

## *In Vitro* Effects of Dexamethasone on Human Serum Paraoxonase-I (PON1) Activity

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### Abstract

Dexamethasone has successfully been used for treatment of some inflammatory diseases. Here, we evaluated the *in vitro* effects of dexamethasone on the activity of human serum paraoxonase (hPON1). PON1 enzyme was purified using simple chromatographic methods: DEAE-Sephadex anion exchanger and Sephadex G 200 gel filtration chromatography from human serum. hPON1 was purified approx. 225-fold with a final specific activity of 4867.3 U x mg<sup>-1</sup> proteins and with a purity up to 39.8%. Dexamethasone dose-dependently decreased *in vitro* hPON1 activity. IC<sub>50</sub> value for dexamethasone was determined as 1.106. Inhibition constant (K<sub>i</sub>) was estimated as 3.284 ± 0.394 mM from Lineweaver-Burk graph. Inhibition type of dexamethasone was determined as noncompetitive. Our results show that dexamethasone inhibits hPON1 activity under *in vitro* conditions.

### INTRODUCTION

Enzymes catalyze almost all chemical reactions in the metabolism of the living organisms. Many chemicals influence the metabolism at low concentrations by decreasing or increasing normal enzyme activity. The results can be vital for specific enzymes [1,2]. Paraoxonase, the calcium-dependent enzyme, (arylesterase, EC 3.1.8.1, hPON1) has an important role in living metabolism. It is an organophosphate (OP) hydrolyser. It also hydrolyses aromatic carboxyl esters such as phenyl

acetate, and it is involved in drug and xenobiotic metabolism. Moreover, it hydrolyses various lactones, including naturally occurring lactone metabolites [3-6]. "PON" name derives from one of its most commonly used *in vitro* substrates, paraoxon. hPON1 also acts as an antioxidant enzyme that is an *in vivo* bioscavenger [7]. So, it protects low-density lipoproteins (LDL) from oxidative modification in metabolism [8-10]. PON1 activity reduces in cardiovascular diseases, diabetes mellitus, chronic renal failure, rheumatoid arthritis, hyperthyroidism and age-related macular degeneration [11-15]. Smoking, oxidative stress, atherosclerosis, genetic factors and fibrinogen have been implicated in the age-related macular degeneration (AMD) [13]. During aging, amount of reactive oxygen species (ROS) have been

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increased in living metabolism. Due to the damaging of the balance of ROS and ROS clearance in aging, it can cause oxidative damage of biomacromolecules and especially membrane phospholipids [13,16,17]. For instance, Baskol and his colleagues studied PON1 activity together with MDA to evaluate the role of oxidative stress in patients with AMD. They found that PON1 activity was lower in the patient group than the control group. Especially, the enzyme is important in atherosclerosis. PON1 is thought to play its anti-atherogenic role by inhibiting the oxidation of LDL, and hydrolyzing lipid peroxides [18]. Indeed, PON1 activity is a predictor of vascular diseases [19]. Because of these, the importance of the paraoxonase activity is well-known in serum. Due to these physiological roles of PON, scientists have performed many studies about activity of this enzyme, up to now. However, there are few studies concerning the effects of medical drugs on PON1 activity. For example, *in vitro* and *in vivo* effects of some diuretic and hypocholesterolemic drugs such as spironolactone, mevastatin, lovastatin, simvastatin, pravastatin and prulifloxacin on paraoxonase activity were investigated [20-22]. Besides, studies of interactions between drugs and different enzymes have been carried out by our laboratory [1,14,23].

Dexamethasone, synthetic glucocorticoid class of steroid hormones, is a powerful drug and, used in the treatment of many inflammatory diseases and certain types of cancer [24,25]. In addition, corticosteroids have been used to control inflammatory and immunological eye diseases [1]. However, we did not encounter any information about effects of this drug on *in vitro* hPON1 activities.

Consequently, we purified PON1 enzyme from human serum with a high yield using the simple three-step procedure that consists of ammonium sulfate precipitation, DEAE-Sephadex anion

exchange chromatography and Sephadex G 200 gel filtration chromatography. In this study, we examined the effects of dexamethasone on hPON1 activity.

## MATERIALS AND METHODS

### Materials

Materials including DEAE-Sephadex A50, Sephadex G-200, Paraoxon, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co (Taufkirchen, Germany). All other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck (Darmstadt, Germany). Dexamethasone was provided from the University Hospital Pharmacy (Atatürk University, Erzurum, Turkey).

