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In vitro effects of pesticide exposure on the activity of the paraoxonase-1 enzyme from sheep liver microsomes

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Abstract: The organophosphate target enzyme paraoxonase-1 (PON1) has been extensively studied in toxicology. Pesticides are organophosphate compounds that are commonly used in agriculture. They are also used as nerve gases, such as sarin and soman. Therefore, the effects of these compounds on enzymatic activity are well known. In the present study, we investigated the in vitro effects of fenoxaprop-p-ethyl, lambda cyhalothrin, imidacloprid, and dichlorvos pesticides on sheep liver microsomal PON1. The enzyme was purified approximately 141-fold with a specific activity of 1822.22 EU/mg proteins. The pesticides inhibited sheep liver PON1 in vitro, and the IC_{50} values for these compounds were 0.0103, 0.069, 0.157, and 0.2 μM, respectively.

Key words: Paraoxonase, fenoxaprop-p-ethyl, lambda cyhalothrin, imidacloprid, dichlorvos, inhibition

1. Introduction

For many decades, pesticides have been increasingly used in agriculture. These materials have been shown to affect the growth of productivity and the specialization of cultures.[1] It is well known that pesticides have neurotoxic effects. They can have crucial effects on the nervous system and metabolism in all living systems. There are many compounds such as carbamates, organophosphates, organochlorines, and bipyridyls that function as pesticides.[1] Pesticides are commonly used to control organisms that are considered harmful, including mosquitoes, insects, mice, and other animals; unwanted plants; fungi; and microorganisms such as bacteria. Although they are helpful in this respect, pesticides can have many dangerous effects in living organisms, including humans, either directly or indirectly. These effects can range from simple irritation of the skin and eyes to more severe effects, such as affecting the nervous system and also causing cancer, Alzheimer's disease, Parkinson's disease, and other metabolic disorders.[1–3] Recently, it has been reported that environmental factors are critical in the regulation of the immune system.[4] Numerous environmental factors, particularly pesticides, can cause oxidative stress. Oxidative stress is determined either by the over-production of free radicals or by the alteration of antioxidant defense mechanisms, including detoxification and scavenging enzymes (Figure 1).[4] The cause of Alzheimer's disease is not known, although it is theorized to be caused by a genetic predisposition and/or certain environmental factors.[4,5] Pesticides may cause Alzheimer's disease because free radicals, which are associated with oxidative stress and formed by pesticides, can cross the blood–brain barrier easily and exert damaging effects on neurons.[1] Similar findings have been reported for cancer and

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Parkinson's diseases. No specific explanations have been proposed for cancer, Parkinson's disease, or Alzheimer's disease.

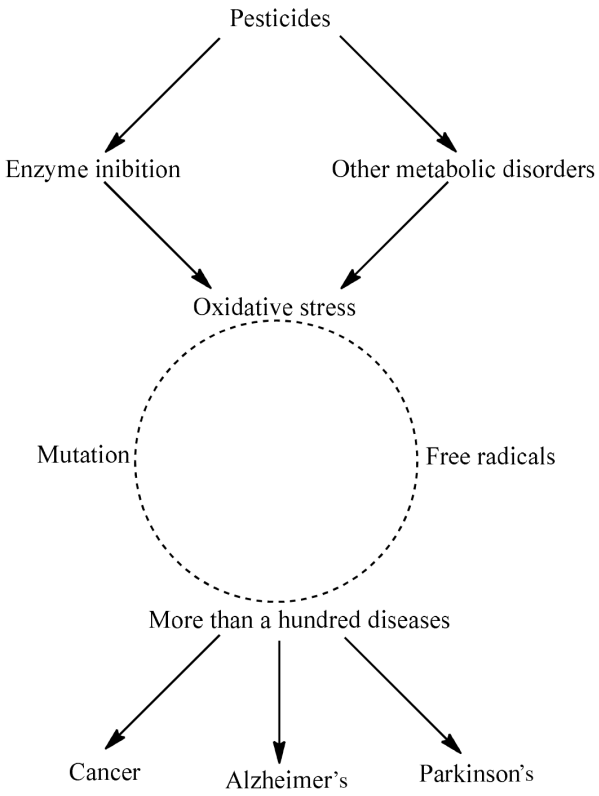


Figure 1. The relationship between pesticides and oxidative stress.

