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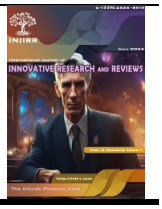

RESEARCH ARTICLE

Purification of Paraoxonase Enzyme from Pumpkin Seeds (*Cucurbitae peponis semen*) and Effect of Some Chemicals and Pesticides on Enzyme Activity

Elife KAYA[1], Nazan DEMİR[2*]

[1] *Kahramanmaraş Sütçü İmam University, Vocational School of Technical Sciences, Department of Food Processing, Kahramanmaraş, Turkiye*
[2] *Muğla Sıtkı Koçman University, Faculty of Science, Department of Chemistry, Muğla, Turkiye*

** Corresponding author E-mail: nazdemir@mu.edu.tr*

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ABSTRACT

An organophosphate-degrading enzyme known as paraoxonase is connected to high-density lipoprotein (HDL) and has a physiological role, such as primarily protecting low-density lipoprotein (LDL) from oxidation. The aim of this study is to purify the paraoxonase enzyme (EC: 3.1.8.1.) from pumpkin seeds and investigate the effect of some chemicals and pesticides on enzyme activity. Paraoxonase enzyme from pumpkin seeds was purified 158.09 fold with a yield of 77.55% using sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. The purity of the purified paraoxonase enzyme was checked by SDS-PAGE electrophoresis. The molecular weight of the purified paraoxonase enzyme was determined as 12942 Da and 20421 Da by gel filtration chromatography. K_m and V_{max} values were calculated as 3.475 mM and 2.935 µmol $L^{-1}.min^{-1}$ for paraoxon substrate, 3.067 mM and 2.647 µmol $L^{-1}.min^{-1}$ for phenyl acetate substrate. Mg^{2+}, Cu^{2+}, Mn^{2+} and Fe^{3+} cations, ascorbic acid, EDTA, β-mercaptoethanol and SDS compounds, diazinon, methyl parathion, ethyl parathion, dimethoate, dichlorvos, fention, acetamiprid and methamidophos pesticides strongly inhibited the enzyme. The analysis results showed that β-mercaptoethanol (IC_{50}: 0.90 mM) was the most potent inhibitor among cations and compounds, dichlorvos (IC_{50}: 0.91 mM) among pesticides.

Contents

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1. Introduction

Having a glycoprotein structure, paraoxonase (PON, EC: 3.1.8.1.) is a calcium (Ca^{2+}) dependent ester hydrolase with both arylesterase and paraoxonase activity [1]. The PON gene family comprises three members in mammals, as identified by PON1, PON2, and PON3. PON1 and PON3 are generated mostly in the liver, but PON2 is synthesized in multiple organs including the brain, liver, kidney, and testes [2, 3]. Despite the presence of the PON2 enzyme in almost all tissues in humans, no PON2 enzyme has been found in serum [4]. The best-known protective function of PON1 is to inhibit the neurotoxicity of circulating organophosphates (OPs) by reverse binding and hydrolysis of OPs such as nerve agents, aromatic carboxylic acid esters and insecticides [5].

Human serum contains the PON1 enzyme, which is capable of hydrolyzing harmful oxon metabolites of numerous insecticides, including parathion, diazinon, and chlorpyrifos, as well as OP nerve agents, including soman, sarin, and tabun. However, OP poisoning is common in agricultural workers as PON1 in mammals has a low affinity for these substrates. Nevertheless, PON1 is reported to be more effective in those chronically exposed to low doses of OP derivatives [6]. OP compounds pose a considerable environmental risk to human health. This is one of the most important reasons why the PON1 enzyme becomes a multifaceted research topic [7]. The PON also has a critical role in the prevention of atherogenesis by inhibiting lipid peroxidation.

The PON enzyme is known to have a protective effect against the formation of lipid peroxides by LDL oxidation, which is found to be bound to HDL [8, 9]. Therefore, the PON enzyme protects cells against oxidative stress and acts as a cellular antioxidant [10]. Reduced levels of PON1 have been identified in patients with atherosclerosis, specifically carotid and cerebral atherosclerosis [11, 12]. Paraoxonase enzyme has been found in fish, frog, turkey, chicken, rabbit, dog, rat, sheep, mice, and many other mammalian species [7]. Nevertheless, few studies are available on the presence of PON enzymes in plants [13]. It is believed that the PON1 enzyme of pumpkin seeds that contain omega fatty acids for cardiovascular health is associated with lipid metabolism.

