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Purification human PON1$_{Q192}$ and PON1$_{R192}$ isoenzymes by hydrophobic interaction chromatography and investigation of the inhibition by metals

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ABSTRACT

In this study, a new purification strategy for human PON1 enzyme was developed using two-step procedures, namely ammonium sulfate precipitation and sepharose-4B-L-tyrosine-9-aminophenantrene hydrophobic interaction chromatography. SDS polyacrylamide gel electrophoresis of the enzyme indicates a single band with an apparent MW of 43 kDa. Overall purification rate of our method was found 901-fold for R isoenzyme and 453-fold for Q isoenzyme. The V_{max} and K_M of the purified enzyme were determined for Q isoenzyme 55 EU and 0.599 mM and for R isoenzyme 50 EU and 0.492 mM, respectively. The *in vitro* effects of some heavy metals (Hg, Cd, Cu, Mn and Ni) were investigated on the purified human serum PON1Q and R isoenzyme, using paraoxon as substrate. Metals were more effective inhibitors on purified human serum PON1$_{R192}$ activity than PON1$_{Q192}$ activity. The kinetics of interaction of metals with the purified human serum PON1$_{R192}$ and PON1$_{Q192}$ indicated a different inhibition pattern. Kinetic constants K_M, V_{max}, and *inhibition type* were determined.

1. Introduction

Paraoxonase (EC 3.1.8.1, PON1) is a calcium dependent serum esterase that is synthesized by the liver. In serum, it is closely associated with high-density lipoproteins [1,2]. Paraoxonase hydrolyze organophosphate compounds are widely used as insecticides and nerve gases. Therefore, PON1 plays 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. In addition, paraoxonase is involved in lipid metabolism, since this enzyme 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].

The physiological substrates of PON1 are still unknown, but structure-reactivity studies [5] and laboratory evolution experiments [6] indicate that the native activity of PON1 is lactonase. 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 [7,8] that have no physiological relevance. It is therefore unlikely that these are PON1's native substrates. Recently, lactonase (lactone hydrolysis) as well as lactonizing (lactone formation) activities of PON1 were described, including those with lactones of potential physiological relevance such as products of fatty acid oxidation [9,10]. These results imply that PON1 might in fact be a lactonase rather than an aryl-esterase or paraoxonase, as traditionally described.

PON1 contains two major polymorphisms as the result of amino acid substitution at position 55 (leucine vs methionine) and at position 192 (glutamine: Q vs arginine: R) [11,12]. The PON1$_{192}$ activity polymorphisms are substrate dependent. The PON1$_{Q192}$ isoform has a higher rate of *in vitro* hydrolysis of diazoxon, sarin, and soman [13], whereas the PON1$_{R192}$ isoform has a higher activity for hydrolyze of paraoxon and chloropyrifos oxon [14]. In addition the ability of HDL to protect LDL against peroxidation *in vitro* is significantly lower in HDL particles containing PON1$_{R192}$ than in those with PON1$_{Q192}$ [15].

Polymorphism of the PON1 gene effects the blood levels PON1 and its catalytic efficiency; both factors strongly effect an individual's susceptible to arteriosclerosis, pollutants and insecticides [16,17]. In addition, it supported the evidence that mice lacking PON1 are highly susceptible to arteriosclerosis and organophosphates poisoning [18].

Its native substrates, its *in vivo* mechanism of action and its molecular target(s) of PON1 remain unknown. PON1 has been recently purified in human but it is not yet commercially available. The partial purification of A-esterase (paraoxonase) was originally carried out from rabbit kidney with overall yield of approximately 13-fold [18]. Further studies on this enzyme improved the purification to 65–100-fold [19]. Sheep serum paraoxonase was purified in 330–385-fold using ethanol, pH and ionic strength fractionation [20]. Rodrigo et al. purified the liver paraoxonase in 415-fold by hydroxyapatite adsorption, chromatography on DEAE–sepharose

