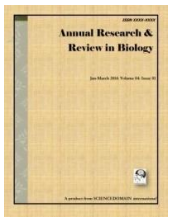

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Purification of Paraoxonase (PON) from Sun Flower (*Helianthus annuus*) and Effects of Some Chemicals on Paraoxonase Activity *In vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Author ND designed the study. Authors HN and YD managed the analyses of the study. Author HN performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In this study, paraoxonase (PON) enzyme was purified from mature seeds of sun flower by using affinity chromatography (Sepharose-4B-L-tyrosine-1-naphthylamine) and the effects of some chemicals were tested on paraoxonase activity as *in vitro.*
Methodology: Paraoxonase was firstly purified from sun flower (*Helianthus annuus*). This enzyme was purified as 427-fold. SDS-polyacrylamide electrophoresis of the enzyme indicates a single protein staining band with an apparent Mr of 35 kDa. The kinetic properties of the purified enzyme were determined.
Results: The enzyme exhibits high activity at broad pH (pH 5.0-9.0) and temperature (40 and

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70ºC). The purified enzyme remains stable at 4ºC for more than 1 year. Paraoxonase is mostly stable at 40ºC. The activity of the enzyme decreases to 55% at a temperature of 60ºC when the treatment was given for a period of 1h. Optimum pH of the purified enzyme was 7.0 and its optimum temperature was 40ºC. Using paraoxon as a substrate, the enzyme shows maximum activity (Vmax) of 7.84μmol.L.min^{-1} with its corresponding Km value of 0.317 mM. The activities was strongly inhibited by Hg2+, Fe3+, β-mercaptoethanol, dithioerythritol, SDS and EDTA while Cu^2+ slightly activates the enzyme activity. As judged by catalytic efficiencies, paraoxon is the preferred substrate.

Conclusion: The present study shows that PON purified from sun flower (*Helianthus annuus*) is stable at wide range of pH and temperature and in the presence of some metal ions.

Keywords: Paraoxonase; purification; sun flower (Helianthus annuus); metal ions.

1. INTRODUCTION

The existence of organophosphatase in mammalian plasma was first reported nearly 50 years ago [1]. Subsequent studies have shown this enzyme to be (paraoxonase/ arylesterase; PON; aryldialkylphosphatase, EC 3.1.8.1), an organo-phospahatase with broad substrate specificity, including aromatic carboxylic acid esters such as phenyl acetate [2].

Initial investigation of PON focused on its ability to hydrolyze organophosphorus (OP) compounds, playing a major role in the detoxification of these compounds and other artificial substrates, so that it may alter significantly an individual's susceptibility to the toxicity of these chemicals [3].

PON might protect against coronary and artery disease by hydrolyzing oxidized phospholipids in LDL and HDL particles as well as against homocysteinylation [4-6]. The mechanism by which PON exerts its action seems to differ in these cases, as the active center for the hydrolysis of oxidized lipids [7].

Paraoxonase enzyme was purified from serum and many tissues such as human, cow, rabbit, rat liver, kidney, small intestine [8-10]. However, there is a little study related to the presence of PON enzyme in plant [11]. It was considered that the PON enzyme which was in seeds of sun flower was directly linked with lipid metabolism. So, PON enzyme was purified by using affinity chromatography (Sepharose-4B-L-tyrosine-1-naphthylamine) from mature seeds of sun flower. It was also investigated if some chemicals have effects on PON enzyme activity *in vitro* and the structure of the PON enzyme in sun flower plant was examined.