It is known that a toxicological mechanism is associated with enzymatic reactions in metabolism. In particular, some enzymes, such as carbonic anhydrase, glucose 6-phosphate dehydrogenase, and other enzymes involved in glucose metabolism, including paraoxonase, are known drug targets. A calcium-dependent esterase, paraoxonase (arylesterase, EC 3.1.8.1, PON1), is a vital enzyme in atherosclerosis. PON1 is a high-density lipoprotein binding enzyme. The gene family of this enzyme contains at least 3 members in mammals: PON1, PON2, and PON3. PON1 and PON3 are expressed primarily in the liver, while PON2 is expressed in various tissues, including the brain, liver, and kidneys. PON1 was the first member of this family to be identified, and it is the most thoroughly studied.[6,7] PON1 is a liver and plasma enzyme that catalyzes the hydrolysis and inactivation of various organophosphates, including paraoxon and the insecticides parathion and chlorpyriphos as well as the nerve agents sarin and soman.[8] In our study, the effects of the pesticides fenoxaprop-p-ethyl, lambda cyhalothrin, imidacloprid, and dichlorvos on PON1 enzyme activity were studied. Fenoxaprop-p-ethyl is a commonly used herbicide. It is one of the members of the aryloxyphenoxypropionate herbicide family, which is used to control certain annual and perennial grass weeds in cereals, certain pulse crops, vegetables, and certain feed and forage crops. It belongs to a class of compounds known to interfere with lipid metabolism in rodents leading to enhanced lipid turnover and peroxisome proliferation in liver cells.[9] Lambda cyhalothrin is an insecticide that belongs to the pyrethroid chemical class of pesticides. It is a mixture of highly active isomers of cyhalothrin and is used to control a wide range of pests in a variety of applications.[10] Imidacloprid is a systemic chloronicotinyl pesticide belonging to the class of neonicotinoid insecticides. It is used on a wide array

of plants, including many major crops. Imidacloprid acts as a neurotoxin and interferes with the transmission of nerve impulses in insects by binding to specific nicotinic acetylcholine receptors.[11] Dichlorvos is a highly volatile organophosphate that is extensively used as an insecticide to control household pests, in public health, and to protect stored products from insects. Additionally, dichlorvos damages the DNA of insects.[12] There are numerous studies on the interactions between specific chemicals and PON1 activity in different mammalian tissues.[13–15] For instance, our laboratory group studied the in vitro effects of heavy metals on the activity of PON1 from human serum.[13] However, there have been few studies on microsomal PON1.

Consequently, this study was conducted to purify PON1 from sheep liver microsomes and investigate the in vitro inhibitory effects of some commonly used pesticides on this enzyme.

2. Results and discussion

PON1 is an aromatic esterase that requires calcium for its activity. It is involved in the detoxification of several organophosphorus insecticides. PON1 is known to play a critical role in the protection of HDL and LDL particles from oxidation, the antioxidant effects against lipid peroxidation on cellular membranes, and the anti-inflammatory process. In addition, it is reported to be associated with protective properties against cardiovascular diseases. Decreased PON1 activity has been associated with atherosclerosis in people with diabetes mellitus, polycystic ovary disease, familial hypercholesterolemia, and renal disease.[16–21]

HDL is referred to as benign cholesterol in living systems, and LDL is considered malignant cholesterol. The PON1 enzyme has a significant role in the benign nature of HDL. With a decrease in the amount of HDL, the amount and activity of the PON1 enzyme drop off. Thus, an increase in the number of atherosclerotic lesions is observed due to oxidation of LDL. In particular, free radicals formed by oxidative stress cause the expression of the PON1 enzyme to be reduced.

