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Study The Chemical Kinetics Properties for Adenosine Deaminase Enzyme (Aminohydrolase EC 3.5.4.4) ADA In Breast Cancer Patients

Muna M. Yaseen Khalid F. Al Rawi Mohammad Q. Al-Ani,

University of Anbar – College Of Science .

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ABSTRACT

This study involved(50) women with newly diagnostic breast cancer and before any treatments, their ages ranges between (26-61) years old, the patients (women*)* subjected to different breast cancer investigations, at this study, we concerned on one of the biochemical tools that used as indicant to breast cancer disease which is Adenosine Deaminase Enzyme (ADA),and study their chemical kinetics properties and with factors that effect on enzyme activity which includes ,The optimal activity were in pH (7.2), as well as the optimal temperatures for activity were 35ºC. and the optimal substrate and enzyme concentration were (0.034Mm) and (0.25µg/ml) respectively, and the incubation time was 30 min. The results of kinetics characterization for ADA results demonstrated Km ,Vmax, Activation energy (Ea) and Temperature coefficient Q10 values were 0.849 mM and 83mMol/l/min, 3927.17cal/mol , 2 respectively.

Introduction

Adenosine deaminase (ADA) is an enzyme that catalyzes the deamination of adenosine and 2-deoxyadenosine to inosine and 2-deoxyinosine. The enzyme is widely distributed in human tissues and works as a marker of cellular immunity, and its activity is found to be elevated in those diseases in which there is a cell-mediated immune response [(1)] .

Studying the enzyme kinetics is very important to give an ideally yield information about the enzyme's activity under vivo conditions, including such Km ,Vmax, Ea and Q10 as kinetics parameters, reaction features as substrate cooperatively, reversibility and Allosteric, and be applicable to enzymatic reactions with multiple substrates as well as detecting the main factors that effect on enzyme activity like PH, Temperature ,Time of incubation, substrate and enzyme concentration [(2)] . These findings might be important for diagnostic purposes in advanced breast cancer. Therefore, the assessment of the levels of serum enzymes may be of significance in early detection of carcinoma breast [(3)] .

* Corresponding author at: University of Anbar – College Of Science
.E-mail address:

Enzyme kinetics is the investigation of how enzymes bind substrates and turn them into products. The rate data used in kinetic analyses are commonly obtained from enzyme assays. Michaels and Menten proposed a quantitative theory of enzyme kinetics, which is referred to as Michaels–Menten kinetics [(4)] .The major contribution of Michaels and Menten was to think of enzyme reactions in two stages. In the first, the substrate binds reversibly to the enzyme, forming the enzyme-substrate complex. This is sometimes called the Michaels-Menten complex in their honor. The enzyme then catalyzes the chemical step in the reaction and releases the product. Enzyme rates depend on solution conditions and substrate concentration. To find the maximum speed of an enzymatic reaction, the substrate concentration is increased until Saturation happens because, as substrate concentration increases, more and more of the free enzyme is converted into the substrate-bound ES complex [(5)].

Km is the Michaels-Menten constant which expresses the substrate concentration at which the reaction rate has half of its maximum value. , the K_m value indicates the affinity of enzyme to the substrate

in reactions catalyst by enzymes If the concentration of a substrate is increased while all other conditions are kept constant, the measured initial velocity Vi, increases to a maximum value Vmax [6].The importance of Km and Vmax values is that their level variations can be presumed to be a diagnostic marker for abnormal cases .This is due to the possessed affinity of the enzyme to catalysis [7].

Measuring reaction rate under standard conditions of substrate, pH and temperature serves as a measure of enzyme activity and concentration [8]. The activity of enzyme is strongly affected by changes in pH and temperature. Each enzyme works best at a certain pH and temperature, its activity decreasing at values above and below that point due to denaturation for enzymes; denaturation can be defined as the loss of enough structure rendering the enzyme inactive [9] . Several factors affect the activity of enzymes. Among these are the temperature and pH. At optimum levels of these factors, enzymes perform their function best. Optimum temperature and pH differ from one enzyme to another [10].