2. MATERIALS AND METHODS

2.1 Preparation of Affinity Gel

After 20 mL of Sepharose 4B and 20 mL of water were combined, 4 g CNBr was added to this suspension. The mixture was kept in an ice bath and stirred magnetically. The pH of the mixture was adjusted to 11 and was maintained for 8-10 min. The reaction was stopped by filtering the gel on a Buncher funnel and washing with cold 0.1 M $NaHCO_3$ buffer (pH: 10). L-tyrosine by using a saturated L-tyrosine solution in the same buffer was coupled to Sepharose-L-tyrosine activated with CNBr. The reaction was completed by stirring with a magnet for 90 min. In order to remove excess L-tyrosine from the Sepharose 4B-L-tyrosine gel, the mixture was drained thoroughly. The affinity gel obtained by diazotization of 1-naphthylamine was coupled to the Sepharose 4B-L-tyrosine. For this purpose, 1-naphthylamine (25 mg) was suspended in 10 mL of ice cold 1 M HCl, and to the suspension was added 75 mg of sodium nitrate in 5 mL ice-cold water. After 10 min. of reaction, the diazotized 1-naphthylamine was poured into 40 mL of the Sepharose-L-tyrosine suspension. The pH was adjusted to 9.5 with 1 M NaOH and, after gentle stirring for 3 h at room temperature, the coupled dark red Sepharose derivative was washed with 1 L of water and then 200 mL of 0.05 M Tris-sulfate, pH 7.5 [12].

2.2 Collection and Treatment of the Plant Material

All purification procedures were carried out at room temperature unless otherwise stated.

Mature sun flower *(Helianthus annuus)* from Erzurum in the full maturity stage were collected. The sun flower *(Helianthus annuus)* was stored at -20^{o}C. Before use, the sun flower was thawed and assayed for total protein and PON and ARE

activities. Thawed sun flower were gently mixed with 1 M $CaCl_2$ and 10 M NaCl to give a final concentration of 10 mM $CaCl_2$ and 3 M NaCl and left for 30 min at room temperature. Any clot was removed by centrifugation (5000 x g, 20 min) at 4ºC.

The homogenate was applied to the affinity column having a structure of Sepharose 4B-L-tyrosine-1-naphthylamine and equilibrated with 25mM Tris/HCl (pH: 8.0)/10mM $CaCl_2$. The affinity gel was washed with the solution 25mM Tris/HCl (pH: 8.0)/10 mM $CaCl_2$/3 M NaCl. Sun flower paraoxonase was eluted with the solution 25mM Tris/HCl (pH: 8.0)/10 mM $CaCl_2$ at 0.5mL/min. Fractions (3mL each) were collected and those with the highest PON activity were pooled [13].

2.3 Paraoxonase Activity Measurements

Paraoxonase activity was determined at 25ºC with paraoxon (diethyl p-nitrophenyl phosphate) (1mM) in 50 mM Tris/HCl (pH 8.0) containing 1 mM $CaCl_2$. The enzyme assay was based on the estimation of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol (ε=18.290 M^{-1} cm^{-1} at pH 10.5) was used to calculate enzyme activity [14]. One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of substrate at 25ºC. Assays were performed using a spectrophotometer (PG Instruments T80 Plus UV/VIS Spectrophotometer) [15-17].

2.4 Arylesterase Activity Measurements

The arylesterase enzyme activity was also measured an arylesterase assay [13]. Arylesterase activity of PON was determined at 25ºC with phenylacetate (1 mM) as the substrate in 50 mM Tris/HCl buffer pH 8.0, containing 1 mM $CaCl_2$. The rate of hydrolysis was measured spectrophotometrically at 270 nm. The molar extinction coefficient of phenol (ε=1310 M^{-1} cm^{-1}) was used for calculation of activity. One unit of arylesterase activity is equal to 1 µmol of phenylacetate hydrolysed/min.

2.5 Inactivation of PON Arylesterase Activity

2.5.1 Removal of calcium ions by a chelex 100 column

One gram of Chelex 100 (200 mesh) was washed once with double-distilled water and packed into a 3.0-mL column. The packed column was equilibrated with 50 mmol/L Tris/HCl buffer, pH 8.0. Subsequently, 1.0 mL of purified PON was passed through the column at a rate of 0.3mL/min. Sequential fractions were collected and assayed for arylesterase activity.

2.5.2 Inhibition with EDTA

The purified PON enzyme from sun flower *(Helianthus annuus)* containing 1.0 mmol/L Ca^{2+} in Tris/HCl buffer, pH 8.0, was diluted with equal volume of 1 mmol/L EDTA. After 18 hours, arylesterase activity of purified PON enzyme was measured.

