Differential hydrolysis of homocysteine thiolactone by purified human serum ^{192}Q and ^{192}R PON1 isoenzymes

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ABSTRACT

Human serum paraoxonase 1 (PON1) is a HDL-associated enzyme that catalyzes the hydrolysis of a variety of aromatic carboxylic acid esters and several organophosphates. Recently it has been suggested that a physiological substrate of serum PON1 is homocysteine thiolactone which is a putative risk factor in atherosclerosis. In this study, human ^{192}Q and ^{192}R PON1 isoenzymes were purified from the respective phenotype human serum, using a protocol consisting of ammonium sulfate precipitation and four chromatography steps: gel filtration, ion-exchange, non-specific affinity, and a second ion-exchange. Using paraoxon as substrate, overall purification fold was found as 742 for ^{192}R PON1 and 590 for ^{192}Q PON1. The final purified enzymes were shown as single protein bands close to 45 kDa on SDS-PAGE and confirmed by Western blot. Substrate kinetics were studied with phenyl acetate, paraoxon and homocysteine thiolactone. Both PON1 isoenzymes showed mixed type inhibition with phenyl acetate. K_m values of ^{192}Q and ^{192}R PON1 for homocysteine thiolactone were 23.5 mM and 22.6 mM respectively. For ^{192}R PON1, the V_{max} was 2.5-fold and k_{cat}/K_m was 2.6-fold higher than those for ^{192}Q PON1 when homocysteine thiolactone is used as substrate. The present data suggest that defining ^{192}Q and ^{192}R PON1 isoforms could be a good predictor and prognostic marker in the cardiovascular risk assessment.

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1. Introduction

Human serum paraoxonase-1 (EC 3.1.8.1, also known as arylesterase), is a 43–45 kDa glycosylated protein comprising of 354 amino acids. Paraoxonase-1 (PON1) is a calcium dependent esterase that hydrolyzes multiple classes of substrates [1,2]. Among these classes are arylesters (including phenyl acetate), lactones, and organophosphate oxons (e.g., chlorpyrifos oxon, diazoxon and paraoxon) [3–5]. Sarin and soman are also hydrolyzed by PON1 [6], and it is thought that PON1 contributes to the detoxification of organophosphate pesticides and nerve gases [7]. However, all of these activities toward man-made chemicals are promiscuous activities of PON1 rather than its primary function.

PON1 exhibits a substrate-dependent polymorphism for the hydrolysis of organophosphorus toxicants in human populations [6,8]. In this regard, most investigations that focused on an amino acid substitution at position Q192R found that some substrates, such as paraoxon, are hydrolyzed faster by the R isoenzyme, whereas other substrates, such as phenyl acetate, are hydrolyzed at the same rate by both isoenzymes. It is also found that other sub-

strates such as diazoxon and the nerve gases soman and sarin, are hydrolyzed more rapidly by the Q isoenzyme [5,6,9,10].

PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoprotein (HDL) particles [11]. PON1 contributes to the protective effect of this lipoprotein on low-density lipoprotein (LDL) oxidation and stimulates cholesterol efflux from macrophages [7,12–14]. But these activities have not been directly linked with PON1's hydrolytic activities [12–14]. Recently, PON1 has been shown to catalyze the hydrolysis of aromatic and aliphatic lactones as well as lactone formation [3,12–17]. Jakubowski [15] suggested that a physiological substrate of serum PON1 is homocysteine thiolactone which is a known risk factor in atherosclerosis. It has been shown that the PON1 protein has homocysteine thiolactonase (HTase) activity and protects against protein homocysteinylation in vitro [15–17]. The anti-atherosclerotic activity has also been associated with phospholipase A2 (PLA2)-like activity [18], but this observation has later been ascribed to contaminations rather than genuine PON1 activity [19,20].

The catalytic mechanism and the physiological function of human PON1 remain unclear, whilst there is consensus that the enzyme has a protective effect. The anti-atherosclerotic activity of PON1 is closely linked to its localization on HDL [11,21,22]. When PON1 and other HDL-associated proteins (PON3, PAFAH, Apo A–I) are considered, it is apparent that purifying PON1 is a challenging

procedure requiring a variety of steps. In addition, the presence of high amount of serum albumin makes the process more difficult. Thus, the objectives of the present study are as follows: (i) to purify [192]Q and [192]R isoenzymes of PON1 from the respective phenotype human serum using a novel and practical method, and (ii) to investigate the kinetic behavior of purified isoenzymes, with particular emphasis on the HTase activity of the enzyme.

2. Materials and methods

2.1. Chemicals

Phenyl acetate, paraoxon (*O,O′*-diethyl-*p*-nitrophenyl phosphothioate), homocysteine thiolactone, bicinchoninic acid protein assay kit (BCA), Coomassie Brilliant Blue G 250, Sephacryl S300HR, DEAE Trisacryl M, and Cibacron Blue were provided by Sigma–Aldrich (USA). All other chemicals were provided from Merck (Germany).

