tissues of the body, LDH is elevated in a variety of disorders, so LDH tests are gaining great clinical importance when separated into isoenzyme fractions, as there are five forms of it in human blood serum and distributed in different tissues [1]. All types of LDH consist of four peptide chains (molecular weight of each = 33,500 Daltons), and each class contains a different ratio of two peptide chains. A chain called M (for muscle) and H (for the heart). In the skeletal muscle, the LDH enzyme consists of four M chains, the nucleus contains the LDH enzyme composed of four H chains, and the rest of the tissues consist of both types [5]. The LDH enzyme is considered a cellular enzyme (cytosolic enzyme), as it works on the transfer of hydrogen ions, as well as works on two essential substances; that is, it catalyzes the oxidation of lactate to pyruvate in the presence of NAD +, which receives hydrogen ions and favors the balance of the reaction towards the reverse direction that reduces pyruvate to lactate [6]. The LDH enzyme plays a significant role in the oxidation process of glucose or glycogen, as it appears in the last step of the glycolysis process, stimulating the reduction of pyruvate in the presence of NAD+ to lactate in the absence of Oxygen [7], during anaerobic conditions, but in the case of aerobic conditions in the presence of Oxygen is not formed, so lactate is not formed, as the process of oxidation of pyruvate continues through the cycle of citric acid (TCA), turning into carbon dioxide and water [8, 9]. About heart diseases, there is an increase in the activity of the LDH enzyme, as was observed in myocardial infarction [10, 11], where there is an increase in the exercise of LDH-2, LDH-1 more than the standard

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limit, and it was also found that the increase in the activity of (LDH) -1/LDH-2) in the blood serum very quickly is an indication of the diagnosis of acute myocardial infarction (Acute myocardial infarction) [12]. The activity of the enzyme LDH also increases in bladder diseases, kidney tumors (kidney malignancy), and Diabetic nephrosclerosis [13]. In general, LDH plays a significant role in diagnosing many common and rare diseases [14]. It has been observed that heart muscle cells that have been treated with inhibition have significantly increased oxygen consumption [15]. This means that inhibition of the LDHA enzyme inhibits aerobic glycolysis and restores the natural dependence of heart muscle cells on oxidative phosphorylation and thus increases cellular ATP production. That is, it improves the metabolic maturation of heart muscle cells [16]. In addition, inhibition of LDHA leads to an increase in the calcium capacity of the heart muscle cells to eliminate the risk of decreased calcium release that causes poor contractility of the heart muscle cells [17], which causes a risk of ventricular arrhythmias or sudden cardiac death [18]. Polyphenols are an essential chemical in many plants and are among the compounds that have antioxidant properties depending on their carbon structure. Pomegranate is one of the fruits richest in phenolic compounds, especially peel. Which constitutes 30% - 50% of the whole fruit, and the percentage of polyphenols in the pomegranate peel was ten times higher (249.4 mg / g) than that found in the pulp (24.4 mg / g) [19, 20], which is represented by urine J phenols in peels with caffeine acid, coumaric acid, chlorogenic acid, gallic acid, ellagic acid, cyanidin, and quercetin [21] Polyphenols had shown therapeutic benefits for many diseases, such as heart diseases, especially vascular diseases (CVD) and metabolic syndrome (MetS) that cause obesity and increase It reduces the risk of cardiovascular disease [22, 23], reduces systolic pressure and diastolic pressure, reduces triglycerides in women with the acute coronary syndrome and provides health benefits against atherosclerosis and consequent cardiovascular disease [24]. The hydroalcoholic extract of pomegranate reduces blood pressure, total cholesterol and LDL cholesterol, which leads to the relaxation of endothelial-dependent coronary arteries and improves cardiovascular parameters as it works to inhibit the enzyme lactate dehydrogenase, which increases in heart patients [25].

2. Experimental

Instruments used and diagnostic kits

Device	Hot plate	Ultra Violet & Visible Spectrophotometer	Water Bath	PH meter	LDH kit
Origin	England	(Cecil Instrument Limited) USA	Gemmy-England	Radiometer-Denmark	Biolabo – France

The kinetic properties of the enzyme lactate dehydrogenase were studied after its separation and partial purification from the serum of heart patients by gel filtration, and they included:

Effect of base material concentration:

The effect of different concentrations of the base substance of Pyruvate on the activity of the partially purified lactate dehydrogenase enzyme was studied using different concentrations (1.4, 1.2, 1, 0.8, 0.6, and 0.4) mmol/L to find out the effect of the attention of the base material on the action of the enzyme lactate dehydrogenase in the presence of NADH. At a concentration of 2.4 mmol/L, the reaction speed was measured according to the method (LDH kit Biolabo) [26], and by drawing the relationship between the reaction speed and the concentration of the substrate to know that the enzyme is subject to the Michaelis-Menten equation. The values of Michaelis-Menten constant Km were obtained using Lenover-Burke method. Graphs linking inverse values of velocity and substance concentration (1/[S] vs 1/v) [27].

