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PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF PARAOXONASE IN THE SERA OF NORMAL AND ECTOPIC PREGNANT WOMEN

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ABSTRACT
Paraoxonase (PON) an enzyme that hydrolyzes paraoxon is located in mammals primarily in the serum and liver. The present work represents the first study on the partially purification of human serum PON in normal and ectopic pregnant women. This enzyme was purified by a three step methods: Ammonium sulfate precipitation (60-80%), dialysis and gel filtration chromatography. The enzyme was purified 402.59-fold with a yield of 46.66% and 358.78 -fold with a yield of 66.66% in normal and ectopic pregnant women respectively on Sephacryl S-200 column. Initial velocity studies for the determination of kinetic constants with paraoxon as substrate revealed a Km and Vmax (using a protocol consisting of three steps). The molecular weight of the enzyme was approximately 44KD which determined by gel filtration. Characterization of partially purified PON was carried out to find the optimum conditions for the enzyme which appear as(10µl optimum enzyme, 1.2mM optimum substrate) concentration, (4min, 2min, 3min, 1min) optimum incubation time, (37ºC, 37ºC, 40ºC, 25ºC) optimum temperature, optimum pH 12 in crude and purified sample for both normal & ectopic pregnancy respectively. Enzyme stability for all studied groups showed that PON is more stable when stored at (-20) ºC than other temperatures. The turn over kinetic parameters (K_m and V_{max}) were determined by Lineweaver-Burk plot. The results indicated that the lowest enzyme affinity towards its substrate was found in purified sample of ectopic group for both 37ºC and 50ºC. The rate constant K_1 value at 50ºC is the higher one than at 37 ºC. Also results showed that K_1 value increased in normal compared to ectopic for both crude sera and purified sample. The time taken for the concentration of a reactant to fall to half initial value ($t_{½}$) for ectopic pregnancy was higher than in normal for both crude sera and purified sample. The values of Hill-coefficient (n) were (0.811, 0.76, 1.06, 0.977) in crude sera and partially purified sample for both normal and ectopic pregnancy respectively.

KEY WORDS: Ectopic pregnancy, Paraoxonase, Purification, Molecular weight, Kinetic and Thermodynamic.

INTRODUCTION

Pregnancy is a stressful condition in which many physiological and metabolic functions are altered to considerable extent[1]. The most common complications of early pregnancy are ectopic pregnancy (EP)[2]. In an ectopic pregnancy, the fertilized egg implants outside of the uterus, usually in the fallopian tube and begins in the rarely, an ectopic pregnancy implants in the women's abdomen, on the outside of the uterus, on an ovary or in the cervix[3].These complications are clinical conditions where biomarkers are urgently needed to improve early diagnosis and where discovery studies must be conducted using human specimens due to the lack of a good experimental model system. There are several studies that investigated PON activity in pregnancy with and without complications and found that serum PON was reduced or did not show any significant changes[4,5]. Paraoxonases have multifunctional roles in various biochemical pathways such as protection against oxidative damage and lipid peroxidation, contribution to innate immunity, detoxification of reactive molecules, bio activation of drugs, modulation of endoplasmic reticulum stress and regulation of cell proliferation/apoptosis[6]. Mammalian gene family of PONs has three members (PON1, PON2, and PON3) is a unique family of calcium-dependent esterases/lactonases[7]. PON1 is a calcium dependent glycoprotein with 354 amino acid residues, and has a molecular mass of approximately 45 kD, depending on the degree of glycosylation. It is synthesized in the liver and bound to HDL in the blood stream [8]. (PON_1) was named after found its ability to hydrolyze the organophosphate substrate paraoxon, which is the toxic metabolite of the insecticide parathion[9]. The purified PON1 appeared as a double band of 39 and 42 kD on SDS-PAGE, whereas PON2 and PON3 appeared as single 39 kD bands [10]. The aim of the present study was to purify and characterize the human serum paraoxonase in normal and ectopic pregnancy women.

MATERIALS & METHODS

Sephacryl S-200, Paraoxon, was purchased from Sigma - Aldrich Chemie (Taufkirchen, Germany) and all other chemicals used in this study were purchased from Sigma-Aldrich or Merck KGaA Darmstadt, Germany and were of analytical grade.

The study included women with (normal and ectopic) pregnancy who was admitted to the units in the medical city, AL-Yarmook and Fatima AL-Zahraa hospitals. Selection of serum samples were based on β-hCG more than1000 Units /ml and ultrasound findings of EP.

Pregnancies of 3–4 weeks duration, based on menstrual dating and pelvic Sonography, were included in the study. The process of collecting specimens by withdraw about 10 ml of vinous blood using plastic disposable syringes, and then left for 20 minutes at room temperature. After coagulation, sera were separated by centrifugation at 1500 ×g for 10 minutes. Hemolysis samples were discarded and sera were stored on -20C° until analysis.

Paraoxonase activity assay

Paraoxonase converts paraoxon (diethyl–*p*-nitro phenyl phosphate) to *p*-nitro phenol. The yellow compound can be measured spectrophotometrically at 405 nm, the molar extension coefficient of p-nitro phenol ($\varepsilon = 33300\ M^{-}.cm^{-}$ at pH= 11.2) was used to calculate the enzyme activity .One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of substrate at 37°C [(11)]. Assays were performed using a spectrophotometer (Cecil, CE10N, England).

Purification methods

Paraoxonase was purified partially from serum of human (normal and ectopic) pregnancy using the following steps:

Ammonium sulfate precipitation

Human crude serum precipitated with (60-80) % ammonium sulfate as mentioned by Dixon and Webb [(12)]. The precipitate was obtained after centrifugation at 10000 xg for 15 min and re dissolved in 1 ml of 0.1 M glycine-Na glycinate buffer at pH 11.2. Both enzyme activity and protein content were determined for each separate fraction.

Dialysis against buffer

The obtained ammonium sulfate precipitate was dialyzed in the presence of 0.1 M glycine-Na glycinate buffer at pH 11.2 overnight at 4°C. Fractions were checked in terms of both protein amount (280 nm) and enzyme activity.

Gel Filtration chromatography

Enzyme solution, which had been dialyzed, was loaded on to the Sephacryl S-200 column after diluted to reach protein concentration (5-10 m gm/ml) then loaded onto the glass column bed (48×1.6) cm which had been pre equilibrated with 0.1M glycine-Na glycinate buffer, pH 11.2. The column washed with 250 ml buffer. Fractions of 1ml/3min were collected and the protein level was monitored by scanning elutes at 280 nm. Enzyme activity and protein concentration of the partially purified samples were checked[(13)]. Tubes having enzyme activity were collected for the kinetic studies.

Determination the molecular weight of partially purified enzyme

The molecular weight of partially purified enzyme was determined by using gel filtration chromatography on Sephacryl S-200 .Column was prepared to determine the void volume of standard proteins and paraoxonase. One milliliter of purified enzyme was loaded onto the column with protein concentration (10 mg/ml), enzyme was eluted with the above conditions. The fractions were analyzed for enzyme activity and protein concentration and elution volume Ve was determined. The constant Kav was determined for each standard protein. The standard curve was drawn between Kav against log molecular weight and use to determine paraoxonase molecular weight.

Total protein determination

The total protein for serum was determined by Lowry *et al*. method [(14)] using bovine serum albumin (BSA) as the standard protein.