2.2. Protein determination

The protein content of fractions collected at the chromatography steps was monitored by measuring A_{280}. Protein concentration in individual samples was estimated by the BCA method [23].

2.3. Paraoxonase activity

Paraoxonase activity towards paraoxon was measured spectrophotometrically at 412 nm. In a typical experiment, a cuvette contained final concentration of 1 mM paraoxon dissolved in methanol in 100 mM Tris–HCl (pH 8.5), 2 mM CaCl$_2$, and 5–50 μl enzyme in a total volume of 1 ml, such that the methanol content of the measurement medium should be under 5%. Assays were followed up to 180 s at 37 °C. The reaction was linear during this period. All rates were determined in triplicate and corrected for non-enzymatic hydrolysis. One unit of paraoxonase activity is equal to 1 nmol of diethyl *p*-nitrophenyl phosphate hydrolyzed/min/mg protein.

2.4. Arylesterase activity

Arylesterase activity towards phenyl acetate was measured spectrophotometrically at 270 nm. Reaction mixtures contained 50 mM Tris–HCl (pH 8.0), 1 mM CaCl$_2$, 1 mM phenyl acetate and 5–20 μl enzyme in a total volume of 1 ml. Assays were measured for up to 60 s at 25 °C. The reaction was linear during this period. All rates were determined in triplicate and corrected for non-enzymatic hydrolysis. One unit of arylesterase activity is equal to 1 μmol of phenyl acetate hydrolyzed/min/mg protein.

2.5. Homocysteine thiolactonase activity

Activity of HTase was determined at 37 °C according to Ellman's method [24]. HTase activity was evaluated by measuring the increase in absorbance of free sulfhydryl groups reacting with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) in 50 mM Hepes buffer (pH 7.4). Reaction mixtures contained 50 mM Hepes buffer (pH 7.4), 1 mM L-homocysteine thiolactone as substrate, and 5–20 μl enzyme in a total volume of 1 ml. Assays were carried out in triplicate and one unit of HTase activity was defined as 1 μmol of DTNB hydrolyzed per minute.

2.6. Substrate and inhibition kinetics with PON1 isoenzymes

Purified [192]Q and [192]R PON1 were subjected to substrate kinetics using paraoxon, phenyl acetate, and homocysteine thiolactone as substrates in the concentration range 0.1–2 mM, 0.125–2.25 mM and 0.5–12 mM, respectively.

The inhibition effect of phenyl acetate on the paraoxonase activity was investigated by measuring the initial velocities at the 0.15, 0.24, 0.48, 0.8 and 1.2 mM paraoxon concentrations against increasing phenyl acetate concentrations.

2.7. Analysis of the kinetic data

Kinetic parameters (V_{max}, K_m, k_{cat}/K_m, and K_i) with phenyl acetate (arylesterase activity), paraoxon (paraoxonase activity), and homocysteine thiolactone (thiolactonase activity) were calculated using non-linear regression module of Systat (version 11, 2005) software. The mode of inhibition was confirmed by Dixon graph by plotting the increasing phenyl acetate concentrations against $1/v$ values and the secondary replot was obtained by using the Dixon plot slope values versus reciprocal of paraoxon concentrations.

2.8. Purification of PON1 enzyme from human serum

All purification procedures were carried out at 4 °C unless otherwise stated.

2.8.1. Enzyme source and phenotyping

PON1 was purified from the serum of healthy human volunteers previously identified as homozygous for [192]Q or [192]R. Phenotyping was done as previously described [25]. To purify PON1, 20 ml serum was pooled and stored at −20 °C. Before use, the serum was thawed and assayed for total protein and paraoxonase/arylesterase activities. The study was approved by the Hacettepe University Faculty of Medicine Ethics Committee (2004/25-13; 04.11.2004).

2.8.2. Ammonium sulfate precipitation

At the first purification step, paraoxonase activity was tested in the range of 20–85% ammonium sulfate. The enzyme was found to precipitate between 35% and 70% interval. The precipitate was collected by centrifugation at $4000 \times g$ for 20 min, redissolved in 20 mM Tris–HCl buffer pH 8.0 and dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM CaCl$_2$. After dialysis, the sample was concentrated to 5 ml using a Centricon 30 microconcentrator (Amicon, USA).

2.8.3. Sephacryl S300HR gel exclusion chromatography

The concentrated sample was loaded on to a column of Sephacryl S300HR (105 cm × 2.44 cm size) at a flow rate of 12.6 ml/h, pre-equilibrated in 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM CaCl$_2$ (buffer A). Each elution fraction was monitored for paraoxonase/arylesterase activity, possible contamination of serum cholinesterase activity and protein concentration. Active fractions (40 ml), eluting between LDL and serum albumin and free of cholinesterase contamination (data not shown), were pooled and used for the next step of the purification.