Determination of the optimum pH: The effect of the pH of the buffer solution ((10mM Tris - HCl pH 7.4) on the reaction rate of lactate dehydrogenase was studied, where solutions with different pH values (4.5, 5.5, 6.5, 7.5, 8.5 9.5) were used in the presence of the base material pyruvate at a concentration of 1.4 mmol/L In the presence of NADH at a concentration of 2.4 mmol/L and a temperature of 37 °C, the activity of the enzyme was measured according to the method (LDH kit Biolabs), and by drawing the relationship between the reaction speed and the pH, and the optimal pH was identified.

Effect of temperature: The method mentioned in the (LDH kit Biolabs) was used to measure the concentration of the enzyme lactate dehydrogenase, where the reaction was carried out at different temperatures (10, 20, 30, 40, 50 and 60) ° C in the presence of NADH at a concentration of 2.4 mmol/L and a pyruvate base substance concentration of 1.4 (mmol). /L, then plotted the relationship between the reaction rate and temperature to determine the optimum temperature for the reaction.

Effect of time: The method mentioned in (LDH kit Biolabs) was used to measure the concentration of the enzyme lactate dehydrogenase, where the reaction was carried out at different time times (1.5, 3, 6, 12 and 24) minutes in the presence of NADH at a concentration of 2.4 mmol/L and a substrate concentration of 1.4 mg/L, then plotted the relationship between reaction speed and time to know the optimal reaction time.

Effect of aqueous extract: The method mentioned in (LDH kit Biolabs) was used to measure the concentration of the lactate dehydrogenase enzyme, where the reaction was carried out by using different concentrations of the extract, which

are (500, 50, 5, 0.5, 0.05, and 0.005) M to find out the effect of the attention of the aqueous extract of pomegranate peels on the action of the enzyme. Lactate dehydrogenase was present in the presence of NADH at a concentration of 2.4 mmol/L and a substrate concentration of 1.4 mg/L. The relationship was drawn between the reaction speed and the extract's attention to find the optimal concentration to inhibit the enzyme.

Effect of alcoholic extract: The method mentioned in (LDH kit Biolabs) was used to measure the concentration of the lactate dehydrogenase enzyme, where the reaction was carried out by using different concentrations of the extract, which are (500, 50, 5, 0.5, 0.05, and 0.005) M to find out the effect of the attention of the alcoholic extract of pomegranate peels on the action of the lactate enzyme. Dehydrogenase in the presence of NADH at a concentration of 2.4 mmol/L and a substrate concentration of 1.4 mg/L. The relationship was drawn between the reaction speed and the extract's attention to find the optimal concentration to inhibit the enzyme.

Water and alcohol extraction of pomegranate peels: Pomegranate peels were collected from medicinal herbs, and the peels were ground using an electric mill to obtain a fine powder:

Alcohol extraction: 6 gm of pomegranate peel powder was added with 30 ml of alcohol (methanol 99%) in a volumetric vial. The volumetric vial with its contents was placed in the Hote plate device with stirring for two hours, after which the solution was left at 4 ℃ for 24 hours, and then the solution was filtered by 150 µm filter paper. The electric oven evaporated the solvent at 60 ℃ for one day, and the active substance was obtained as a residue.

Water extraction: 80 gm of pomegranate peel powder was added with 500 ml distilled water. The volumetric vial with its contents was placed in the Hote plate with stirring for two hours, after which the solution was left at 4 ℃ for 24 hours, and then the solution was filtered using 150 µm filter paper. Electric oven evaporated the solvent at 60 ℃ for two days, and the active substance was obtained as a residue.

Reagent of the active compounds of the aqueous and alcoholic solution

Reagent of phenols (Ferric chloride reagent): A ferric chloride reagent was prepared by adding 0.5 g of ferric chloride to 50 ml of distilled water.

Drops of iron chloride reagent were added to 15 ml of each alcoholic and aqueous extract, where a residue and colour change were observed indicating the presence of phenolic compounds [28].

Reagent Wakener (Reagent of alkaloids): The reagent was prepared by dissolving 2 gm of potassium iodide KI in 5 ml of distilled water, then adding 1.27 gm of iodine to it and mixing the solution until dissolution, then filling the volume with distilled water to 100 ml. Put 3 ml each of the alcoholic extract and the aqueous extract in a test tube, then add 2 ml of the reagent. A brown precipitate was obtained for both solutions, indicating the presence of alkaloids.

Reagent Meyer (Reagent of alkaloids): Prepare by dissolving 1.58 gm of mercury chloride $HgCl_2$ in 60 ml of distilled water, and in another container, dissolve 5 gm of potassium iodide KI in 10 ml of distilled water, then mix the two previous solutions and complete the volume to 100 ml of distilled water and put 3 ml each of the alcoholic extract and the aqueous extract in a tube Then, 2 ml of the reagent was added. A white precipitate was obtained, indicating the presence of alkaloids.