The goal of this study was to purify and characterize the PON1 enzyme using affinity chromatography from pumpkin seeds containing a high concentration of essential fatty acids, as well as to evaluate the effects of certain chemicals and pesticides on enzyme activity in vitro.

2. Material and Method

2.1. Chemicals

Sepharose-4B, CNBr, Sephadex G-100, protein assay reagent, paraoxon and electrophoresis chemicals were used as the materials, were supplied by Sigma- Aldrich. The additional chemicals were by Sigma or Merck and were of analytical grade.

2.2. Preparation of Affinity Gel

CNBr (4 g) was added to a suspension made up of Sepharose 4B (20 mL) and water (20 mL). In an ice bath with magnetic stirring, the mixture's pH was brought to 11 and maintained there for 15 minutes. The mixture was then passed through a Buchner funnel, washed with cool $NaHCO_3$ buffer (0.1 M, pH:10, 250 mL) and poured into a beaker. A cold solution tyrosine (80 mg) dissolved in 20 mL of the same buffer was added to the beaker and mixed. Sepharose-L-tyrosine was activated with CNBr and then was linked with L-tyrosine through a saturated L-tyrosine solution in the same buffer. It was mixed using a magnet for 90 minutes so that the reaction was completed.

The Sepharose 4B-L-tyrosine gel was washed with a lot of water to get rid of any remaining L-tyrosine. The compound 1-naphthylamine was diazotized and then linked to the Sepharose 4B-L-tyrosine, resulting in the formation of a Sepharose 4B-L-tyrosine-1-naphthylamine affinity gel. To do this, 1-naphthylamine (25 mg) was dissolved in HCl (1M, 10 mL) at about 0°C. 5 mL of solution containing $NaNO_2$ (75 mg) at 0°C was added dropwise to the 1-naphthylamine solution. Diazotized 1-naphthylamine was added to the Sepharose-4B-L-tyrosine (40 mL) solution after the reaction was realized for 10 minutes. The pH was set to 9.5 with NaOH (1M) and gently mixed for 3 hours at room temperature. After that, Tris-sulfate (0.05 M, pH 7.5, 200 mL) were added to 1 L of water to washed the linked dark red Sepharose derivative [14].

2.3. Preparation of Pumpkin Seeds (*Cucurbitae peponis semen*) Homogenate

Pumpkin seeds were obtained from the province of Erzurum. Pumpkin seeds (10 g) were first mechanically disintegrated through several steps using liquid nitrogen. Then, they were placed in 0.1 M Tris-HCl /2 mM $CaCl_2$/0.5 M NaCl buffer with a pH of 8.0 and mixed with a magnetic stirrer for 30 min at room temperature. Homogenates were centrifuged at 5000 rpm at +4 °C for 30 min.

2.4. Affinity Chromatography (Sepharose 4B-L-tyrosine-1-naphthylamine)

The homogenate was balanced with a solution of Tris-HCl (0.1 M, pH: 8.0) and $CaCl_2$ (2 mM), and then transferred to the affinity column composed of sepharose 4B-L-tyrosine-1-naphthylamine. The affinity gel was rinsed with a solution containing Tris-HCl (0.1 M, pH: 8.0), $CaCl_2$ (2 mM), and NaCl (0.5 M). After, elution of pumpkin seeds paraoxonase was realized using the solution Tris-HCl (0.1 M, pH: 6.8) / $CaCl_2$ (2 Mm) at 0.5 mL min^{-1}. The highest PON1 activity fractions (5 mL each) were obtained [15].

2.5. Paraoxonase Activity Assay

In Tris-HCl (50 mM, pH 8.0) with $CaCl_2$ (1 mM) at 25 °C, 1 mM paraoxon (diethyl p-nitrophenyl phosphate) was used to test the paraoxonase activity. The absorbance of p-nitrophenol was measured at 412 nm. Calculation of enzyme

activity was performed using the p-nitrophenol molar extinction coefficient (ε=18000 M^{-1} cm^{-1} pH 8.0). The enzyme unit of paraoxonase refers to the number of micromoles of paraoxon hydrolyzed in 1 minute [16].