2.8.4. First DEAE-Trisacryl M ion-exchange chromatography

The pooled fractions were mixed with 40 ml (packed volume) of anion exchanger DEAE-Trisacryl M, which was pre-equilibrated overnight with 20 mM Tris–HCl, pH 8.0 buffer containing 2 mM CaCl$_2$ (buffer B). The suspension was mixed and centrifuged at $1500 \times g$ for 10 min to pack the gel, and the supernatant was decanted and discarded. The packed DEAE-Trisacryl M was resuspended in 40 ml of 2 mM CaCl$_2$ solution, mixed and respun. The gel was washed 4 times by this batch technique and then transferred to a 20 cm × 2 cm Pharmacia column as slurry for subsequent elution. This was accomplished using a linear gradient of 0.0–0.4 M NaCl in the buffer B. The elution fractions with the highest

arylesterase/paraoxonase activity were pooled for further purification.

2.8.5. Cibacron Blue 3GA non-specific affinity chromatography

The pooled fractions from DEAE-Trisacryl M chromatography were mixed with an equal volume of blue agarose (Cibacron Blue 3GA Type 3000-CL), which was pre-equilibrated overnight with buffer B containing 4 M NaCl. The mixture was transferred to a column (15 cm × 2.5 cm size) and then separately washed four times with 100 ml of the buffer B, containing 4 M, 3 M, 2 M and 1 M NaCl, respectively. By the fourth wash, absorption at 280 nm should be less than 0.02. The blue agarose gel was then washed twice with 100 ml of buffer B to reduce the ionic strength. The bound enzyme was eluted from the column by using buffer B containing 0.1% sodium deoxycholate. Fractions with the highest arylesterase/paraoxonase activities were pooled and used for second DEAE Trisacryl M chromatography.

2.8.6. Second DEAE-Trisacryl M ion-exchange chromatography

The active fractions containing paraoxonase/arylesterase activity were pooled and Triton X-100 concentration was adjusted to 0.1%. To this mixture, an equal volume of anion exchange DEAE-Trisacryl M, which was equilibrated overnight with buffer B containing 0.1% Triton X-100 was added. The gel was washed 3 times by the batch technique described above and then transferred to the column (20 cm × 2 cm size). The column was washed with buffer B containing 0.1% Triton X-100 and 15 mM NaCl until the absorbance of the eluate at 280 nm was close to zero. Then the enzyme was eluted with a linear gradient of 15–400 mM NaCl in the buffer B containing 0.1% Triton X-100. Fractions with the highest PON1 activity were pooled, dialyzed and concentrated by using a Centricon 30 microconcentrator (Amicon, USA).

2.8.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Protein fractions from different purification steps were subjected to SDS-PAGE in a discontinuous system of Laemmli [26]. The polyacrylamide concentration was 10% (w/v) for the separating gel and 4% for the stacking gel. The samples were run at room temperature at 100 V for 90 min. The gels were stained with Coomassie Brilliant Blue R-250.

The protein sample from the last purification step was electrophoresed and blotted onto a nitrocellulose membrane at 80 V for 2 h. The membrane was blocked by 5% dry milk in TBS (Tris-buffered saline) and incubated with goat polyclonal anti-PON1 (1:1000, Santa Cruz Biotechnology) for 1 h. Following wash with TBS, the membrane was incubated with anti-goat-HRP (1:5000, Santa Cruz Biotechnology) for 1 h as secondary antibody. Enhanced chemiluminescence (ECL) kit from Amersham [27] was used for protein visualization.

3. Results and discussion

3.1. Purification of PON1 enzyme from human serum

Although there are numerous publications on PON1, there is a lack of a consensus on its substrate range and specificity. Thus the requirement for novel purification procedures which enable the identification of functional roles of PON1 keep their actuality. In addition to PON1, HDL includes several enzymes such as lecithin:cholesterol acyltransferase (LCAT), platelet-activating factor acetylhydrolase (PAFAH) and PON3. PON1 is also closely associated with apolipoprotein A–I (Apo A–I) on HDL. Therefore, difficulties related to the elimination of these potentially contaminant proteins are encountered during purification procedures. Another difficulty exists in the elimination of the highly
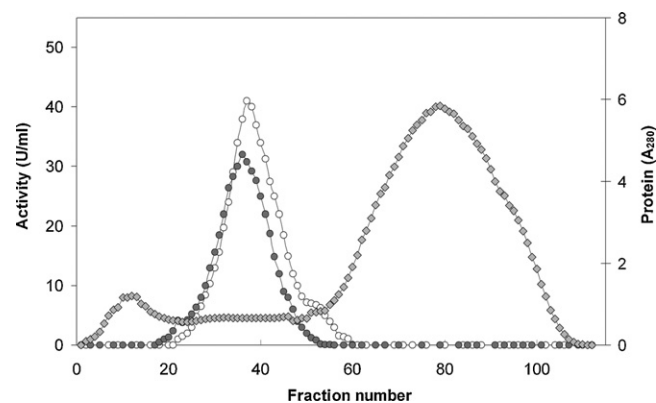