3. Results and Discussion

Optimal concentration of pyruvate and finding Km

The effect of the concentration of the pyruvate base material on the rate of the partially purified enzyme reaction was studied using a Sephadex G-100 column and finding the optimal concentration of the base material pyruvate. Concentrate the pyruvate until the maximum speed is reached at the attention (0.8 M). Then the reaction speed decreases at dilute concentrations until the effectiveness is proven at the optimal concentration.

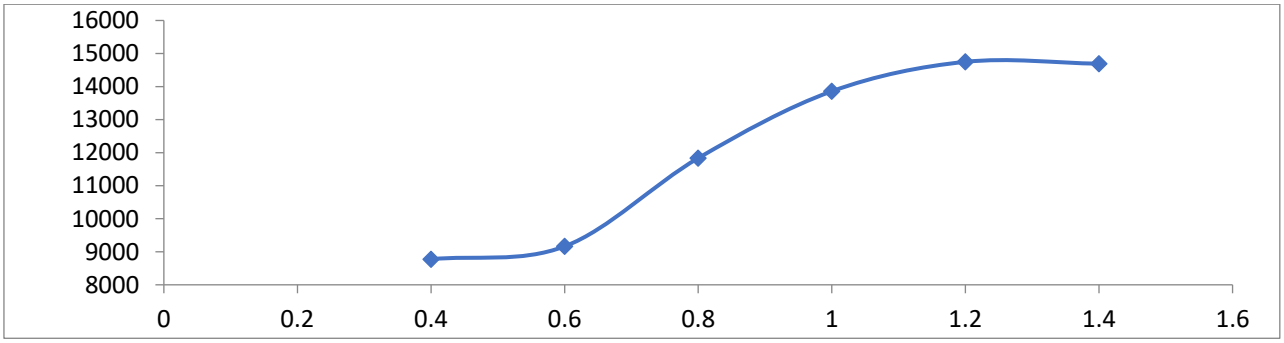

Figure (1): The effect of the concentration of the base substance on the activity of the enzyme lactate dehydrogenase purified from the serum of heart patients

It is clear from figure (1) that the enzyme is subject to the Michaelis-Menten equation, and there are several ways to calculate the value of the Michaelis-Menten constant Km, defined as the affinity between the enzyme and the substrate. To stimulate strong reactions and find out the stability

of enzymes and the effect of inhibitors and activators on enzymatic activity [29], or is the concentration of the base substance when the rate of velocity is half the value of the maximum speed [30] when the enzyme is subject to the Michaelis-Menten equation. Pools are the most accurate and best because of their ease of use, lack of mathematical operations and their efficiency in showing the experiment's accuracy.

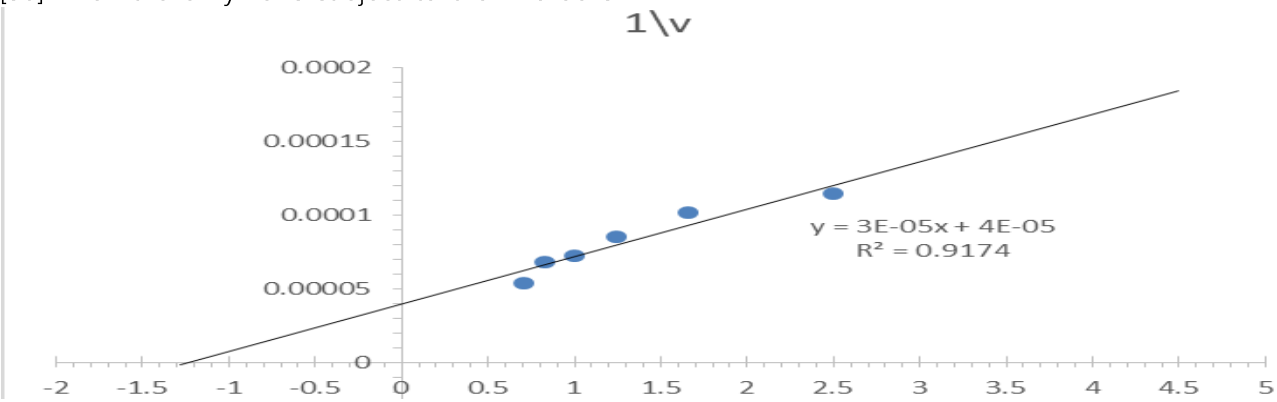

Figure (2): Lineweaver-Burk relationship to calculate the Michaelis constant for lactate dehydrogenase enzyme purified from heart patients

To calculate the values of the constant Km for the enzyme purified by the gel filtration method, the above procedure was followed as in figure (2). The value of Km for the enzyme lactate dehydrogenase was (M 0.243), as well as its maximum speed at the same base material equal to (M / min 1.5197), and these results varied with Some previous studies were conducted to determine the kinetic parameters of the enzyme. The differences between these studies were clear and almost natural due to the different sources from which the enzyme was purified and the different methods used in purification.

