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Purification and kinetic properties of rabbit liver paraoxonase 1

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ABSTRACT

Paraoxonase 1 (PON1) is synthesized in the liver and secreted into the blood, where it is associated exclusively with HDL. In this study, rabbit liver PON1 enzyme was purified to homogeneity using a new purification approach, and the kinetic properties of the enzyme were investigated using phenyl acetate and homocysteine thiolactone as substrates. Rabbit liver PON1 was purified through the preparation of liver microsomal fraction, Sephacryl S300 HR gel filtration chromatography, DEAE Trisacryl M ion-exchange chromatography and hydroxyapatite chromatography steps. Using this method, rabbit liver PON1 was purified 576 times with a specific activity of 2726 U/mg protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed the obtained enzyme as a single protein band close to 40 kDa. The Km of the this enzyme was found as 0.55 ± 0.024 mM for phenyl acetate and 17.31 ± 1.2 mM for homocysteine thiolactone. In this study, a new approach was used to purify PON1 enzyme from rabbit liver and for the first time in the literature, its kinetics was studied with homocysteine thiolactone as substrate.

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1. Introduction

Paraoxonase 1 (PON1) is a Ca^{2+}-dependent serum esterase synthesized in the liver [1–3]. Human paraoxonase gene family has three members and they show a high similarity at the amino acid level among mammalian species [3–5]. PON1 is the most studied enzyme in this group and it has been shown to hydrolyze several synthetic ester substrates, including organophosphorous insecticides [1–5].

Mackness et al. suggested that serum PON1 associated with HDL is a possible key enzyme in lipid metabolism [6]. Further studies have indicated that PON1 decreases the susceptibility of low-density lipoprotein (LDL) to lipid peroxidation *in vitro*, so it may prevent the development of atherosclerosis [7–13]. The antiatherogenic role of PON1 is strongly supported by transgenic mice studies [14–16]. PON1-knockout mice are more susceptible to develop atherosclerotic lesions, compared to their wild type counterparts and high-density lipoprotein (HDL) in knockout mice fails to prevent LDL oxidation in cultured artery wall cells in contrast to HDL in wild type mice [14]. *In vitro* studies showed that PON1 protects both HDL and LDL from oxidation. Also PON1 increases HDL's efflux capacity in macrophages [8,10,17]. However both of these activities are independent from its hydrolytic activity. Recently, PON1 has been shown to catalyze the hydrolysis of variety of aromatic and aliphatic lactones as well as lactone formation [2,3,18–20]. Recent studies also showed that PON1 is able to lactonize fatty acid oxidation products and metabolize the produced lactone. Moreover, structure-reactivity studies of PON1 indicated that PON1 is primarily a lactonase [2–5,18–20]. These studies defined PON1 as "Ca^{2+}-dependent lipophylic lactonase". Jakubowski [21] suggested that a physiological substrate of serum PON1 is homocysteine thiolactone which is a known risk factor in atherosclerosis.

PON1 is synthesized mainly in the liver and a portion of it is secreted into the blood, where it is associated exclusively with HDL. Compared with serum PONs, little is known about the liver enzyme. Since liver is the main site of organophosphate detoxification by PON1, liver PON1 activity should be of greater significance than the serum activity [22]. Serum PON1 has been purified from several mammalian species, but only human and rabbit PON1 proteins have been characterized [5,23–34]. The PON1 enzymes from these two species show 85% amino acid homology and similar substrate specificity [23,24]. Moreover, rabbit serum PON1 activity was found to be higher than the other mammalian species and to have a higher Ca^{2+} binding affinity [25,35]. Since liver PON1 is an essentially microsomal enzyme, the purification consists of a multistep procedure. To date, several studies with rat and mouse liver PON1 defined solubilization and subcellular distribution of the enzyme but there are very few studies describing the purification of PON1 from rabbit liver [26–31,36]. In these studies, usually more than three chromatographic steps are used including ion exchange and hydroxyapatite non-specific affinity chromatographies. In our study, gel filtration chromatography is performed as the first step of procedure. This unusual application allowed us to eliminate a large portion of proteins, and further ion exchange and affinity

