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Kinetic and Thermodynamic Study of Catalase Enzyme in Iraqi Patients with Active Acromegaly

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Abstract: *Acromegaly is a rare, chronic, progressive disease characterized by an excess secretion of growth hormone (GH) and increased circulating insulin-like growth factor 1 (IGF-1) levels. It is caused by a pituitary adenoma in the vast majority of cases. Catalase is a tetrameric protein of 244 kDa, comprising four identical subunits of 59.7 kDa. Each subunit contains 527 amino acid residues. CAT is responsible for degradation of hydrogen peroxide (H_2O_2). It is protective enzymes that coexist in nearly all animal cells. The aim of this study is Estimation of oxidative stress in serum by measuring catalase enzyme in acromegalic group, Study of the optimum conditions of the catalase enzyme in sera of patients with acromegaly and comparing them with the optimum conditions of control group and determining the kinetic and thermodynamic parameters of catalase enzyme in acromegaly patients and comparing them to the control group. 30 male control and 30 male patients with active acromegaly was selected for this study. The results in this study revealed a significant decrease in mean serum levels of CAT activity in acromegaly group compared to control group. The optimum conditions for CAT enzyme are (65 mmol / L, pH=7.0, 45ºC) for patients and control, 80 sec for patients and 60 sec for control. Kinetic parameters of patients group (Ka, K+1, K-1 and n [Et]) are lower than in the control group and (Km and $t_{0.5}$) of patients are higher when compare with control group. Thermodynamic parameters (ΔHo, ΔSo, ΔH*, ΔG* are positive and ΔGo, ΔS* are negative) for patients and control groups. Ea for patient is higher than Ea for control group. Acromegaly patients group have large activation energy so need large time to give the product than control group. Decrease CAT enzyme affinity to substrate and rate of reaction of CAT for acromegaly patients group. CAT is a spontaneous and endothermic process through negative value of (ΔG^o) and positive value of (ΔH^o).*

Keyword: *Acromegaly, Catalase, kinetics, thermodynamic.*

• Introduction

Acromegaly is a rare, chronic, progressive disease characterized by an excess secretion of growth hormone (GH) and increased circulating insulin-like growth factor 1 (IGF-1) concentrations. It is caused by a pituitary adenoma in the vast majority of cases [1] the therapeutic options for acromegalies include surgery, medical therapies and radiotherapy. However, despite all these treatments option, approximately 50% of patients are not adequately controlled [2]. Catalases (CAT, H2O2 : H2O2 – oxidoreductase, EC 1.11.1.6) are enzymes with a long history that goes back to the 19th century, when they became one of the first sources of valuable information about the nature and behavior of enzymes. Catalase is a tetrameric protein of 244 kDa, comprising four identical subunits of 59.7 kDa. Each subunit contains 527 amino acid residues, one haem group, namely iron (III) protoporphyrin IX, and a tightly bound molecule of NADPH [3], CAT is responsible for degradation of hydrogen peroxide (H_2O_2). It is a protective enzyme that coexists in nearly all animal cells [4]. The activities of CAT have been investigated in various pathophysiological states associated with increased oxidative stress. Oxidative stress is implicated in the pathogenesis of some diseases like atherosclerosis, diabetes mellitus, and hypertension, hyperlipidemia, and neurodegenerative diseases [5].

• Materials And Methods

2.1 Subjects

The patients were selected from attended the National Diabetes Centre /Baghdad during the period from December 2015 to June 2016.This study includes 30 male patients with active acromegaly, their ages ranged (30-40) years ,and 30 normal healthy males, their ages ranged (30-40) years as a control group. Patients and volunteers with osteoporosis, osteomalesia, cardiovascular disease, renal failure, diabetic mellitus, hypertension alcoholics and smokers were excluded from the study. All patients were diagnosed by physicians.

2.2 Samples Analysis

2.2.1 Specimen collection: About 10 mL of venous blood samples were taken using plastic disposable syringes. Blood samples were left for 30 minutes at room temperature. After coagulation, the sera were separated by centrifugation at 3000 rpm for 10 minute. Hemolysis samples were discarded and the sera were stored and frozen about -20 ºC until analysis.