For instance, Marsillach et al. (2009) observed alterations in PON1 in rats treated with CCl_4. They suggested that liver impairment due to free radical production causes a decrease in peroxisome proliferator-activated receptor (PPARδ) gene expression and an inhibition of ATP-binding cassette transporter (ABCA1). Therefore, HDL synthesis will be insufficient and, consequently, the level of PON1 gene expression and the PON1 concentration in the serum will be reduced.[22] Accordingly, an increase in atherosclerotic lesions is observed.

The pharmaceutical and toxicological mechanisms in metabolism are carried out by the activities of a wide variety of enzymes. Some of these enzymes are known drug targets in the literature. In particular, some enzymes involved in glucose metabolism, including glucose 6-phosphate dehydrogenase and carbonic anhydrase, are known drug targets.[23,24] In recent years, the PON enzyme family, particularly PON1, has been studied as a drug target. For instance, Alıcı et al. (2008) reported that intravenous anesthetics, such as propofol, ketamine, and etomidate, significantly inhibit human PON1 activity both in vitro and in vivo. The authors determined the rank order based on the effects of drugs to be as follows: etomidate > propofol > ketamine in vitro, and propofol > etomidate > ketamine in vivo.[25] Another study investigated the impacts of teicoplanin, rifamycin, tobramycin, ceftriaxone sodium, cefuroxime sodium, ceftazidime, ornidazole, and amikacin sulfate on PON1. In that study, it was observed that some antibiotics inhibited PON1 activity at very low doses and they had different inhibition mechanisms.[26] Conversely, other chemicals, such as metal ions, pesticides, and fungicides, may also affect PON and other enzymes at very low concentrations. One study examined the effects of EDTA, Mg^{2+}, $Co^{2+,}$ Ba^{2+}, La^{3+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, p-hydroxymercuribenzoate (p-OH-MB), and phenyl mercuric acetate (PMA) on paraoxonase activity from human liver microsomes. The authors found that the metals and other inhibitors showed different inhibition patterns. While EDTA, Ba, La, Cu, p-OH-MB, and PMA were

competitive inhibitors, Zn was noncompetitive, and the results were mixed for Hg.[27] Moreover, genetic disorders related to pesticides, particularly organophosphates (OPs), have critical effects on almost all enzymes, including paraoxonase. For instance, in one study, the authors investigated the effect of PON1 genotypes and phenotypes variations on DNA damage in 230 workers exposed to organophosphates. They reported that variations in the expression and catalytic activity of the PON1 enzyme are associated with polymorphisms of the PON1 gene.[28]

It is well known that environmental conditions have important effects on all living things, including humans. These unsuitable factors lead to genetic disorders and thus mutations and lack of expression of the enzymes, and they result in more than 100 diseases, including Alzheimer's disease, cancer, and Parkinson's diseases, that are specifically associated with oxidative stress (Figure 1). In particular, pesticides play a critical role. Neurotoxic agents may be a source of many diseases, such as cancer, Alzheimer's disease, and many other dangerous diseases.[29,30] In this respect, studies on the structures and activities of the affected enzymes are important. More than 10 enzymes have been purified and examined for drug–enzyme interactions and inhibition by metals and other compounds in our laboratory.[31–34] In the present study, PON1 was purified with a specific activity of 1822.22 EU/mg proteins at an approximately 141-fold concentration from sheep liver microsomes (Table 1). In the purification steps, we included the preparation of a microsomal pellet, ammonium sulfate precipitation, DEAE Sephadex A-50 anion exchange chromatography, and Sephadex G-100 gel filtration chromatography. Our purification results are similar to those of other studies.[35–37] To identify the enzyme, SDS-PAGE was performed and a single protein band was obtained (Figure 2). Finally, the impact of the pesticides fenoxaprop-p-ethyl, lambda cyhalothrin, imidacloprid, and dichlorvos on PON1 activity was determined. The IC_{50} values of these pesticides were 0.0103, 0.069, 0.157, and 0.2 μM, respectively (Figure 3; Table 2).