Molecular characterization of crude and partially purified paraoxonase

1- Optimum conditions for crude and partially purified Paraoxonase

The selected samples were classified into four groups for optimization study as below:

Optimum enzyme concentration

Paraoxonase enzymatic reaction was carried out using different enzyme concentrations by adding different volumes (5, 10, 15, 20, 25 and 30) µl from crude and partially purified enzyme, assay buffer was used to complete volume.

Optimum substrate concentration

The procedure was followed as above with optimum enzyme concentration (10µl) and different substrate volumes (133, 166, 200, 233 and 266) µl which added separately to all set tubes. Mixtures volume was completed by adding the assay buffer.

Determination PON reaction order (n) and rate constant (k_1)

Relation time determines the advanced rate constant (k) and half life time ($t_{1/2}$) by using pseudo first order reaction in the normal and ectopic pregnancy (crude and partially purified) samples using liner curve of enzyme activity values for a period of times (1 ,2, 3, 4 ,5)min. Plotting the relation between ln(Vmax/Vmax-V) opposite time (minutes) and from the slope can obtain the value of k while the value of $t_{1/2}$ can be obtained from the equation ($t_{1/2} = \ln 2/k = 0.693/k$) [(15, 16)]. From the relation between log (V/Vmax-V) opposite log[S] we can determine Hill coefficient by the equation (Log (V/Vmax-V)= n log [S] – log k)[(17)].

Optimum incubation time

The procedure was followed as steps above with constant optimum (enzyme, substrate) concentration at different incubation times (0.5, 1, 2, 3, 4 and 5) minutes.

Optimum temperature

The procedure was followed as above with constant optimum (enzyme, substrate) concentration and incubation time for the normal and ectopic pregnancy (crude and purified)samples using different incubation temperatures (15, 25, 37, 40, 50)°C .

Optimum pH

The procedure was followed as above with constant optimum (enzyme, substrate) concentration, incubation time & temperature for the normal and ectopic pregnancy (crude and purified) samples with different pH value (5, 7, 9, 11.2, 12) have been added to all set of tubes.

The stability of PON

Enzyme stability in crude and purified samples were monitored after incubate for one day and one week at different temperatures (20, 4, 25, 37, 40) °C, the activity of PON was measured as above.

Thermodynamic studies for the crude and partially purified enzyme

The same above steps of enzyme assay were followed, including measurement of enzyme activity at high (50C°) and moderate (37C°) temperatures in the crude and

purified samples of ectopic and normal pregnancy to determine the energy parameters: equilibrium constant Keq, Gibb´s energy ΔG, enthalpy change ΔH, entropy change ΔS, activation energy Ea, stereo-frequency temperature coefficient Q10. The value of Keq was collision factor PZ, heat of activation ΔH⋆ and calculated at moderate (M=37°C) and high (H=50°C) temperatures from the equation (Keq= 1/Km) [18], Energetic parameters were determined by applying the equation (ΔG= -2.303 RT log Keq). The change in enthalpy ΔH was calculated from the integral Van´t Hoff equation between the limits of Keq at H and M temperatures: {ΔH= 2.3 R (log KeqH /log KeqM)(TH*TM/TH-TM)}.

The Gibb´s Helmholtz equation (ΔG =ΔH -T ΔS) was used to calculate the entropy change: The activation energy (Ea) for the reaction was calculated based on the integrated form of the Arrhenius equation {log kH /logkM = Ea /2.303 R *(TH-TM/TH*TM)}. The PZ factor was determined applying following equation (Ln PZ= (Ea/RT)+ln Keq) Where Z is the number of collisions occurring between reacting molecules and enzyme in one unit volume per unit time ,(Ea/RT) is the frequency factor which measures the probability that any molecule will have sufficient energy to react , and P is the stearic potency for that fraction of molecules which success in getting of enough energy during collisions. Heat of activation (ΔH) was calculated using the equation p (Ea= ΔH⋆ + RT).The temperature coefficient, Q10 (which is the factor by means of which the rate constant is increased by raising the temperature 10°C) was calculated using the following equation (Ea=(2.3 RTM *TH log Q)/10).

Discontinuous Polyacrylamide Gel-Electrophoresis (Lammeli) Polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli[19] using a 12% separating gel, 3% stacking gel, but without sodium dodecyl sulfate and mercapto ethanol. The protein bands were localized with Coomassie brilliant blue R250. In order to locate the position of PON bands, poly acryl amide gel was cut to two pieces: the first was stained using staining protein method. The second unstained portion of the gel is used to determine PON activity using paraxon as substrate. The relative mobility of each protein was measured from the equation (Rm= distance moved by the protein/ distance moved by the bromophenol blue *100).

RESULTS & DISCUSSION

Paraxonase was purified from the serum of healthy human previously using blue agarose and DEAE chromatography [20,21,22]. Consequently, another study purified PON enzyme from human serum with a high yield using the simple three steps [23]. Demir *et al*.[24] purified PON from human serum using sepharose-4β-I-Tyrosine-1-naphylamine affinity chromatography. In this study PON1 was purified by using three sequential steps: ammonium sulfate precipitation, dialysis and Gel filtration chromatography. Table (1) summarized the results when PON was subjected to purification process.

TABLE 1: Steps of PON purification

Steps	PON activity (U/ml)	Tatal volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield %	Fold
1-Crude sera								
a-Ectopic P	133.2	3ml	80.6	241	399.6	1.65	-------	-------
b-Normal P	166.5	3ml	86	258	499.5	1.93		
2-Ammonium sulfate								
a-Ectopic								
*Supernatant	183.15	2ml	50	100	366.3	3.66	91.66	2.21
*precipitate	49.95		12.4					
b-Normal								
*Supernatant	199.8	2ml	50.18	100.36	399.6	3.98	80	2.01
*precipitate	99.9		18					
3-Dialysis								
a-Ectopic P	49.65	1ml	37.2	37.2	349.65	9.39	87.5	5.69
b-Normal P	283.05	1ml	21.8	21.8	283.05	12.98	56.66	6.7226
4-Gel filtration								
a-Ectopic P	26.64	10ml	0.045	0.45	266.4	592	66.66	358.78
b-normal P	23.31	10ml	0.03	0.3	233.1	777	46.66	402.59

Different concentrations of ammonium sulfate were used after conducting some preliminary trials; 60-80% concentration was selected for the enzyme precipitation. Each of the purification step resulted in increasing specific activity; the maximum activity was calculated after gel chromatography through Sephacryl S-200. Correspondingly, there was a decrease in the total protein contents from crude to partially purify by gel chromatography through Sephacryl S-200 in ectopic and normal group respectively. This decrease in protein content indicates the separation of protein impurities from the desired enzyme fraction (PON). Fold of purification was calculated after each step and found to be equal to (2.21, 2.01) after ammonium sulfate precipitation, (5.69, 6.72) fold after dialysis, whilst the maximum (358.78, 402.59) fold of purification was observed after gel filtration in (ectopic and normal) groups respectively. Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield reduced to (91.66, 80) % after ammonium sulfate precipitation, (87.5, 56.66) % after dialysis and reached to its minimum level (66.66, 46.66) %, after gel filtration in (ectopic and normal) groups respectively. The results cleared that every step in the enzyme purification resulted in the removal of undesirable fractions of protein therefore, decline in enzyme yield was

found after the process of purification. Also, table (1) cleared that PON was finally purified with a fold of purification and specific activity in normal higher than in ectopic sample. The differences may be due to essentially lowest PON activity that noticed in ectopic patients and highest found in normal pregnant women from the beginning, these results can attributed to the fact that the presence of inflammatory factors in patients sample may effect PON purified and activity. Paroxnase has been purified from different sources with different yields and purification folds. For instance in a one study, human serum PON enzyme was purified approximately 62.1 fold using Agarose blue, Sephadex G200, DEAE-trisacryl M,. Sephadex G75 chromatography techniques[25].