2.6. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The enzyme purity was evaluated by performing SDS-PAGE based on Laemmli's procedure [17]. The stacking and separating gels had acrylamide concentrations of 3% and 10%, respectively, and contained 1% SDS. 20 µg sample was subjected to the electrophoresis. A staining solution consisting of 0.1% Coomassie Brilliant Blue R-250 in a mixture of 50% methanol, 10% acetic acid, and 40% distilled water was applied to the gel and left for a duration of 2 hours. The same solution was washed again but without the dye until the removal of protein bands. Photographs of the electrophoretic pattern were taken (Figure 2).

2.7. Measurement Molecular Weight using Gel Filtration

Sephadex G-100 (3 g) was put into a buffer solution (200 mL). It was kept at 90 °C for 5 hours. Sephadex G-100 was filled into a column (1,3×30 cm). The column was balanced for a duration of 24 hours using a buffer solution containing 0.05 M Na_3PO_4 and 1 mM dithioerythritol (pH:7). Equilibration was maintained until the solution taken from the bottom of the column did not absorb at 280 nm. After the standard protein solutions (bovine serum albumin (66 kDa), egg albumin (45 kDa) carbonic anhydrase (29 kDa), α-lactalbumin (14 kDa) and trypsinogen (24 kDa) were placed into the column, the calibration curve was obtained. The purified PON1 enzyme was passed through the column and subsequently eluted under identical conditions. Results were matched with those of standard proteins [18].

2.8. Protein Determination

Bradford's technique [19] was employed by taking bovine serum albumin as the reference to determine protein content of the samples.

2.9. *In vitro* Some Metal Ions and Compounds Studies

The effects of purified PON1 enzyme on several metal ions (Mg^{2+}, Cu^{2+}, Mn^{2+} and Fe^{3+}) were studied. Each metal ion solution was prepared at five concentrations. Each solution was placed into an enzyme-containing cuvette. The IC_{50} values were determined using activity (%) vs. metal in concentration graphs.

The impact of several compounds on the activity of the pure PON1 enzyme was assessed using thiol-specific inhibitors, non-specific agents, and detergent. Ethylenediaminetetraacetic acid (EDTA), ascorbic acid, β-mercaptoethanol, sodium dodecyl sulfate (SDS) were used as the compounds in the study.

2.10. *In vitro* Pesticides Studies

The effects of diazinon, methyl parathion, ethyl parathion, dimethoate, dichlorvos, fention, acetamiprid and methamidophos pesticides at concentrations of 100 ppm, 10 ppm and 1 ppm on the PON1 enzyme activity that was purified from pumpkin seeds were determined. Each pesticide solution was prepared at five concentrations. Using plots of activity (%) against metal ion concentration, the IC_{50} values were determined.

3. Results and Discussion

Pumpkin seeds provide a good amount of the important of the essential fats omega-3, omega-6 and many minerals needed for hormonal balance, brain functions, skin quality, and heart health. It has been observed that lifestyle and dietary patterns affect the PON1 activity and concentration in cardiovascular diseases [20, 21]. There are a limited number of studies on the purification of paraoxonase enzyme, also known as an antioxidant enzyme, in plants [22, 23]. This study is of great importance for the characterization of the PON enzyme identified and purified in plants.

Using the Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography technique, the PON1 enzyme was purified and characterized from pumpkin seeds. As a result of the study, PON1 enzyme was purified 158.09-fold with a yield of 77.55% from pumpkin seeds. Table 1 shows how many folds this enzyme was purified.

Figure 1 shows the elution results of the PON1 enzyme purified from pumpkin seeds in the activity-absorbance graph. The molecular weights of PON1 enzyme purified from pumpkin seeds were observed to be 12942 Da (≈13000 Da) and 20421 Da by gel filtration chromatography. The enzyme's purity was assessed using SDS polyacrylamide gel electrophoresis. The enzyme was found to be pure and the gel was photographed and is shown in Figure 2.