Table 1. Purification steps of sheep liver microsomes PON1.

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield %	Purification fold
Homogenate	55.768	20	4.32	86.40	1115.36	12.90	100	1
Ammonium sulfate precipitation	59.058	7	3.98	27.86	413.330	14.83	37	1.149
Ion exchange chromatography	71.077	5	1.10	5.500	315.385	57.34	28	4.444
Gel filtration chromatography	9.840	4	0.0054	0.0216	39.3600	1822.22	3.5	$\sim$ 141

Although there are benefits associated with the use of pesticides, they are also potentially hazardous to humans, animals, other organisms, and the environment. The inhibitory effects of fenoxaprop-p-ethyl, lambda cyhalothrin, imidacloprid, and dichlorvos on the activity of PON1 from sheep liver microsomes were determined in this study. The pesticide with the greatest effect was fenoxaprop-p-ethyl, which is commonly used as an herbicide. However, other pesticides also exhibited inhibitory effects at very low concentrations. The usage of pesticides is inevitable in modern agriculture. However, it is important to consider their role in the balance of nature. Living things act without waiting. This process can be with occurrence of all reactions of the metabolism, quickly. It is well known that enzymes catalyze almost all chemical reactions in the metabolism of the living systems. Pesticides may cause negative effects on enzyme activities or the 3-dimensional structures of enzymes. This could result in some changes in the metabolisms of living organisms, and cause detrimental effects for both the life kingdoms and the environment.

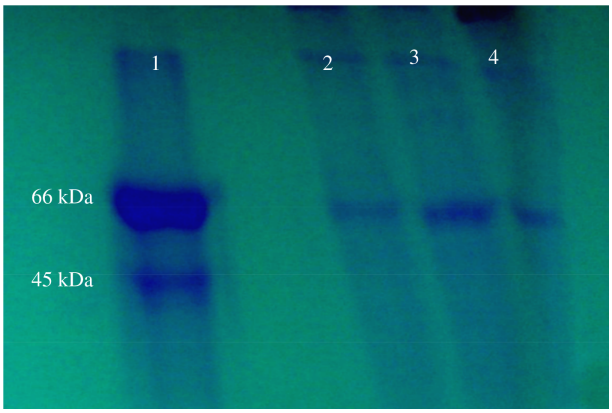


Figure 2. Purification of the paraoxonase-1 enzyme was confirmed by SDS-PAGE analysis. The samples were applied as 20 μg to the electrophoresis. Lane 1, standard proteins (Bovine serum albumin 66 kDa, Ovalbumin 45 kDa), Lane 2–4, purified paraoxonase-1.