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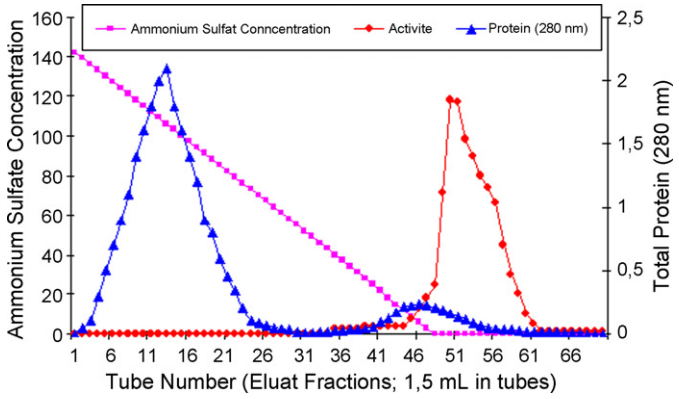

Fig. 1. Purification of human serum PON1 by hydrophobic interaction chromatography. Fractions from the ammonium sulfate extraction were pooled as described in Section 2. This material was eluted by increasing the ammonium sulfate concentration. Protein concentration was determined by measuring an absorbance of 280 nm and PON1 activities of fractions were assayed activity using paraoxon substrate. 1 unit = 1 μmol min^{-1} per ml. Abbreviation: U, units.

CL-6B, non-specific affinity chromatography on Cibacron Blue 3 GA and anion exchange on Mono Q HR 5/5 [21].

In this study, we developed a new strategy for the purification of the PON1 enzyme. Specifically, human serum PON1 was purified by two-step procedures using ammonium sulfate precipitation and sepharose-4B-L-tyrosine-9-aminophenantrene hydrophobic interaction chromatography which was specifically designed to the retained N-terminal hydrophobic signal peptide for PON1 enzyme.

However, to our knowledge, no study is available on the *in vitro* effects of metals on paraoxonase Q and R allozymes activity. In this study, we aimed to determine any possible effect of some metals on pure PON1Q and pure PON1R activity.

2. Materials and methods

The materials used include sepharose-4B, L-tyrosine, 9-aminophenantrene, paraoxon, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All

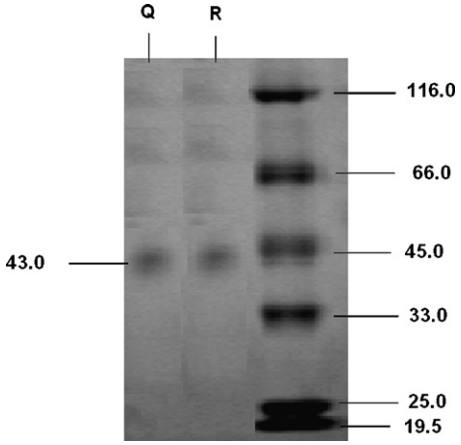

Fig. 2. SDS-PAGE of human serum paraoxonase. The pooled fractions from ammonium sulfate precipitation and hydrophobic interaction chromatography (sepharose-4B, L-tyrosine, 9-aminophenantrene) were analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 3 contained 3 μg of various molecular mass standards: β-galactosidase, (116.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase, (33.0 kDa), β-lactoglobulin (25.0 kDa), lysozyme (19.5 kDa). 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 43.0 kDa.

Table 1
Summary of the purification of human serum paraoxonase Q and R isoenzymes.

	Volume (ml)	Activity (U ml^{-1})	Total activity (U ml^{-1})	Protein amount (mg ml^{-1})	Total protein (mg)	Specific activity (U mg^{-1})	Overall yield (%)	Overall purification (fold)
Q type								
Serum	36	12.9	464.4	7.5	262.5	1.76	100	–
Ammonium sulfate fractionation	16	18.5	296.0	7.9	126.4	2.34	63.7	1.47
Hydrophobic interaction chromatography	1.5	45	67.5	0.021	0.032	2109	14.5	901
R type								
Serum	33	38.0	1254	7.3	240.009	5.21	100	–
Ammonium sulfate fractionation	11	61.0	671	8.3	1.30	7.35	55.3	1.5
Hydrophobic interaction chromatography	1.5	60.0	90	0.018	0.027	3333	6.9	453

Units: 1 μmol 4-nitrophenol formed per minute. Purification (fold): specific activity, *n* purification step/specific activity in serum. Yield: activity of fractions combined for the next purification step/total activity in serum × 100. Yields figures do not include all of the activity actually recovered. Usually, three tubes were pooled for hydrophobic interaction chromatography.