The results of this study are in agreement with the findings of Sukru and his colleagues [23], they found an increase in the enzyme activity and decrease in total protein contents when PON1were purified from human serum with a final specific activity of 4867.34 U/mg and yield 39.8.They used four steps including: Triton x-100-treated serum, ammonium sulfate precipitation, Ion exchange and Gel filtration chromatography. Thus compared with other mentioned studies, present purification procedure takes less time and good specific activity, yield and fold of purification. During the present study, maximum fold of purification were (358.78, 402.59) in (ectopic and normal) groups respectively was attained after gel filtration. The procedure of purification was considered imperative as it resulted in a significant increase in the specific activity of PON. The relation between number of fractions of apposite protein concentration (absorbance at 280nm) against enzyme activity for both normal and ectopic samples were presented in figure1 (a&b) and PON elution volume Ve was estimated from this figure.

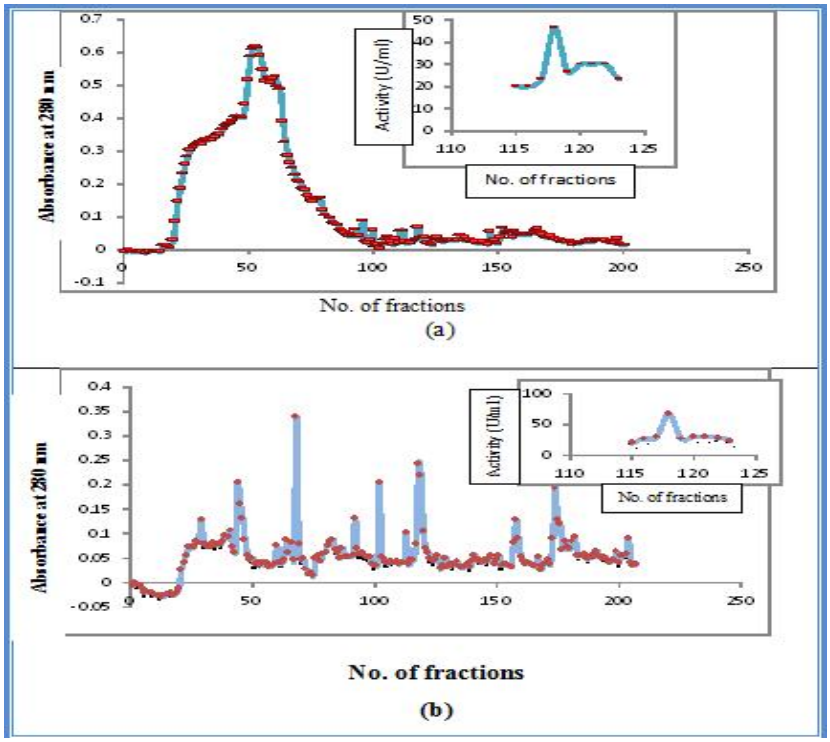

FIGURE 1: Elution volume of human PON1 from a-normal and b-ectopic samples by using sephacryl S-200

Four standard proteins with known molecular weights were used to estimate the molecular weight of PON. The elution volume Ve for each protein was estimated from figure (2).

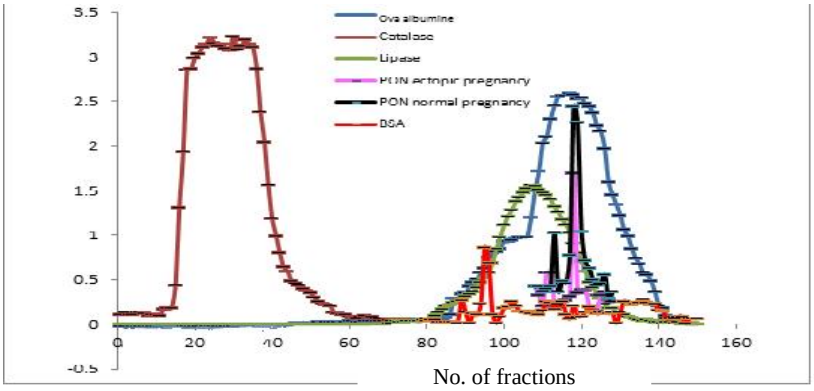

FIGURE 2: Elution volumes of standard proteins

Eluted fractions were followed by measuring absorbance of protein content at 280nm. The K_{av} Values of the standard proteins and partially purified enzyme were determined and calibration curve have been drawn between K_{av} against molecular weight logarithm as cleared in figure (3):

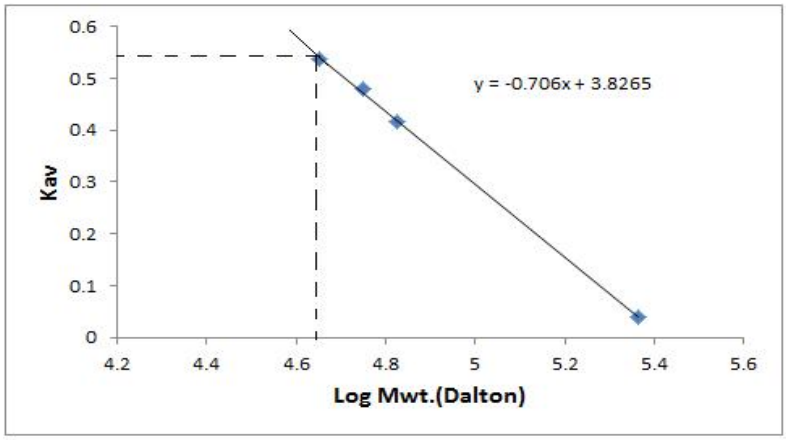

FIGURE 3: Determination of PON molecular weight

On application of Kav=4.64 value on partially purified PON1 for both ectopic & normal samples on the calibration curve in figure (3), the molecular weight was found to be 44000 Dalton = 44K Da. It is obvious from figure (4) that upon the comparison among protein profile of the crude sera and the purified enzyme of the studied groups, the proteins in crude sera separated into several protein bands and the purified samples show less protein bands which reflect that there are other protein present in the purified sample i.e.; PON1 is partially purified. Figure (4a) showed the double blue bands were demonstrated in each of crude samples and the purified enzyme of the studied groups. Dragomir et.al. [25] concluded that the purified PON1 appeared as double band, whereas PON2 and PON3 appeared as single bonds using SDS-PAGE electrophoresis. When the R_m value of the purified enzyme was compared with that of the crude sera, It is clear that the purified enzyme have the greater motilities (37.5) than that present in its corresponding crude sera samples (31.25) which mean the free motilities of partially purified enzyme from the crude that contain many proteins and compartment elements delayed enzyme motion. The glycoprotein stain for both the crude and purified samples Figure(4b) showed that the glycoprotein band in crude sample (ectopic and normal) is more clear from that in purified sample, and this reflects the low concentration of the glycoprotein enzyme in purified sample.