Table 1 Summary of the PON1 purification procedure from pumpkin seeds

Purification step	Activity (EU mL^{-1})	Total volume (mL)	Protein (mg mL^{-1})	Total Protein (mg)	Total Activity (EU)	Specific activity (EU mg^{-1})	Yield (%)	Purification fold
Homogenate	1.926	20	26.04	520	38.52	0.074	100	-
Sepharose-4B-L-tyrosine-1-naphtylamine	2.134	14	0.182	2.551	29.87	11.72	77.55	158.09

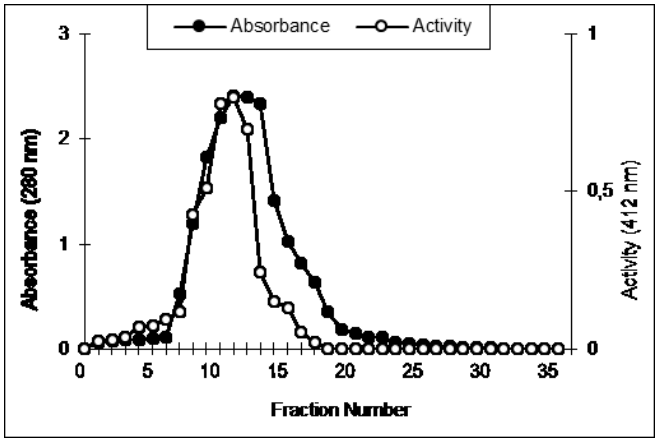

Figure 1 The activity-absorbance graph on the PON1 enzyme purified from pumpkin seeds.

Using Lineweaver-Burk graphs, values for V_{max} and K_m were calculated. The analysis on pumpkin seeds showed that the V_{max} and K_m values for paraoxon substrate were 2.935 µmol/L.min and 3.475 mM, respectively, and the V_{max} and K_m values for phenylacetate substrate were 2.647 µmol/L.min and 3.0674 mM, respectively.

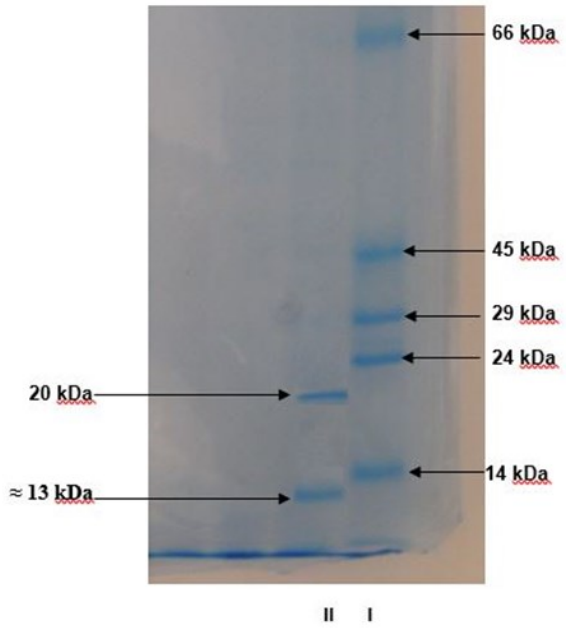

Figure 2 SDS-PAGE bands of PON1 enzyme from pumpkin seeds. Lane I: standard protein; α-lactalbumin (14 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa) Lane II: PON1 enzyme purified from pumpkin seeds.

Many factors such as pH, temperature, enzyme and substrate concentration, ionic strength, and the presence of inhibitors and activators affect the rate of enzymatic reactions, i.e., activity [24].

The pH and temperature values that provide the best conditions for the paraoxonase enzyme, which was obtained from pumpkin seeds and purified, were determined. The PON1 enzyme's optimum pH was determined to be 7, and it maintained activity in the pH range of 4-8. The optimum temperature was 39 °C, and the temperature range in which it demonstrated activity was 20-60 °C (Figure 3 - Figure 4).