Table 2
IC_{50} values (mM) of metals on paraoxonase enzyme Q and R type.

Type	Cu	Hg	Ni	Cd	Co	Mn
Q	0.310	0.891	1.144	0.218	3.91	0.609
R	0.061	0.106	1.026	0.152	0.781	0.304

other chemicals used were analytical grade. The metal chlorides were of commercial origin and at the highest available purity (99%). They were dissolved in bidistile water (pH: 8 at 25 °C).

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

In order to classify individual phenotypes, two parameters were used. According to Eckerson et al., phenotypic distribution of the paraoxonase activity was determined by the basal and stimulation of paraoxonase activity by 1 M NaCl.

$$\frac{\text{Paraoxonase activity with 1 M NaCl} - \text{basal paraoxonase activity}}{\text{Basal paraoxonase activity}} \times 100\%$$

Individuals were classified for paraoxonase phenotype using the antimode at 60% stimulation as the dividing point between the non-salt-stimulated, Q type, and the salt-stimulated, QR (60–200%) and R (200%-up) types [22].

2.1.1. Ammonium sulfate precipitation

Human serum was isolated from fresh human blood taken to 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 sulfate precipitation (60–80%) [23]. The precipitate was collected by centrifugation at 15000 rpm for 20 min, and redissolved in 100 mM Tris–HCl buffer (pH 8.0).

2.1.2. Hydrophobic interaction chromatography

The pooled precipitate obtained from human serum by using ammonium sulfate precipitation was subjected to hydrophobic interaction chromatography. The final saline concentration of precipitate was adjusted to 1 M ammonium sulfate, prior to that it was loaded onto the hydrophobic column prepared from sepharose-4B-L-tyrosine-9-aminophenantrene. The preparation of hydrophobic column is as follows. 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 with cold 0.1 M $NaHCO_3$ buffer pH 10. L-Tyrosine by using saturated L-tyrosine solution in the same buffer was coupled to sepharose-4B-L-tyrosine activated with CNBr. The reaction was completed by stirring with a magnet for 90 min. In order to remove excess of L-tyrosine from the sepharose-4B-L-tyrosine gel, the mixture was washed with distilled water. The hydrophobic gel was obtained by diazotization of 9-aminophenantrene and coupling of this compound to the sepharose-4B-L-tyrosine. The pH was adjusted to 9.5 with 1 M NaOH and, after gentle stirring for 3 h at room temperature; the coupled red sepharose derivative was washed with 1 l of water and then 200 ml of 0.05 M Tris–sulfate pH 7.5. The column was equilibrated with 0.1 M Na_2HPO_4 buffer pH 8.00 including 1 M ammonium sulfate. The paraoxonase was eluted with ammonium sulfate gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulfate pH 8.00. The purified PON enzyme was stored in the presence of 2 mM $CaCl_2$ at +4 °C, in order to maintain activity.

2.1.3. Total protein determination

The absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulfate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [24], with bovine serum albumin standard.

2.1.4. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed in order to verify the purified enzyme. It was carried out in 12% and 3% acrylamide concentrations, containing 0.1% SDS, for the running and stacking gel, respectively, according to Laemmli [25].

2.1.5. Paraoxonase enzyme assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al. [26]. The reaction was followed for 2 min at 37 °C by monitoring the appearance of p-nitrophenol at 412 nm in Biotek 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 the calculation. PON1 activity (1 $U\,l^{-1}$) was defined as 1 μmol of p-nitrophenol formed per minute.

2.1.6. In vitro inhibition kinetic studies and determination of inhibition types

For the inhibition studies of metals different concentrations were added to the enzyme activity. Paraoxonase activity with metals was assayed by following the hydration of paraoxon. Activity % values of paraoxonase for five different concentrations of each metal were determined by regression analysis using the Microsoft Office 2000 Excel. Paraoxonase activity without a metal was accepted as 100% activity. The inhibitor concentration causing up to 50% inhibition (IC_{50} values) on metals were determined from the graphs.

