IN VITRO EFFECTS OF SOME PESTICIDES ON PON1Q192 AND PON1R192 ISOENZYMES FROM HUMAN SERUM

Nahit Gençer* and Oktay Arslan

Balikesir University, Science and Art Faculty, Department of Chemistry/Biochemistry Section, 10100 Balikesir, Turkey

ABSTRACT

Human serum paraoxonase 1 (PON1) contains two polymorphic forms, Q (glutamine) or R (arginine) located at codon 192. The Q isoform has a higher activity for hydrolysis of diazoxon, sarin, and soman; whereas the R isoform has a higher activity for hydrolysis of paraoxon and chlorpyrifos oxon. In this study, the *in vitro* effects of commonly used pesticides (Purtapyr, Roundup, Agrofarm and Practicur) on purified human serum Q and R isoenzymes were investigated. Q and R isoforms were separately purified with ammonium sulphate precipitation and hydrophobic interaction chromatography, and the *in vitro* effects of the pesticides on purifying Q and R were determined using paraoxon as a substrate. IC_{50} values of these pesticides ex-hibiting inhibition effects were found from graphs of paraoxonase activity percentage by plotting their concentrations. The inhibition kinetics (Ki) interaction of these pesticides with the human serum Q and R isoenzymes were also determined.

KEYWORDS:
PON1; phenotype; inhibition; pesticides.

1. INTRODUCTION

PON 1 is a calcium-dependent HDL-associated (apoprotein A-1) glycoprotein, catalyzing the hydrolysis of aromatic carboxylic acid esters, organophosphate (OP) insecticides and nerve gases [1, 2]. In addition, PON1 is involved in lipid metabolism, since it probably hydrolyzes multiple oxygenated forms of polyunsaturated fatty acids of low-density lipoproteins associated with phospholipids. For this reason, paraoxonase can be defined as an antioxidant enzyme [3, 4]. But its native substrates, *in vivo* mechanism of action, and also molecular target(s) of PON1 remain unknown.

The gene for PON1 shows two common polymorphisms: Q (glutamine) or R (arginine) at codon 192 [5, 6]. This polymorphism is substrate-dependent. The PON1Q192 isoform has a higher rate of *in vitro* hydrolysis of diazoxon, sarin, and soman [7], whereas the PON1R192 isoform has higher activity for the hydrolysis of paraoxon and chlorpyrifos oxon [8]. Both hydrolyze phenylacetate approximately at the same rate [9]. In addition, the ability of HDL to protect LDL against peroxidation *in vitro* is significantly lower in HDL particles containing PON1R192 than in those with PON1Q192 [10]. Polymorphism of the PON1 gene in-fluences the blood levels of PON1 and its catalytic efficiency; both factors strongly affect individuals susceptible to arteriosclerosis, pollutants and insecticides [11, 12]. In addition, it showed that mice lacking PON1 are highly susceptible to arteriosclerosis and organophosphate poisoning [12].

Many pesticides are being used in agriculture to improve the yields. Although the use of these chemicals had a positive effect on crop production, certain pesticides, their residues, metabolites and/or contaminants created many unforeseen adverse effects on the environment [13].

The purpose of this study was to investigate the *in vitro* effects of 4 commonly used pesticides: (RS)-5-ethyl-2-(4isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid (Purtapyr), N-phosphonomethyl-glycine (Roundup), 2.4-dichlorophenoxyacetic acid (Agrofarm) and propyl-3-(dimethylamino) propylcarbamate monohydrochloride (Practicur) on purified human serum paraoxonase Q and R allozymes activity. However, to our knowledge, no study is available on the *in vitro* effects of these pesticides on paraoxonase Q and R allozymes activity.

2. MATERIALS AND METHODS

The materials used (Sepharose 4B, L-tyrosine, 9-aminophenanthrene, paraoxon, protein assay reagents and chemicals for electrophoresis) were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade. The pesticides were of commercial origin, and at the highest available purity level (99%).

2.1. Paraoxonase enzyme assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically with the method de-

* Corresponding author

scribed by Adkins et al. [5]. The reaction was followed within 2 min at 37 °C by monitoring the appearance of p-nitrophenol at 412 nm in a Biotech automated recording spectrophotometer. A molar extinction coefficient (ε) of p-nitrophenol at pH 8.0 in 100 mM Tris–base buffer of 17,100 M^{-1} cm^{-1} was used for calculation. PON1 activity (1 U /L) was defined as 1 μmol of p-nitrophenol formed per min.

2.2. Phenotyping and purification of human PON1 types Q and R

In order to classify individual phenotypes, two parameters were used. According to Eckerson et al. [14], phenotypic distribution of the paraoxonase activity was determined through the basal stimulation of paraoxonase activity by 1 M NaCl. Individuals were classified for paraoxonase phenotype using the antimode at 60 % stimulation as the dividing point between the non-salt-stimulated Q type as well as the salt-stimulated QR (60-200 %) and R (>200 %) types.