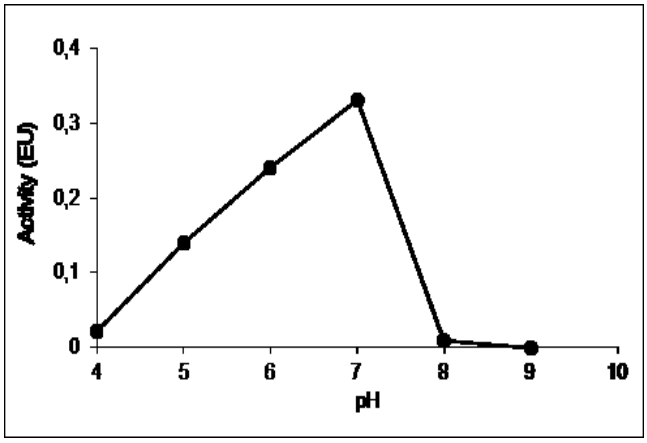

Figure 3 The impact of pH on the purified PON1 enzyme from pumpkin seeds.

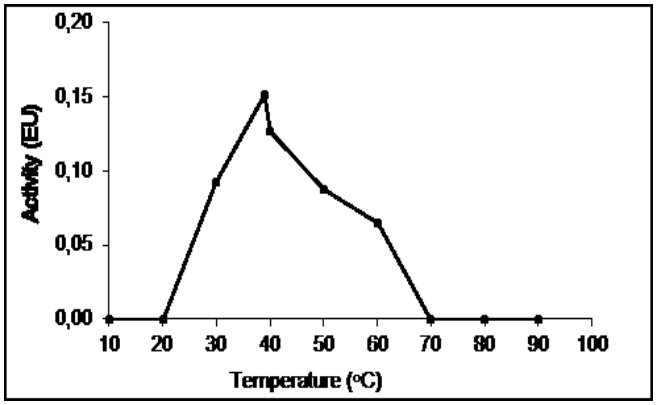

Figure 4 The impact of temperature on the purified PON1 enzyme from pumpkin seeds

Both inhibition and stability of enzymatic activity establish a control mechanism in biological systems. Studies on stability reveal details about the composition and operation of enzymes. Many drugs and toxic compounds function in this way. It is also useful to analyze the enzyme action mechanisms in order to figure out enzyme inhibition. There are also studies on metal interaction with the PON1 enzyme.

For example, it was reported that manganese, cobalt, zinc, barium, copper, mercury and magnesium inhibited liver microsomal PON1 enzyme [25]. In this study, the effects of Mg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+} and cations and EDTA, ascorbic acid, β-mercaptoethanol and SDS compounds on PON1 enzyme activity purified from pumpkin seeds were determined. It was found to inhibit the enzyme activity at all concentrations of Mg^{2+}, Cu^{2+}, Mn^{2+} and Fe^{3+}. It was found to inhibit the enzyme activity at all concentrations of EDTA, ascorbic acid, β-mercaptoethanol and SDS compounds (Table 2).

Table 2 IC_{50} values of metal ions and compounds on PON1 enzyme purified from pumpkin seeds

Metal ions and compounds	IC_{50} (mM)
Mg^{2+}	3.05
Cu^{2+}	2.11
Mn^{2+}	4.47
Fe^{3+}	1.66
EDTA	2.38
ascorbic acid	1.74
β-mercaptoethanol	0.90

SDS	0.97

Inhibition effects of metal ions and compounds on purified PON1 activity, IC_{50} values of β-mercaptoethanol, SDS, Fe^{3+}, ascorbic acid, Cu^{2+}, EDTA, Mg^{2+} and Mn^{2+} were found to be 0.90, 0.97, 1.66, 1.74, 2.11, 2.38, 3.05, 4.47 mM, respectively (Table 2).

A low IC_{50} value indicates a high inhibitory effect. The results showed that β-mercaptoethanol was stronger inhibitor than the other metal ions and compounds. These results are similar to those from other sources that have been acquired. For example, Demir et al. [13] investigated the *in vitro* effects of Fe^{3+}, Mn^{2+}, and Mg^{2+} metal ions on PON1 purified from olive (*Olea europaea* L) and discovered that they were inhibitory effect on the PON1 enzyme. Another study by Demir et al. [23] on the PON1 enzyme purified from sunflower (*Helianthus annuus*) determined the inhibitory properties of Fe^{3+}, Mn^{2+}, β-mercaptoethanol, SDS and EDTA metal ions and compounds. Ekinci and Beydemir [26] examined the *in vitro* inhibitory effects of some metal ions (Zn^{+2}, Cr^{+2}, Fe^{+2}, and Pb^{+2}) on PON1 enzyme purified from human serum and reported that metals had inhibitory effects.