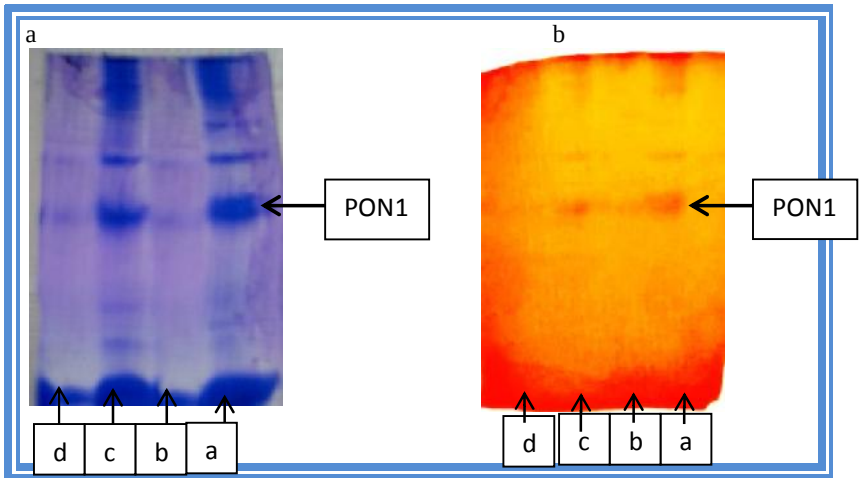

FIGURE 4: Conventional – PAGE 7.5% profile of purified and crude sera PON (*a- Crude sera from NP b- Purified).*

Samples from NP c- Crude sera from EP d- Purified sample from EP)

Figure (5) A, B, C, D illustrated the effect of the different enzyme concentrations on its activity in the sera of normal and ectopic pregnancy (crude and partially purified) samples, it's clear from this figure that the optimum PON concentration equal to (10µl) for both (crude, partially purified) enzyme of normal and ectopic samples and there is slightly decrease in enzyme velocity with increased enzyme concentration beyond the optimum concentration.

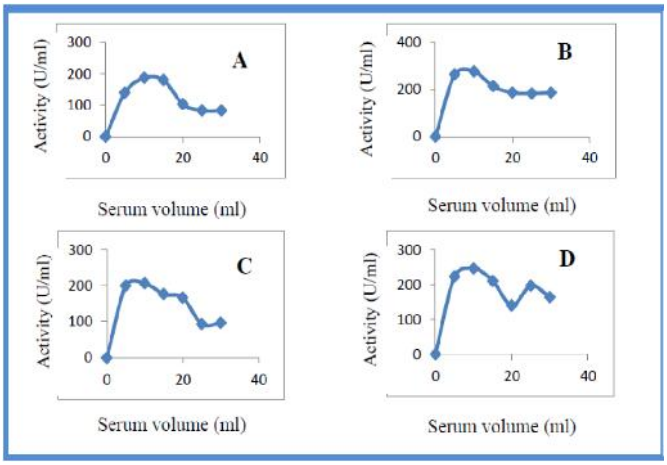

FIGURE 5: Effect of [E] concentrations on activity of PON in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

Figure (6) A, B, C, D indicated that PON enzyme obeys Michalis-Menton equation in its esterase activity in all studied groups. The curvature of the figure is hyperbolic and the optimal substrate concentration for the enzymatic activity was found to be 1.2mM for all groups.

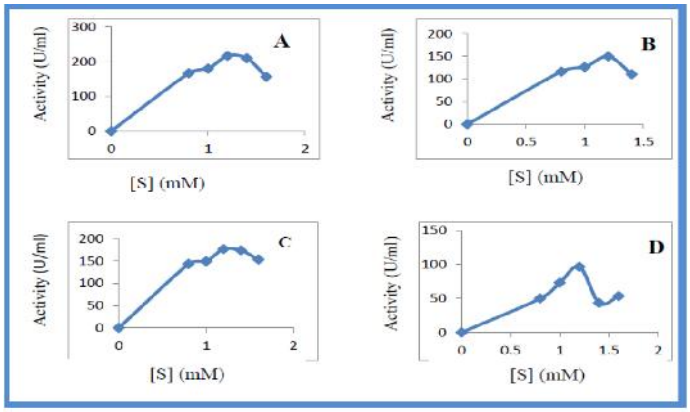

FIGURE 6: The effect of different substrate concentration on the enzymatic activity in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

The results indicated that PON in all four groups were saturated by the substrate paraoxon in the same enzyme concentration and the decline in velocity may be due to the accumulated of enzyme molecules that are delayed and steric the binding of substrate to active site[26]. The curvature of the figure is hyperbolic and the optimal substrate concentrations for the enzymatic activity were found to be 1.2mM for all groups. Figure (7) A, B, C, D represents Lineweaver-Burk plot for PON activity in the four studied groups at 37ºC and 50ºC. The turnover kinetic parameters (Km and Vmax) were determined from these plots and are presented in table (2).

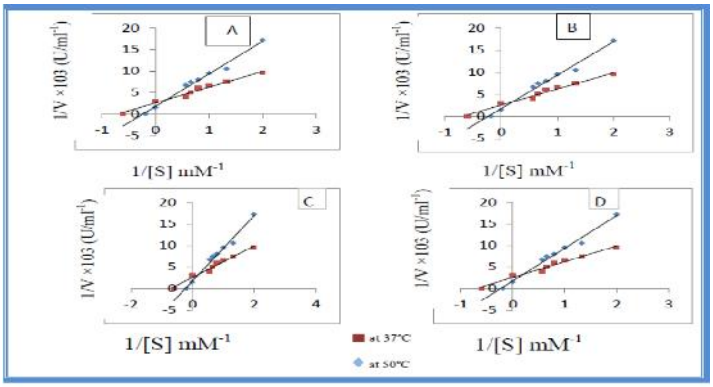

FIGURE 7: Determination of Km and Vmax at 37ºC and 50ºC for PON by using Lineweaver – Bruk plot in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

TABLE 2: Km and Vmax values of PON activity in the four studied groups as determined by Lineweaver-Burk plot

Groups	*Lineweaver-Burk*			
	K_m (mM) 37ºC	*V_{max}×1000 (nmol/L/min)*	*K_m (mM) 50ºC*	*V_{max}×1000 (nmol/L/min)*
Crude sample of NP	1.6	0.333	5.55	0.666
Crude sample of EP	1.6	0.25	5.5	0.5
Purified sample of NP	1.6	0.25	5	0.5
Purified sample of EP	2.5	0.25	7.14	0.5

The results from table (2) indicated that the lowest enzyme affinity towards its substrate was found in partially purified enzyme in EP at both 37ºC and 50ºC, this may be attributed to the present of some effectors in the sera of patients (which has been clear of some effectors in the purified sample) that decrease enzyme affinity to its substrate. This means that the infection by disease may affect the enzyme affinity to its substrate and this may be due to the charge in the chemical structure (the ionic state of the active sites) that become less suitable for conjunction with substrate[27]. Crude and partially purified samples of normal pregnancy in both 37ºC and 50ºC show the highest activity of enzyme, figure (8).This means probability of change in active sites number of PON in ectopic pregnancy leads to decrease its activity [22].