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chromatography steps resulted in a higher yield and purification fold. Thus, here we present a three-step practical and effective purification method for rabbit liver PON1 and for the first time the kinetic properties of the purified enzyme is investigated using phenyl acetate and homocysteine thiolactone as substrates

2. Materials and methods

2.1. Chemicals

Phenyl acetate, eserine, homocysteine thiolactone, Tris base, Tris–HCl, calcium chloride ($CaCl_2$), acetic acid, methanol, ethanol, glycerol, SDS, *N*,*N*,*N*′,*N*′-tetra-methylethylenediamine, ammonium persulphate, sucrose, 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), bicinchoninic acid protein assay kit (BCA), Sephacryl S300 HR, DEAE Trisacryl M, and hydroxyapatite (Bio-Gel HTP) were provided by Sigma–Aldrich (USA). Other reagents were obtained from Merck (Germany).

2.2. Enzyme assays

Arylesterase activity was measured according to Gan et al. [37], using phenyl acetate as substrate at a final concentration of 1 mM. Formation of phenol at 25 °C was monitored at 270 nm, in the presence of 50 mM Tris–HCl (pH 8.0) containing 1 mM $CaCl_2$ and 40 μM eserine. Enzymatic activity was calculated from the molar extinction coefficient of phenol ($\varepsilon_{270} = 1310\,M^{-1}\,cm^{-1}$) and corrected for the non-enzymatic hydrolysis. One unit of arylesterase activity is defined as 1 μmol of substrate hydrolyzed per min, under the defined assay conditions.

Homocysteine thiolactonase activity was measured spectrophotometrically using a modification of the method described by Billecke et al. [18]. Homocysteine thiolactone was used as substrate at a final concentration of 2 mM in 50 mM Hepes buffer (pH 7.4). Thiolactone hydrolysis was measured at 37 °C, using Ellman's procedure [38] for monitoring at 412 nm the accumulation of free sulfhydryl groups reacting with DTNB. Enzymatic activity was calculated from the molar extinction coefficient of DTNB ($\varepsilon_{412} = 13600\,M^{-1}\,cm^{-1}$) and corrected for the non-enzymatic hydrolysis. One unit of homocysteine thiolactonase activity is defined as 1 μmol of substrate hydrolyzed per min, under the defined assay conditions.

2.3. Protein assay

Due to the high UV absorbance of Triton X-100, protein concentrations of the fractions were determined by the bicinchoninic acid protein assay (Sigma–Aldrich, USA), using bovine serum albumin as a standard [39].

2.4. Enzyme purification and kinetics

2.4.1. Preparation of microsomal fraction

New Zealand male rabbits were obtained from Hacettepe University Experimental Animals Laboratory, with the approval of the Hacettepe University Animal Ethics Committee (2005/21-9; 07.04.2005). Rabbits were sacrificed by decapitation and the livers were immediately removed, washed and perfused with ice-cold 20 mM Tris–HCl (pH 7.4) containing 0.9% NaCl and stored at −80 °C prior to microsomal preparation.

Microsomal fractions were prepared by a modification of the method of Rodrigo et al. [30]. Frozen livers were allowed to thaw for 5 min and minced with scalpel in a Petri dish on ice. Mincing was carried out until the tissue had a doughy consistence and then homogenized in (1:4, w/v) ice-cold 5 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose using a mechanically driven Teflon pestle in a glass homogenizer. Homogenate was centrifuged at 460 × *g* for 10 min and 12,500 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 105,000 × *g* for 1 h. The microsomal pellets were resuspended in 20 mM Tris–HCl (pH 7.4) containing 1 mM $CaCl_2$. Solubilization of PON1 from microsomal membranes was achieved by the addition of Triton X-100 at a final concentration of 0.1% and gently stirring the sample for 40 min. Subsequently the sample was kept on ice for 30 min and recentrifuged at 105,000 × *g* for 1 h after which the supernatants were pooled. The pooled supernatants were concentrated to 6 ml using an Amicon ultrafiltration system with a 30 kDa cut-off membrane filter.