Table 3 IC_{50} values of pesticides on PON1 enzyme purified from pumpkin seeds

Pesticides	IC_{50} (mM)
Methyl parathion	1.44
Dichlorvos	0.91
Dimethoate	1.03
Diazinon	2.30
Methamidophos	1.84
Ethyl parathion	1.12
Fention	1.21
Acetamiprid	1.63

According to Table 3, it was determined that the order according to IC50 values for pesticides was as follows: dichlorvos > dimethoate > ethyl parathion > fention > methyl parathion > acetamiprid > methamidophos > diazinon. The results showed that dichlorvos was stronger inhibitor than the pesticides.

PON1 is an enzyme hydrolyzing OP that also features antioxidant properties in metabolism. In recent years, there have been studies on the interactions between paraoxonase and pesticides. One study in Turkey found that PON1 activity was 30% lower in patients who suffered from acute organophosphate poisoning compared to controls. However, when PON1 activity was examined again six months after exposure to OP, it was found that the level of PON1 activity reverted to normal. It has been argued that the cause of low PON1 activity in the patients who were poisoned is either direct inactivation of PON1 by OPs or competition of the OPs that cause intoxication with the substrate used in the measurement of the activity of PON1 [27]. Cebeci et al. [28] examined the *in vitro* effects of pesticides (dichlorvos, fenoxaprop-p-ethyl, imidacloprid, and lambda-cyhalothrin) on the microsomal PON1 in sheep liver and reported that pesticides displayed an inhibitory effect on PON1 enzyme. In another study, Demir et al. [22] examined the effects of dichlorvos, chlorpyrifos-methyl parathion-methyl, chlorpyrifos ethyl, dimethoate, acetamiprid, mancozeb, trifluralin and lufenuron pesticides on human serum paraoxonase *in vitro* and reported that pesticides inhibited the enzyme.

4. Conclusion

Inhibition of enzyme activities by toxic compounds such as metals, drugs, pesticides and gases can cause threatening consequences for living organisms. Therefore, the number of toxicological studies that investigated the effects of metals and pesticides on enzyme activities has recently increased. In the present study, it was observed that all the chemicals studied inhibited the PON1 enzyme.

Reduced activity of the PON1 enzyme may lead to dangerous consequences. Especially people exposed to compounds and people at risk of vascular disease are reported to have altered activity of this paraoxonase enzyme. Therefore, it is important investigate by which substances the PON enzyme is inhibited. Both the deficit and inhibition of PON activity have been associated with a diverse array of illnesses including diabetes mellitus, cardiovascular disease, rheumatism, and Alzheimer's disease.

Therefore, identification, purification and characterization of the PON1 enzyme from pumpkin seeds, from which many benefits are provided to human health, is vital. This study is believed to guide further studies by examining the three-dimensional structure of the enzyme purified from pumpkin seeds and comparing it with the PON1 enzyme structure purified from plants in different studies.

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Conflict of Interest

The authors declared no conflict of interest.

References

[1] Durrington PN, Mackness B, Mackness MI. Paraoxonase and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **21**(4):473–480.

[2] La Du B, Aviram N, Billecke S, Navab M, Primo-Parmo S, Sorenson RC, et al. On the physiological role(s) of the paraoxonases. *Chemico-Biological Interactions* (1999):119–120, 379–388.

[3] Primo-Parmo SL, Sorenson RC, Teiber J, Du BN. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. *Genomics* (1996) **33**(3):498–507.

[4] Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR. PON2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *Journal of Biological Chemistry* (2001) **276**(48):44444–44449.

[5] Costa LG, Cole TB, Furlong CE. Polymorphisms of paraoxonase (PON1) and their significance in clinical toxicology of organophosphates. *Journal of Toxicology: Clinical Toxicology* (2003) **41**(1):37–45.