Fig. 1. Sephacryl S300HR gel filtration of PON1 from human serum. A 5 ml sample of human serum was fractionated on 105 cm × 2.44 cm Sephacryl S300HR column in 20 mM Tris–HCl buffer (pH 8.0) including 1 mM CaCl$_2$, and 2 ml fractions were collected. Paraoxonase activity was assayed with *p*-nitrophenyl phosphate as substrate (○). Arylesterase activity was measured from hydrolysis of phenyl acetate (●). Diamond squares (♦) represent A_{280} profile.

abundant serum protein, albumin. In this study we present a purification method for human serum PON1 that differs from previously reported methods and that yields a highly pure enzyme with substantial activity. Purification of PON1 from human serum of [192]Q and [192]R isoenzymes is summarized in Table 1. In our method, the volume of human serum used is as low as 20 ml. The purification procedure involved five sequential steps: ammonium sulfate precipitation, gel filtration chromatography, ion-exchange chromatography, non-specific affinity chromatography and a second ion-exchange chromatography.

Prior to the application of gel filtration chromatography, ammonium sulfate precipitation was conducted as a pre-purification method. A considerable amount of paraoxonase enzyme with high activity was obtained at 35–70% ammonium sulfate. Later this fraction was concentrated up to 5 ml, the optimal volume required for the gel filtration chromatography sample.

There are mainly four lipoprotein classes in the serum, namely; HDL, LDL, VLDL and chylomicrons with respective diameters of 5–17 nm, 18–25 nm, 25–80 nm and 80–1200 nm [28]. The main reason for preferring gel filtration as the first chromatography step is that serum PON1 enzyme is bound to HDL and since there is a significant size difference between HDL and the other lipoproteins, gel filtration allows a better resolution between lipoproteins.

When the 5 ml sample was applied to the column of 105 cm × 2.24 cm with a flow rate of 12.6 ml/h, HDL was found to be significantly eluted between LDL and albumin (Fig. 1). It has been reported in the previous studies that PON1 purification products were sometimes contaminated with small amounts of cholinesterase, albumin and/or Apo A–I. It has been shown that cholinesterase contributes slightly to 'arylesterase' activity of PON1 due to its well-established phenyl acetate hydrolyzing capacity, whereas serum albumin to a certain degree contributes to 'paraoxonase' activity [1,29]. Gan et al. [1] reported that a small portion (less than 5%) of total paraoxonase activity might be due to serum albumin. In our procedure, cholinesterase and albumin were significantly eliminated at the gel filtration step due to their molecular sizes.

The pooled active fractions from this gel filtration chromatography were applied to DEAE Trisacryl M ion-exchange column and eluted in a single narrow peak at 243 mM NaCl concentration. The pooled sample from this first DEAE column (about 21 ml) was applied to Cibacron Blue. This chromatography is based on the hydrophobic interaction between apolar ligand of the matrix and the target biomolecule at high salt concentration. A recent study

Table 1
Summary of the purification of human serum ^{192}Q and ^{192}R PON1 isoenzymes.

	Arylesterase				Paraoxonase			
	Specific activity (U/mg protein)	Total activity (U)	Fold	Yield	Specific activity (U/mg protein)	Total activity (U)	Fold	Yield
^{192}Q PON1								
Serum	1.22	1592	1.0	100.0	1.88	2415	1.0	100.0
Ammonium sulfate	1.83	1492	1.5	93.7	2.75	2114	1.5	87.7
Sephacryl S300HR	26.00	992	21.7	62.3	32.00	1216	16.2	50.5
DEAE-Trisacryl M 1	72.00	666	59.0	41.8	87.50	809	44.2	35.5
Cibacron Blue 3GA	122.00	378	100.0	23.7	138.50	429	70.0	17.8
DEAE-Trisacryl M 2	970.00	146	795.0	9.2	1167.60	176	589.7	7.3
^{192}R PON1								
Serum	1.34	1700	1.0	100.0	4.34	5512	1.0	100.0
Ammonium sulfate	2.01	1578	1.5	92.8	6.15	4830	1.4	87.6
Sephacryl S300HR	28.85	1053	21.5	61.9	76.12	2778	17.5	50.4
DEAE-Trisacryl M 1	80.57	709	60.0	41.7	211.80	1864	48.8	33.6
Cibacron Blue 3GA	143.50	402	107.0	23.6	387.14	1084	89.2	17.9
DEAE-Trisacryl M 2	1048.00	152	783.0	8.9	3221.00	467	742.0	8.5

reports a faster and simpler purification method, which does not require any non-specific affinity chromatography step, such as the Cibacron blue, stating that such a procedure has multiple steps and longer purification time [30]. However, given the particularly high amount of serum albumin in addition to the complex structure of HDL, we thought that it was not likely to purify PON1 to homogeneity without the non-specific affinity chromatography step.