Material and Method

Five ml of blood were collected in plane tubes from (50) patients, newly diagnostic and before any treatment. The blood sample tubes were centrifuged at 3000 rpm for 2-5 minutes then the serum extracted to be stored or used immediately in the process of enzyme assay.

Enzymatic activity assay

The protocol of (Guisti & Galanti Method) was adopted for the determination of SADA activity [11] . ADA activity was determined according to the reaction: Adenosine deaminase hydrolyses adenosine to ammonia and inosine. The ammonia formed further reacts with a phenol and Hypochlorite in an alkaline medium to form a blue indo phenol complex with indophenol complex formed is directly proportional to the amount of Adenosine Deaminase present in the sample.

Characterization of ADA enzyme

Enzyme specificity

Solutions of 1% (w/v) Adenosine, 2-Deoxyadenosine (reaction substrate), Formycine, Adenine, Cytidine, and Guanosine, were prepared by dissolving in 50mM sodium acetate buffer pH 5.

Amount of 1.8 ml reaction substrates were added separately to test tubes, then 0.2 ml of the purified enzyme was added to each test tube and incubated at 30^{o}C for 20 minutes. The reaction was arrested and enzyme activity was determinated.

Optimal pH for enzyme activity

Wide ranges of pH (5-9) has been used in order to determine the highest serum adenosine deaminase (S-ADA) activity at optimum pH value. Adenosine(50 mM) was prepared with pH ranges using sodium acetate 0.05 M for pH ranges (5.0, 6.0), while Tris-HCl 0.05 M buffer was used for pH ranges (7.0, 8.0, 9.0), the pure enzyme was added, the enzyme activity was estimated after incubation period. The optimum pH was estimated by scheming the relationship between the enzyme activities versus the pH values.

Optimal temperature for enzyme activity

The Adenosine 50 mM was prepared in tubes using Tris-HCl buffer 0.1 M, the tubes was incubated in different temperatures (20, 25, 30, 37, 42, 45, 50, 60) to 10 minutes for mixing, then the enzyme was added and incubated for 20 minutes to start the reaction, then the activity was estimated and the optimum temperature was evaluated by plotting the correlation between the enzyme activities versus the temperature values.

Determination of the activation energy and temperature coefficient

Activation energy of the purified enzyme were calculated from the relationship between inverse absolute temperature (1/K^{o}) as X-axis versus logarithms of observed reaction rate (Vmax) as Y-axis according to Arihinus following formula [13].

Log (Vmax) = Ea /2.3 RT + Log A

The determination of temperature coefficient (Q10) between two temperatures degree from the following relation:

Ea = 2.3 R T_2T_1 log Q10/10

Effect of Incubation time on ADA activity

The activity is determined by measuring the amount of product produced or the amount of substrate used up per unit of time under high concentrations or saturating conditions of substrate. [12]

Effect of Enzyme Concentration on ADA Activity

It is important to establish that the activity varies with enzyme concentration. The activity of enzyme was measured in the presence of different concentrations of purified enzyme from serum between (0.05-0.35) µg/ml, The activity is essentially the measurement of the initial velocity under conditions that make it the maximum velocity, the activity should be proportional to the amount of enzyme added. That is, if two or three times as much enzyme is added the activity should be two or three times as great [(12)].

Effect of Substrate Concentration on the Enzyme Activity.

Michaels –Menten constant (Km) and maximum velocity (Vmax) of ADA were determined the effect of substrate concentration on the enzyme activity, measuring the activity of enzyme in the presence of different concentrations of Adenosine as a substrate between($7.3\times10^{-7}$and 0.5×10^{-2} Mm/L)by using the same procedure in above. the enzymatic activity was determined according to adenosine amount that liberated in one minute at condition assay.[(11)]

The correlation between each substrate concentration and the enzyme activity was plotted in order to determine the optimum substrate concentration for each enzyme activity, then the values of Km and Vmax for ADA reaction with substrate were determined by using the Line weaver-Burk plot (the relationship between 1/V versus 1/Substrate).[(14)]

Results and Discussion

Kinetics calculation of the enzyme and standard conditions of ADA were defined by studying ADA activity dependence on: pH, Temperature, Enzyme concentration, Time of incubation, Substrate concentration.