2.2.2 Estimation of catalase enzyme activity

2.2.2.1 Principle: Spectrophotometric assay for serum catalase activity was estimated by methods of Goth, L. et.al.[6]. It was based on formation of hydrogen peroxide a stable complex with ammonium molybdate and measured at 405 nm.

2.2.2.2 Reagents: -Substrate: (65 µmol per mL hydrogen peroxide in 60 mmol/L sodium-potassium phosphate buffer, pH 7.4)

-Stop solution: 32.4 mmol/L ammonium molybdate ($(NH_4)_6\ Mo_7O_{24}\ .4H_2O$).

2.2.2.3 Method: The assay was as follows: 0.2 mL serum was incubated in 1.0 mL substrate at 37 o C for 60 s. The reaction was stopped with 1.0 mL of stope solution and the yellow complex was measured at 405 nm against blank 3

Blank 1 :(1.0 mL substrate +1.0 mL stop solution + 0.2 mL serum)

Blank 2: (1.0 mL substrate + 1.0 mL stop solution + 0.2 mL buffer)

Blank 3 :(1.0 mL buffer + 1.0 mL stop solution + 0.2 mL buffer)

2.2.2.4 Calculated

$$\text{Serum catalase activity (kU/L)} = \frac{\text{A (sample)} - \text{A (blank 1)}}{\text{A (blank 2)} - \text{A (blank 3)}} \times 271$$

2.2.3 Estimation of the most appropriate conditions for catalase enzyme activity

The procedure was followed as the same steps that were described in Goth, L. *et al* [6]. Method by using different:

- Concentration of substrate. The optimum substrate concentration was estimated by plotting the relationship between the enzyme activities versus the substrate concentration values.
- Incubation times with optimum substrate concentration. The optimum time was estimated by plotting the relationship between the enzyme activities versus the time values.
- Incubation temperatures with optimum conditions of substrate concentration and incubation time .The optimum temperature was estimated by plotting the relationship between the enzyme activities versus the temperature values.
- PH at optimum conditions of substrate concentration, incubation time and incubation temperature. The optimum pH was estimated by plotting the relationship between the enzyme activities versus the pH values.
- Measuring the kinetic parameters of catalase enzyme

Michaelis-Menten constant Km and maximum velocity Vmax values were measured by using different substrate concentrations at optimum values conditions. The values of Km and Vmax were calculated from Lineweaver-Burk plot using the reverse values of V and [S].

- ***The thermodynamic study of catalase enzyme***

According to the steps of the experiment explained in Goth, L. *et al* [6], and measuring the kinetic of CAT, the thermodynamic parameters were calculated by using different concentration of enzyme substrate at different temperatures for each concentration.

- ***The thermodynamic parameters of standard state***

The thermodynamic parameters of standard state are obtained from Vant't-Hoff plot; the values of the natural logarithm of equilibrium constant [Keq or Ka] is obtained at different temperatures; they plotted against the reciprocal values of absolute temperature in Kelvin (1/T), according to the following equations:

Keq=1/Km, Slope= - $\Delta H^o/R$, G= - RT ln keq

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad \ln Keq = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT}$$

- ***The thermodynamic parameters of transition state***

The thermodynamic parameters of transition state are obtained from Arrhenius plot of ln [K] values against (1/T) that gives a linear relationship according to the following equation:

$$\Delta H^* = Ea - RT$$

$$\Delta G^* = -RT \ln K + RT \ln \left[\frac{KT}{h} \right]$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T}$$

• Statistical Analysis

Data were presented as mean ±SD using SPSS program version 20. The differences between two groups were analyzed by independent t-test, P-value ≤0.05 considered significant.

