Identification of paraoxonase 3 in rat liver microsomes: purification and biochemical properties

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Three paraoxonase genes (*PON1*, *PON2* and *PON3*) have been described so far in mammals. Although considerable information is available regarding *PON1*, little is known about *PON2* and *PON3*. *PON3* has been isolated recently from rabbit serum [Draganov, Stetson, Watson, Billecke and La Du (2000) *J. Biol. Chem.* **275**, 33435–33442] and liver [Ozols (1999) *Biochem. J.* **338**, 265–275]. In the present study, we have identified the presence of *PON3* in rat liver microsomes and a method for the purification to homogeneity is presented. *PON3* has been purified 177-fold to apparent homogeneity with a final specific activity of 461 units/mg using a method consisting of seven steps: solubilization of the microsomal fraction, hydroxyapatite adsorption, chromatography on DEAE–Sephacel CL-6B, non-

specific affinity chromatography on Cibacron Blue 3GA, two DEAE-cellulose steps and a final affinity chromatography on concanavalin A–Sephacel. SDS/PAGE of the final preparation indicated a single protein-staining band with an apparent molecular mass of 43 kDa. The isolated protein was identified by nanoelectrospray MS. Internal amino acid sequences of several peptides were determined and compared with those of human, rabbit and mouse *PON3*, showing a high similarity. Some biochemical properties of *PON3* were also studied, including optimum pH, *K_m* and heat and pH stability.

Key words: paraoxonase, *PON3*, purification, rat liver.

INTRODUCTION

Paraoxonase (aryldialkylphosphatase, EC 3.1.8.1, *PON1*) is a calcium-dependent serum esterase that is synthesized by the liver and exhibits a broad-substrate specificity [1–4]. In serum, *PON1* is closely associated with high-density lipoproteins [5,6] and recent studies have demonstrated the contribution of *PON1* to the antioxidant protection conferred by high-density lipoproteins on low-density lipoprotein oxidation [4–11].

A few years ago, it was established [12] that *PON1* is a member of a multigene family in mammals that includes at least three members, *PON1*, *PON2* and *PON3*. All three *PON* genes are located adjacent to each other on chromosome 7 in humans and on chromosome 6 in mice and seem to be a result of gene duplication. The three *PON* genes show a high similarity at the amino acid level within a given species and between the mammalian species [12,13]. *PON1* and *PON3* are expressed primarily in the liver. In contrast, *PON2* is widely expressed in a number of tissues, including brain, liver, kidney and testis and may have multiple mRNA forms [14]. *PON1* and *PON2* polymorphisms have been associated with a higher risk of coronary heart disease [15–18], but to date there are no reports on polymorphisms in the *PON3* gene. Recent research [19,20] suggests that *PON2* and *PON3* possess antioxidant properties similar to those of *PON1*.

The ability to hydrolyse paraoxon (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphate; referred to as POX hereafter) and phenylacetate (PA) is routinely used for measuring *PON1* activity *in vitro* and in serum samples. Recently [3,4], it has been reported that purified human and rabbit serum *PON1* and *PON3* also hydrolyse a variety of lactones and cyclic carbonate esters, including naturally occurring lactones and pharmacological agents.

Compared with serum paraoxonase, little is known about the hepatic enzyme, perhaps due to the fact that liver paraoxonase

is essentially a microsomal enzyme associated with vesicles derived from the endoplasmic reticulum [21]. The presence of *PON1* activity in liver microsomes has been reported by several laboratories [22–24]. We reported the partial purification of rat liver paraoxonase [25] and, later, we developed a method for the purification of *PON1* from rat liver microsomes to homogeneity [24]. In an independent study, Huang et al. [22] reported the purification of an A-esterase from mouse liver microsomes.

In a previous study [26], we detected the presence of two peaks with POX hydrolytic activity (*PON1*) after non-specific affinity chromatography of rat liver microsomes on Cibacron Blue 3GA, suggesting that two proteins capable of hydrolysing POX are present in rat liver microsomes. Recently, methods for the isolation of *PON3* from rabbit serum [4] and rabbit liver [27] have been reported.

In the present study, we have identified the presence of *PON3* in rat liver microsomes on the basis of the differential hydrolysis of selected substrates [4]. A method for the purification of *PON3* to homogeneity is also presented. The identity of purified *PON3* was confirmed by sequencing of several peptides by MS. Also, some biochemical properties of rat liver *PON3* are presented in this paper. This is the first report describing an enzyme in rat liver identified as *PON3*.

EXPERIMENTAL

Chemicals

Hydroxyapatite (Bio-Gel HTP), Tris base, SDS, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulphate, DEAE-Biogel A, Coomassie Brilliant Blue R-250, acrylamide and bisacrylamide were provided by Bio-Rad (Richmond, CA, U.S.A.).

Abbreviations used: ConA, concanavalin A; DHC, dihydrocoumarin; MALDI-TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; mSPON, microsomal paraoxonase; PA, phenylacetate; POX, paraoxon (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphate).