Effect of temperature on the reaction rate of lactate dehydrogenase enzyme

Figure (3) illustrates the effect of temperatures on the activity of the lactate dehydrogenase enzyme purified from the serum of heart patients. higher or slightly lower) than the temperature of the cell that contains it, and the speed of the enzymatic reaction increases with the increase in temperature (40 ℃), because the increase in temperature at a specific range will increase the kinetic energy of the molecules and thus increase the rapprochement and collisions between the molecules of the base substance and the enzyme, and the reaction speed increases until it reaches The optimum degree of reaction, and then the speed begins to gradually decrease due to the process of destruction or deformation of the enzyme molecule, and it occurs due to the dissolution of the hydrogen bonds and other bonds responsible for the stability of the enzyme [29], where a decrease or stop occurs in the effectiveness of the enzyme, and the deformation is explained by the state of ionization of the aggregates on the surface of the enzyme and its substance Basically, since enzymes are complex protein molecules, their catalytic activity is affected by the regular triple structural structure. Therefore, high temperatures change the normal geometry of the enzyme, which causes the enzyme to lose its activity [31].

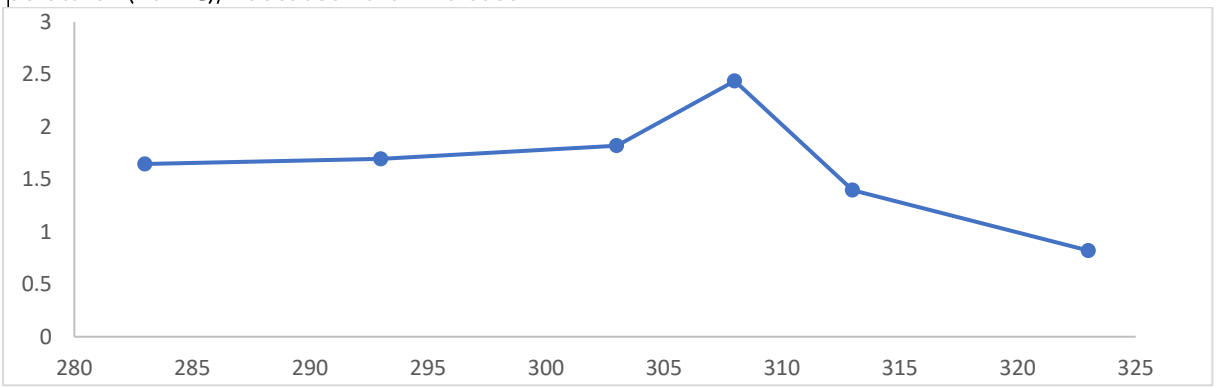

Figure (3): Effect of temperature on the reaction rate of the lactate dehydrogenase enzyme purified from the serum of heart patients

Arrhenius equation

According to the Arrhenius equation, the rate of enzymatic reaction rate changes with the change in temperature. $K=A\ e^{-Ea/RT}$

A and Ea are fixed quantities that depend only on the nature of the studied reaction. These constants are the activation energy and the Arrhenius coefficient, respectively. K is the reaction rate constant.

The relationship can be drawn between logK versus the inverse of the absolute temperature, as it gives

a straight-line subject to the Arrhenius rule to a degree (37°C), where the activation energy can be calculated from the slope of the resulting straight line as shown in figure (4).

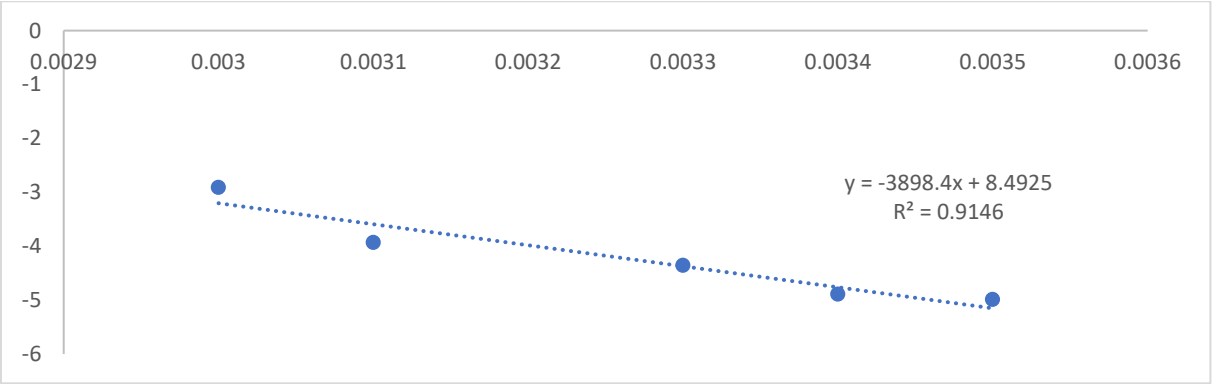

Figure (4): A diagram of the Arrhenius equation

slope=-Ea/2.3R

From the graph, it was found that the activation energy is equal to (745.45KJ.K^{-1}.mol^{-1}). The enzymes work to form a transition state, which is a complex intermediate compound (ES*), to form the products. It is a complex with a lower energy barrier compared to uncatalyzed reactions. This state results in the transitional transition, significantly decreasing the activation energy Ea for the catalyzed enzymatic reactions. The decrease in the energy barrier of the enzyme results in an increase in the reaction speed equally for the forward and reverse reactions, meaning that it does not change the equilibrium position of the enzymatic reaction [32].