• Results

Table 1 displayed a highly significant decreased ($p \leq 0.01$) in CAT activity of acromegalic patients compared to control group. Table (2) and figure (1) show that the most appropriate conditions for catalase enzyme activity of patients and compare with control group which was described that optimum concentration of substrate :The activity of CAT enzyme opposite different concentrations of substrate was studied for control and patients group and showed a hyperbolic shape in figure1 (A and B), also it show that CAT obey Michaelis-Mentin kinetics. Optimum incubation time, the enzyme exhibited its maximum activity at 60 sec of reaction time in control group and 80 sec in patients group figure (1) (C and D). Optimum temperature, figure 1 (E and F) show that the enzyme activity was increased with temperature and it showed highest activity at temperature 45°C. Optimum pH, figure 1 (G and H) show that the enzyme has the highest effective enzymatic at pH 7 in patients and control groups. Table (3) showed the value of K1 and t1/2, KM, Vmax, K+, K-1 and n[Et] of CAT in acromegalic patients and control groups. Table (4) showed the value of K, K-1 of CAT for acromegaly and control groups at four different temperatures. Figure (2) showed Kinetic study of catalase enzyme. Table (5) showed the value of ΔHº, ΔGº, ΔSº, ΔH*, ΔG*, ΔS* of CAT in different temperature for acromegaly patients group. Table (6) showed the value of ΔHº, ΔGº, ΔSº, ΔH*, ΔG*, ΔS* in different temperature for control group. Figure (3) showed thermodynamic study of catalase enzyme.

Table 1: CAT level in control and acromegalic patients

* Significant at $p \leq 0.05$ in comparison between acromegalic group and control group

Parameter	**Controls, N=30 (mean ± SD)**	**Patients, N=30 (mean ± SD)**	**t-test**	**P- value**
Catalase (Ku/L)	**36.82±9.9**	**13.10± 1.3**	**1.09**	**0.007***

Table (2): Most appropriate condition for CAT enzyme activity.

Optimum condition	Control	Patients
Substrate concentration (mmol /mL)	65	65
pH	7.0	7.0
Temperature ºC	45	45
Time(Sec)	60	80

Table (3): the value of K_1 and $t_{1/2,}$ KM, Vmax, K+, K-1 and n[Et] of CAT in the study groups.

Kinetic parameters	Control	Patient
Km(mmol. L^{-1})	13.33	33.33
Ka (L. $mmole^{-1}$)	75 $x10^{-3}$	30 $x10^{-3}$
K+1(L. $mmole^{-1}.min^{-1}$)	66.6	14
K-1(min^{-1})	8.8 $x10^{2}$	4.6 $x10^{2}$
$t_{0.5}$ =0.69/K_{-1} (min)	7.8 x 10^{-4}	15 x 10^{-4}
n[E_t] =p0 (mmol. L^{-1})	45	35

Table (4): K, K-1 of CAT for acromegaly and control groups at four different temperatures

T °C	T k	1/T x10^3	K (M^{-1}) control	ln K control	K-1 control	ln K-1 control	K (M^{-1}) patient	ln K patient	K-1 patient	ln K-1 patient
5	278	3.5	12	2.48	1.1	0.0953	20	2.99	1.8	0.587
25	298	3.3	22	3.09	1.3	0.262	30	3.4	2	0.69
37	310	3.2	28	3.33	1.6	0.47	35	3.55	2.4	0.875
45	318	3.1	35	3.55	2	0.69	40	3.68	3.6	1.28

Table (5): ΔHº, ΔGº, ΔSº, ΔH*, ΔG*, ΔS* of CAT in different temperature for acromegaly patients group

T °C	ΔHº (kJ/mol)	ΔGº (kJ/mol)	ΔSº (J/mol)	Ea (kJ/mol)	ΔH* (kJ/mol)	ΔG* (kJ/mol)	ΔS* (kJ/mol)
5	14.46	-6.910	76.87	22.6	20.28	66.574	-0.158
25	14.46	-8.423	76.78	22.6	20.12	71.281	-0.163
37	14.46	-9.149	76.15	22.6	20.02	73.776	-0.165
45	14.46	-9.729	76.06	22.6	19.95	74.676	-0.167

Table (6): of ΔHº, ΔGº, ΔSº, ΔH*, ΔG*, ΔS* in different temperature for control group