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DEAE–Sephacrose CL-6B, low-molecular-mass standards and Mono Q HR 5/5 were obtained from Amersham Biosciences. POX, BSA (fraction V), Cibacron Blue 3GA, ConA (concanavalin A)–Sephacrose, Laemmli sample buffer electrophoresis reagent, DHC (dihydrocoumarin) and PA were supplied by Sigma (St. Louis, MO, U.S.A.). Milli-Q (Millipore, Bedford, MA, U.S.A.)-grade water was used throughout, and all buffers were degassed and adjusted to their respective pH values at 25 °C. Others reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Animals

Male Wistar rats weighing 180–200 g at the time of death were used. Animals were maintained on lab chow (Paulab, Barcelona, Spain) and tap water *ad lib.* with a 12 h day/night cycle (light cycle, from 7:00 to 19:00). Rats were starved for 16 h before decapitation. Spanish regulations for experimental animals were observed in the present study.

Enzyme activities

A-esterase activity towards POX was quantified spectrophotometrically by a modification of the method of Reiner and Radic [28] using 100 mM Tris/HCl buffer (pH 7.4) and 1 mM CaCl₂. The reaction was studied for 2 min at 37 °C by monitoring the appearance of *p*-nitrophenol at 405 nm in a PerkinElmer Lambda 2 automated recording spectrophotometer. Final substrate concentration during the enzyme assay was 2 mM, and all rates were determined in duplicate and corrected for the non-enzymic hydrolysis.

Hydrolytic activity against PA was determined by the method described by Junge and Klees [29] using 100 mM Tris/acetate buffer (pH 7.4) and 10 mM CaCl₂. The reaction was studied at 37 °C by monitoring the appearance of phenol at 270 nm. The final substrate concentration in the cuvette was 3.8 mM and all rates were determined in duplicate. Non-enzymic hydrolysis was negligible.

Hydrolysis of DHC (an aromatic lactone) was measured by the method described by Draganov et al. [4] with minor changes. The assay medium contained 1 mM substrate (from a 100 mM stock solution, dissolved in methanol) in 50 mM Tris/HCl buffer (pH 7.0) and 1 mM CaCl₂ in a final volume of 1 ml. The reaction was studied at 37 °C by monitoring the increase in UV absorbance at 270 nm. All rates were determined in duplicate and corrected for the non-enzymic hydrolysis.

Buffers used in rat liver PON3 purification

The following buffers were used. Buffer A: 5 mM Tris/HCl (pH 7.4) and 0.25 M sucrose. Buffer B: 5 mM Tris/HCl (pH 7.4). Buffer C: 20 mM potassium phosphate (pH 7.5) and 0.1 % (v/v) Triton X-100. Buffer D: 400 mM potassium phosphate (pH 7.5). Buffer E: 20 mM Tris/HCl (pH 7.7), 2.5 mM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 20 mM NaCl. Buffer F: 20 mM Tris/HCl (pH 7.7), 2.5 mM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 50 mM NaCl. Buffer G: 20 mM Tris/HCl (pH 7.7), 2.5 mM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 500 mM NaCl. Buffer H: 20 mM Tris/HCl (pH 7.7), 2.5 mM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 2 M NaCl. Buffer I: 50 mM Tris/HCl (pH 8.0), 100 µM CaCl₂ and 0.1 % (v/v) Triton X-100. Buffer J: 50 mM Tris/HCl (pH 8.0), 100 µM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 1 M NaCl. Buffer K: 100 mM Tris/HCl (pH 8.5) and 0.5 M NaCl. Buffer L: 100 mM sodium

acetate (pH 4.5) and 0.5 M NaCl. Buffer M: 20 mM Tris/HCl (pH 7.7), 2.5 mM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 10 mM NaCl. Buffer N: 20 mM Tris/HCl (pH 7.7), 2.5 mM CaCl₂ and 0.1 % (v/v) Triton X-100 containing 500 mM NaCl. Buffer O: 20 mM Tris/HCl (pH 7.7), 1 mM CaCl₂ and 0.1 % (v/v) Triton X-100. Buffer P: 25 mM Tris/HCl (pH 7.4), 1 mM CaCl₂, 0.1 % (v/v) Triton X-100 and 0.15 M NaCl. Buffer Q: 25 mM Tris/HCl (pH 7.4), 1 mM CaCl₂, 0.1 % (v/v) Triton X-100 and 0.15 M NaCl containing 0.5 M methyl α -D-mannopyranoside.

Purification of rat liver PON3

All purification procedures were performed at 0–4 °C unless otherwise noted. The initial steps in the purification of rat liver PON3 essentially followed the procedure for purification of rat liver PON1 developed previously in this laboratory [24].