In addition, inhibition types of metals were determined on paraoxonase activity. In order to obtain inhibition types, K_M and V_{max} values of the enzyme using paraoxon as a substrate were measured at seven different substrate concentrations at pH 8.0 and 37 °C. K_M and V_{max} values were determined by means of Lineweaver–Burke graphs. The seven different substrate concentrations, 0.2, 0.4, 0.6, 0.8, 1 and 1.2 mM, were added to reaction with or without metals.

3. Result and discussion

In order to classify individual phenotypes, two parameters were used. According to Eckerson et al., phenotypic distribution of the paraoxonase activity was determined by the basal and stimulation of paraoxonase activity by 1 M NaCl [22]. PON1 was purified from the healthy human volunteers previously identified as homozygous for PON1Q or for PON1R.

In this study, a new strategy for the purification of the PON1 enzyme was developed. Human serum paraoxonase was purified by two sequential procedures, ammonium sulfate precipitation followed by hydrophobic interaction chromatography specifically designed for PON1 enzyme.

Table 3
Type of inhibition of metals on paraoxonase enzyme Q and R type.

Type	Cu	Hg	Ni	Cd	Co	Mn
Q	Competitive	Noncompetitive	Competitive	Noncompetitive	Competitive	Competitive
R	Competitive	Noncompetitive	Competitive	Uncompetitive	Competitive	Competitive