T °C	ΔHº (kJ/mol)	ΔGº (kJ/mol)	ΔSº (J/mol)	Ea (kJ/mol)	ΔH* (kJ/mol)	ΔG* (kJ/mol)	ΔS* (kJ/mol)
5	22.4	-5.732	101.19	12.16	9.84	67.711	-0.1998
25	22.4	-7.655	100.85	12.16	9.68	72.341	-0.2019
37	22.4	-8.582	99.94	12.16	9.58	74.820	-0.2021
45	22.4	-9.385	99.95	12.16	9.51	76.236	-0.2014

A-Effect of substrate	**B-Effect of substrate**
C-Effect of time	**D- Effect of time**
E-Effect of temperature	**F-Effect of temperature**
G-Effect of pH	**H-Effect of pH**

Figure (1): Most appropriate conditions on CAT activity

A-Lineweaver-Burk plot	B-Lineweaver-Burk plot
C-Pseudo first order reaction	D-Pseudo first order reaction
E- Scatchard plot	F-Scatchard plot

Figure (2): Kinetic study of CAT enzyme.

A-Lineweaver-Burk graphs at four different temperature	B-Lineweaver-Burk graphs at four different temperature
C-Van't Hoff plot	D-Van't Hoff plot
E-Van't Hoff plot	F-Van't Hoff plot

Figure (3): Thermodynamic study of CAT enzyme

- **Discussion**

We suggest that reduced levels of catalase enzyme confirm an increased susceptibility to oxidative damage and this observation is in agreement with the reports that inverse relationship exist between lipid peroxidation and catalase status. Catalase enzyme depletion can lead to impair the cell defense against the toxic action of catalase enzyme and leading to cell injury and death, this lead to increased lipid peroxidation and

reduced antioxidant status in acromegaly patients. The decrease in the activity of CAT enzyme observed could be due to less availability of NADH and hence, no beneficial effect on CAT activity to decrease lipogenesis / oxidative stress [7]. Góth L. *et al* [8] examined the possible origin of the increased serum catalase activity. Due to the poor catalase content and small mass of pancreatic tissue it could not be released from the pancreas into the serum. From these results it may be concluded that the toxic damage of erythrocytes due to zymogen activation and ROS yielded a diagnostic tool of acute pancreatitis Góth L. *et al* [9] showed serum catalase activity was moderately increased in fatty liver, acute alcoholic hepatitis and in accordance with their results, the assay of serum catalase activity is suggested for the detection of severe liver cell damage. Ilieva V. *et al* [10] found that chronic obstructive pulmonary disease (COPD) and those with asthma, pneumonia and other kind of pulmonary diseases had a significant increase ($P<0.001$) in antioxidant enzyme CAT as compared with control group. Anagnostis P. *et al* [11] found that acromegalic as compared with controls had significantly lower levels of catalase activity (8.2±5.8 vs. 51.3±29.1 mmol/mL/min, $p<0.001$). Also a study by Faassen M.V. *et al* [12] showed a trend towards reduction in acromegalic patient's catalase efficiency and this agreement with our present study. The optimum concentration of substrate is (65 mmol/L) for patients and control groups. The results demonstrated that the increase in the enzymatic activity is proportional to substrate concentrations in the range of concentrations (5-65 mmol/L).Under these conditions, entire enzyme bound to substrate as complex and no further increasing in the reaction rate is possible [13,14]. Increased incubation time at the beginning of reaction would allow enzyme to act more extensively results in higher CAT activity obtained so increased activity of CAT with increased the time of incubation. CAT activity was decline even when the reaction reached to equilibrium and hence loss of catalytic activity with time, losing a significant amount of activity over the period of incubation. The optimum temperature depends on the assay time chosen. The true optimum temperature for an assay is the maximum temperature at which the enzyme exhibits a constant activity over a time period at least as long as the assay time [15]. Less than 45°C temperature activity was also decreased CAT activity enzyme was obtained from these curves that the reaction rate is enhanced due to the increase of reaction activation energy as a result of molecules collision until the temperature reaches the optimum value 45 °C and the enzyme lost its activity more than 45 ºC, raising the temperature above the optimum degree, causes a decline in the velocity indicating a disruption of the compact three dimensional structure that is required for catalytic activity.