Enzyme specificity against different substrates

The relative substrate specificity of ADA was determined as shoe in Table (7). Adenosine and 2-deoxyadenosine were substrates for ADA which presenting the activities(0.77)IU/l and(0.80) IU/l respectively. While Formycine, Adenine, Cytidine, and Guanosine, were not deaminated. these mean that the specificity of ADA enzyme for two main substrates as show below in Table (1).Our result which observed with Adenosine and 2-Deoxyadenosine agrees with [(15)] and [(16)] which found the same specificity for ADA with adenosine and 2-Deoxyadenosine.

Table (1): Specificity of ADA enzyme on different substrates

Substrate	Activity	Activity U/ml
Adenosine	1	0.77
2-Deoxyadenosine	1	0.80
Formycine	0	0
Adenine	0	0
Cytidine	0	0
Guanosine	0	0

Effect of pH on enzyme activity

The different effect of pH on the Adenosine deaminase enzyme activity was studied. as show in **figure (1).**The results showed that maximum enzyme activity was at(pH 7.2) for purified ADA.

The velocity of enzyme-catalyzed reactions depends on pH, it's also act as a factor in the stability of enzymes. Enzymes have optimum pH and frequently give bell-shaped curves of velocity against pH, even though other shapes have been observed [(12)]. Extremely high or low pH values generally result in complete loss of activity for most enzymes ,decreasing in ADA activity at low pH may be due to consequence of pH environment of reaction in ionic groups of active site, or changing in ionic state for substrate, or complex enzyme-substrate at the concentration of substrate above than Km, if the substrate concentration is small , it will depend on enzyme [(17)].

Generally at pH below 6 , enzyme protonates and loses its negative charge thus loss its activity to bind to the substrate , whereas , at pH higher than 6 , the substrate ionizes and loses its positive charge , thus loose its affinity to bind to the enzyme to form enzyme –substrate complex , and decrease the reaction velocity, There are many studies in the effect of PH on ADA activity, The results of [(18)] showed that the optimum pH for ADA was 7.5,Other study found that the optimum (PH =6.8) for ADA in Hemolytic Anemia, and (PH=6.5) in Leukemia and thalassemia patients[(19)] .While in the study was carried on acute renal failure optimum pH value (6.5)[(7)].

Effect of temperature on enzyme activity

Different temperatures were tested to determine optimal temperature for ADA enzyme activity (from 15 to 50) ºC, the optimal temperature was(35 ºC) and the results showed that there was elevate in ADA enzyme activity with increasing the temperature followed by reducing in ADA activity as shown in **figure (2) .**

The result of the present study showed that the change in activity of serum ADA enzyme above and below optimum temperature, This might be due to the alterations in the ratio of isoenzymes in the serum of patient for the reason that isoenzymes have not the same stability to temperature. The tertiary structure of an enzyme is kept principally by amounts of non-covalent links, When molecule absorbs much energy the tertiary structure will broke, and enzyme will be denatured, which loses enzymes activity [(20)] while below 37^{o}C. ADA activity decreases due to insufficient energy that required to perform enzyme substrate complex , this result agree with previous studies [(21,7)] other study found optimum temperature for ADA was 37 ^{o}C .[(22)]

Calculation of Activation Energy (Ea) and Temperature coefficient (Q10)

Activation energy (Ea) was calculated for transformation of substrate to product by enzyme acting, the Temperature coefficient (Q10) also estimated from the value of (Ea) as the equation noted previously, The activation energy of the binding reaction was estimated from Arrhenius plot which gives a linear correlation according to the Arrhenius equation calculated from the slope of the straight line ,plot of Log V(max) Vs. 1/T K^{-1} was investigated as: Log V(max) = - Ea/2.3 R . 1/T + Log A ------ (Arrhenius equation)

The thermodynamic parameters of the transition state for ADA reaction were estimated from (**Figure 3).** A linear relationship was obtained with the activation energy equal (3927.17) cal/mol and the temperature coefficient equal (2) for purified enzyme.