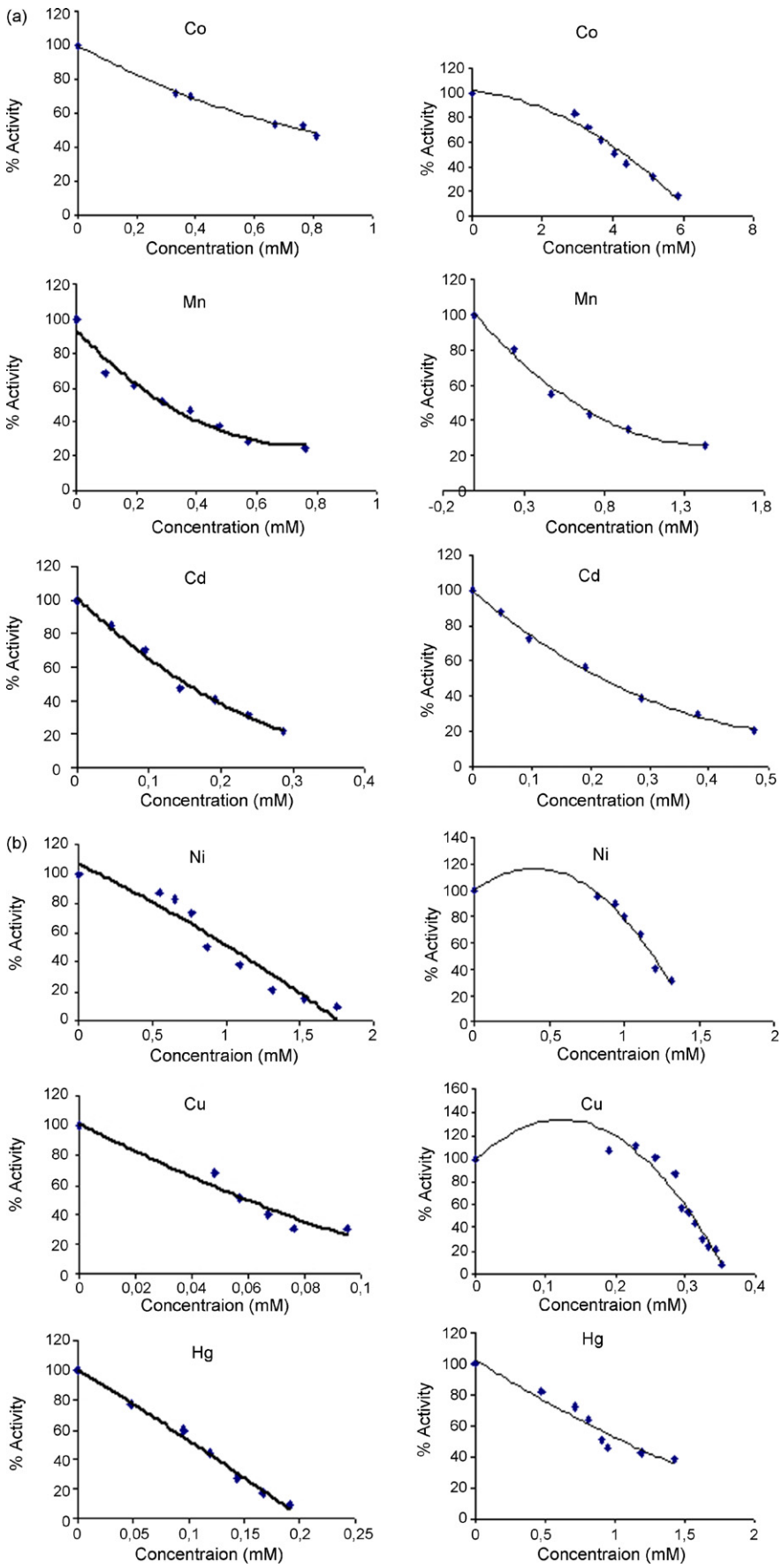

Fig. 3. (a) Inhibition of metals on paraoxonase enzyme Q and R type, respectively. (b) Inhibition of metals on paraoxonase enzyme Q and R type, respectively.

Subsequently, prior to loading onto hydrophobic interaction column; the precipitate was saturated with 1 M Ammonium sulfate in order to improve its efficiency for binding to hydrophobic gel of the column. A new hydrophobic gel has been synthesized in order to reduce the number of the purification steps of paraoxonase enzyme. The hydrophobic gel was designated based on the retained N-terminal hydrophobic signal peptide for PON1 enzyme. 9-Aminophenantrene, which is a hydrophobic group, was added to sepharose-4B gel matrix with the extension of L-tyrosine arm.

Fig. 1 shows the typical elution pattern of the enzyme activity on hydrophobic column. The enzyme activity and total protein concentration were determined from all fractions collected from each purification step. The fractions with the highest paraoxonase activity and the lowest protein contents, 51, 52 and 53 tubes were pooled. Finally, PON1R 901-fold and PON1Q 453-fold was purified.

In another study, paraoxonase activity from pooled plasma of Q and R phenotypes shows considerable variation 122.7 and 737 units, respectively [26]. As seen in Table 1, each purification step yielded excellent results compared to the final specific activity and purification values reported for other purification procedures [27,28].

Different purification protocols have been used for PON enzyme from different sources. Furlong et al. (1991) [30] reported 62.1-fold PON purification from human serum using four-step purification protocols, namely Agarose Blue, Sephadex G-200, DEAE–Trisacryl M Sephadex G-75. Sheep serum paraoxonase was purified in 330–385-fold using ethanol, pH and ionic strength fractionation [20]. Rodrigo et al. [21] purified the liver paraoxonase in 415-fold by hydroxyapatite adsorption, chromatography on DEAE–sepharose CL-6B, non-specific affinity chromatography on Cibacron Blue 3 GA and anion exchange on Mono Q HR 5/5. In addition, liver PON3 has