It is thought that certain phospholipids and complexes structurally bearing apolipoproteins (in particular Apo A–I and Apo J) provide the optimal medium for the activity and stabilization of serum PON1 and its interaction with physiologic substrates [10,31]. Besides, PON1 can also interact with amphipathic complexes such as phospholipid micelles and detergent molecules [32]. Triton X-100 is a non-ionic detergent used for the solubilization of PON1 from HDL. Micelle solutions obtained with an appropriate concentration of Triton X-100 such as 0.1% maintains the enzyme activity by stabilizing PON1 in a manner similar to HDL [33]. Thus in our study, solubilization of PON1 was performed at the final step of the purification process. This was based on the fact that PON1 activity and stability can be affected from the potential changes (in particular during gel filtration) in the size and form of PON1 micelles solubilized from HDL. Incubation of the enzyme with Triton X-100 overnight helps to separate the enzyme from lipoproteins such as HDL which affect the enzyme binding to ion-exchange column. The bound enzyme was eluted in a single peak at 206 mM NaCl concentration. Purified PON1 was stable for at least two weeks at 4 °C.

Paraoxonase and arylesterase activities co-eluate at all chromatographic steps of the purification procedure. However, a slight difference was found in the chromatographic pattern of ^{192}Q and ^{192}R phenotypes. Table 1 clearly shows that each purification step of our procedure yielded better final specific activity and purification fold values as compared to previous purifications from different
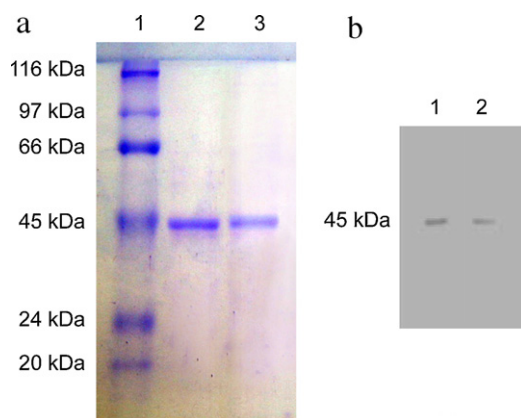

Fig. 2. Characterization of the purified human serum PON1. (a) The sample from the second ion-exchange chromatography was analysed by SDS-PAGE and revealed by Coomassie Blue staining. Line 1 contained 20 μg of various molecular-mass standards: β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa). Lines 2 and 3 contained 10 μg of purified human serum ^{192}Q PON1 and ^{192}R PON1 respectively. (b) Western blot analysis of the purified human serum ^{192}Q PON1 (line 1) and ^{192}R PON1 (line 2).