### Paraoxonase activity assay

Paraoxonase activity of the enzyme was determined at 25°C with paraoxon (diethyl p-nitrophenyl phosphate) (1 mM) in 50 mM glycine/NaOH, pH 10.5 buffer containing 1 mM CaCl<sub>2</sub>. Enzyme assay was based on the estimation of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol ( $\epsilon = 18,290 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 10.5) was used for calculation of the enzyme activity [26]. One enzyme unit is defined as the amount of enzyme that catalyzes hydrolysis of 1  $\mu\text{mol}$  of substrate at 25°C. Assays were performed using a spectrophotometer (CHEBIOS UV-VIS, Rome, Italy).

### Ammonium sulfate precipitation

26 ml of Triton X-100 treated human serum was applied ammonium sulphate precipitation. The precipitation intervals for paraoxonase enzyme were 60–80% [27]. The precipitate was collected by centrifugation at 15 000 rpm for 20 min, redissolved in 100 mM Na-phosphate buffer pH 7.0.

## **DEAE-Sephadex A50 Anion Exchange**

### **Chromatography**

Enzyme solution, which had been dialyzed in the presence of 1 mM Na-phosphate buffer pH 7.0 at 4°C, was loaded onto the DEAE-Sephadex A50 anion exchanger column (3 cm<sup>2</sup> x 30 cm) that had been equilibrated with 100 mM Na-phosphate buffer (pH=7.0). The column was washed with 100 mM Na-phosphate buffer (pH=7.0), and then elution was performed with a linear gradient of 0-1.5 M NaCl. Eluted fractions were collected as three milliliters and enzyme activity was checked at 412 nm. Tubes having enzyme activity were combined. All purification procedure was carried out at 4°C

### **Sephadex G 200 gel filtration chromatography**

Fractions from DEAE-Sephadex column were mixed with glycerol and loaded onto the Sephadex G-200 column (60 cm x 2 cm) that had been equilibrated with 100 mM Na-phosphate buffer (pH=7.0). Elution was performed with the same buffer [28]. Fractions were checked in terms of both protein amount (280 nm) and enzyme activity (412 nm). Tubes having enzyme activity were combined for other kinetic studies.

### **Protein Determination**

During the purification steps, protein quantity was determined spectrophotometrically at 595 nm according to the Bradford method using bovine serum albumin as the standard [29].

### **SDS polyacrylamide gel electrophoresis**

SDS polyacrylamide gel electrophoresis was performed after purification of the enzyme. It was carried out in 10% and 3% acrylamide concentrations for the running and the stacking gels, respectively, containing 0.1% SDS according to Laemmli [30]. 20 µg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with

several changes of the same solvent without the dye. The electrophoretic pattern was photographed.

### ***In vitro* studies for the drugs**

We examined the *in vitro* inhibitory effects of dexamethasone. The drug was tested in triplicate at each concentration used. hPON1 enzyme activities were measured in the presence of different inhibitor concentrations. Concentrations are 0.324, 0.647, 1.00, 1.5 mM for dexamethasone. Control cuvette activity was acknowledged as 100% in the absence of inhibitor. For dexamethasone, an Activity %-[Inhibitor] graph was drawn. For determination of value, three different inhibitor concentrations were tested for each substance. In these experiments, paraoxone was used as substrate at five different concentrations (0.150, 0.300, 0.450, 0.600 and 0.750 mM). Obtained Lineweaver–Burk curve was used for determination of  $K_m$  and the inhibitor type [31].

## **RESULTS AND DISCUSSION**

HDL-associated enzyme (PON1) has no known natural substrates [32]. It can use several non-physiological substrates, including aryl esters, organophosphates (OPs) and lactones [33,34]. Serum PON1 protects LDL and HDL from oxidation by various free radical generators. hPON1 has free thiol groups in its three dimensional structure which contribute to its antioxidant activity. Interestingly, a free sulfhydryl at cysteine 284 is used by PON to prevent LDL oxidation and is the active region for its antioxidant activity [35]. Sorenson *et al.* observed that a PON1 (wild-type) of the cysteine sulfhydryl group (Cys283) was inactivated by para-hydroxymercuribenzoate (PCMB) but a PON1 mutant of C283S (Cys283 was replaced with serine) and C283A (Cys283 was replaced with alanine) were not inactivated by PCMB [36]. They saw that C283S and C283A mutant enzymes retained both

paraoxonase and arylesterase activities, and the  $K_m$  values for paraoxon and phenyl acetate were similar to those of the wild type. From these results, it is seen that Cys283 residue is free in active PON. For these physiological roles of the calcium-dependent enzyme, scientists have performed many studies about activity of this enzyme, until now. Especially, the importance of the enzyme with the protection against atherosclerotic lesions has been well known. The antioxidant role of HDL is mostly results from PON1 [12,15] and genetic experiments proved its potential of protection against atherogenesis [37]. However, activity of this enzyme has recently gained importance in eye metabolism. Lipids in the outer segments of photoreceptors may be damaged by the reactive oxygen species. It may also cause the progressive deterioration of the retinal pigment epithelial cells. Indeed, the macula has a high polyunsaturated fatty acid content, high  $O_2$  consumption, and is exposed to visible light. So, it is highly susceptible to oxidative stress. In this respect, it is clear that PON activity is vital in eye metabolism.