2.4.2. Sephacryl S300 HR gel filtration chromatography

The concentrated sample was loaded on to a column of Sephacryl S300 HR (105 × 2.44 cm) at a flow rate of 21.5 ml/h, pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.4) containing 0.1% Triton X-100 (Buffer A). During the wash with buffer A fractions of 2.4 ml/tube were collected. Each fraction was monitored for arylesterase activity and protein concentration. Fractions with PON activity were pooled and used for next step of the purification.

2.4.3. DEAE Trisacryl ion-exchange chromatography

The pooled fractions from the previous steps were loaded on to a DEAE Trisacryl column (15 cm × 2 cm size) at a flow rate of 25 ml/h, pre-equilibrated with buffer A containing 1 mM $CaCl_2$ and 10 mM NaCl. Following a wash of the column with the same buffer, bound enzyme was eluted by a linear gradient of 10–300 mM NaCl in buffer A. Each fraction was assayed for enzyme activity. Active fractions were pooled and dialyzed for 4 h against two successive changes of 3 L of 10 mM phosphate buffer (pH 7.4) containing 0.1% Triton X-100, 100 μM $CaCl_2$ and 20% glycerol (buffer B).

2.4.4. Hydroxyapatite chromatography

Following dialysis, the sample was applied to a hydroxyapatite column (25 cm × 2.5 cm size), pre-equilibrated with buffer B containing 100 μM $CaCl_2$ at a flow rate of 2.5 ml/h. The enzyme was eluted by a linear gradient of 10–300 mM phosphate buffer following a wash with buffer B. Fractions were assayed for protein and enzyme activity. Active fractions were pooled and dialyzed for 4 h against two successive changes of 3 L of 20 mM Tris–HCl buffer (pH 7.4) containing 0.1% Triton X-100.

2.4.5. 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 [40]. 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 profile from the last purification step was electrophoresed and blotted to a nitrocellulose membrane at constant 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 [41] was used for protein visualization.

2.4.6. Kinetics of purified rabbit liver PON1

Phenyl acetate and homocysteine thiolactone were used as substrate in kinetic studies. For phenyl acetate, initial rates were measured in 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 25 °C. Assay mixture contained 0.14 μg of enzyme. The reaction

was started by adding phenyl acetate at different concentrations (0–1.6 mM) and monitored spectrophotometrically for 1 min. The reaction was linear during this period. Assays were carried out in triplicate and corrected for spontaneous hydrolysis of phenyl acetate. For homocysteine thiolactone, initial rates were measured in 50 mM Hepes buffer (pH 7.4) at 37 °C. The final concentration of enzyme used was 2 μg in assay mixture. The reaction was started by the addition of homocysteine thiolactone at different concentrations (0.5–14 mM) and monitored spectrophotometrically for 2 min. Assays were carried out in triplicate and the interfering reactions have been eliminated by using a blank tube containing all the compounds of except enzyme.

The kinetic data were analyzed and kinetic constants were calculated by means of the non-linear curve-fitting program of the statistical software package Systat (Version 11, 2005).

3. Results and discussion

Rabbit liver PON1 enzyme was purified from detergent-solubilized microsomes in steps involving Sephacryl S300 HR gel filtration, DEAE Trisacryl M ion-exchange chromatography and hydroxyapatite chromatography, a summary of which is presented in Table 1. Although it has been reported that the solubilization step might decrease the activity of the enzyme [27,28], we considered this step indispensable to obtain a better yield at further chromatographic procedures. We used 0.1% Triton X-100 for the solubilization of PON1 from the membrane, as suggested by Gil et al. [27], to keep the activity loss at minimum. Thus, Table 1 shows that the specific activity of the sample was increased about nine times after this application.

After solubilization, the sample was loaded onto a gel filtration column and collected fractions were analyzed for protein content and arylesterase activity. Size exclusion chromatography has not been generally used as the first step of PON1 purification [24–26,30–37]. However, this initial step was required to eliminate a higher percentage of proteins present in the extract. In a previous study of PON1 purification from rat liver, only 72% of the proteins were eliminated using hydroxyapatite non-specific affinity chromatography [31]. In our study, as the first chromatography step we preferred to use a larger size gel filtration column, resulting in the removal of 80% of the protein. A good elution profile was obtained with a specific activity of 132 U/mg protein of the active fraction and an overall yield of 53% (Fig. 1A, Table 1).