sources [30,34–37]. Fig. 2 displays the results of SDS-PAGE analysis that shows the final purified enzyme as a single protein band close to 45 kDa (Fig. 2a). The specificity of the band is further confirmed by Western blot (Fig. 2b). This finding is in accordance with previous studies that demonstrate PON1 band at 45 kDa [1,38,39], but purified PON1 has also been shown to be a doublet within the 37–48 kDa range, depending on the degree of protein glycosylation [2].
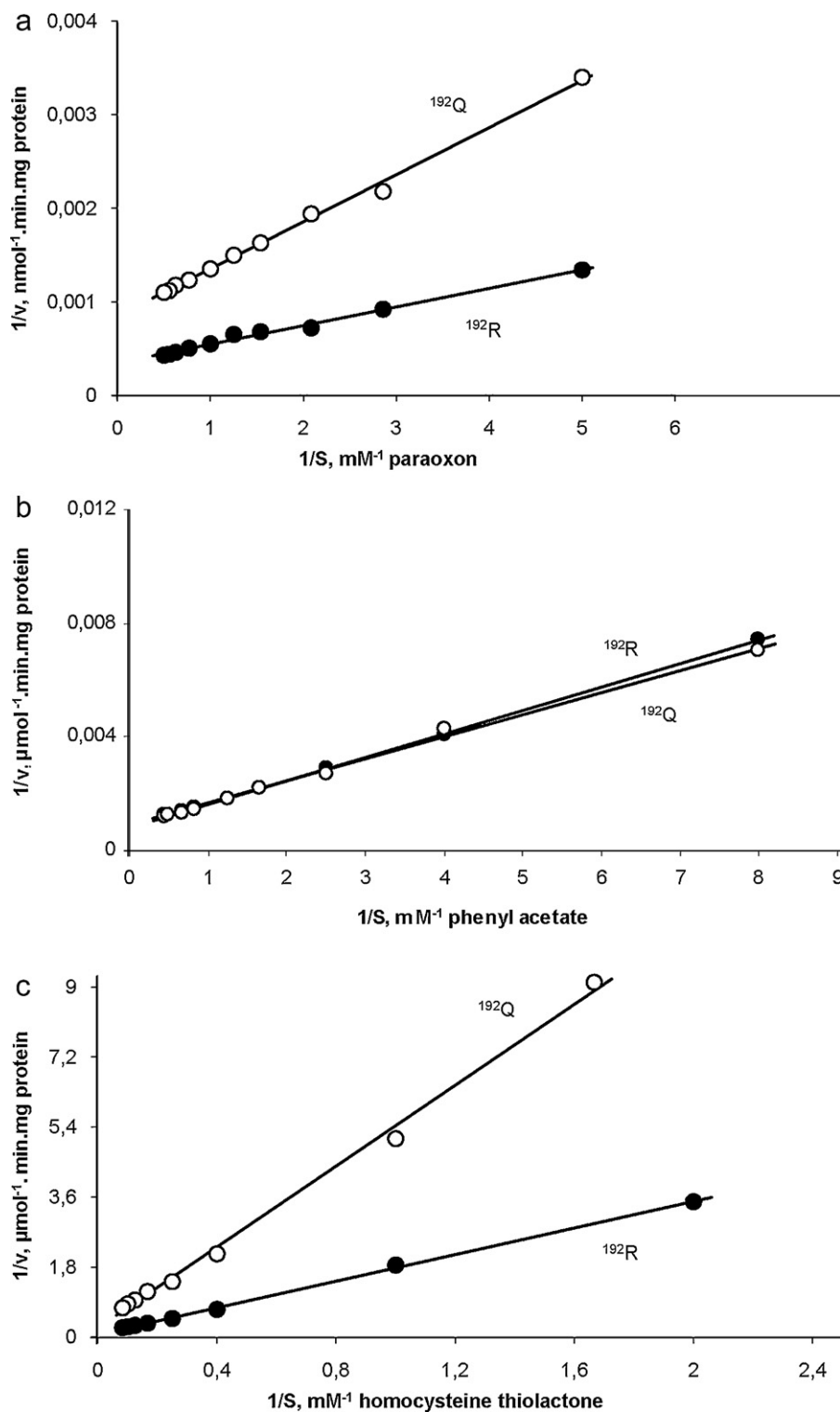
Table 2
Kinetic data for purified human serum ^{192}Q and ^{192}R PON1 isoenzymes.

Substrate		^{192}Q PON1	^{192}R PON1
Phenyl acetate	V_{max} (μmol/min mg protein)	1167	1208
	K_m (mM)	0.98	0.92
	k_{cat}/K_m (M^{-1} min^{-1})	50×10^6	55.2×10^6
Paraoxon	V_{max} (μmol/min mg protein)	50	2.88
	K_m (mM)	0.96	0.56
	k_{cat}/K_m (M^{-1} min^{-1})	0.067×10^6	0.22×10^6
Homocysteine thiolactone	V_{max} (μmol/min mg protein)	0.0045	0.011
	K_m (mM)	23.5	22.6
	k_{cat}/K_m (M^{-1} min^{-1})	8	21.2

Fig. 3. Lineweaver-Burk plot of purified ^{192}Q PON1 (○) and ^{192}R PON1 (●) for (a) paraoxon hydrolysis (r=0.998 and 0.995 respectively), (b) phenyl acetate hydrolysis (r=0.998 and 0.992 respectively) and (c) homocysteine thiolactone hydrolysis (r=0.985 and 1.0 respectively).

3.2. Analysis of the kinetic data of purified ^{192}Q and ^{192}R PON1

The kinetics of substrate hydrolysis for paraoxon, phenyl acetate, and homocysteine thiolactone were determined for the purified ^{192}Q and ^{192}R PON1. Fig. 3a–c show typical Lineweaver-Burk plots [40] of paraoxon, phenylacetate and homocysteine thiolactone hydrolysis by purified ^{192}Q and ^{192}R PON1. The kinetic data are summarized in Table 2. Kinetic parameters (K_m and V_{max})

were analysed by means of non-linear regression module of Systat (version 11, 2005). It is reported that although ^{192}R isoenzyme displays a higher activity against paraoxon than ^{192}Q isoenzyme, their activity towards phenyl acetate is not so different [6]. For phenyl acetate, K_m and V_{max} values for ^{192}Q and ^{192}R PON1 found in our study were almost identical, which is compatible with other reports [41,42]. ^{192}R PON1 showed higher hydrolytic activity (V_{max} = 2.88, k_{cat}/K_m = 0.22) against paraoxon than ^{192}Q PON1