3.5. Reaction order kinetics of an enzyme analogue

The equation log[s]= -kt/2.303+log[s]$_o$ has been used to calculate the reaction rate constant towards the formation of the enzyme complex, where the reaction is considered to be of the first order if a straight line is obtained from drawing the relationship between ln[A] against time (t), and from the slope of the straight line, the value of (k) can be found, as the slope of the straight line is equal to (-k1/2.303), as shown in figure (5), as it is seen that the interaction is of the first order.

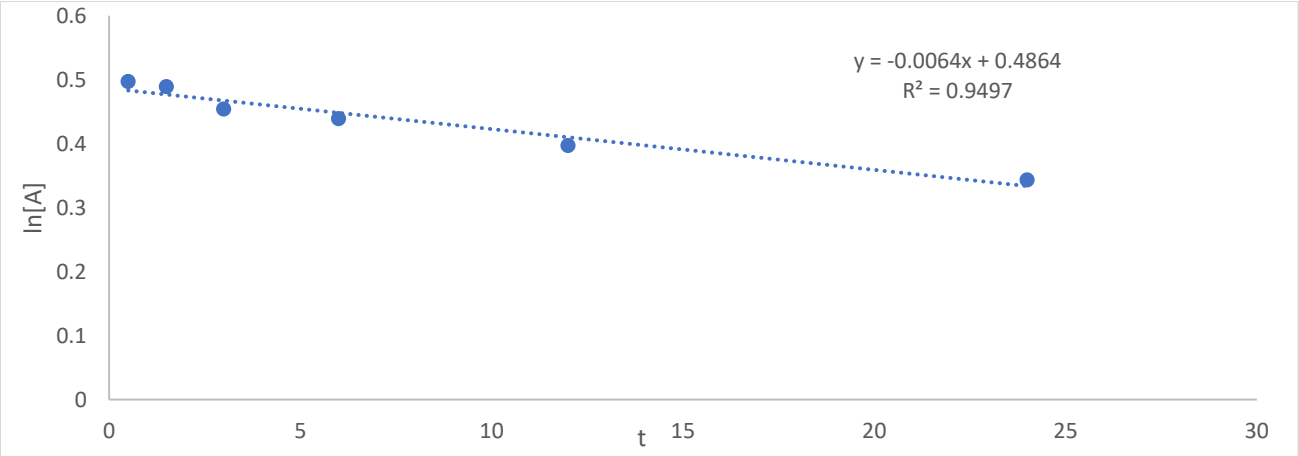

Figure (5): Average reaction rate for the first order at 37°C

3.6. Thermodynamic studies of the enzyme lactate dehydrogenase

Thermodynamic coefficients were calculated to find out the direction of the course of the reaction, the spontaneity and randomness of the system, and the type of reaction, whether it was exothermic or endothermic.

The enthalpy value of the transition state was calculated from the Eyring Polanyi equation, which shows the effect of temperature on the reaction rate for all chemical reactions in different phases. The Eyring-Polanyi equation is a theoretical structure based on the transition state model. There is an energy barrier during the interaction path between the reactants and the products, representing the energy threshold or the minimum energy required for the reaction to occur or the activation enthalpy. Particles that possess considerable kinetic energy approach each other while the potential energy is constant, and with time, the speed of the particles begins to decrease gradually. From each other more to form the active enzyme complex ES *, which has the highest potential energy, as it has an unstable molecular arrangement. The bonds quickly disintegrate and form the product or disintegrate to return to the reactants. The reaction rate is proportional to the concentration of molecules with sufficient energy to form the active complex. The enthalpy value of the transition state is the difference between the enthalpy of the transition state and the total enthalpy of the enzyme and the

substrate in the steady state. Figure (6) shows a drawing of the Eyring Polanyi relationship between lnT and the absolute temperature inverse.