Preparation of the microsomal fraction

Microsomal fractions were prepared essentially as described previously [21]. Briefly, rat livers (20 g) were removed, placed in beakers on ice, rinsed with ice-cold homogenization buffer (buffer A), minced with scissors and then placed in 4 vol. of ice-cold buffer A. They were then homogenized (6 strokes at 1100 rev./min) using a mechanically driven Teflon pestle in a glass homogenizer (Potter–Elvehjem-type homogenizer) with 1.02 mm clearance. The homogenate was transferred to a power-driven close-fitting (0.045 mm clearance) Perspex [poly(methyl methacrylate)]/glass homogenizer and homogenized as before. After diluting the homogenate to 10 % (w/v) with the homogenization buffer, nuclei and mitochondria were removed by successive centrifugation at 460 g for 10 min and at 12 500 g for 10 min in a Beckman J2–21 refrigerated centrifuge. The post-mitochondrial supernatant fraction was then centrifuged at 105 000 g for 60 min in a Beckman 55.2 Ti rotor operated in a Beckman L8–55 refrigerated centrifuge. The microsomal pellet derived from 10 g of liver tissue was suspended in 20 ml of buffer B.

Solubilized microsomal membranes

Paraoxonase was extracted by the addition of Triton X-100 [25]. The microsomal fraction was adjusted to 0.75 % Triton X-100, vortex-mixed, stored at 4 °C for 30 min and then centrifuged at 105 000 g for 60 min.

Batchwise hydroxyapatite

The supernatant (28 mg of protein/ml) was gently stirred for 30 min in the presence of 40 g (dry wt) of hydroxyapatite, which had been pre-equilibrated in buffer C. The hydroxyapatite adsorption was performed at 4 °C for 30 min with gentle agitation. The exchanger was recovered by centrifugation at 1080 g for 1 min. The matrix was washed four times with 1 vol. of buffer C. After washing, each supernatant was centrifuged as above, and then the four supernatants were pooled and centrifuged again at 4300 g for 2 min to eliminate residual particles of the exchanger [25]. The supernatants were monitored for PA, POX and DHC hydrolyses to localize PON1 and PON3 activities. The pooled supernatants were concentrated to 20 ml using a Filtron ultrafiltration system (Filtron Technology Corp., Clinton, MA, U.S.A.) fitted with Omega™ Serie Membrane of 30K. After use, hydroxyapatite was regenerated by washing thoroughly with buffer D and then re-equilibrated in the starting buffer (buffer C).

DEAE–Sephacrose CL-6B/ion-exchange chromatography

The concentrated hydroxyapatite supernatant was loaded at a flow rate of 0.5 ml/min on to a DEAE–Sephacrose CL-6B column (column bed 1.6 cm × 22 cm) pre-equilibrated in buffer E. The column was connected to a peristaltic pump P-3 (Amersham Biosciences), then washed with buffer E until the absorbance A_{280} decreased to approx. 0.1 (approx. 100 ml), and the bound material was eluted from the column using a linear salt gradient of 50–500 mM NaCl in the same buffers (50 ml each of buffers F and G). Fractions of 4 ml were collected during washing and gradient elution. Each fraction was monitored for the three activities as indicated above. In this case, the three activities co-eluted. Peak activity fractions were pooled and concentrated to approx. 10 ml using a Filtron ultrafilter. The column was regenerated with buffer H.

Cibacron Blue 3GA/non-specific affinity chromatography

The concentrated eluate was adsorbed/pumped at 0.5 ml/min on to a Cibacron Blue 3GA column (1 cm × 10 cm) pre-equilibrated with buffer I. The column was washed (8–10 bed vol. of the same buffer) and the protein eluted with buffer J (containing 1 M NaCl). Fractions (4 ml) were collected. Two peaks with the three activities were detected (M_1 and M_2). The first peak (M_1) was obtained during the washing of the column and M_2 was eluted with 1 M NaCl. The active fractions of M_2 containing PON1 [24] were discarded. The active fractions of M_1 (6–15) were pooled, concentrated by ultrafiltration (Filtron) to a final volume of 10 ml and then dialysed overnight against buffer O. After use, the column was washed with 5 vol. of buffer K followed by 5 vol. of buffer L and then re-equilibrated in the starting buffer (buffer I).

First DEAE anion-exchange chromatography

The concentrated M_1 fractions were loaded at a flow rate of 0.5 ml/min on to a DEAE–Biogel A column (column bed, 1.6 cm × 22 cm) pre-equilibrated in buffer M. The column was connected to a peristaltic pump P-3 (Amersham Biosciences), washed with buffer M until A_{280} decreased to approx. 0.1 (approx. 100 ml), and the bound material was eluted from the column using a linear salt gradient of 15–500 mM NaCl in the same buffers (50 ml of buffer M and 50 ml of buffer G respectively). Fractions of 4 ml were collected during washing and gradient elution. Each fraction was monitored for the three activities as indicated above. Two peaks with activity were detected. The first peak (D_1 , that hydrolysed PA and DHC) was obtained during the washing of the column (fractions 8–18) and the bounded protein was eluted with the gradient. A second activity peak (D_2) that hydrolysed the three substrates was eluted in fractions 43–50. Active fractions corresponding to D_1 (PON3) were pooled and concentrated to approx. 10 ml using a Filtron ultrafilter.