($V_{max} = 0.96$, $k_{cat}/K_m = 0.067$), and this trend is similar to those reported by Josse et al. [43]. The reported K_m values in the literature are various probably due to differences in enzyme source, pH, substrate concentration, salt and detergent content (type and concentration) of the assay. It is not clear whether the catalytic efficiency of PON1 towards the organophosphates, arylesters and lactones occurs at a single or multiple active centers. The exact molecular basis of substrate selectivity is not known, either. The kinetic data of paraoxonase activity in the presence of increasing phenyl acetate [0.1–1 mM] concentrations were plotted on a $1/v$ versus [phenyl acetate] graph for both isoenzymes (Fig. 4a and b). In this Dixon graph it can be observed that the lines intersect at the second quadrant, which is observed in both competitive and mixed type inhibitions. Replot of slopes versus paraoxon concentrations (Fig. 4c) revealed the inhibition type as linear mixed type. The K_i values obtained with phenyl acetate for the pure ^{192}Q and ^{192}R PON1 phenotypes were 0.78 and 0.73 mM, respectively. In mixed type inhibition, the inhibitor is bound both to the ES complex and the free enzyme. Since phenyl acetate is a substrate of PON1, it may use the same substrate binding site with paraoxon and competitively
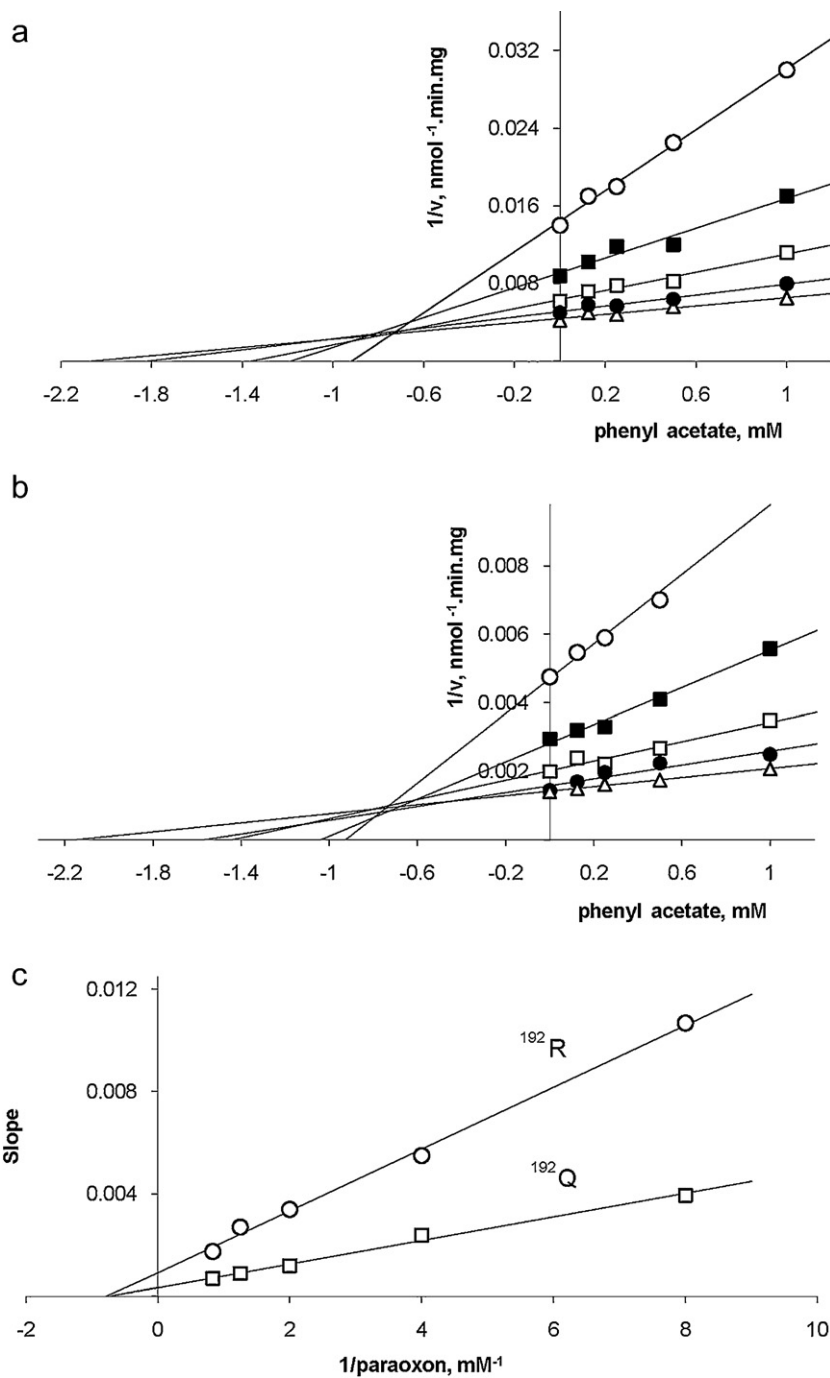