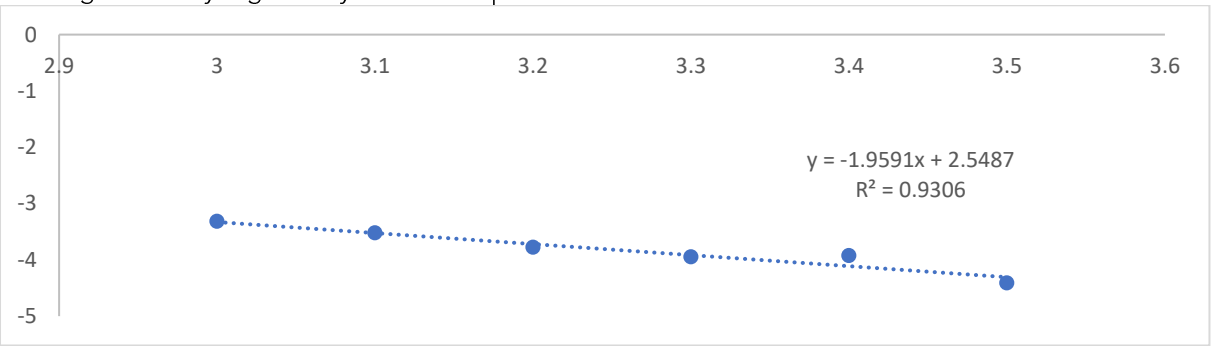

Figure (6): Drawing of the Erying-Polanyi equation to calculate the thermodynamic functions of the enzyme transition state

Where the enthalpy of the transition state was calculated from the slope of the straight line resulting from drawing the Eyring-Polanyi relationship as: Slope = -ΔH*/R

The entropy value was calculated from the intersection of the straight line with the y-axis.

Intercept = lnKB h+ΔS*R

Entropy represents the value of regularity or randomness of the system. It represents the difference between the entropy of the transition state and the total entropy of the enzyme and the substrate.

Table (1): The values of the Erying-Polanyi equation for calculating the thermodynamic functions of the enzyme transition state

1/T *1000	Ln V/T	Velocity/T (K)	T (K)
3.51	3.619-	0.0268	283
3.41	3.623-	0.0267	293
3.3	3.579-	0.0279	303
3.22	3.349-	0.0351	313
3.10	3.927-	0.0197	323
3	4.414-	0.0121	333

The free energy of the standard state is important in determining the spontaneity of a reaction and can be calculated from the equation ΔG* = ΔH*-TΔS*

Table (2): Values of change in the free energy of the transition state at different temperatures

T(K)	ΔG* K.J.mol^{-1}	ΔG* K.J.mol^{-1}	ΔS* J.mol^{-1}.K^{-1}	ΔH* J.mol^{-1}
293	-64.608	-14.568	+57.557	9818.0
303	-70.464			
313	-76.221			
323	-81.970			
333	79.590			

The enthalpy value of the transition state ΔH* of the Enzyme is equal to J.mol-1 9818, where the positive value of the enthalpy indicates that the enzymatic reaction is endothermic, meaning that it needs the energy to form the enzyme complex ES* because the energy of the enzyme complex ES* is higher than that of the Enzyme and base material. As for the resulting entropy value of the transitional state ΔS* for the Enzyme was found to be equal to J.mol^{-1}.K^{-1} +57.557. It is clear from the positive value of entropy that the enzymatic reaction tends towards an increase in randomness and irregularity of the system when forming the active complex, meaning that the structural structure of the complex the formed ES* is less regular than the structural composition of the reactants (Enzyme and substrate) and this is consistent with [33] where the entropy increases in the enzyme lactate dehydrogenase in pig serum. The Gibbs free energy of the transition state determines the extent to which the reaction takes place and the formation of the active complex. The free energy of the transition state ΔG* for the Enzyme under study was equal to KJ.mol-1 -14.568, where the negative value of the Gibbs energy indicates that the enzymatic reaction towards the formation of the complex ES* takes place. Automatically, it does not need to be equipped with a card. That is, the reaction does not include the association of the enzyme lactate dehydrogenase with ATP for the enzymatic reaction to occur. The reason for its complex stability can be attributed to hydrophobic interactions, where the stability of the complex is attributed to electrostatic interactions, hydrogen bonds, and Vander Waals interactions, as hydrophobic interactions contribute to the stability of the complex by the positive value of the change in standard enthalpy [34].

3.7. Effect of pH on the reaction rate of the enzyme lactate dehydrogenase

The effect of pH on the activity of the LDH enzyme

purified from heart patients was studied at pH values that ranged between (4.4-9.4). This is through its effect on the side chains of amino acids in the protein that is necessary to maintain the triple structure of the Enzyme and the ionization of the aggregates in the base material. It also affects the active site's shape and the base materials' binding. Therefore, each Enzyme has an optimal pH with the highest reaction speed, reflected in the Enzyme's effectiveness. A high or low pH above certain limits can lead to enzyme deformation and loss of its effectiveness. Figure (7) shows the use of different degrees of pH for the buffer solution (Tris-HCl) used in the enzymatic reaction, as it causes an increase in the reaction speed with the increase in the pH levels until the maximum speed is reached at the optimal pH, which was at pH 7.4, after which it decreased. Enzyme activity at pH degrees higher and lower than (7.4) [30].