Second DEAE anion-exchange chromatography

The concentrated pooled fractions from the first DEAE step were dialysed overnight against buffer O and loaded on to a column of DEAE–Biogel A under the same conditions described previously for the first DEAE. Fractions of 4 ml were collected during washing and gradient elution. Each fraction was monitored for the hydrolyses of DHC, PA and POX. A single peak hydrolysing DHC and PA (but not POX) was obtained during the washing of the column. Active fractions (10–23) were pooled and concentrated to approx. 10 ml using a Filtron ultrafilter.

ConA–Sephacrose affinity chromatography

A suitable quantity of ConA was packed in a column (1 cm × 10 cm; Amersham Biosciences) and equilibrated overnight with buffer P. The pooled fractions from the second DEAE were applied on to the column at 0.35 ml/min. The column was washed with the same buffer, and fractions of 4 ml were collected. The bound enzyme was eluted with a linear gradient of 80 ml of buffer P and 80 ml of a buffer containing 0.5 M methyl α -D-mannopyranoside (buffer Q) at 0.35 ml/min. Fractions of 4 ml were collected and those with the highest DHC hydrolytic activity were pooled (29–31) and concentrated by using Microsep 30 microconcentrator (Filtron Technology Corp.). The Microsep was also used to remove most of the contaminating lectin fragments from the pooled fractions. After use, the column was washed with 5 vol. of buffer K followed by 5 vol. of buffer L and then re-equilibrated in the starting buffer (buffer P).

Protein determination

The protein contents of fractions from the column chromatographic separations were monitored by measuring A_{280} . Protein concentration was estimated by the method of Lowry et al. [30], with BSA fraction V as a reference standard. A modification of this procedure [31] was used to assay protein in the presence of Triton X-100.

SDS/PAGE

Protein samples obtained during the different purification steps were analysed by SDS/PAGE as described by Laemmli [32], using a Bio-Rad Miniprotean III electrophoresis unit. The final monomer concentration in the 0.75-mm-thick slab gels was 12 % (w/v) for the separating gel and 4 % (w/v) for the stacking gel. Before loading, all samples were incubated in the presence of 1 % (w/v) SDS and 100 mM dithiothreitol for 5 min at 100 °C. The samples were run at a constant voltage of 200 V applied for 45 min. The proteins were visualized by silver staining and Coomassie Blue staining.

Amino acid sequence analysis

In-gel digestion and extraction

The approx. 43 kDa band from one lane in a Coomassie Blue gel was excised and minced into small pieces. The gel pieces were washed with 50 mM NH_4HCO_3 , dehydrated with acetonitrile and dried under vacuum. The sample was re-hydrated and digested overnight at 30 °C, with a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 $\mu\text{g/ml}$ trypsin (Promega modified trypsin; Promega, Madison, WI, U.S.A.). The supernatant was collected and the peptides were extracted from the gel pieces with 50 % acetonitrile/0.5 % trifluoroacetic acid.

Matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI–TOF–MS)

The tryptic digest was analysed by MALDI–TOF (Voyager DE-PRO, Applied Biosystems, Foster City, CA, U.S.A.). The mass analyser was scanned over the m/z range 750–4500 amu and the resulting spectrum was used to search for matching proteins in the NCBI database (National Center for Biotechnology Information, U.S. National Library of Medicine, Rockville Pike,

Bethesda, MD, U.S.A.) and SWISS-PROT Protein Sequence database using the Protein Prospector search program.

Nanoelectrospray mass spectrometry (MS/MS)

Experiments were performed on a Finnigan LCQ ion-trap mass spectrometer (ThermoQuest, Finnigan MAT, San Jose, CA, U.S.A.) equipped with a nanospray interface (Protana, Odense, Denmark). The spray voltage applied was 0.85 kV and the capillary temperature was 120 °C. For MS/MS experiments, the isolation window was 3 mass units wide and the relative collision energy was 35 %.

Characterization of the purified enzyme

Biochemical properties of PON3 were determined using DHC as the substrate. The effect of pH on the activity of rat liver PON3 was measured at 37 °C over the pH range 5–10 in 50 mM buffers [sodium acetate/acetic (pH 5.0–6.0); Tris/HCl (pH 7.0–8.0); and glycine/NaOH (pH 9.0–10.0)] containing 1 mM Ca^{2+} . Spontaneous hydrolysis rate was determined in each case and subtracted from the total enzyme rate. pH stability was determined by assaying the residual activity after preincubation of the enzyme in the buffers mentioned above for 0–30 min at 37 °C. The final pH was checked in parallel control experiments, and the deviation from the nominal pH was 0.10–0.45 unit at the extreme pH values. DHC hydrolysis was then measured at pH 7.0 as described in the Enzyme activities section.

Heat inactivation was performed at 40.0, 45.0, 50.0, 52.5 and 60.0 °C in a constant temperature water bath for a set interval over the range 0–180 min. The tubes were then placed on ice and assayed for residual DHC hydrolysis by using the standard procedure. Results were corrected for non-enzymic hydrolysis. The first-order rate constants for enzyme inactivation were calculated by plotting the logarithm of the percentage of the original remaining activity against preincubation time using a computer program (Enzfitter, Biosoft).