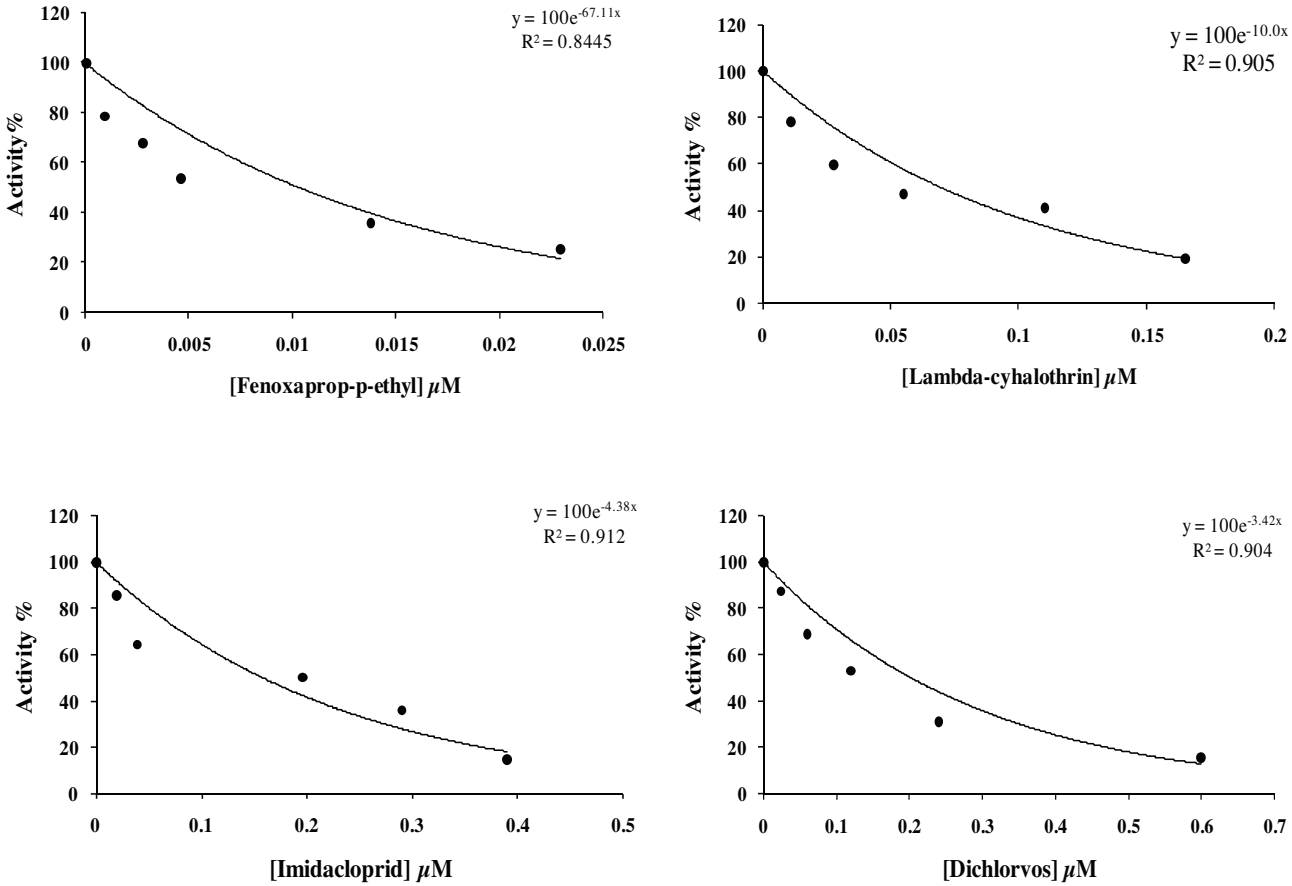
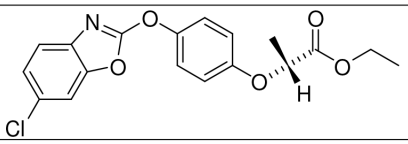
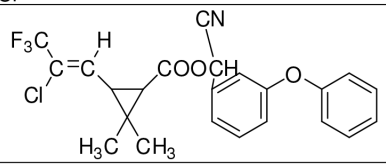
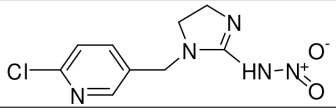
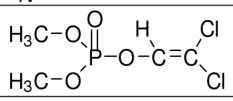


Figure 3. Determination of IC_{50} values of each drug performed using Activity%-[Inhibitor] graphs. To obtain these values, the experiments were performed at 5 different drug concentrations. Paraoxone was used as a substrate in the studies. A blind tube contained all of the reaction mixtures except for the enzyme solution.

Table 2. IC_{50} values for inhibition of sheep liver microsomal PON1 by pesticides.

Pesticidet	Structure	IC_{50} (μM)
Fenoxaprop-p-ethyl		0.0103
Lambda cyhalothrin		0.0690
Imidacloprid		0.1570
Dichlorvos		0.2000

3. Experimental

3.1. Materials

DEAE-Sephadex A50, 1-naphthylamine, paraoxon, the protein assay reagents, and the chemicals for electrophoresis were obtained from Sigma-Aldrich (Germany). Other chemicals were obtained from either Sigma-Aldrich or Merck (Germany).