It is known that many drugs and chemicals influence enzyme activities at low concentrations by decreasing or increasing, especially by inhibiting specific enzymes [38] with critical function. Sometimes, these enzymes can be key role in a pathway or catalysis of a crucial reaction. They are important drug target enzymes. However, we encountered a few inhibition studies on PON activity. For instance, Tomás et al. investigated the effect of lipid-lowering drug simvastatin therapy on serum PON activity with familial hypercholesterolemia (FH). They found that serum PON1 activity toward paraoxon significantly increased during treatment with simvastatin. So, they expressed simvastatin might have important antioxidant properties [22]. Besides, Sinan and colleagues were showed that gentamycin sulfate and cefazolin sodium dose- and time-dependently inhibited human serum PON1, with  $C_{50}$  values of 0.887 and 0.0084 mM,

respectively, but did not affect liver PON1 activity in human hepatoma HepG2 cells [27]. In addition, the effects of several metal ions such as Co (II), Cu (II), Mn (II), Hg (II), p-hydroxymercury benzoate (pOHMB) on PON1 and PON3 activities from rat liver were investigated for *in vitro* conditions [32]. In the study, Pla and colleagues determined inhibition effects of all compounds on PON1 and PON3 activities. Due to the these results, they think that active sites of PON1 and PON3 may be residues of lysine, histidine, phenylalanine, cysteine, tryptophan, aspartic acid, glutamic acid and asparagines since the residues have the ability for binding to metals. As regards to the  $K_m$  values, the range of potent inhibitors for PON1 were  $Hg^{2+} > pOHMB > Co^{2+} > Mn^{2+} > Cu^{2+}$  and for PON3 were  $Hg^{2+} > Cu^{2+} > pOHMB > Mn^{2+} > Co^{2+}$ . They saw from this data that the inhibitory potency is different for both enzymes. So, they expressed that more extensive inhibition studies were necessary for a better understanding of the protective role of PONs against the toxic effects of xenobiotics, including environmental heavy metals and oxidative stress by-products. Considering the statements above, we think that our present study contributes to understanding of the protective role of PONs.

We purified PON1 enzyme from human serum using only three stages, ammonium sulfate fractionation (60-80%), DEAE-Sephadex anion exchange and Sephadex G 200 gel filtration chromatography. After overall purification, hPON1 enzyme was obtained with a recovery of 39.8 % and a specific activity of 4867.3 U x  $mg^{-1}$  proteins. This enzyme was also purified approx. 225-fold (Table 1 and Figure 1). Up to now, PON enzyme has been purified from different sources with different yields and purification folds. For instance, in a study, human serum PON enzyme was purified approx. 62.1-fold using Agarose blue, Sephadex G 200, DEAE-Trisacryl M, Sephadex G 75 chromatography techniques [39]. Pla and his colleagues purified

Table 1. Summary of the PON1 purification procedure.

Purification step	Activity (U/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Triton X-100-treated serum	108	26	5	130	2808	21.6	100	1
Ammonium sulphate precipitation (60-80%)	113.2	19	4.1	77.9	2150.8	27.6	76.5	1.27
Ion exchange chromatography	195.1	7	0.18	1.26	1365.7	1083.8	48.6	50.17
Gel filtration chromatography	223.9	5	0.046	0.23	1119.5	4867.3	~39.8	~225

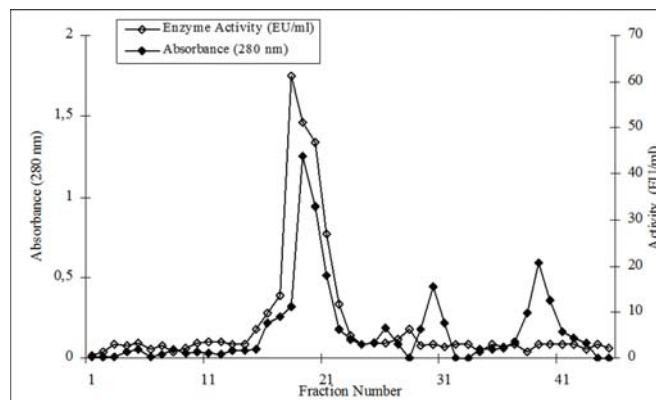


Figure 1. Purification of human serum PON1 by Sephadex G-200 gel filtration chromatography. Qualitative protein concentration was determined by measuring an absorbance at 280 nm and hPON1 activities of fractions were assayed using a paraoxon substrate.