Fig. 4. Inhibiton of purified PON1 isoenzymes at different concentrations of phenylacetate using paraoxon as substrate. The paraoxon concentrations used in the assay were: (○) 0.15 mM; (■) 0.24 mM; (□) 0.48 mM; (♦) 0.8 mM; (△) 1.2 mM. The inhibitor phenyl acetate was used in the range of 0.1–1 mM. (a) Dixon plot of ^{192}R PON1 [0.15 mM paraoxon ($r = 0.993$); 0.24 mM paraoxon ($r = 0.990$); 0.48 mM paraoxon ($r = 0.995$); 0.8 mM paraoxon ($r = 0.990$); 1.2 mM paraoxon ($r = 0.992$)]. (b) Dixon plot of ^{192}Q PON1 [0.15 mM paraoxon ($r = 0.995$); 0.24 mM paraoxon ($r = 0.997$); 0.48 mM paraoxon ($r = 0.990$); 0.8 mM paraoxon ($r = 0.990$); 1.2 mM paraoxon ($r = 0.985$)]. (c) Dixon slope replots for typical PON1 ^{192}Q and ^{192}R isoenzymes. The slopes of the Dixon plots were replotted as a function of 1/[paraoxon] ($r = 0.993$ for ^{192}R PON1); ($r = 0.996$ for ^{192}Q PON1).

inhibit its hydrolysis. Alternatively, it may as well use a different binding site and induce a conformational change that renders the enzyme inactive.

PON1 hydrolyzes a wide range of substrates, such as esters, thioesters, phosphotriesters, carbonates, lactones, and thiolactones. The highest activities observed thus far are with synthetic substrates such as phenyl acetate and dihydrocoumarin ($k_{cat}/K_m \geq 10^6 \, M^{-1} \, s^{-1}$) [10,44], which have no physiological relevance. It is therefore unlikely that these are PON1's native substrates. Recently, thiolactonase activity of PON1 was described, including those with thiolactones of potential physiological relevance such as products of protein-N-homocysteinylation [45]. HTase is actually identical with paraoxonase/arylesterase activity. Its natural function is likely to detoxify homocysteine thiolactone by hydrolyzing it to homocysteine [45]. Homocysteine thiolactone reacts with amino groups of lysine residues forming homocysteine-N-lysine-protein. Protein N-homocysteinylation is a novel example of protein modification that may explain the involvement of homocysteine and its metabolite, homocysteine thiolactone in the pathology of human disease [46]. In our study, ^{192}R isoenzyme had higher HTase activity (k_{cat}/K_m) than ^{192}Q isoenzyme (Table 2). K_m value for homocysteine thiolactone of ^{192}Q and ^{192}R PON1 was 23.5 and 22.6 mM respectively. Although K_m for homocysteine thiolactone is relatively high, other enzymes also have high K_m values for physiologically significant substrates (such as carbonic anhydrase (32 mM) and cystathionine β-synthase (25 mM)) [45]. This is the first study in literature describing the native HTase activity of purified human ^{192}Q and ^{192}R PON1 isozymes.

PON1 polymorphism at codon 192 can be considered as the basis of the apparent differences observed in enzyme activity among different individuals and ethnic groups. Several studies reported that the QQ genotype was more common in European and Turkish populations than the RR genotype. Also individuals with QQ genotype displayed significantly lower serum paraoxonase activity as compared to the RR genotype [47,48]. However, there are few studies where the effects of these polymorphisms on HTase activity in human serum were investigated. A study on subjects with cardiovascular disease and healthy controls, high HTase activity was associated with ^{192}R, whereas low HTase activity was associated with ^{192}Q [49]. Similar findings were also reported by Kerkeni et al. [50]. High activity form of HTase provides better protection against protein homocysteinylation than the low activity form [51]. Because PON1 is a component of HDL, these data offer a possible explanation for the inverse relationship between HDL levels and the coronary heart diseases in the relevant populations. In fact, *in vitro* studies showed a reduced ability of ^{192}Q PON1-HDL to protect LDL from oxidative modification [51].

In conclusion, this is the first study investigating the kinetics of purified PON1 ^{192}Q and ^{192}R isoenzymes using homocysteine thiolactone as a substrate. Our data showed that the HTase activity of ^{192}R isoenzyme was higher than the ^{192}Q isoenzyme. We suggest that, defining ^{192}Q and ^{192}R PON1 isoforms could be a good predictor and prognostic marker in the cardiovascular risk assessment.

Acknowledgements

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