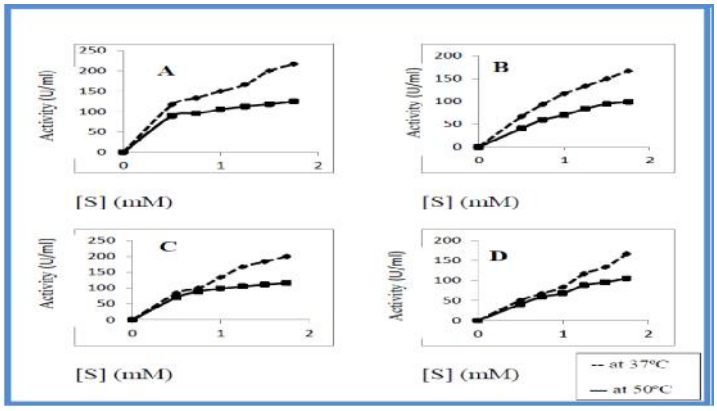

FIGURE 8: Michael's- Mention curve at 37ºC and 50ºC for PON in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

In addition, It is clear that Km was high at a higher temperature (50ºC), this indicated that the affinity of PON to paraoxone decrease at high temperature (50ºC) due to an increase in the kinetic energy of the interacting molecule that is not favored in these way to increase the probability of PON interaction with paraoxon. In contrast it can be assumed that at 37ºC when Km is low (high affinity of PON to its substrate), rotation of various atoms took place in such a way, which facilitate the entrance of paraoxon molecule to the catalytic site through the mouth of the deep gorge. It is possible that phospho ester part of paraoxone could fit more favorably into the His 115-His134 and anionic pockets of the catalytic center of the PON [24].A comparative pattern of the Vmax values was also obtained at regular intervals table (2). These data verified that the amount of active enzyme was higher in 50ºC and lower in 37ºC. Poh *et.al.*[28] estimated the value of Km and Vmax for PON and found it to be equal to Km=0.335mM, Vmax=194 u/L. Alireza *et.al.*(312) investigated the PON1 kinetic parameters and found the following results : Km=0.4mM, Vmax=0.255mol/ml/min.

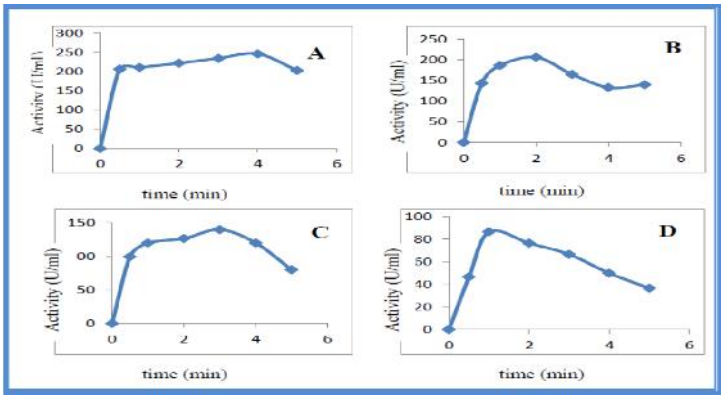

FIGURE 9: Incubation time of PON activity in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D-Partially purified sample of EP

Figure (9) A,B,C,D showed the effect of incubation times on the enzyme activity in the four studied groups which were (4,2,3,1) minutes in the crude and partially purified sample for both normal & ectopic . The activity of PON increases by increasing the incubation time for all studied groups allowing the enzyme to be completely saturated with the paraoxon until reaching to the optimum time course, then incubation of binding mixture for time periods longer than that required for maximum binding resulted in a decrease in binding. This may be due to reversible dissociation of complex after reaching to the equilibrium state [29]. Results also indicated that the crude sample needs longer time to reach maximum activity than in partially purified sample for both normal and ectopic pregnancy; this means that the partially purified enzyme has more freedom for motion and less steric effect. Michael *et.al*.[30] reported 15 minutes as maximum time course of PON activity. Lourdes *et.al*.[31] showed that optimum PON velocity was at 30minutes of incubation.

In this study, the rate of paraoxon (1.2mM) esterification by the action of PON was measured at different temperatures as shown in figure (10) A, B, C, D.

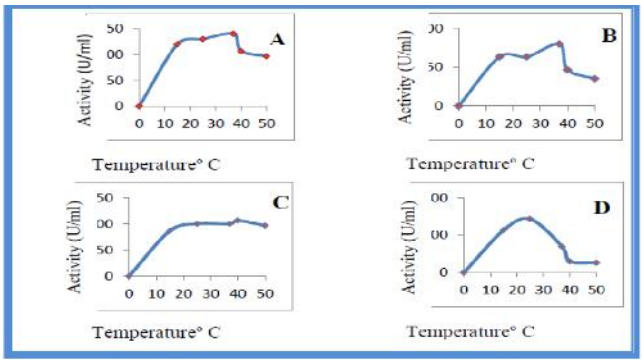

FIGURE 10: the effect of different temperatures on PON activity in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP.

Figure (10) demonstrated that upon increasing the temperature, the esterase activity increases in the all studied groups, where the optimum temperatures that observed were (37ºC, 37ºC, 40ºC, and 25ºC) in crude, partially purified samples for normal and ectopic pregnancy respectively. But above optimum temperature, a decline in the activity was observed indicating a disruption of the compact three dimensional structures that is required for catalytic activity, which may due to the denaturation occurring for some enzyme molecules that lead to make changes in catalytic site, which decrease active site number [30]. Partially purified enzyme in normal pregnancy appeared optimum temperature (40ºC) to reach maximum activity, whereas, ectopic group show low optimum temperature (25ºC) at optimum activity, these data may explained by that in normal group (partially purified sample) enzyme structure is a good form that is stable until 40ºC, while in ectopic the enzyme give maximum activity at near temperature. However, enzymes are complex protein molecules, they act as a biological catalysts enable the living organisms to obtain and use energy very rapidly through changing the rate of chemical reaction, but they don`t affect the final equilibrium. Only small quantities are needed to bring about the transformation of large number of molecules[32]. The catalytic activities of enzymes result from a precise and high ordered tertiary structure. The tertiary structure of an enzyme is maintained primarily by a large number of weak non-covalent bonds. This means that the structure is easily affected when high temperature is used. A high temperature increased the enzyme and substrate collision and this is offset by the increasing rate of the denaturation [33]. Mechael *et al*. [30] presented that PON has high activity at 60ºC . Lowrdes *et.al*.[31] appeared that 47.5ºC was the optimum temperature of PON. Maria *et.al* [34] studied effect of temperature on PON activity and found maximum activity at 55ºC. A change in pH alters the rates of enzyme catalyzed reaction with many enzymes exhibiting a bell shaped curve when enzyme activity is plotted against pH. Figure (11) A,B,C,D shown that the optimum pH for each four groups was 12.

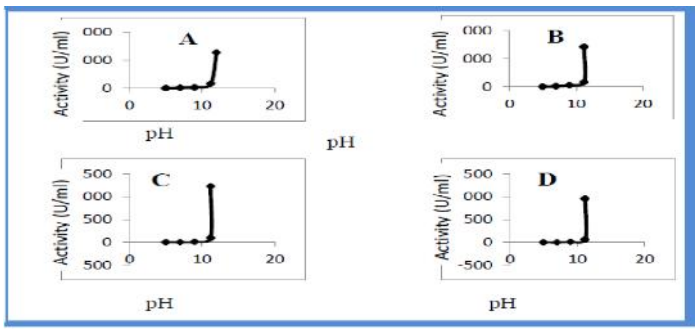

FIGURE 11: The effect of pH on PON activity in: A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP.