PON3 from rat liver with a final specific activity of  $461 \mu\text{mol min}^{-1} \text{mg}^{-1}$  and a yield of 0.4% [32]. They obtained an overall purification factor of 177-fold using six steps, including hydroxyapatite adsorption, DEAE-Sepharose CL-6B chromatography, Cibacron Blue 3GA non-specific affinity chromatography, anion exchange on Mono Q HR 5/5, DEAE-cellulose, and a final affinity chromatography on Concovalin A-Sepharose. Thus, compared with other mentioned studies, our purification procedure both takes less time and has higher specific activity, yield, and purification. The final purified hPON1 had only one protein band on SDS-PAGE (Figure 2). We also examined the *in vitro* effects of dexamethasone on human serum PON1 activity. Both the  $IC_{50}$  and  $K_i$  parameters were determined (Figure 3 and 4).  $IC_{50}$  value was 1.106 mM for dexamethasone (Table 2 and Figure 3). Lineweaver-Burk graph shows that dexamethasone inhibits the enzyme in a noncompetitive manner and inhibition constant was estimated as  $3.284 \pm 0.394 \text{ mM}$  (Table 2 and Figure 4). In addition, Alici and his colleagues reported the

Table 2.  $K_i$  values and inhibition type.

Drug	$IC_{50}$ (mM)	$K_i$ (mM)	Average $K_i$ (mM)	Inhibition Type
Dexamethasone	1.106	4.063	$3.284 \pm 0.394$	noncompetitive
		2.780		
		3.011		



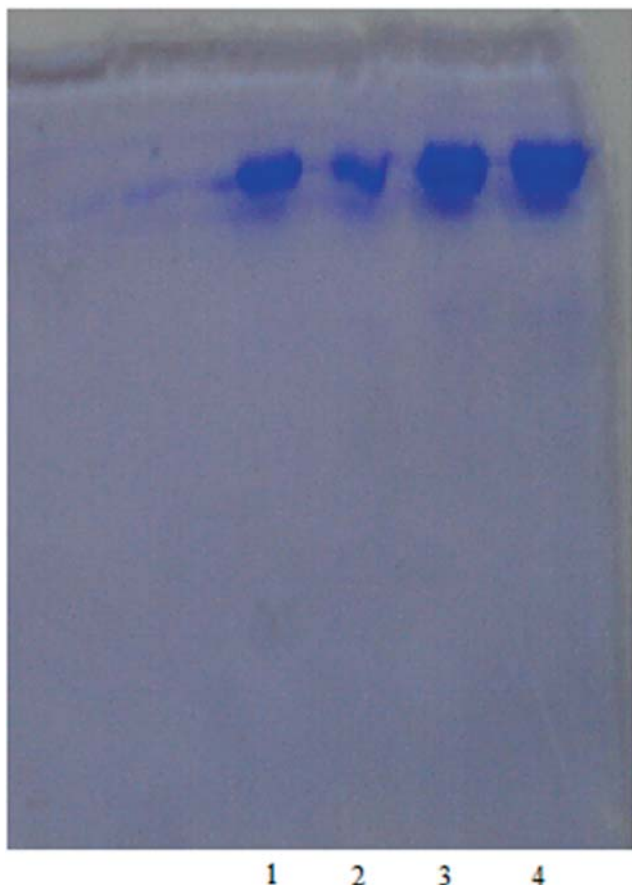


Figure 2. SDS-PAGE analysis of purified PON1. Lane 1, 2, 3 and 4 contain a human serum sample.

some intravenous anesthetics such as propofol, etomidate and ketamine inhibited *in vitro* hPON1 activity. They found to  $K_i$  values for propofol, etomidate and ketamine  $0.322 \pm 0.111$ ,  $0.059 \pm 0.014$  and  $6.480 \pm 0.963$  mM, respectively [4]. Besides, Sinan et al. was determined  $K_i$  values for gentamycin sulfate and cefazolin sodium as  $0.026 \pm 0.015$  and  $0.012 \pm 0.00065$ , respectively [27]. When these studies were compared to our study, it is seen that propofol, etomidate, gentamycin sulfate and cefazolin sodium have more inhibition effects than dexamethasone. However, value of ketamine is more than dexamethasone. So, dexamethasone is potent inhibitor for hPON1 enzyme.