The activation energy in our study for ADA reaction reached to (3927.17) cal/mol , This result falls within the range of the most enzymes (6000-18000 cal/mol) as mentioned by [(23)] .The low activation energy of the purified enzyme suggests a high efficiency for accelerating reaction to convert the substrate (adenosine) to product such as peptide and amino acid[(24)]. and this indicated that enzyme plays an important role in decreasing of activation energy. Despite the significance of activation energy in determining the catalytic ability of the enzyme, however most of the available works that characterized adenosine deaminase were not pay attention to this parameter, a previous study[(25)]showed that the activation energy for ADA was equal to (9617.8cal/mol) in normal and (17111 cal/mol) in Thalassemia patients. Other study[(26)]showed that the Ea value equal(4854.64 cal/mol) and the temperature coefficient (Q10) was (1.84) in patients with renal stone.

Effect of incubation time on enzyme activity

The figure**(4)**shows enzyme activity dependence on incubation time(0 – 60 minutes). thirty minutes (30 min) was adopted as the standard incubation time throughout the work because it relates to a linear region of the curve and presents reliable absorbance values.

Effect of enzyme concentration on enzyme activity

By using different amount of ADA enzyme and studying their activity on adenosine as substrata, we get the higher activity of ADA enzyme which was (0.25µg/ml) as shown in figure (5) .The effect of enzyme concentration on the velocity of reaction makes it the maximum velocity .In addition, the activity must be relatively to the amount of enzyme that added[(12)].Previous study[(27)] found that the optimum ADA enzyme concentration was (0.2µg/ml) inpatients with colon tumor.

Effect of substrate concentration on enzyme activity

The activity of the ADA was measured in the presence of different concentration of adenosine as a substrate. It was found that the maximum activity of the ADA enzyme was obtained by using (0.034) Mm of adenosine as show in figure (6).

Table(2): The effect of substrate conc. on velocity for ADA reaction

[S]mMol/l	[V]mMol/l/min
7.3×10^{-7}	10
6.35×10^{-6}	25
5×10^{-5}	40
1.5×10^{-4}	53
0.9×10^{-4}	60
0.5×10^{-3}	71
0.5×10^{-2}	83

The present study was planned to clarify ADA kinetics in purified enzyme, Km is the Michaels-

Menten constant , it expresses the substrate concentration at which the reaction rate has half of its maximum value [28] .Kinetic constant of ADA catalyzed the conversion of adenosine to inosine were found to be readily obtained by analyzes curve of a single reaction by conversional initial velocity analysis [29]

Calculation of K_m and V_{max}

Results in our study showed from Line weaver–Burk plot by plotting in figure (7) the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. A linear relationship was obtained with a Km and Vmax value of the enzyme were 0.849 mMol and 83mMol/L/min ,respectively.

Many other researches shows that the values of *Km* of ADA enzyme in many abnormal cases is lower than in normal individuals [20, 30] In a study carried out by [19] Km and Vmax were actually found to be ($11x10^{-3}$)mM and (17.54) $mM.min^{-1}$ respectively in the serum of normal individuals. While [31] found that they were ($8x10^{-3}$M and 4.4m $M.min^{-1}.mg^{-1}$),($10x10^{-2}$M and 3mM.$min^{-1}.mg^{-1}$),($13x10^{-3}$M and 2.25m $M.min^{-1}.mg^{-1}$), ($15x10^{-3}$M and 19mM.$min^{-1}.mg^{-1}$) in normal, Anima, Rheumatoid Arthritis and in both respectively. Previous studies [32,33] pointed out that the high K_m indicates weak binding but a low K_m indicates strong binding of the enzyme to substrate. While Vmax values are higher in patients than in normal individuals, this may be due to the change in the ratio of isoenzymes in patients or may be due to change in the structure or conformation of the enzyme, the important marker for abnormal cases [34, 19] .

The important of Km and V max values is that their level variations can be presumed to be a diagnostic marker for abnormal cases .This is due to the possessed affinity of the enzyme to catalysis.