2.5.3 Heat Inactivation

PON was incubated in 0.01 M phosphate buffer (pH:8.0) at 60ºC for 15 minutes.

2.5.4 Blockage of free sulfhydryl groups

p-Hydroxymercuribenzoate (PHMB) (1 to 10 mmol/L) was incubated with PON for 1 hour at 37°C in 0.01 M phosphate buffer (pH:8.0). Excess sulfhydryl agent was removed before incubation, by dialysis, using a Centricon 100 microconcentrator (Amicon).

2.6 SDS Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Electrophoresis under denaturing conditions was performed at different polyacrylamide gel concentrations of 10% according to the discontinuous buffer system of Laemmli [15]. Electrophoresis was carried out in vertical slab gels and the runs were performed at a constant current intensity of 15 mA/plate in the stacking gels and 30 mA/plate in the running gels. The gel was stabilized in the solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. The staining was performed for about 2 h in a solution of 0.1% Coomassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, the washing was carried out in a solution of 50% methanol + 10% acetic acid + 40% distilled water until the protein bands were cleared [18].

2.7 Molecular Weight Determination with Gel Filtration

Sephacryl S-100 was filled into a column (3 x 70 cm). The column was balanced for 4 h with the

buffer (0.05 M Na_3PO_4, 1 mM ditihioerythreitol, pH: 7) until the absorbance at 280 nm was no longer obtained. The standard protein solutions (bovine serum albumin (66 kDa, pepsin 34.7 kDa, trypsinogen 24 kDa, β-lactoglobulin and lysozyme 14.3 kDa) were added to the column. The purified protease enzyme was added into the column and then eluted under the same conditions. The flow rate through the column was 20 mL/h. The results were compared with standard proteins [19].

2.8 Determination of Protein Amount

Protein content of the samples was quantified according to the method of Bradford (1976) using bovine serum albumin as standard [20].

2.9 *In vitro* Studies for Some Chemicals

Working solutions of the following compounds were prepared from stock solutions in 0.1 M Tris-HCl pH 8.1 free of calcium and added to samples to obtain the desired final concentration (1 mM) in the range showed for each compound, as indicated in brackets for PON respectively: $CaCl_2$, $MgCl_2$, $Hg(NO_3)_2$, $MnCl_2$, $ZnSO_4$, $Cu(NO_3)_2$, $FeCl_3$, ethylene diaminetetraacetic acid, (EDTA), dithioerythritol, β-mercaptoethanol, SDS.

2.10 Statistical Methods

The SAS TM for Windows TM 6.11 computer program was used to perform statistical analyses. Data were presented by descriptive analysis (case number, mean, standard deviation). The comparisons between groups were performed by Student's *t*-test and ANOVA. The $p < 0.05$ probability was accepted as the significance level.

3. RESULTS AND DISCUSSION

Essential fatty acids cannot be synthesized by the body and some essential vitamins (A, D, E, F) that can dissolve only in oil is the source. Sun flower is a great value as nutritious food and a source of essential vitamins and essential fatty acids. LDL is a major risk factor, but HDL is a useful component to arteries for cardiovascular diseases [21]. It was considered that there could be the paraoxonase enzyme which was also known as the antioxidant enzyme, in sun flower, and then this research was done. This study is important for the firstly identified and purified PON enzyme in a plant [22-24].

Paraoxonase (PON) enzyme from sun flower *(Helianthus annuus)* was purified by Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. Specific activities were calculated in both extract and purified enzyme solution for paraoxonase. Paraoxon and phenyl acetate were used as substrates in the determination of activity and the activity-absorbance graph was drawn (Fig. 1).

Furthermore, purification fold was determined for purified PON enzyme, and it was shown in Table 1. The specific activity of the enzyme was increased with purification step and the final purification fold was 427.1 (Table 1).