Michaelis constants (K_m) were determined from Lineweaver–Burk and Hane plots using a weighted non-linear regression program (Enzfitter, Biosoft). Assays were performed at 37 °C and pH 7.0.

RESULTS

Purification of rat liver PON3

Procedure for the purification of PON3 from rat liver is summarized in Table 1. Differential hydrolysis of three substrates (POX, PA and DHC) was used to study the purification of PON1 and PON3 by the method of Draganov et al. [4]. Fractions showing DHC and PA hydrolyses, but lacking POX hydrolysis, were considered as PON3. The purification procedure involved the following seven sequential steps: solubilization of the microsomal fraction, hydroxyapatite adsorption, chromatography on DEAE–Sephacrose CL-6B, non-specific affinity chromatography on Cibacron Blue 3GA, two DEAE–cellulose steps and a final affinity chromatography step using ConA–Sephacrose.

The first chromatographic step on DEAE–Sephacrose (Figure 1) yielded only one peak containing the three activities. Chromatography on Cibacron Blue (Figure 2) showed two peaks (M_1 and M_2) with the three activities. The first peak (M_1) was obtained during the washing of the column and the retained material was eluted with 1 M NaCl, yielding a second peak (M_2). Only peak M_1 was used to follow the purification procedure. This fraction

Table 1 Summary of the purification and yields of rat liver PON3

Data are for a typical purification run. M_1 , see Figure 2; D_1 , see Figure 3.

Step	Volume (ml)	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Overall purification	Overall yield (%)
Crude extract	81.0	3896.1	10234.0	2.6	1	100.0
Solubilization	13.5	400.4	6835.0	17.1	6.6	66.8
Hydroxyapatite	192.0	94.3	2875.0	30.5	11.7	28.1
DEAE–Sephacrose	13.5	38.9	1612.5	41.5	15.9	15.7
Cibacron Blue (M_1)	13.0	12.3	728.0	59.2	22.8	7.1
First DEAE (D_1)	36.0	1.1	119.3	108.4	41.7	1.2
Second DEAE	75.0	0.4	57.3	143.2	55.1	0.6
ConA	12.0	0.08	36.9	461.2	177.4	0.4

* Using dihydrocoumarin as substrate.

† The crude extract contained 20 g of rat liver.

Figure 1 DEAE–Sephacrose CL-6B chromatography of rat liver PON3

Pooled fractions from the batchwise hydroxyapatite were applied on DEAE–Sephacrose CL-6B chromatography. The active material was eluted by increasing the NaCl concentration. A_{280} (x) was monitored and fractions were collected and assayed for POX (■), PA (○) and DHC (△) hydrolyses.

showed a specific activity of 59.2 units/mg with a purification index of 22.8.

The pooled fractions were chromatographed on a DEAE column. The elution profile (Figure 3) revealed two peaks (D_1 and D_2) with enzyme activity. Peak D_1 showing DHC and PA hydrolyses was obtained during the washing of the column. The material retained (D_2) was eluted with NaCl and showed the three activities. According to the definition of PON3 activity adopted in the present study, D_1 was considered as PON3 and peak D_2 as PON1B. The fraction obtained after this treatment (D_1) had a specific activity of 108.4 units/mg and showed an overall purification of 41.7-fold. The low yield of this step (1.2% of the initial activity) can be explained by the fact that hydrolysis of DHC in D_1 is due exclusively to PON3, whereas in previous steps PON1 and PON1B, in addition to PON3, contributed to the DHC hydrolysis. A second pass of D_1 on DEAE cellulose was introduced to eliminate contaminating proteins (Figure 4). The elution profile showed a single peak with DHC and PA hydrolytic activities during the washing of the column.

Figure 2 Non-specific affinity chromatography on Cibacron Blue 3GA of rat liver PON3

Fractions 36–40 from the DEAE-Sephacel CL-6B chromatography were pooled, and concentrated by ultrafiltration and applied to a Cibacron Blue 3GA column. The material was eluted with 50 mM Tris/HCl (pH 8.0), 100 μ M CaCl_2 and 0.1 % (v/v) Triton X-100 containing 1 M NaCl. Two peaks (M_1 and M_2) with the three activities were detected. A_{280} ($\times$) was monitored and fractions were collected and assayed for POX ($\blacksquare$), PA ($\circ$) and DHC ($\triangle$) hydrolyses.

Figure 3 First anion-exchange chromatography on DEAE of rat liver PON3

Fractions 6–15 (M_1) from the Cibacron Blue 3GA chromatography were pooled. This material was dialysed and concentrated by ultrafiltration before being applied on to a DEAE column. The material was eluted by increasing the NaCl concentration. Two peaks (D_1 and D_2) with activity were detected. Fractions 8–18 (D_1), which hydrolyse DHC and PA, were pooled. A_{280} ($\times$) was monitored and fractions were collected and assayed for POX ($\blacksquare$), PA ($\circ$) and DHC ($\triangle$) hydrolyses.