3.2. Methods

3.2.1. Paraoxonase activity assay

The PON1 activity assay was performed according to our previous study[14] at 25 °C, and diethyl p-nitrophenyl phosphate (paraoxon) was used as a substrate. The molar extinction coefficient of p-nitrophenol was 18.290 M^{-1} cm^{-1} at pH 10.5 and it was used to calculate the enzyme activity.[38]

3.2.2. Preparation of the microsomal samples

Briefly, sheep livers (20 g) were removed, placed in beakers on ice, rinsed with an ice-cold homogenization buffer (including 0.25 M sucrose, 5 mM Tris/HCl pH 7.4), minced with scissors, and then placed in 4-fold of the same ice-cold buffer. They were then homogenized using an ULTRA TURRAX. The homogenate was centrifuged at 460 × g for 10 min, and the precipitate was discarded. The supernatant was centrifuged at 12,500 × g for 60 min, and the precipitate was discarded. The mitochondrial supernatant fraction was then centrifuged at 105,000 × g for 60 min. The microsomal pellet derived from the sheep liver tissue was suspended in a 5 mM Tris/HCl buffer (pH 7.4).

3.2.3. Ammonium sulfate precipitation

The microsomal pellet suspension was precipitated with ammonium sulfate. The precipitation intervals for PON1 were 20%–70%. The precipitate was collected by centrifugation at 13500 rpm for 20 min and redissolved

in a 50 mM Tris/HCl buffer at pH 7.7. The solution was dialyzed against 10 mM Tris/HCl buffer (pH 7.7) containing 1 mM 2-mercaptoethanol.

3.2.4. Purification of sheep liver microsomes PON1

3.2.4.1. DEAE-Sephadex A50 anion exchange chromatography

A DEAE-Sephadex A50 anion exchange column (3 cm^2 × 30 cm) was prepared according to our previous study.[26] The dialyzed enzyme solution was loaded onto a column. The column was washed with equilibration buffer, and then elution was performed with a linear gradient of 0.5–1 M NaCl. The eluted fractions were collected, and the enzyme activity was checked at 412 nm. The tubes with enzyme activity were combined. All purification procedures were performed at 4 °C.

3.2.4.2. Sephadex G-100 gel filtration chromatography

Active enzyme fractions from the DEAE-Sephadex column were loaded onto the Sephadex G-100 column (60 cm × 2 cm), which had been equilibrated with 20 mM Tris/HCl (pH 7.7). Elution was performed with the same buffer. The fractions were analyzed for both the protein amount (280 nm) and the enzyme activity (412 nm). The tubes with enzyme activity were combined for other kinetic studies.

3.2.5. Protein determination

During the purification steps, the protein quantity was determined spectrophotometrically at 595 nm according to a previous study.[39]

3.2.6. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was conducted according to our previous studies.[40,41] Protein standards and PON1 samples from sheep liver microsomes were loaded into each slot of the stacking gel (slab gel dimensions: 16 × 18 cm). Initially, a voltage of 80 V was applied until the bromphenol blue reached the running gel. The voltage was then increased to 200 V for 3–4 h (Hoefer Scientific Instruments SE 600). The gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid and destained with methanol/acetic acid. The electrophoretic patterns were photographed (Figure 2).

3.2.7. In vitro inhibition studies

We examined the inhibitory effects of several commonly used pesticides: fenoxaprop-p-ethyl, lambda cyhalothrin, imidacloprid, and dichlorvos. All pesticides were tested in triplicate at each concentration used. The PON1 activities were measured in the presence of different concentrations of pesticides. The control activity was assumed to be 100% in the absence of any inhibitor. For each pesticide, a graph of the percent of activity versus the drug concentration graph was drawn, and the IC_{50} values were calculated.

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