SDS polyacrylamide gel electrophoresis was performed after the purification of the PON enzyme and the electrophoretic pattern was photographed (Fig. 2). Then, the molecular weight of the enzyme was determined as 35 kDa by using the gel filtration chromatograph. These results show that the enzyme has one subunit at 35 kDa.

V_{max} and K_M values are calculated by using Lineweaver-Burk graphs. K_M and V_{max} values of purified PON are 3.76 mM and 131.5μmol/L.min., respectively

The properties of purified enzyme from sun flower *(Helianthus annuus)* are determined. For this aim, optimum pH, optimum temperature and stability of the enzyme has been investigated. In addition, the effects of some substances on enzyme activity are found out. Enzyme inhibition and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes.

The stability of PON is affected by both physical parameters (pH and temperature) and chemical parameters (inhibitors or activators). The enzymatic hydrolysis of paraoxon also depends on several physicochemical factors. It depends on contact time, enzyme concentration, temperature of incubation and pH.

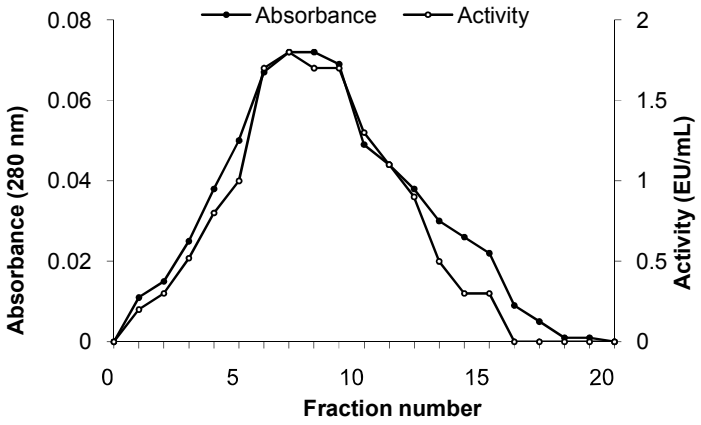

Fig. 1. The graphs of absorbance, paraoxonase activity of paraoxonase enzyme from sun flower *(Helianthus annuus)*

Table 1. The purification of paraoxonase from sun flower *(Helianthus annuus)*

Enzyme Fraction	Volume	activity	Total activity		Protein	Specific activity	Purification
	mL	EU/mL	EU	%	(mg/mL)	EU/mg protein	Fold
Homogenate	38	12.9	490.2	-	1.34	0.096	-
Sepharose-4B-L-tyrosine-1-naphtylamine	30	8.2	246	50.2	0.2	41	427.1

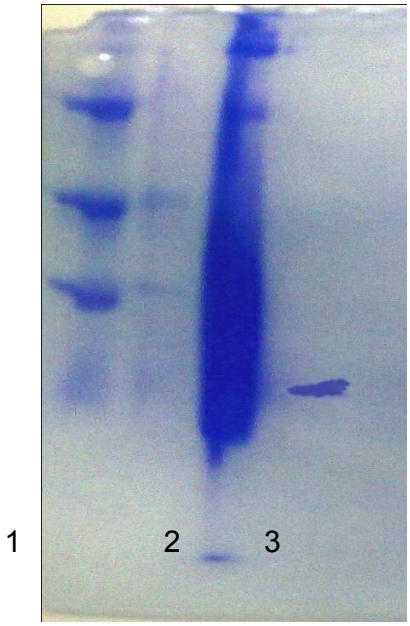

Fig. 2. SDS-PAGE bands of paraoxonase (Lane 1: Standard proteins (glyceraldehyde-3-phosphate dehydrogenase (36 kDa), chicken egg ovalbumine 845 kDa), bovine serum albumin (66 kDa), phosphorylase B (97 kDa), lane 2: Sun flower *(Helianthus annuus)* homogenate and lane 3: purified paraoxonase from sun flower

Similarly, optimal pH studies are investigated for PON with 1 pH unit increments between 4.0 and 11.0 (Fig. 3). The optimal reaction pH for PON was 8.0, and it was active between pH 5.0 and 10.0.