These data showed that human PON was somewhat sensitive to the effect of acid function pH. Study of the pH dependence of the enzyme-catalyzed reaction can provide important information about the identity of the prototropic groups at the active site of the enzyme[35,36]. Part of the decline in activity below pH 6.0 could result from irreversible enzyme inactivation [35]. An estimation of pKa values was made according to Wilkinson *et al.* [37], they showed pKa1 values of 8.4-8.7 and pKa2 values of 11.3-11.6. These data suggest that probably sulfhydryl group of cysteine residue (pka 8-8.5) and guanidine group of arginine residue (pka 11.6-12.6) may responsible for the catalytic activity, and these is in agreement with the results of this study. Gilf *et al.*[38] showed that at extreme pH values (5,11,12), PON activity showed biphasic kinetics. The pH profile of PON in human liver microsomal fraction showed optimum activity at 10.0-10.5.Mc. Craken *et al.*[39] have reported an optimum pH of 9 for PON human liver microsomes, and similar value (pH 9.5) have been reported for PON human plasma had pericardial fluid[40]. The optimum pH values found by other researchers for human serum PON are in the range of 10-11[41,42]. Alici *et al.*[43] showed that the PON optimum activity at pH 10.5 may due to the presence of cysteine, tyrosine and lysine near the proposed active site. Figure (12) showed that in the crude sera and partially purified samples in both normal and ectopic pregnancy the enzyme lose some activity when stored for one week compared to the storing for one day at (-20,4,25,37,40) ºC respectively. Additionally PON is more stable when stored at (-20) ºC than other temperatures in all studied groups. According to these results, it's a clear reasonable state that human PON have a resistance to heat in activation [44]

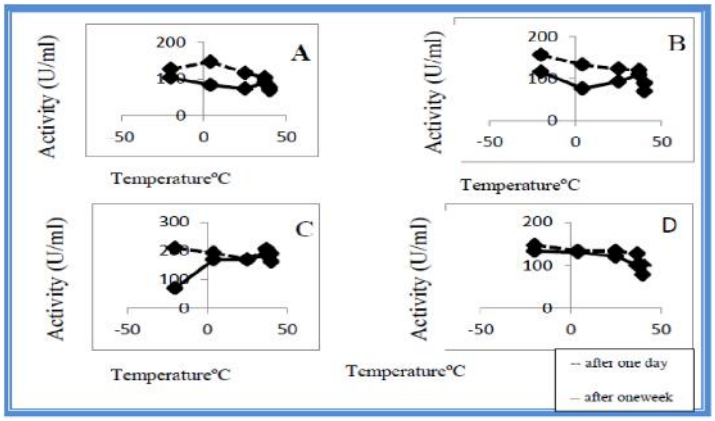

FIGURE 12: PON stability after incubation one day & one week at different temperatures in : A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

Rochua *et al.*[45] estimated the effect of storage of PON for up to 3 days at room temperature on its activity in plasma samples, the results showed no significant change in the activity compared to the activity on samples stored for 16-18 months at -70ºC did, however, there is a significant decrease in PON activity level. Samples stored under these conditions for 1 year or less did not significantly differ in its activity. Karen *et al.*[46] Studied the effect of storage duration up to 2 years (–80) of PON, and found the decrease in enzyme activity level was minimal. However, after 7years, PON activity decreases more noticeably. Similarly freeze – thaw cycles did not affect the PON1 activity in sample stored < 2 years. Marchegiani *et al.*[44] calculated a half – life of 9.5 years for PON activity stored at -20ºC by fitting an exponential function to their data; thus it may be possible to adjust for storage duration in longitudinal studies by fitting a similar model.

Kinetic and Thermodynamic Studies on PON Reaction in the Sera of Crude and Partially Purified Samples of Normal and Ectopic Pregnancy

Determination of the Forward Rate Constant (K_1) and The Half-Life ($t_{1/2}$) of PON Reaction

The rate law or rate equation for a chemical reaction is an equation that links the reaction rate with concentrations of the presence of reactants and constant parameters. In a reaction of the type[47]:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$

The rate of the reaction is proportional to [E], [S]

$$\frac{d[ES]}{dt} = K_1[E]^1[S]^1 - K_2[ES].$$

To derive an expression that relates the reaction velocity, (V) to the concentrations of the substrate and enzyme, and the rates of the individual steps. The reaction velocity V can be expressed as: V = K2 [ES]. Since ES is an intermediate and hence its concentration unknown, we have to express [ES] in terms of known values. The rates at which [ES] is formed Rate of formation of ([ES] =K1 [E][S])The use of the steady–state approximation to express V in terms of known quantities. Under the steady-state approximation, the concentra -tion of intermediate [ES] stays constant while the concentrations of reactant and product change.

$$V = V_{max}\frac{[S]}{[S]+Km} \ldots\ldots (4), \quad Km = \frac{K-1+K2}{K1}.$$

These equations describe the kinetic behavior of enzyme as modeled by the kinetic scheme of enzyme. The maximum reaction velocity, Vmax is reached when all enzyme sites are saturated with the substrate (Vmax=K2 [E] Vmax=K2 [E]).Because of most enzyme kinetic

studies concentrate on the initial approximately linear part of enzyme reaction and if the concentration of one of the reactants remains constant (because it is a catalyst or it is in great excess with respect to other reactants, its concentration can be grouped with the rate constant obtaining a pseudo constant. So, the study attempt to use of pseudo first order reaction to determine the rate constant K1 of PON reaction at 37ºC, 50ºC , linear curve improved the correct chosen. Figures (3-14) and (3-15) showed the slope which represents the value of forward reaction rate constant K1 (min-1) at 37ºC, 50ºC respectively.

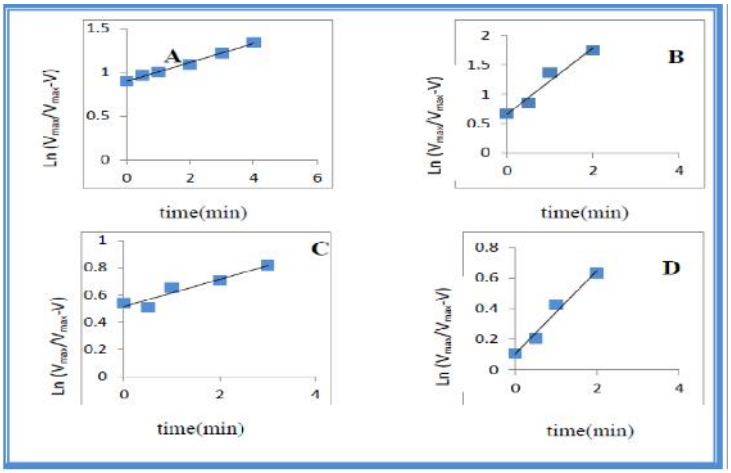

FIGURE 13: Pseudo first order reaction for PON enzyme in four studied groups at 37ºC. A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