2.3. Ammonium sulphate precipitation

Human serum was isolated from fresh human blood taken to a dry tube. The blood samples were centrifuged at 1500 rpm for 15 min, and the serum was removed. Firstly, serum paraoxonase was isolated by ammonium sulphate precipitation (60–80%) [15]. The precipitate was collected by centrifugation at 15000 rpm for 20 min, and redissolved in 100 mM Tris–HCl buffer (pH 8.0).

2.4. Purification of PON1 Q and R isoforms from human serum by hydrophobic interaction chromatography

The pooled precipitate obtained from human serum by using ammonium sulphate precipitation was subjected to hydrophobic interaction chromatography. The final saline concentration of the precipitate was adjusted to 1 M ammonium sulphate. Prior to that, it was loaded onto the hydrophobic gel column, prepared from Sepharose-4B/L-tyrosine/9-aminophenanthrene. The preparation of hydrophobic column for the purification of human serum paraoxonase is as follows [16]: 10% CNBr was prepared in 1:1 dilution of Sepharose-4B and water. The mixture was titrated to pH 11 in an ice-bath and maintained at that pH for 8–10 min. The reaction was stopped by filtering the gel on a Buchner funnel and washing it with cold 0.1M $NaHCO_3$ buffer (pH 10). L-Tyrosine (by using a saturated L-tyrosine solution) in the same buffer was coupled to Sepharose-4B activated with CNBr. The reaction was completed by magnetic stirring for 90 min. In order to remove excess L-tyrosine from the sepharose-4B/L-tyrosine gel, the mixture was washed with distilled water. The hydrophobic gel was then obtained by diazotization of 9-aminophenanthrene, and coupling of this compound to Sepharose-4B/L-tyrosine. The pH was adjusted to 9.5 with 1M NaOH and, after gentle stirring for 3 h at room temperature; the coupled red Sepharose derivative was washed with 1 L water and then, 200 ml of 0.05 M Tris–sulphate (pH 7.5). The column was equilibrated with 0.1 M Na_2HPO_4 buffer (pH 8.0) including 1 M ammonium sulphate. The paraoxonase was eluted with ammonium sulphate gradient using 0.1 M Na_2HPO_4 buffer, with and without ammonium sulphate pH 8.0. The purified PON1 enzyme was stored in the presence of 2 mM $CaCl_2$ at +4 °C, in order to maintain activity.

2.5. Total protein determination

The absorbance at 280 nm was used for monitoring the protein in the column effluents and ammonium sulphate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [17], with bovine serum albumin standard.

2.6. 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 stacking gel, respectively, containing 0.1% SDS according to Laemmli [18]. A 20-mg sample was applied to the electrophoresis medium. Gel was stained overnight in 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol and 10 % acetic acid, and then destined by frequently changing the same solvent, without dye. The electrophoretic pattern was photographed with the system to produce an image of the gel.

2.7. In vitro inhibition kinetic studies and determination of Ki values

For the inhibition studies of the above pesticides, different concentrations of them were added to the enzyme. Q and R isoenzymes activities with pesticides were assayed by following the hydration of paraoxon. Activity % values of paraoxonase for 5 different concentrations of each pesticide were determined by regression analysis using Microsoft Office 2000 Excel. Paraoxonase activity without a pesticide was accepted as 100 % activity. For the pesticides having an inhibition effect, the inhibitor concentration causing up to 50 % inhibition (IC_{50} value) was determined from the graphs. In addition, Ki values of these pesticides were determined relative to Q and R activity. In order to obtain K_M and Vmax values, the enzyme for paraoxon at optimum pH (pH 8.0) and temperature (37 °C) was measured at 7 different substrate concentrations (0.2, 0.4, 0.6, 0.8, and 1 mM). K_M and Vmax values were determined by means of Lineweaver–Burke graphs. Two different concentrations of these pesticides were added to the mixture, and reactions resulting were determined. Ki values were calculated from Lineweaver–Burke graphs.

3. RESULTS AND DISCUSSION

In this study, the effects of commonly used pesticides, namely (RS)-5-ethyl-2-(4isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid, N-(phosphonomethyl)-glycine, 2.4-dichlorophenoxyacetic acid and propyl-3-(dimethylamino) propylcarbamate monohydrochloride, on purified

human serum Q and R isoenzymes were investigated *in vitro*. In order to investigate these effects, human serum paraoxonase Q and R were purified by ammonium sulphate precipitation at 60—80% intervals, and subjected to hydrophobic interaction chromatography. We previously reported a purification strategy designed for the human PON1 enzyme consisting of 2-step procedures resulting in a shorter and more straightforward approach, in contrast to other purification procedures [16].