The effect of temperature was investigated between 0ºC and 90ºC with 10 degree increments (Fig. 4) and the optimum temperature was found to be 50ºC. Enzyme has activity between 20ºC and 80ºC. The thermo stability study of PON was carried out at temperatures ranging from 40ºC to 80ºC (Fig. 5).

The purified enzyme appears to be stable and retains its full activity after 1 h incubation from 40ºC to 50ºC, but the activity is reduced to 20% after 1 h at 60ºC. PON activity decreases dramatically when the temperature increases above 70ºC with only 13% and 8% activity remaining at 70ºC and 80ºC, respectively. Thermo stability of PON was at 40ºC as maximally, because it was remained nearly full active at 40ºC for at least 24h.

To assess the PON and arylesterase (ARE) activities of purified PON enzyme from sun flower *(Helianthus annuus)* related to the presence of Ca^{2+} ion, the following experiment was done. Experiment was carried out with balanced previously with 50 mmol Tris/HCl buffer column packed with (pH:8.0) Chelex 100 (200 mesh) and PON and ARE activities were measured before and after purified PON enzyme was passed through this column. The PON and ARE activities were lost at a rate of 84.2% and 78.8%, respectively.

Purified PON enzyme was incubated at 60 ^{o}C for 15, 30 and 60 minutes and then PON and arylesterase activities were determined. It was observed that PON and ARE activities were lost at a rate of 75.8%, 95.1 and 96.7%, respectively at 15, 30 and 60 minutes.

When the purified PON enzyme from sun flower *(Helianthus annuus)* was treated with EDTA for 18 hours at room temperature, it almost fully lost its arylesterase activity.

PON contains 3 cysteines; 2 of them form an intramolecular disulfide bond, while the third, at position 283, is free [13]. Cys283 was hypothesized to play a role in PON esterase activity, but earlier site-directed mutagenesis from this laboratory showed that substitution with either serine or alanine results in the retention of PON arylesterase activity. In the current study, reaction of the PON Cys283 with the sulfhydryl reagent PHMB was caused a dose-dependent reduction in PON arylesterase activity, by 28%, 32%, or 87% for PON using PHMB concentrations of 0.1, 1.0, or 10.0mmol/L, respectively.

PON activity was assayed in the presence of different reagents (Table 2). Among the salts tested, considerable loss of activity was observed only with Hg^{2+}, Fe^{3+}, whereas Ca^{2+} and Cu^{2+}, acting somewhat as an enhancer. Inhibition of enzyme activity in presence of Hg^{2+} and Fe^{3+} might be indicative of essential vicinal sulfhydryl groups of the enzyme for productive catalysis. The enzyme activity was retained 3.1% at 1.0 mM Hg^{2+}, 18.6% at 1 mM Mn^{2+}, 8.6% at 1 mM Fe^{3+}, 2.0% at 1 mM EDTA, 23.3% at 1 mM β-mercaptoethanol, 21.5% at 1 mM dithioerytritol. Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+} ions activated the purified PON enzyme activity at rates of 165.2%, 127.2%, 137.7%, 161, 8%, respectively (Table 2).

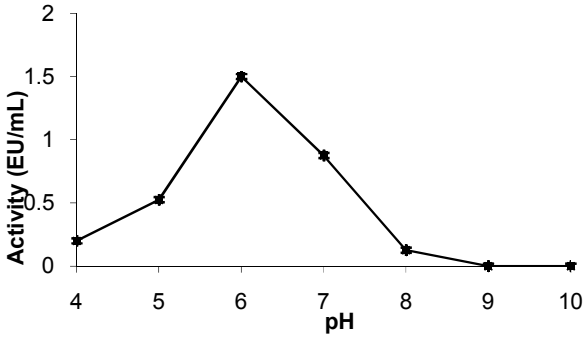

Fig. 3. The effect of pH on the activity of purified paraoxonase from sun flower *(Helianthus annuus)*