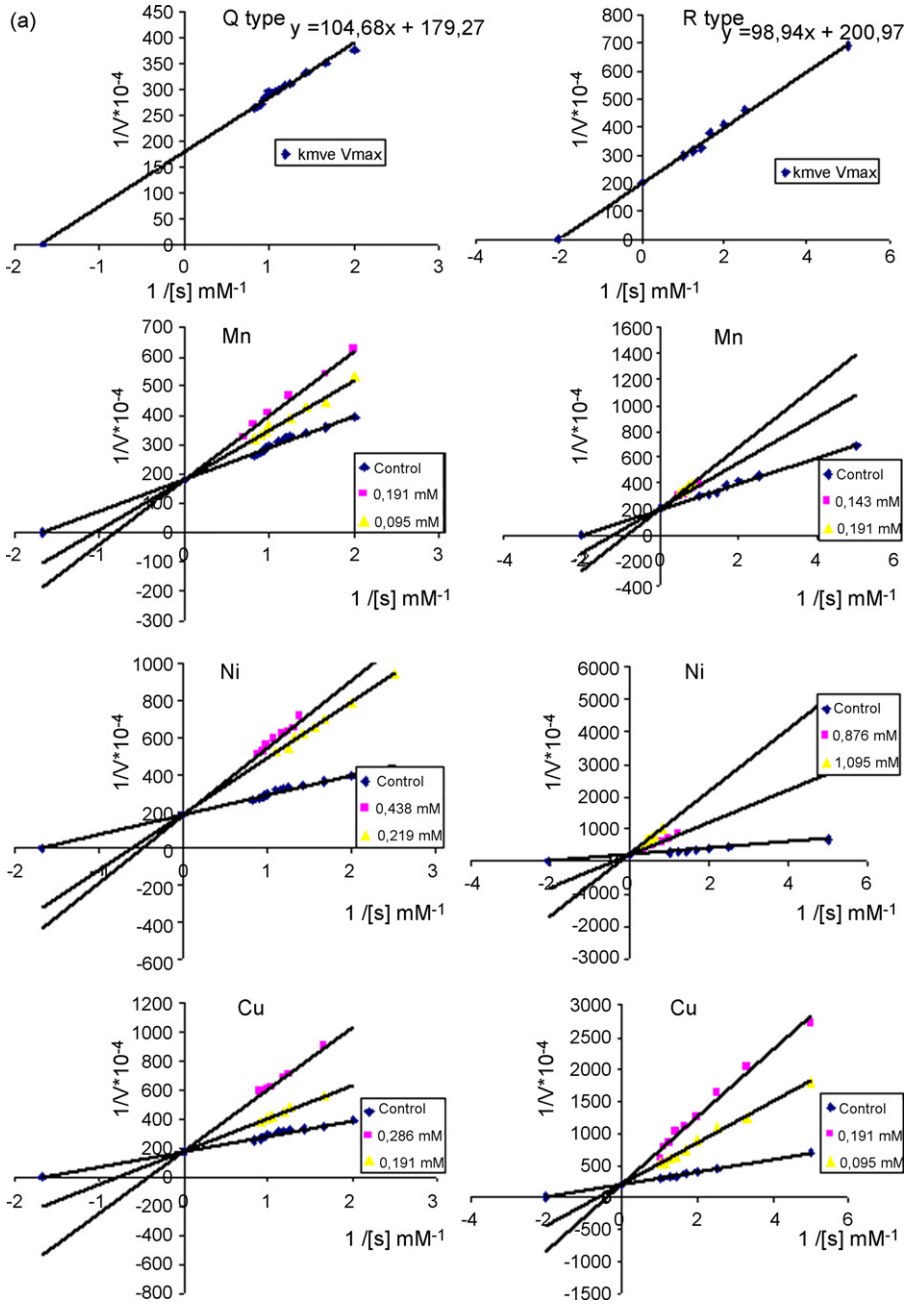

Fig. 4. (a) Type of inhibition of metals on paraoxonase enzyme Q and R type, respectively. (b) Type of inhibition of metals on paraoxonase enzyme Q and R type, respectively.