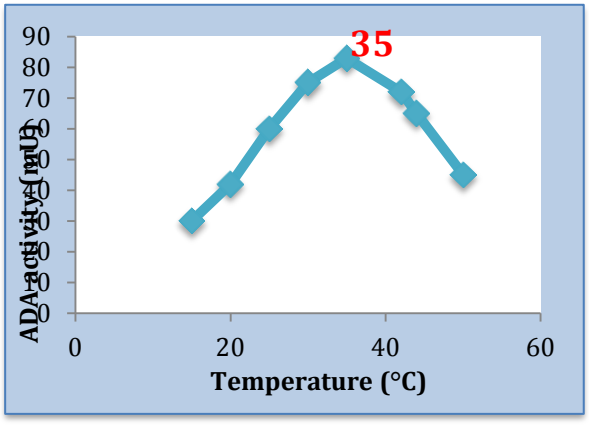
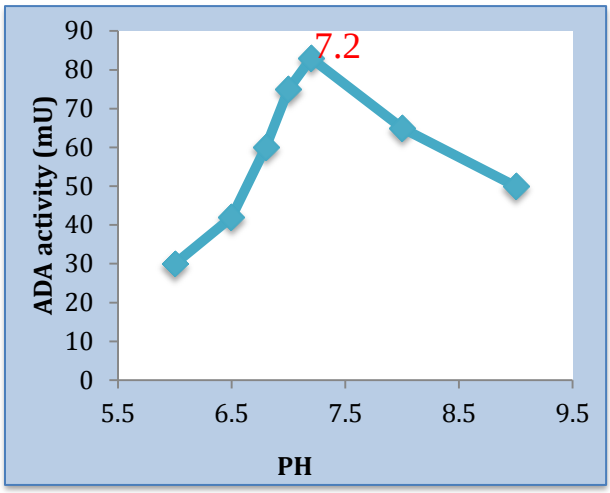


Fig (1)Effect of temperature on ADA activity.



Fig(2): Effect of PH value s on ADA activity

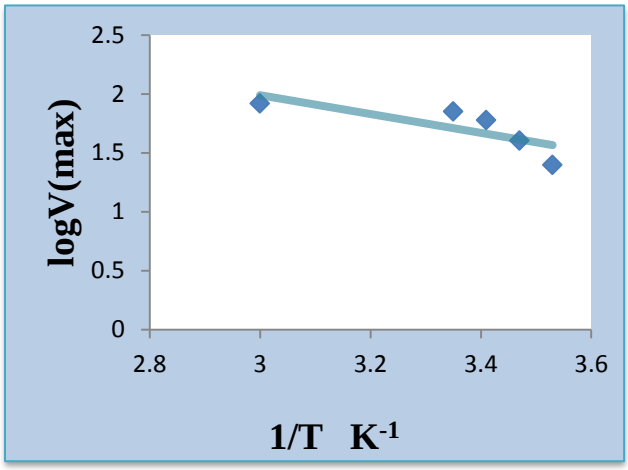
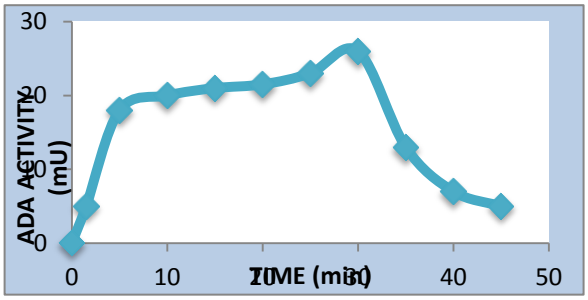
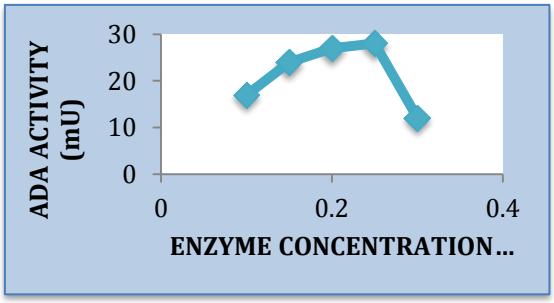


Fig (3):Arrhenius plot for activation energy (Ea)calculation plot of (Log V) Vs. (1/T K-1)