The gel for hydrophobic interaction chromatography was synthesized using Sepharose 4B, L-tyrosine and 9-aminophenantrene. Overall purification rate of our method was found to be 901-fold for R isoenzyme and 453-fold for Q isoenzyme. The Vmax and K_M values of purified enzyme were determined to be 55 EU and 0.599 mM for Q isoenzyme, but 50 EU and 0.492 mM for R isoenzyme, respectively [16]. Several studies also reported that K_M values for paraoxon from different labs can show quite similar values. Eckerson et al. [14] found K_M values of 0.43 and 0.46 mM for paraoxonase type Q and type R enzymes. The purity of the isoenzymes was confirmed by SDS-PAGE. As seen in Fig. 1, a single band, 43 kDa, was obtained, which corresponds to the results of previous studies [19-21].

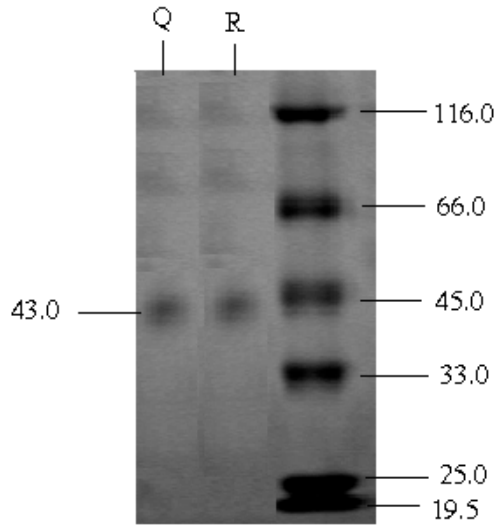

FIGURE 1 - SDS-PAGE of human serum paraoxonase. The pooled fractions from ammonium sulfate precipitation and hydrophobic interaction chromatography (Sepharose-4B, L-tyrosine, 9-aminophenanthrene) were analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 3 contained 3 µg of various molecular mass standards: ß-galactosidase, (116,0), bovine serum albumin (66.0), ovalbumin (45.0), carbonic anhydrase, (33,0), ∞-lactoglobulin (25.0), lysozyme (19.5). Thirty microgram of purified human serum paraoxonase Q type (lane 1) and paraoxonase R type (lane 2) migrated with a mobility corresponding to an apparent Mr (relative molecular mass) of 43.0 kDa.

As can be seen in Fig. 2, all of the selected pesticides inhibited the human serum PON1Q and R activities *in vitro*. The kinetic parameters for the various pesticides are presented in Table 1. The IC_{50} values for the pesticides on purified Q and R isoenzymes were determined to be quite different because they were more inhibiting purified human serum PON1Q192 activity than that of PON1R192.

The next step was to study the kinetics of interaction of these pesticides with the purified human Q and R isoenzymes. Two different concentrations of these pesticides were used for the determination of inhibition types. Inhibition properties of purified Q and R isoenzyme solution by the pesticides were investigated with paraoxon as substrate at pH 8.0 (see Table 1). The corresponding inhibition types were determined by the method of Lineweaver–Burk (Fig. 3). The Lineweaver-Burk double-reciprocal plot was analyzed with a range of paraoxon concentrations (0.6 –1.2 mM). The data indicate different inhibition of PON1 activity by the pesticide types (Table 1). Agrofarm is a competitive inhibitor and the other pesticides are non-competitive inhibitors for R isoenzyme, whereas Purtapyr is non-competitive and the others are competitive for Q isoenzyme. Relative studies have not reported investigations of the inhibition of paraoxonase Q and R isoenzymes as substrate using paraoxon.

Individual response to pesticide exposure may be affected by polymorphisms in genes affecting pesticide metabolism. Animal studies suggested that changes in serum paraoxonase activity alter susceptibility to organophosphate (OP) toxicity [22]. The role of genetic polymorphisms as determinants of health was explored in many areas of public health research, including epidemiological and toxicological studies [23]. The evidence of the wide variation in serum PON1 activity among individuals [9] raised the hypothesis that subjects with low PON1 activity might be more susceptible to the toxicity of some OPs, such as methyl parathion and chlorpyrifos, which are hydrolyzed faster by the R alloenzyme. Epidemiological studies have explored the role of PON1Q192R genotype or phenotype on the susceptibility to OP exposure to develop neurological toxicity and health problems [24-27], and DNA damage in somatic cells [28].

The Vmax and K_M values (Q isoenzyme: 55 EU and 0.599 mM; R isoenzyme: 50 EU and 0.492 mM) were quite similar as in several studies from different labs [16]. Eckerson et al. [14] reported 0.43 mM for the paraoxonase type Q enzyme and 0.46 mM for the paraoxonase type R enzyme.

Many pesticides, at relatively low dosages, affect the metabolism of biota by altering normal enzyme activity. The pesticides employed in this present study showed inhibitory effects on the activity of Q and R isoenzymes to various degrees. Turan et al. [29] reported that some pesticides (Folidol, Amina, Trimidal, Fusilade and Rubigan) were effective inhibitors for carbonic anhydrase enzyme. Verep [30] reported from bioassay tests of *Leuciscus cephalus* with some pesticides; deltamethrin was the most

toxic compound in that study, when compared to malathion and carbaryl (1-naphthyl methylcarbamate).