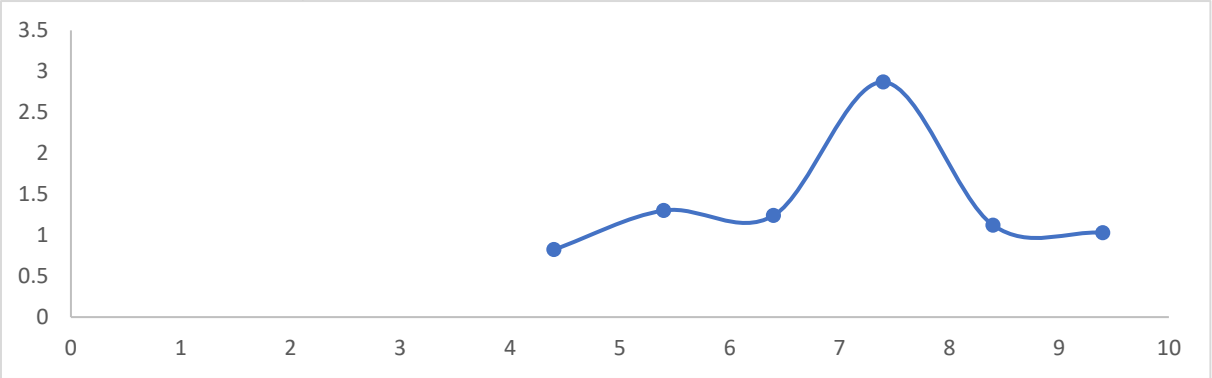

Figure (7): The effect of pH on the reaction rate of lactate dehydrogenase purified from the serum of heart patients

Determination of the optimal time for lactate dehydrogenase activity

The enzyme activity was measured at different times (from 1.5 to 40) minutes. It was found that the optimal time to measure the activity of the lactate dehydrogenase enzyme purified from heart patients is (24) minutes, as the speed of the enzymatic reaction increases with increasing time until reaching the highest effectiveness at the time [30]. Minutes, as the enzyme is saturated with the base substance during this period, the activity decreases gradually. The reason for this decrease is the enzyme's thermodynamic characteristics, as the enzyme's hydrogen bonds are broken due to its dynamic activity. Thus, the stability of the three-dimensional shape of the enzyme decreases, and thus the effectiveness decreases.

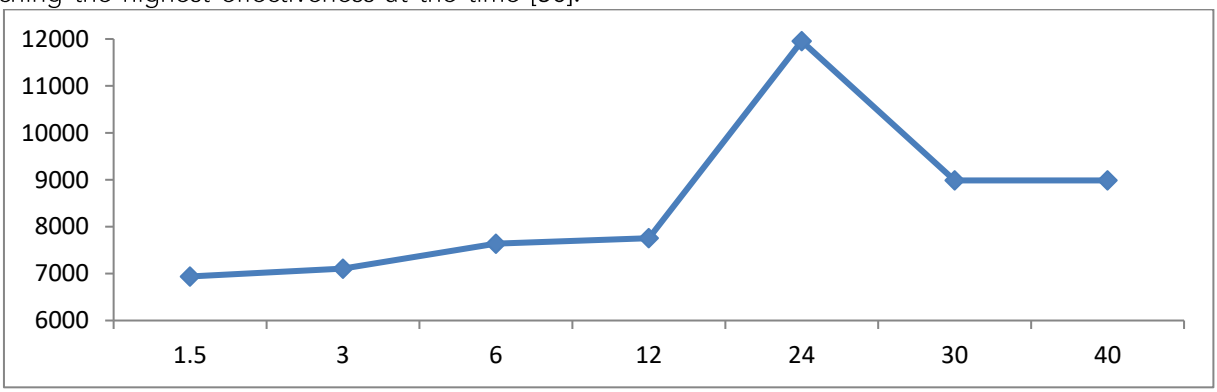

Figure (8): Effect of time on the reaction rate of lactate dehydrogenase purified from the serum of heart patients

Diagnosis of aqueous and alcoholic pomegranate extract

The extracts used in the study were identified using the reagents shown in table (3).

Table (3): Reagents, sediment colors, and the result of the used extracts

Reagent	The color of precipitate formed for the aqueous and alcoholic extract	Result
Ferric chloride reagent	Brownish yellow in color	Indication of the presence of phenolic compounds
Wakener reagent	Brown	Indication of the presence of alkaloids
Meyer reagent	White	Indication of the presence of alkaloids

Studying the effect of compounds extracted from pomegranate peels on the inhibition of the lactate dehydrogenase enzyme purified from heart patients

Different concentrations of the aqueous and alcoholic extract containing phenolic compounds were used (from 500 to 0.005), and it was found that the optimal concentration of the extract was (5 M). It is shown from Figure (10) that the inhibition is of the type (noncompetitive inhibitor) for the aqueous extract. At the same time, Figure (9) shows

that the alcoholic extract of pomegranate peels inhibits the enzyme type (uncompetitive inhibitor).