In the second chromatographic step, the pooled active fraction was loaded onto a DEAE Trisacryl M ion-exchange column and the bound enzyme was eluted with a linear gradient of 10–300 mM NaCl. A single narrow peak was obtained at 108 mM NaCl (Fig. 1B). This chromatography step resulted in a 122-fold purification with a 30% loss of activity (Table 1). The pooled fractions were dialyzed. The dialyzed sample was further purified using hydroxyapatite chromatography.

Human serum PON1 requires the presence of calcium for enzymatic activity. Calcium facilitates the formation of an enzyme-substrate complex and accelerates the breakdown of this intermediate into enzyme and product. Using purified human serum esterase, Eckerson et al. showed a parallel dependence of arylesterase and paraoxonase activities on calcium concentration [42]. Calcium ion is not only important for the catalytic activity of serum PON1, but it also serves to stabilize the enzyme in its native molecular structure. Interestingly, rabbit PON1 has 25.7 times higher binding affinity for Ca^{2+} in the Ca^{2+} binding site, which is essential for enzyme stability, and thus rabbit PON1 is considered as a more stable enzyme than human PON1 [25,35]. It has been reported that during purification, the presence of Ca^{2+} at a given concentration (1–2 mM) is required in some mammalian species for the activity and stability of enzyme [22,25,31,35]. In our study, we only added Ca^{2+} during the preparation of microsomal fraction, and we did not observe any activity loss at subsequent steps. This suggests that Ca^{2+} remained bound to the enzyme during the whole procedure. In accordance with that, when we studied the inhibitory effect of EDTA, we did not observe any inhibition of the liver enzyme at EDTA concentrations that inhibit human serum PON1 (data not shown). At the hydroxyapatite chromatography step, we have achieved the optimal elution profile by adding 100 μM Ca^{2+} to the phosphate buffer (Fig. 1C). Higher calcium concentrations resulted in poor yield, probably due to the interaction of Ca^{2+} with phosphate. At this step the purification fold was found to be 576 times, with a specific activity of 2726 U/mg protein and a yield of 14% (Table 1). The final purified enzyme was found as a single protein band close to 40 kDa on SDS-PAGE by Coomassie Blue staining (Fig. 2A) and we confirmed by Western blot that it was specific to PON1 (Fig. 2B). In previous studies, purified PON1 has been shown to be a doublet within 37–48 kDa, depending on the glycosylation state of the enzyme [28,31,36,37,43].

In our study, rabbit liver PON1 enzyme was purified to homogeneity using a new purification approach with a better yield than other studies in the literature. Rodrigo et al. used a four-step procedure (hydroxyapatite, DEAE sepharose and Cibacron Blue) that resulted in a 415-fold purification of the sample [31]. Huang et al. have reported a solubilization procedure with sodium cholate and Triton N-101 and a method for purification of PON1 from mouse hepatic microsomes involving gel filtration chromatography, followed by ion-exchange chromatography and non-specific affinity chromatography. They obtained a 1531-fold purification with a recovery of 10% [28]. Ozols also used a four-step method where he utilized DEAE-cellulose chromatography as first and second steps which were followed by hydroxyapatite and Bio-Pro Q chromatographies and finally carried out an amino acid analysis