Fig(4):Incubation time of ADA enzyme



Fig(5): ADA activity Effects with enzyme concentration

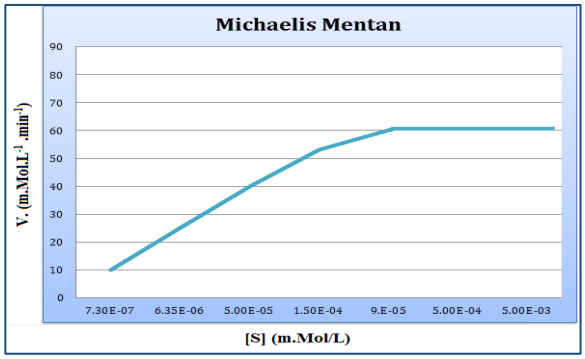


Fig (6) : (Michaelis-Mentin) plot show effect of substrate concentration on ADA activity.

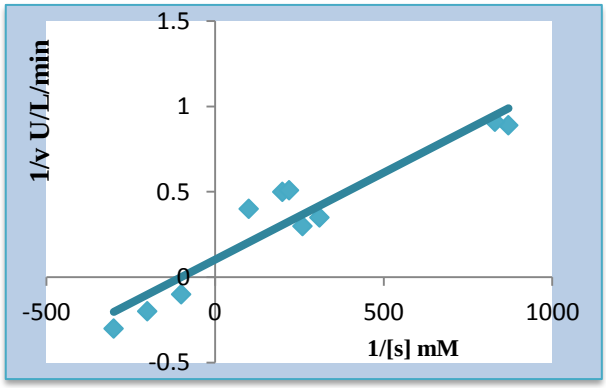


Fig (7): Kinetic constant (Km and Vmax) by Line weaver–Burk plot for ADA enzyme purified from serum of breast cancer patients.

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دراسة بعض الخصائص الحركية لأنزيم الادينوسين نازع الأمين Adenosine Deaminase(Aminohydrolase EC 3.5.4.4) لدى مريضات سرطان الثدي

منى محمد ياسين خالد فاروق الراوي محمد قيس العاني

E.mail:

االخلاصة

تضمنت الدراسة (٥٠) امرأة مصابه بسرطان الثدي مشخصه حديثا وقبل الخضوع لأي برنامج علاجي، تتراوح اعمارهم ما بين (٢٦- ٦١) سنة، النساء المريضات خضعوا لفحوصات مختلفه خاصة لتشخيص سرطان الثدي. تركزت الدراسه على واحدة من اهم المتغيرات الكيموحيوية التي تستخدم في الكشف عن وجود اصابه بمرض سرطان الثدي والاشارة اليه وهو أنزيم أدينوسين نازعة أمين(ADA) تمت دراسة بعض حركيات الإنزيم الكيميائية بالإظافة الى تحديد بعض العوامل المؤثرة في نشاط الانزيم والتي تضمنت تحديد خصوصية الانزيم باستخدام مركبات مختلفة تضمنت (الادينوسين، ٢ – هيدروكسي أدينوسين ،فورماسين، أدينين، سايتيدين، وغوانوسين) وبعد اختبار نشاط الانزيم أظهرت النتائج أن الادينوسين و ٢ –هيدروكسي أدينوسين هي المواد الاساس للأنزيمكما وجد ان درجة الحموضة المثلى لنشاط الانزيم هو(٧.٢)، وكذلك درجة الحرارة المثلى كانت ٣٥°م و التركيز الأمثل للماده الاساس والانزيم هو (٠.٠٣٤)ملي مول و (٠.٢٥) مايكروغم / مل على التوالي، كما وجد ان الزمن الامثل للحضن (٣٠) دقيقه. أظهرت نتائج دراسة الخصائص الحركية لأنزيم والتي شملت ثابت ميكايلس (Km)، السرعة القصوى (Vmax) ،طاقة التنشيط (Ea) وقيمة المعامل الحراري له هي كالاتي (٠.٨٤٩ ملي مول/لتر) و(٨٣ ملي مول/لتر/دقيقه) و(٣٩٢٧.١٧ كلاري /مول) و(٢) على التوالي.