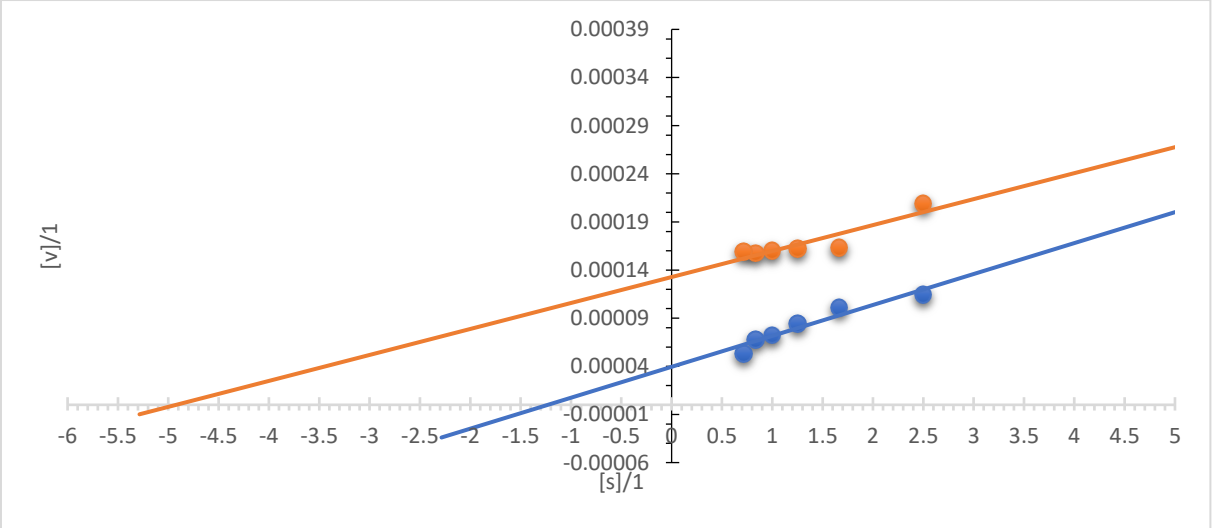

Figure (9): Lineweaver-Burke equation with or without alcohol inhibitor of pomegranate extract

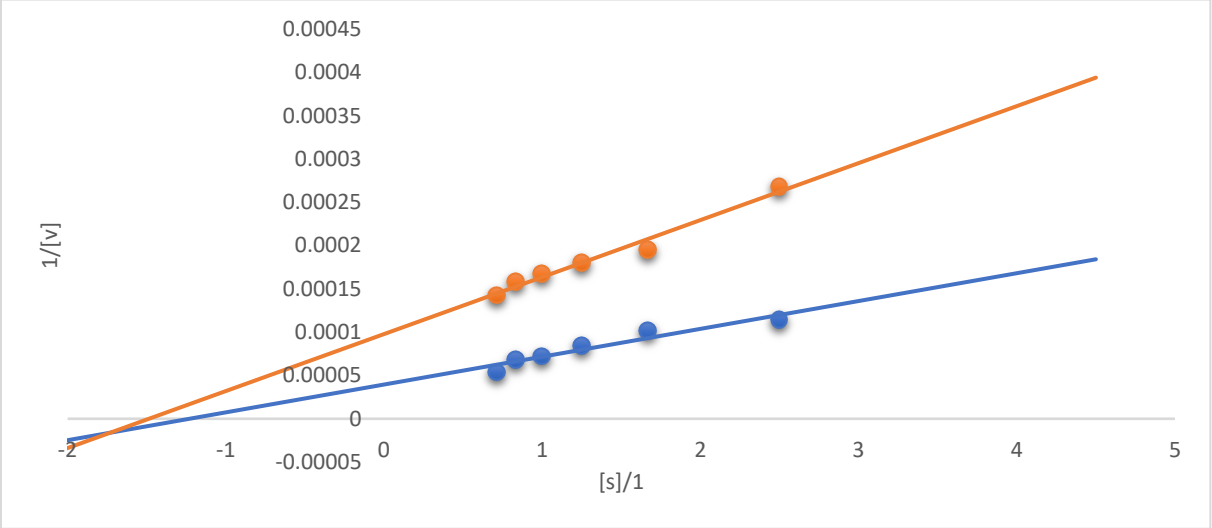

Figure (10): Lineweaver-Burke equation with or without aqueous inhibitor of pomegranate extract

This is consistent with [35], where a significant decrease was found in the level of LDH after giving rats with myocardial infarction phenol glycoside, a phenolic compound found in plants. *Enzyme inhibition* is defined as the enzyme-substrate reaction affected by the presence of any organic chemical, inorganic metal, or bio composite due to its covalent or non-covalent interactions with the enzyme's active site [36]. The phenolic inhibitors can compete with the adenine-containing substrate, which is a second-order reaction in phenol concentration. When adenine is replaced in NADH by hypoxanthine, it becomes a first-order reaction in phenol concentration [37-42].

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