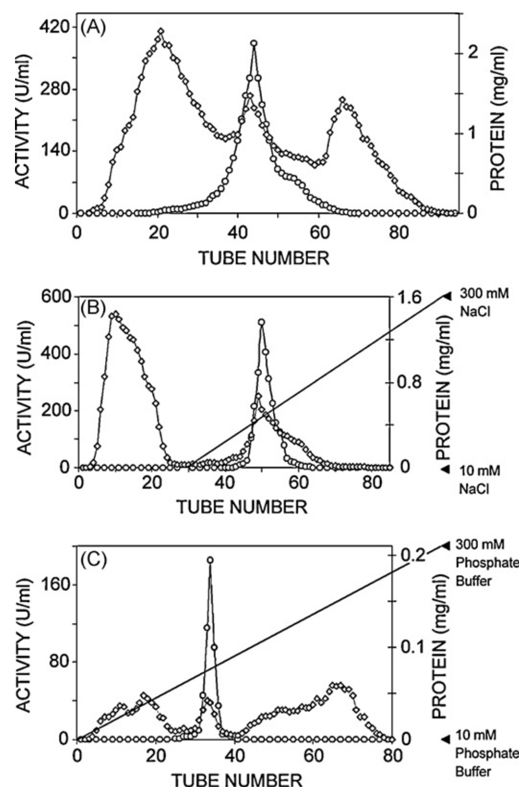

Fig. 1. Elution profiles of chromatography steps related to the purification of rabbit liver paraoxonase 1. Gel filtration chromatography (A), ion-exchange chromatography (B), hydroxyapatite chromatography (C). (–○–) activity, (–◇–) protein.

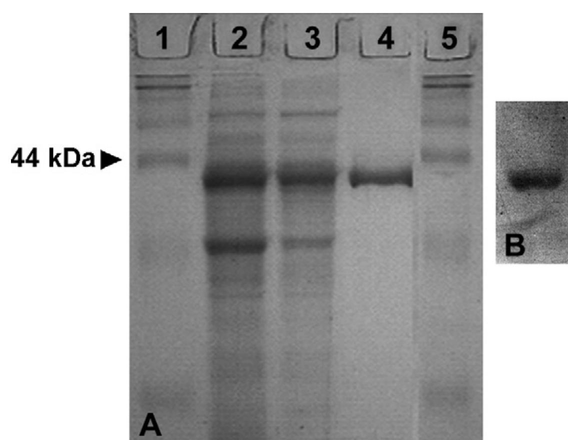

Fig. 2. Characterization of the purified rabbit liver paraoxonase 1. (A) The SDS-PAGE of the samples at different purification steps. Lanes 1 and 5: molecular weight standard (myosin 215 kDa, galactosidase 116 kDa, phosphorylase B 97 kDa, BSA 86 kDa, ovalbumine 44 kDa, carbonic anhydrase 32 kDa, trypsin inhibitor 26 kDa), lane 2: 15.4 μg of gel filtration chromatography sample, lane 3: 15 μg of ion-exchange chromatography sample, lane 4: 6 μg of hydroxyapatite chromatography sample. (B) Western blot analysis of the purified enzyme sample.

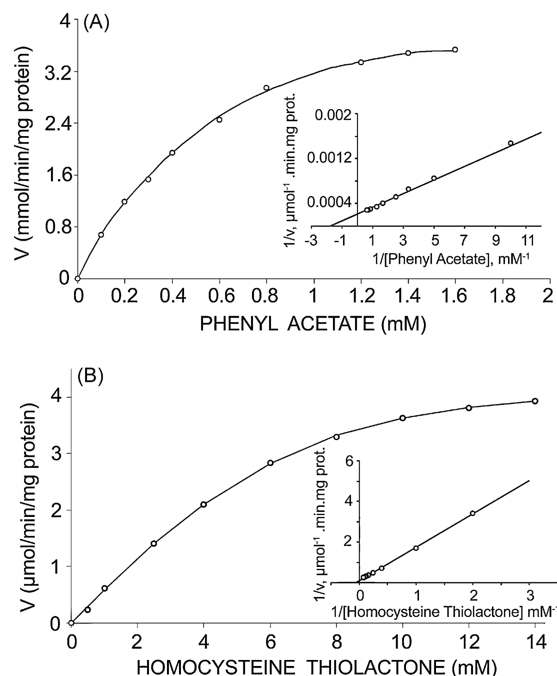

Fig. 3. Kinetic analysis of purified rabbit liver paraoxonase 1. (A) Michaelis–Menten plot for arylesterase activity of purified rabbit liver paraoxonase 1 assayed at various concentrations of phenyl acetate. The inset shows the Lineweaver–Burk plot. Km and Vm were calculated as 0.55 mM and 5054 μmol/min/mg protein, respectively, by using the curve-fitting program of the statistical software package Systat (Version 11, 2005) (B) Michaelis–Menten plot for homocysteine thiolactonase activity of purified rabbit liver paraoxonase 1 assayed at various concentrations of homocysteine thiolactone. The inset shows the Lineweaver–Burk plot. Km and Vm were calculated as 17.31 mM and 10.55 μmol/min/mg protein, respectively, by using the curve-fitting program of the statistical software package Systat (Version 11, 2005).

on the purified sample [36]. Ozols reported a specific activity of 5–10 nmol/min/mg protein for rabbit liver PON1 but gave no information on yield.