A final affinity-chromatography step was introduced to purify PON3 further. ConA binds structures containing an α -linked mannose [33], so that it is widely used in the purification of membrane and other glycoproteins [34]. The elution profile of protein and DHC hydrolysis from the ConA column are shown in Figure 5. The retained material was eluted with a linear gradient of α -D-mannopyranoside, collected and washed by using a Centricon 30 microconcentrator to remove contaminating lectin fragments.

At the end of the purification procedure, PON3 was purified 177-fold to a final specific activity of 461 units/mg. An overall recovery of approx. 0.4 % was obtained. By following this procedure, approx. 80 μ g of purified PON3 can be obtained from 20 g of rat liver.

Figure 4 Second anion-exchange chromatography on DEAE of rat liver PON3

Fractions 8–18 from the first DEAE-cellulose chromatography were pooled. This material was dialysed and concentrated by ultrafiltration before being applied on to a second DEAE column. The material was eluted by increasing the NaCl concentration. A_{280} ($\times$) was monitored and fractions were collected and assayed for POX ($\blacksquare$), PA ($\circ$) and DHC ($\triangle$) hydrolyses. A single peak hydrolysing DHC and PA (but not POX) was detected. Fractions 10–23 were pooled for further chromatographic steps.

Figure 5 ConA-Sephacel 4B chromatography of rat liver PON3

Fractions obtained during washing of the second DEAE chromatography step, were concentrated by ultrafiltration and applied on to a ConA column. The material was eluted by increasing the methyl α -D-mannopyranoside (α -DMMP) concentration. A_{280} ($\times$) was monitored and fractions were collected and assayed for DHC ($\triangle$) hydrolyses.

The presence of 2.5 mM Ca^{2+} (as cofactor) and 0.1 % (w/v) Triton X-100 (as detergent) in the buffers throughout the purification procedure was essential for maintaining the activity of the enzyme. In the absence of calcium and Triton X-100, the enzyme activity was quickly lost. Purified PON3 was quite stable, showing no decrease in specific activity after storage for 1 month at 4 $^{\circ}\text{C}$.

Molecular mass of rat liver PON3

Figure 6 shows that the purified product separated by SDS/PAGE appears as a single band (lanes C and D), after Coomassie Blue staining, with an apparent molecular mass of 43 kDa using a standard curve.

Figure 6 SDS/PAGE of rat liver PON3

The pooled fractions from the first DEAE (D_1) and the ConA column were analysed by SDS/PAGE (15% gel) and revealed by Coomassie Blue staining. Experimental conditions were as described in the text. Lanes A and E samples contained 3 μ g of various molecular-mass standards: phosphorylase *b* (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa). Lane B contained 10 μ g of D_1 , and lanes C and D contained 5 and 10 μ g of the ConA eluate respectively. Purified rat liver PON3 (lanes C and D) migrated with a mobility corresponding to an apparent molecular mass of 43 kDa. O, origin; TD, tracking dye front.

Table 2 Identification of tryptic peptides by MS/MS analysis

Peptide sequence	Position	Species
YPGMPAFAPDKPGR	71–84	PON3 mouse
IFLM(I)DL(M)NEQ(K)NSR	85–96	PON3 mouse
VVAQGFSSANGITVSLDQK	214–232	PON3 mouse
LLIYNPEDPPGSEVLR	290–305	PON3 rabbit

Amino acid sequence analysis

To characterize the isolated protein, MALDI-TOF-MS and MS/MS analyses were performed. The purified protein after SDS/PAGE was digested by trypsin followed by MALDI-TOF-MS analysis. The resulting spectrum was used to search for matching proteins in the NCBI and SWISS-PROT databases using the Protein Prospector program. This search did not produce any significant result. Several tryptic peptides were then subjected to MS/MS analysis followed by a search of the fragmentation spectra in the NCBI and SWISS-PROT databases using the Protein Prospector program. The tryptic peptides were identified as belonging to the PON3 gene product, as shown in Table 2.

Enzyme characterization

The pH profile showed optimum activity at 8.8 (Figure 7). Rat liver PON3 showed a high stability against pH. Almost 100% activity was maintained over the pH range 5.0–10.0 during the incubation period (0–180 min). Rat liver PON3 showed the maximum activity at 52.5 °C, but was quite stable between 40 and 55 °C. Inactivation occurred at 60 °C, approx. 50% of its activity remaining after 90 min. The K_m value for DHC hydrolysis was 0.748 ± 0.132 mM and the catalytic rate constant K_{cat} was 1321 ± 88 m-units/ml.