In conclusion, PON1 enzyme was purified from human serum using the simple three-step procedure that consists of ammonium sulfate precipitation, DEAE-Sephadex anion exchange chromatography and Sephadex G 200 gel filtration chromatography with a high yield and in a short time. Besides, the *in vitro* effects of dexamethasone on the activity of

hPON1 were investigated and the results showed that dexamethasone is potent inhibitor. So, the drug must be used carefully and the dosage closely monitored to decrease side effects.

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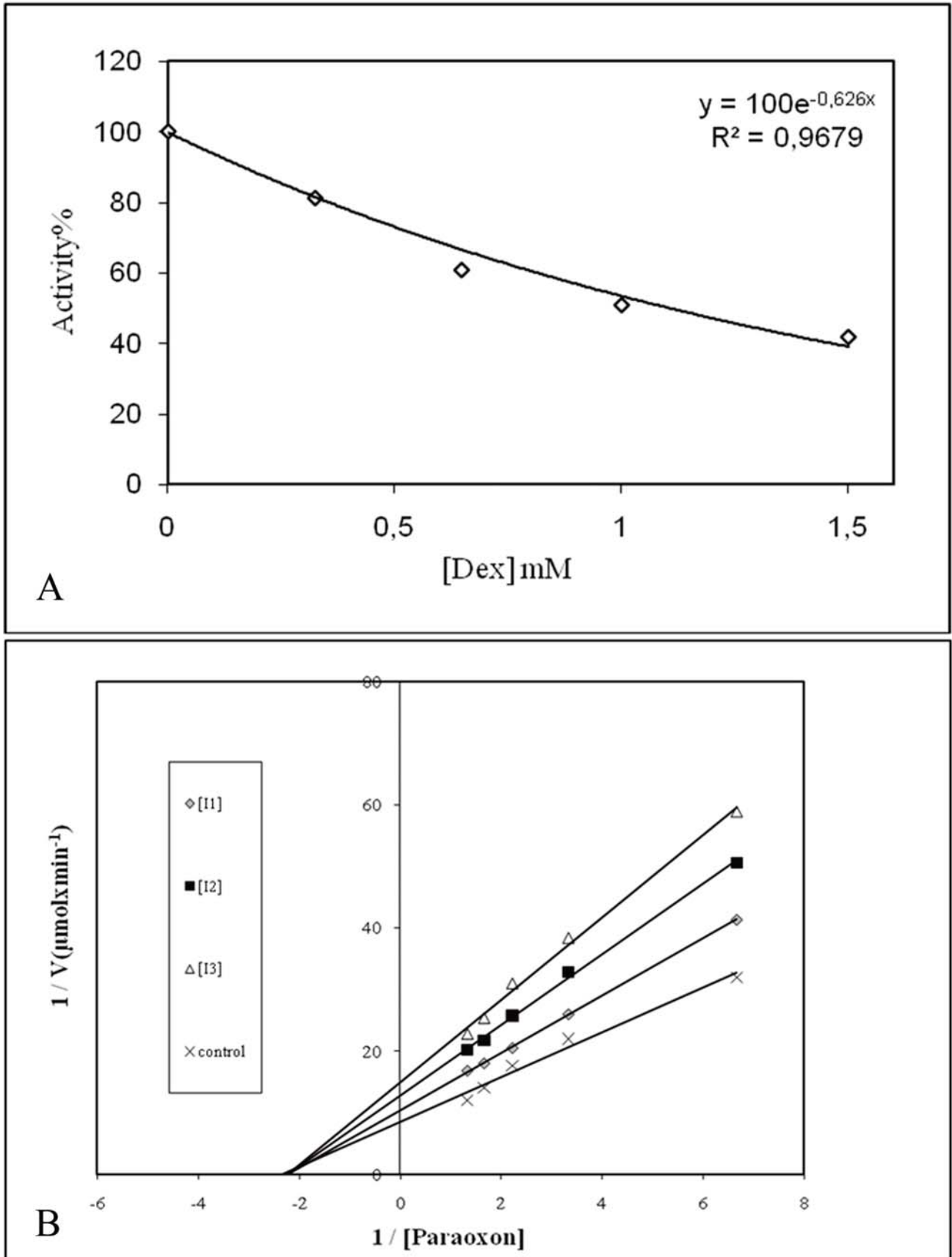


Figure 3. (A) *In vitro* effect of dexamethasone and (B) Lineweaver-Burk graphs at 5 different substrate (paraoxon) concentrations and at 3 different dexamethasone.

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