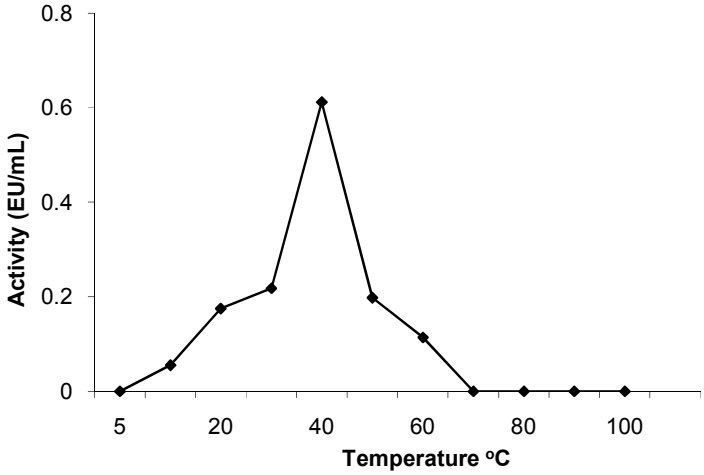

Fig. 4. The effect of temperature on the activity of purified paraoxonase from sun flower *(Helianthus annuus)*

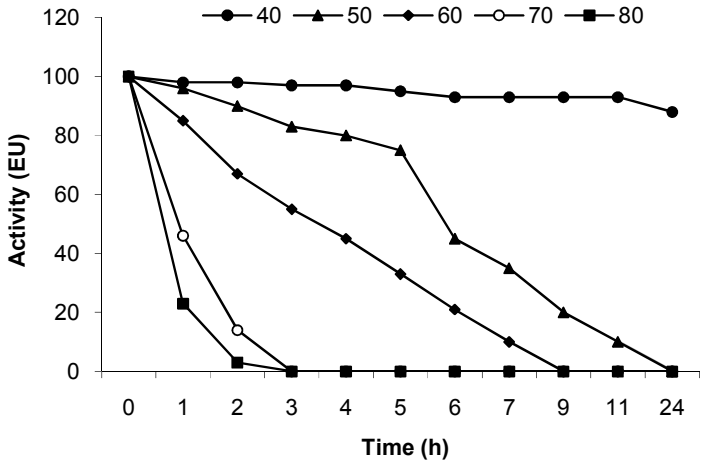

Fig. 5. The temperature stability of the purified PON from sun flower *(Helianthus annuus)*

Table 2. The effect of all kinds of compounds on the activity of purified paraoxonase from sun flower *(Helianthus annuus)*

Chemical compounds	Concentration (mM)	Relative activity (%)
Control	-	100
$CaCl_2$	1	165.2±3.2
$MgCl_2$	1	127.2±1.7
$Hg(NO_3)_2$	1	3.1±0.5
$MnCl_2$	1	18.6±4.4
$ZnSO_4$	1	137.7±0.4
$Cu(NO_3)_2$	1	161.8±0.1
$FeCl_3$	1	8.6±2.1
EDTA	1	2.0±0.2
Dithioerythritol	1	21.5±1.1
β-mercaptoethanol	1	23.3±2.3
SDS	1	12.2±3.3

4. CONCLUSION

In conclusion, it was observed high paraoxonase activity in the sun flower, paraoxonase has firstly been purified by Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography and some of its kinetic parameters have been investigated. Crude extracts from freeze-dried sun flower showed higher paraoxonase activity (12.9 EU/mL). Then, the paraoxonase enzyme was further characterised by SDS-PAGE and its molecular weight was estimated as 35 kDa. Results of this research is very valuable to both scientific and industrial products as well as on the quality of sun flower oil.

Plants which were taken with a diet and has the antioxidant effects could be suggested to exhibit PON enzyme activity. It was determined that the PON enzyme had positive effect on atherosclerosis and vascular occlusion and heart disease, Alzheimer's disease, blood pressure, etc in several studies. These plants could be used for the same aims. It was extremely important to identify, purify and characterize the PON enzyme from sun flower. Sun flower is widely used in the production of sun flower oil and it is useful for human health.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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