DISCUSSION

Previous studies in rabbit have reported the purification of PON3 in serum [4] and the isolation of a liver MsPON (microsomal

Figure 7 Optimum pH profile of rat liver paraoxonase

Hydrolysis of DHC by purified rat liver PON3 was determined over the pH range 5.0–10.0. Results are from one representative experiment of three experiments performed. The conditions of the experiment were as described in the Experimental section.

paraoxonase), 84% identical with the sequence predicted by a human cDNA for PON3 [27]. However, to our knowledge, there are no additional results regarding the purification of PON3 in other species. In the present study, we have provided evidence that PON3 is present in rat liver microsomes and we have developed a method for the purification of PON3 to homogeneity from that source.

We have suggested previously the presence of more than one enzyme hydrolysing POX and PA in rat liver [24,35]. When purifying rat liver PON1, we detected the presence of two peaks with POX hydrolytic activity (M_1 and M_2) in the non-specific affinity chromatography on Cibacron Blue 3GA [24]. The retained fraction (M_2) showed the highest POX hydrolytic activity, and it was further used to purify a protein identified as PON1 to homogeneity [24]. The second POX hydrolytic activity was not retained by Cibacron, probably due to small differences in the structure or amino acid composition. Both fractions (M_1 and M_2) hydrolysed POX, PA and phenyl thioacetate [26], but showed a different elution profile. Unlike M_2 , where the three activities showed a complete overlapping, in M_1 (the first peak) PA and phenyl thioacetate hydrolyses were partially but not clearly separated from POX hydrolysis. These results pointed out the presence of more than one enzyme activity in M_1 fraction.

Recently, it has been reported that lactones could be a physiological substrate for paraoxonase [4]. Similar to PON1, PON3 is primarily expressed in the liver. On the basis of this fact, as well as owing to the presence of other PONs in rat liver microsomes, we speculated that PON3 might be present in the M_1 fraction obtained from Cibacron Blue. The problem was to differentiate between PON1 and PON3.

The presence of PON3 was checked by using an indirect method on the basis of the differential hydrolysis of three selected substrates, POX, PA and DHC. According to Draganov et al. [4], PON1 hydrolyses the three substrates, whereas PON3 fails to hydrolyse POX but hydrolyses DHC at approximately the same rate and PA at a lower rate. This strategy was applied throughout all the purification steps reported previously for PON1 [24], with the aim of detecting the presence of PON3. It also allowed us to distinguish PON1 from PON3 due to the overlapping hydrolysis of the substrates used (namely DHC).

The results on Cibacron Blue gave us the key for the localization of PON3 (Figure 2). The higher DHC hydrolysis in M_1 raised the possibility that PON3 could be present in this fraction. Nevertheless, evidence for the presence of PON3 in M_1 was

Table 3 Amino acid sequence of several tryptic peptides of rat liver PON3, compared with mouse, rabbit and human PON3

Fragments 1–4 were generated after hydrolysis with trypsin and sequenced by MS. Differences in amino acid residues are shown in bold.

Species	Fragment	Amino acid sequence
Rat	1	71 Tyr-Pro-Gly-Met-Pro-Ala-Phe-Ala-Pro-Asp-Lys-Pro-Gly-Arg 84
Mouse		Tyr-Pro-Gly-Met-Pro-Ala-Phe-Ala-Pro-Asp-Lys-Pro-Gly-Arg
Rabbit		Tyr-Pro-Gly-Met-Pro- Asn -Phe-Ala-Pro-Asp- Glu -Pro-Gly- Lys
Human		Tyr-Pro-Gly-Met-Pro- Asn -Phe-Ala-Pro-Asp-Lys-Pro-Gly- Lys
Rat	2	85 Ile-Phe-Leu-Met (Ile)-Asp-Leu (Met)-Asn-Glu-Gln (Lys)-Asn-Ser-Arg 96
Mouse		Ile-Phe-Leu-Met- -Asp-Leu- -Asn-Glu-Gln- -Asn- Pro-Glu
Rabbit		Ile-Phe-Leu- -Ile-Asp- -Met-Asn-Glu- -Lys-Asn- Pro -Arg
Human		Ile-Phe-Leu-Met- -Asp-Leu- -Asn-Glu-Gln- -Asn-Ser-Arg
Rat	3	214 Val-Val-Ala-Gln-Gly-Phe-Ser-Ser-Ala-Asn-Gly-Ile-Thr-Val-Ser-Leu-Asp-Gln-Lys 232
Mouse		Val-Val-Ala-Gln-Gly-Phe-Ser-Ser-Ala-Asn-Gly-Ile-Thr-Val-Ser-Leu-Asp-Gln-Lys
Rabbit		Val-Val-Ala- Lys -Gly-Phe-Ser-Ser-Ala-Asn-Gly-Ile-Thr-Val-Ser-Leu-Asp- Lys -Lys
Human		Val-Val-Ala- Lys -Gly-Phe- Cys -Ser-Ala-Asn-Gly-Ile-Thr-Val-Ser- Ala -Asp-Gln-Lys
Rat	4	290 Leu-Leu-Ile-Tyr-Asn-Pro-Glu-Asp-Pro-Pro-Gly-Ser-Glu-Val-Leu-Arg 305
Mouse		Leu-Leu-Ile-Tyr-Asn-Pro-Glu- Gly -Pro-Pro-Gly-Ser-Glu-Val-Leu-Arg
Rabbit		Leu-Leu- Asn -Tyr-Asn-Pro-Glu-Asp-Pro-Pro-Gly-Ser-Glu-Val-Leu-Arg
Human		Leu-Leu- Asn -Tyr-Asn-Pro-Glu-Asp-Pro-Pro-Gly-Ser-Glu-Val-Leu-Arg

lacking, probably because it might be present along with other PONs.