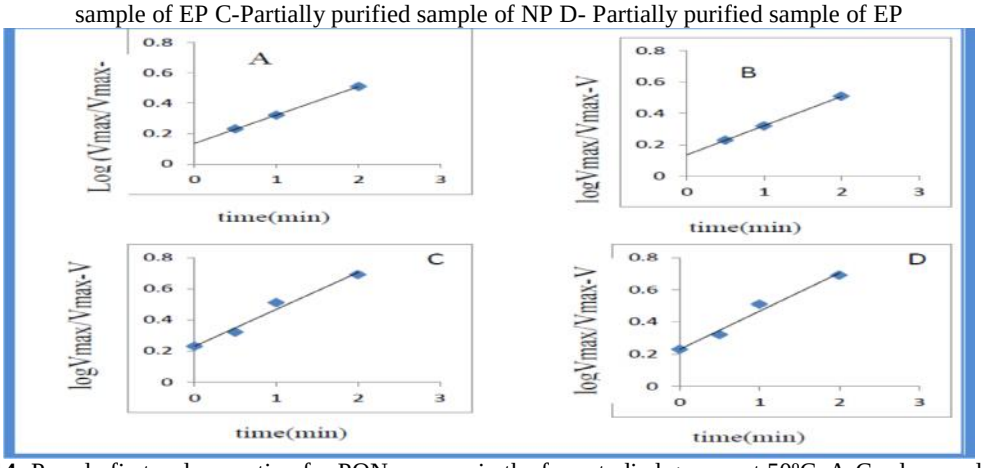

FIGURE 14: Pseudo first order reaction for PON enzyme in the four studied groups at 50ºC. A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP d- Partially purified sample of EP

Also the half life time ($t_{1/2}$), which represents the time needed for the formation of half amount of the ES complex at equilibrium was calculated from:

$$t_{1/2} = ln\frac{2}{K_1} = \frac{0.693}{K_1}$$

The values of K and $t_{1/2}$ at 37ºC and 50ºC were summarized in table (3).

TABLE 3: Rate constant and half-life time for PON in Crude sera and purified samples for both normal and ectopic pregnancy

Groups	37ºC		50ºC	
	$K_1(min^{-1})$	$t_{1/2}$(min)	$K_1(min^{-1})$	$t_{1/2}$(min)
Crude sera of N.P	0.377	1.83	0.916	0.756
Crude sera of E.P	0.13	5.33	0.176	3.93
Purified sample of N.P.	0.107	6.47	0.378	1.833
Purified sample of E.P	0.114	6.07	0.28	2.47

Table (3) Revealed that the rate constant K1 value at 50ºC is higher than at 37ºC. Results also shown that K1 value increased in normal compared to ectopic for both crude sera and partially purified samples. So the rate constant K1 changes with temperature. This indicates that the rate of formation of ES complex depends on the temperature. More clear suggestion is the effect of patients group on Keq for ES formation depends on the following equation:

$$K_{eq} = \frac{K1}{K-1}$$

Table (3) also indicated that the time taken for the concentration of a reactant to fall to half initial value at 37ºC and 50ºCfor ectopic pregnancy was higher than in normal for both crude sera and partially purified sample. These can be explained as the enzyme in patients groups is less active and needs more time to convert the reactant to the product.

Determination of Hill Coefficient (n) of PON Reaction

The values of (logV/Vmax-V) against log[S] were plotted according to Hill equation, figure (15).The value of Hill-coefficient (n) equals to the slope of the resulting straight line. The values of Hill-coefficient (n) were (0.811, 0.76, 1.06, and 0.977) in crude sera and partially purified sample for both normal and ectopic pregnancy respectively. These values cleared the number of binding sites per enzyme molecule and illustrated also that when n=1, the binding sites act independently of one another [(331)]. From the results of this study, it's clear that the binding site in partially purified enzyme was higher than enzyme in crude sera, and in normal, higher than in patient samples.

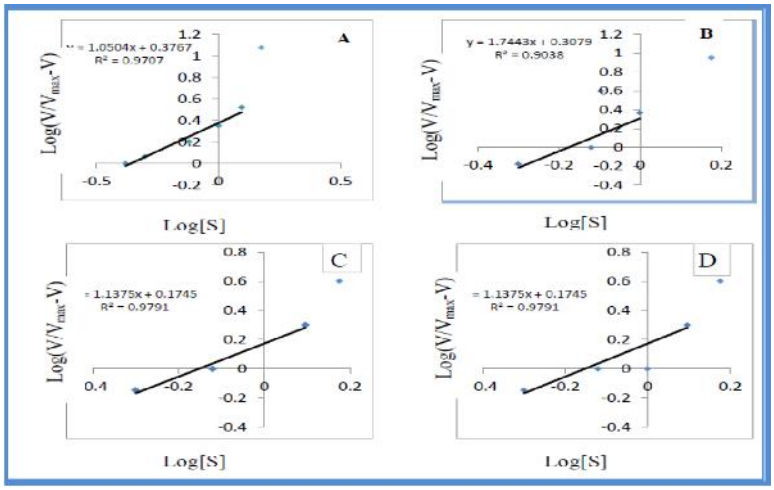

FIGURE 15: The Hill coefficient for PON enzyme in the four studied groups at 37ºC. A-Crude sample of NP B-Crude sample of EP C-Partially purified sample of NP D- Partially purified sample of EP

REFERENCES

[1]. Saladin, Kenneth S: "Anatomy and Physiology: the Unity of Form and Function", 6th Ed., New York, 2012; McGraw-Hill.

[2]. Stuart, I.F., "Human Physiology ", 10th Ed., McGraw Hill Medical, China, 2008; 20,707-708.

[3]. Beata, E., Seeber, M.D. & Kurt, T. Suspected Ectopic Pregnancy**.** *Obstet Gynecol* .2006, 107,399–413.

[4]. Emre, S. and Zehra S. Serum PON and Aryl esterase Activities throughout the Normal Pregnancy. *Nobel Med*. 2011, 7(1), 49-55.

[5]. Harun Toy, Hakan Camuzcuoglu, Hakim Celik, Ozcan Erel Nurten Akso.Assessment of Serum Paraoxonase and Aryl esterase Activities in Early Pregnancy Failure. *Swiss. Med. Weekly.* 2009, 39(5-6), 76-81.

[6]. Martinelli, N., Consoli, L., Girelli, D., Grison, E., Corrocher, R., Olivieri, O. Paraoxonases: ancient substrate hunters and their evolving role in ischemic heart disease. Adv Clin Chem. 2013, 59, 65–100.

[7]. Himbergen, T.M., van Tits, LJ., Roest, M., Stalenhoef, A.F. The story of PON1: how an organophosphate-hydrolyzing enzyme is becoming a player in cardiovascular medicine. *Neth. J. Med.* 2006, 64 (2), 34–8.

[8]. Lu, H., Zhu, J., Zang, Y., Ze, Y., Qin, J. Cloning, purification, and refolding of human paraoxonase-3 expressed in Escherichia coli and its characterization Protein. *Expr Purif.* 2006, 46(1), 92-9.

[9]. Ekinci, D., Beydemir, S. Evaluation of the impacts of antibiotic drugs on PON 1; a major bio scavenger against cardiovascular diseases. *Eur J Pharmacol*. 2009, 617(1-3), 84-9.

[10]. Huen, K., Harley, K., Bradman, A., Eskenazi, B., Holland, N. Longitudinal changes in PON1 enzymatic activities in Mexican-American mothers and children with different genotypes and haplotypes. *Toxicol. Appl. Pharmacol*.2010, 244 (2), 181–9.

[11]. Mueller RF, Hornung S, Furlong CE, Anderson J, Giblett ER, Motulsky AG. Plasma PON Polynorphism: A newenzyme assay, population, family, Biochemical and linkage studies. *Am. J. Hum. Gene*. 1983,35,393-408.