In the kinetic studies of the purified enzyme phenyl acetate and homocysteine thiolactone were used as substrates. Michaelis–Menten and Lineweaver–Burk graphics were plotted and the Km value of the enzyme was determined for each substrate. Rabbit liver PON1 displayed Michaelis–Menten kinetics with both substrates (Fig. 3A and B). For phenyl acetate, the maximum rate of the enzyme (Vmax) and Km value were found to be 5054 ± 125 μmol/min/mg protein and 0.55 ± 0.024 mM, respectively.

There is no kinetic data available on rabbit liver PON1 but there are few studies on human and rabbit serum enzyme [25,43]. Kuo and La Du compared the activities of human and rabbit serum PON1 and found that the activity of rabbit serum PON1 was 3–4 times higher than that of human serum enzyme [25]. Likewise in our study, the Km of purified rabbit liver PON1 for phenyl acetate as substrate was lower than that of human serum PON1 (data not shown). These data indicate that the affinity of rabbit PON1 to phenyl acetate is twice higher than the affinity of human PON1.

PON1 has a wide range of substrate specificity, hydrolyzing esters, thioesters, phosphotriesters and carbamates. So far, the highest specific activity of PON1 was found to be for phenyl acetate and paraoxon. But these substrates are not considered as natural substrates of PON1. Jakubowski defined the presence of a specific enzyme that catalyzes the hydrolysis of homocysteine thiolactone and his further studies indicated that this enzyme is identical to PON1 [21]. Thus, PON1 has thiolactonase activity, hydrolyzing homocysteine thiolactone to homocysteine [21].

On the basis of this data, we also investigated the kinetic properties of our purified PON1 using homocysteine thiolactone as substrate. We found that the Km of the enzyme for homocysteine thiolactone was 17.31 ± 1.2 mM and the Vmax was 10.55 ± 0.58 μmol/min/mg protein. Jakubowski [21] obtained a Km value of 23 mM in 100 mM K-Hepes buffer containing 2 mM Ca^{2+} (pH 7.4) for human serum PON1. The difference between two Km values may be due to the difference in the assay methods and/or the source of the enzyme. The lower Km value for homocysteine thiolactone that we found in our study is comparable to other Km values found in the literature for rabbit serum PON1. Human epidemiological studies suggest that low PON1 activity is a risk factor for the development of coronary heart disease. In baboons, it has been shown that chronic thiolactone infusion or elevated serum homocysteine levels promoted atherosclerotic plaque formation. In contrast, a similar procedure did not generate atherosclerosis in rabbits that have higher serum homocysteine thiolactonase levels than primates [21]. Thus, hydrolyzing homocysteine thiolactone may be an important if not the principal mechanism of the protective activity of PON1 against atherosclerosis.

Table 1
Summary of purification and yields of rabbit liver PON1.

Step	Volume (ml)	Total protein (mg)	Activity[b] (U/ml)	Total activity (μmol/min)	Specific activity (U/mg prot)	Fold	Yield
Crude extract[a]	82	3485.00	200.80	16465.60	4.73	1	100
Solubilization	40	336.00	345.60	13824.00	41.10	9	84
Sephacryl S300 HR	70	66.50	125.80	8806.00	132.40	28	53
DEAE Trisacryl M	25	10.75	248.40	6210.00	577.67	122	38
Hydroxyapatite	25	0.83	90.50	2262.50	2725.90	576	14

[a] The crude extract contains 20 g of rabbit liver.
[b] Phenyl acetate is used as substrate.

In summary, we purified the rabbit liver PON1 enzyme using four chromatography steps with an approach different from that in the literature, and this study is the first one for liver PON1 in which both phenyl acetate and homocysteine thiolactone substrate kinetics were studied. We suggest that in further studies, determination of the kinetic behavior of rabbit liver PON1 towards other lacton substrates, and investigation of the substrate specificity of rabbit liver PON1 in comparison to the serum enzyme of the same species would be beneficial for a better understanding of liver PON1 function.

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