At this point, the introduction of a purification step through DEAE-cellulose efficiently resolved PON3 from other PONs (Figure 3). When the active fractions of M_1 were applied on to a column of DEAE-cellulose, PON3 was fully separated from other fractions capable of hydrolysing the three substrates. SDS/PAGE of this preparation showed the presence of contaminant proteins (Figure 6, lane B). Consequently, a second pass through DEAE-cellulose and an affinity-chromatographic step on ConA were introduced to improve the purification of rat liver PON3.

The introduction of anion-exchange chromatography on DEAE-cellulose and affinity chromatography on ConA yielded excellent results and a good final specific activity (see Table 1). PON3 represents approx. 10% of the PON1 content reported in rat liver microsomes [24]. The overall yield of the purification of rat PON3 is lower (one-third) than the yield reported [4] for rabbit serum PON3. However, the specific activity of rat PON3 using DHC as substrate falls within the range reported by Draganov et al. [4] for the same substrate ($220 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

The purity of the final enzyme preparation was confirmed by the appearance of a single band in SDS/PAGE with a molecular mass of approx. 43 kDa (Figure 6). Ozols [27] described MsPON from rabbit liver as a single polypeptide consisting of 350 residues and a calculated molecular mass of 39,049 kDa. Draganov et al. [4] reported that PON3 is a 40 kDa protein associated with the high-density lipoprotein fraction of rabbit serum. A similar molecular mass for PON3 has been proposed by Reddy et al. [19]. The molecular mass for PON3 is quite similar to the molecular mass for PON1 found in our laboratory [24] as well as by others [22,36,37].

The identity of the purified protein was confirmed to be PON3 by MALDI-TOF-MS and nanoelectrospray MS (see Table 2). As shown in Table 3, rat PON3 is 95% identical with the deduced cDNA sequence of the mouse PON3 gene. A comparison of rat PON3 with MsPON (rabbit liver PON3) and human PON3 indicates 88.5 and 90% identity respectively. The degree of identity between rat PON1 and PON3 is 67%, which is very similar to that reported for humans [38] and rabbit [27].

To our knowledge, no results on biochemical properties of PON3 have been reported. The optimum pH found for PON3 is similar to that reported previously for PON1 from rat liver microsomes [23,39], sheep serum [41] and human serum [42]. Diazoxonase has also been reported to have a similar optimum pH [40]. Preincubation of PON3 samples over the pH range 5.0–10.0 had no effect on the activity measured at pH 7.0, indicating a high pH stability. The strong stability against pH contrasted with the inactivation studies reported previously for PON1 in rat liver microsomes [23,24,26]. pK_a values were determined by the method of Wilkinson [43]. pK_{a1} and pK_{a2} values calculated were 6.87 and 10.8 respectively. These results suggest that the imidazolium group of histidine (pK_a 5.5–7.0) and the ϵ -amino group of lysine (pK_a 9.5–10.6) or the phenolic OH group of tyrosine (pK_a 9.8–10.5) may be responsible for the catalytic activity of PON3. The same amino acid residues have been reported for PON1 from rat plasma and liver [23]. Most of the residues essential for the hydrolytic activity in human serum PON1 are conserved in rabbit serum PON3 [4]. Sorenson et al. [44] have suggested that three out of ten histidine residues described in serum PON1 from different sources (human, rabbit and mouse) could be implicated in the catalytic-centre activity. These results are in accordance with the implication of a histidine residue in the activity of rat liver PON3 as deduced from pH studies.

The time-dependent heat inactivation of rat liver-purified PON3 showed a monophasic pattern over the range of temperatures assayed. A similar pattern has been described for rat plasma PON1 [23] and purified rat liver PON1 [24]. However, PON3 was always more resistant than PON1 to heat inactivation over the range of temperatures assayed. The K_m value calculated for purified rat liver PON3, using DHC as substrate, was 0.748 mM. This value is of the same order as those found for the hydrolysis of POX by rat liver PON1 [24–26]. Lower K_m values have been reported for the hydrolysis of DHC by PON1 isoenzymes (Q and R) [3].

In summary, our results show a good purification method for rat liver PON3 and indicate high similarities between several internal amino acid sequences of rat PON3 and human, rabbit or mouse liver PON3. Some biochemical properties of the purified rat

PON3 are presented in this paper, suggesting a slightly different behaviour from rat liver PON1. Further experiments by using a more specific substrate for PON3 are therefore necessary to achieve the complete characterization of PON3 and to establish the relative toxicological importance of PON3 with respect to PON1.

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