[6] Başkol G, Köse K. Paraoxanase: Biochemical features, functions and clinical importance. *Erciyes Medical Journal* (2004) **26**(2):75–80.
[7] Dragonov DI, Du NB. Pharmacogenetics of paraoxonases: a brief review. *Naunyn-Schmiedeberg's Archives Pharmacology* (2004) **369**:78–88.
[8] Mackness B, Davies GK, Turkei W, Lee E, Roberts DH. Paraoxonase status in coronary heart disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* (2001) **21**(9):1451–1457.
[9] Carey J, Shih DM, Hama SY, Villa N, Navab M, Reddy TS. The paraoxonase gene family and atherosclerosis. *Free Radical Biology and Medicine* (2005) **38**(2):153–163.
[10] Ceja-Gálvez HR, Torres-Sánchez ED, Torres-Jasso JH, Ornelas AV, Salazar-Flores J. Effect of structure and function of paraoxonase-1 (PON-1) on organophosphate pesticides metabolism. *Biocell* (2020) **44**(3):363–370.
[11] Medina-Díaz IM, Ponce-Ruiz N, Ramírez-Chávez B, Rojas-García AE, Barrón-Vivanco BS. Downregulation of human paraoxonase 1 (PON1) by organophosphate pesticides in HepG2 cells. *Environmental Toxicology* (2017) **32**(2):490–500.
[12] Costa LG, Giordano G, Guizzetti M, Vitalone A. Neurotoxicity of pesticides: a brief review. *Frontiers in Bioscience-Landmark* (2008) **13**(4):1240–1249.
[13] Demir N, Nadaroglu H, Demir Y. Purification of paraoxonase (PON) from olive (Olea europaea L.) and effect of some chemicals on paraoxonase activity in vitro. *Asian Journal of Chemistry* (2011) **23**(6):2588.
[14] Sinan S, Kockar F, Arslan O. Novel purification strategy for human PON1 and inhibition of the activity by cephalosporin and aminoglikozide derived antibiotics. *Biochimie* (2006) **88**(5):565–574.
[15] Gan KN, Smolen A, Eckerson HW, Du BN. Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metabolism and Disposition* (1991) **19**(1):100–106.
[16] Renault F, Chabrière E, Andrieu JP, Dublet B, Masson P, Rochu D. Tandem purification of two HDL-associated partner proteins in human plasma, paraoxonase (PON1) and phosphate binding protein (HPBP) using hydroxyapatite chromatography. *Journal of Chromatography B* (2006) **836**(1-2):15–21.
[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (1970) **227**(5259):680–685.
[18] Whitaker, JR. Determination of molecular weights of pro-teins by gel filtration of Sephadex. *Analytical Chemistry* (1963) **35**(12):1950–1953.
[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* (1976) **72**(1-2):248–254.
[20] Kudchodkar BJ, Lacko AG, Dory L, Fungwe TV. Dietary fat modulates serum paraoxonase 1 activity in rats. *The Journal of nutrition* (2000) **130**(10):2427–2433.
[21] Kleemola P, Freese R, Jauhiainen M, Pahlman R, Alfthan G. Dietary determinants of serum paraoxonase activity in healthy humans. *Atherosclerosis* (2002) **160**(2):432.
[22] Demir N, Nadaroglu H, Demir Y, An GA, Vitro I. Study of some Pesticides on the Activity of Human Serum Paraoxonase (PON1. *Jordan Journal of Chemistry* (2011) **6**(4):439–451.
[23] Demir N, Nadaroglu H, Demir Y. Purification of Paraoxonase (PON) from Sun Flower (Helianthus annuus) and Effects of Some Chemicals on Paraoxonase Activity In vitro. *Annual Research & Review in Biology* (2015) **5**(1):25–33.
[24] Bisswanger H. Enzyme assays. *Perspectives in Science* (2014) **1**(1-6):41–55.
[25] Cornish-Bowden A. *Fundamentals of Enzyme Kinetics*. London: Portland Press (2004).
[26] Ekinci D, Beydemir Ş. Purification of PON1 from human serum and assessment of enzyme kinetics against metal toxicity. *Biological trace element research* (2010) **135**(1):112–120.
[27] Sözmen EY, Mackness B, Sözmen B, Durrington P, Girgin FK. Effect of organophosphate intoxication on human serum paraoxonase. *Human & Experimental Toxicology* (2002) **21**:247–252.
[28] Cebeci B, Alim Z, Beydemir Ş. In vitro effects of pesticide exposure on the activity of the paraoxonase-1 enzyme from sheep liver microsomes. *Turkish Journal of Chemistry(2014* (2014) **38**(3):512–520.