The loss of activity at the higher temperatures is due to the thermal conformational changes of the enzyme, but lost its activity in high and low at pH 7 because denaturation of protein molecule . The pH optimum for different enzymes varies depending on the nature of the catalytic groups [16]. If the environment of the enzyme is very acidic or very basic, the enzyme may irreversibly denature, or unravel, until it no longer has the shape necessary for proper functioning .The activity of enzyme is profoundly affected by pH, the decrease in activity of the enzyme on either side of pH optimum can be due to 2 general cases, the first is that pH may affect the stability of the enzyme, causing it to become irreversibly inactivated through changing the state of ionization of enzyme as a protein [17] ,the second is that the pH may affect the kinetic parameters of an enzymatic reaction, it may affect the stability of enzyme-substrate complex (ES), the velocity of the rate limiting step, or both([18] .Islamovic S. *et al* [19] found that catalase operates the best at conditions around pH 7.1 in the absence of inhibitor. Maehly AC. *et al* [20] found the optimum pH for human catalase is approximately 7. The affinity of CAT enzyme is decrease in acromegaly patient's scince Km (Ka) higher in acromegaly group value than control group. The km value is an important constant in enzyme studies. It's the substrate concentration which gives half the maximum velocity, so it is indicator of the enzyme affinity to words it substrate. An enzyme with a high affinity for its substrate has a low km value, while the Vmax refers to the amount of active enzyme present [21, 22]. Vmax(K-1) is lesser value in acromegaly group than control group, that's mean activity is low in acromegaly group than control group, the enzyme has a greater capacity to link to substrate in control group more than acromegaly patients group therefore acromegaly patients has a low affinity enzyme to substrate (high Km value) and low activity than control group .

We suggest that oxidative damage effect on CAT activity in acromegaly patients group more than control group. Our present study attempt to use of pseudo first order reaction to determine the rate constant K1 of CAT reaction at 37 °C, linear curve improved the correct choice. K1 for acromegaly patients less value than control, these results mean the formation of ES in patients less than control so we suggest enhance of K1 because activity of the enzyme. The activity of enzyme also decreased the half life time ($t_{1/2}$), where t1/2 is, 15 x 10-4, 7.8 x 10-4 for acromegaly patients and control group respectively. Total concentration of binding substrate to active sites of CAT enzyme n[E]t for control group more than n[E]t for acromegaly patients when calculated from scatchard equation. There were increase in the activation energy of CAT in acromegaly patients group compared to control group, this indicated the presence of change that increased the thermodynamic barrier in acromegaly patients group leading to delay in the breakdown ES complex and the formation of products. ΔG^o have a negative value in two studied group, that's mean the formation of activated complex is a spontaneous process. ΔS^o is a positive value and the reaction rate will be greater than normal. The positive entropy change

(ΔS^o) is the main factor in the unfolding of proteins that yields a negative ΔG^o in spite of the positive (un favorable) ΔHo values, Moreover the differences in the value of ΔS^o among the studied groups are an indication of variation in their conformational stability, molecular flexibility, complexity and structure rigid, ΔH^o value of CAT enzyme in two studied groups were positive and this indicated that the enzymatic reaction is endothermic. ΔH* in the present investigation was calculated [22, 6] KJ/mol for acromegaly patients group and [12,16] KJ/mol for control group. This demonstrated that acromegaly enzyme increased the value of ΔH* from control sera enzyme. These large values of ΔH* indicated that a large amount of stretching , squeezing or even breaking of chemical bonds occurred during the formation of the transition state [23]. The large changes in ΔG*, Ea, ΔH*, ΔS* confirm the effect of pathological case on enzyme mechanism and activity.

• Conclusion

We conclude that the decreased level of serum CAT activity in patients with acromegaly when compare with control group, it may be to have a large activation energy and need a large time to give the product than control group. So decrease CAT enzyme affinity to substrate and rate of reaction of CAT for acromegaly patients group. CAT is a spontaneous and endothermic process through negative value of (ΔG^o) and positive value of (ΔH^o).

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