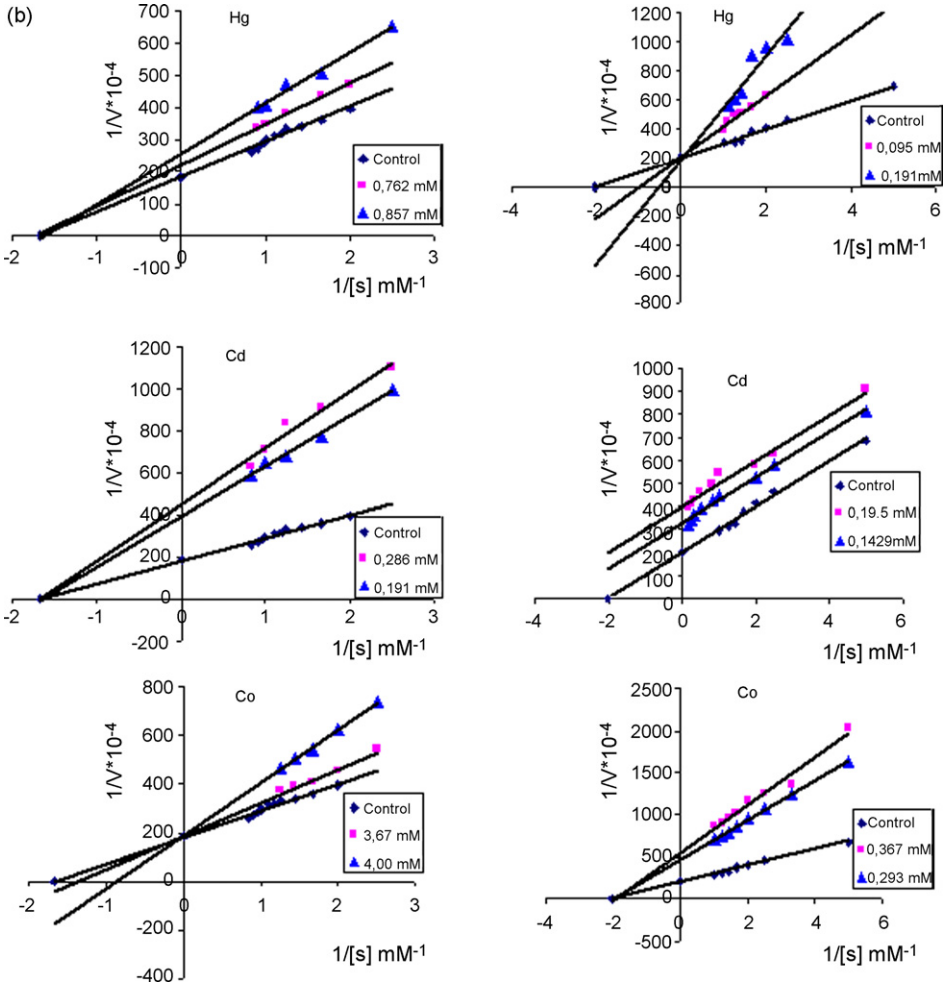

Fig. 4. (*Continued*).

been purified in 177-fold that uses a protocol consisting of seven steps [29].

Fig. 2 illustrates the final purification patterns determined by SDS gel electrophoresis. The purified human serum A-esterase gives a single band on SDS-PAGE with a weight of 43 kDa. This corresponds to the previous studies [26,27,29].

Some purification studies indicate the differences of the migration of PON bands in SDS gel electrophoresis [26]. Furlong et al. [30] demonstrated two PON bands purified from rabbit serum. Gan et al. [26] report that human paraoxonase contains 15.8% carbohydrate. Sequence analysis [31] indicates five potential N-glycosylation sites in rabbit paraoxonase and four in humans. Therefore a minimum molecular weight of human PON1 was 43,000 Da [31]. Moreover, a molecular weight of PON enzyme may increase up to 47–54 kDa in case of the contamination of albumin and ApoA1 [32]. However, our purification protocol yielded a single 43 kDa band suggesting the purification free from contaminants.

It has been shown that calcium is required for enzyme stability and activity, and that the enzyme is inhibited by many other metals, especially transition metals [33]. However, most studies have been performed with unpurified isoenzyme. The purpose of the present paper is to compare the sensitivity of purified for Q and R isoenzyme towards metal ion inhibition.

The kinetic parameters for the various metal chlorides are presented in Table 2. The IC_{50} values obtained with purified Q and R isoenzyme are in different value (Fig. 3). Metals were more effective inhibitors on purified human serum $PON1_{R192}$ activity than $PON1_{Q192}$ activity. The next step was to study the kinetics of interaction of heavy metals with the purified human Q and R isoenzyme. Two different concentrations of heavy metals were used for the determination of inhibition types (Fig. 4). Inhibition properties of purified Q and R isoenzyme solution by Hg, Cd, Co, Ni, Mn and Cu were investigated with paraoxon as substrate at pH 8.0 (Table 3).

Several studies have also reported that K_M values for paraoxon from different labs could show considerable similarities. Eckerson et al. [34] reported 0.43 mM K_m value for the paraoxonase type Q enzyme and 0.46 mM the paraoxonase type R enzyme.

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 concentration (0.6–1.2 mM). The data indicates that the inhibition of PON1 activity by metals different type. Relatively studies have not reported on investigations of the inhibition of paraoxonase Q and R isoenzyme as substrate using paraoxon.

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