[12]. Dixon, M., Webb, E.:Enzymes, 2nd ed. New York. 1964,Academic press.Unc.

[13]. Ammar, M.S. Studies on the rmophilic bacteria Ph.D,Thesis, Botany department, Facutty of Science, Al-Azhar university,Cairo, Egypt, 1975.

[14]. Lowry, O., Rose bergh, N., Farr, L. and Ronall, J. *J.Biol.Chem*.1951,193-265.

[15]. Aziz, R.S. An MSc. Thesis , College of Medcine , University of Salahaddin, Erbil,2003.

[16]. Fields, Z.W.R., Roeske, E. Morkin and H.I. Yamamura. 1978. Cardiac muscarinic cholinergic receptors: biochemical Identification and characterization. J. Biol. Chem. 253:3251-3258..

[17]. Segal, I.H. Biochemical calculations.2nd ed.,John Wiley of Sons , Inc. Printed in USA, 1976.

[18]. Mohammed A., Abdullah S., Ali A., Abdulaziz A. Thermodynamic analysis of human retinal acetyl cholinesterase inhibittion using an anti-Alzheimer`s drug. Tacrin,through the dvelopment of a dual substrate and temperature model . proc. NAtl. Sci. Conc. ROC (B),2000, 24(3),108-115.

[19]. Amersham Biosciences: Protein electrophoresis technical Manual. Hand book, U.S.A., 1999.

[20]. Mackness, M., Arrol S., Mackness B., During ton P. The alloenzymes of PON determine the effectiveness of HDL in protecting LDL against lipid peroxidation. *Lancet.* 1997, 349, 851-852.

[21]. Gan, K., Smolen, A., Eckerson, H. Purification of human serum PON. *Drug. Metab. Dispar*. 1991, 19, 100-106.

[22]. Aviram, M. Plasma lipoprotein seperation by discontinous denity gradiant ultra centerinfugation in hyper lipoproteinemia patients. *Biochem.Med*. 1983,30,111-118.

[23]. Sukru B., Deniz E., Orhan A. :In vitro effects of dexamethasone on human serum PON_1 activity. *Hacette. J. Biol. Chem*. 2009, 37(3),197-205.

[24]. Demir, N., Nadaroglu, H., Demir, Y. :Purification of human serum PON and effect of acetylsalicylic acid on PON activity. form.*Inter.J. Pharma*. 2008, 46 (6), 393-399.

[25]. Dragomir, I., John, F., Audrey, S., Yoichi, O., Roger, S. and Bert, N. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *Journal of Lipid Research*. 2005 (46),1239-1247.

[26]. Gan, K., Smolen, A., Eckerson, H. Purification of human serum PON. *Drug. Metab Dispar*. 1991, 19, 100-106.

[27]. Furlong, C.E., Costa, L.G., Hassett, C., Richter, R.J., Sundstrom, J.A., Adler, D.A., Disteche, C.M., Omiecinski , C.J., Chapline, C., Crabb, J.W. Human and rabbit PON. Purification, cloning, sequencing, mapping and role of poly morphism in organophosphate detoxification, *Chem. Biol. Interact*. 1993,87,35.

[28]. Poh, R., Muniandy, S. Evaluation of polymorphin at codon 192 of PON1 on its kinetic behavior. *Scien*. 2009, 9, 5 41-548.

[29]. Stenzel, J., Worek F., Eyer P. Preparation and characterization of di alkyl phosphorylobidoxime conjugates, potent anticholinesterase derivatives that are quickly hydrolyzed by human PON. *Biochem. Pharamacol*. 2007, 74, 1390-400.

[30]. Michael Aviram, Scott Billecke, Robert Sorenson, Charles Bisgaier, Roger Newton, Mira Rosenblat, John Erogul, Cary Hsu, Cristina Dunlop, Bert La DuPON active site required for protection against LDL oxidation involves its free sulfhydryl group. *Arter. Thromb. Vase. Bil*. 1998,18,1617-1624.

[31]. Lourdes Rod, Fernando Gil, Antonio F. Hernandez Jerez, Anabel Marina, Jesus Vazquez and Antonio PLA. Purification and characterization of paraoxon hydrolase from rat liver. *Biochem. J.* 1997,321,595-601.

[32]. Tamara C. Otto, Christina K. Harsch, David T. Yeung, Thomas J. Magliery, Douglas M. Cerasoli, and David E. Lenz. Dramatic differences in organ ophophorus hydrolase activity between human & chimeric recombinant mammalian PON .*Biochem. J*. 2009, 48(3),10416-22.

[33]. HuX., JiangX., Lenz, D., Cerasol, D., Wallqvist, A. Insilic analyses of substrate interactions with human serum PON1. *Proteins*. 2009,75,486-498.

[34]. Maria, C., Fernando, G., Antonio, F., Lourdes, R. Human liver PON1 subcel- lular distribution and characterization. *J. Biochem. Mole. Toxi*. 1998, 61-69.

[35]. Segal, I.H.: Enzyme Kinetics.Behavious of analysis of lipid equilibrium and steady state enzyme systems, Jhon Wiley and Sons. New York, 1965.

[36]. Bowden, Warton, C.: Enzyme kinetics .IRL Press, Oxford, 1988.

[37]. Wilkinson, G.N. Statical estimations in enzyme kinetic. *Biochem.J*. 1961, 80,324-332.

[38]. Gil, F., Gonzalvo, M.C., Hernández, A.F., Villanueva, E., Pla, A. Differences in the kinetic properties, effect of calcium and sensitivity to inhibitors of paraoxon hydrolase activity in rat plasma and microsomal fraction from rat liver. *Biochem. Pharmacol*.1994, 48, 1559-1568.

[39]. McCracken, N., Blain, P., Williams, F. Human xenobiotic metabolizing estera -ses in liver and blood Biochem. *Pharmacol*. 1993,46,1125-1129.

[40]. Hernandez,A., Pla A., Valenzuela A.Characterization of Paraoxonase activity in pericardial fluid .*Chem. Biol. Interact*. 1993,87,173-177.

[41]. Krisch, V.K. Enzymatic hydrolase on di ethyl P-nitro phenyl phosphate durchmenschliches serum. *Z.Klin. Chem. Klin. Biochem*. 1968,1,6-10.

[42]. Mueller. R.F., Hornung, S., Furlong, C.E., Anderson, J., Giblett, E.R., Motulsky, A.G. Plasma paraoxonase polymorphism: a new enzyme assay *Am. J. Hum. Genet*.1983, 35, 393-408.

[43]. Alici, H., Ekinci, Beydemir S. Intravenous anesthetics inhibit human PON1 activity in vitro and in vivo. *Clin.Biochem.* 2008, 41(17),1384.

[44]. Olivieri, F., Cardelli, M., James, R.W., Boemi, M., Franceschi CMarchegiani F Marra M. PON: genetic and activities dur -ing aging. *Rejven. Res*. 2008, 11,113-27.

[45]. Rochua, D., Chabriere, E., Massona, P. Human PON: A promising approach for pretreatment and therapy of organophosphorus poisoning. *Toxi.* 2007, 233,47.

[46]. Karen, H., Rebecca, R., Clement, F., Brenda, E., Nina, H. Validation of PON1 enzyme activity assay for longitudinal studies .*Clinica.Chimica*. Acta. 2009, 402,67-74.

[47]. Cornish –Bowden,Warton C. Enzyme kinetics .IRL Press, Oxford ,1988.