Purification and characterization of paraoxon hydrolase from rat liver

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Paraoxonase (paraoxon hydrolase), an enzyme that hydrolyses paraoxon (*O*,*O*-diethyl *O*-*p*-nitrophenyl phosphate), is located in mammals primarily in the serum and liver. Although considerable information is available regarding serum paraoxonase, little is known about the hepatic form of this enzyme. The present work represents the first study on the purification of rat liver paraoxonase. This enzyme has been purified 415-fold to apparent homogeneity with a final specific activity of 1370 units/mg using a protocol consisting of five steps: solubilization of the microsomal fraction, hydroxyapatite adsorption, chromatography on DEAE-Sepharose CL-6B, non-specific affinity chromatography on Cibacron Blue 3GA and anion exchange on Mono Q HR 5/5. The presence of Ca^{2+} and Triton X-100 in the buffers throughout the purification procedure was essential for maintaining enzyme activity. SDS/PAGE of the final preparation indicated a single protein-staining band with an apparent M_r of 45000. N-terminal and internal amino acid sequences were determined and compared with those of paraoxonases from human and rabbit serum and mouse liver, showing a high similarity. The pH profile showed optimum activity at pH 8.5. The pH stability and heat inactivation of the enzyme were also studied. The K_m for liver paraoxonase was 1.69 mM.

INTRODUCTION

Aldridge [1] defined the A-esterases as a group of enzymes that hydrolyse organophosphorus esters, in contrast with B-esterases, which are inhibited by organophosphorus compounds. The first demonstration of an A-esterase was by Mazur [2], who showed that rabbit serum was able to hydrolyse paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphate) and di-isopropylphosphorofluoridate. The hydrolysis of paraoxon was studied in detail by Aldridge [3], and many A-esterases have been identified in different tissues of several animal species. Much of the early work was summarized by Aldridge and Reiner [4], and updated reviews have been published [5,6].

Mammals tend to exhibit high A-esterase activity in the blood and the liver, and this is apparently an important factor in determining the resistance of these organisms to organophosphate toxicity [7]. On the other hand, birds have very low levels of A-esterase activity and are more susceptible to organophosphorus compounds [8]. The relationship between serum A-esterases and organophosphate toxicity in man has been reviewed by Lotti and co-workers [9].

The substrate specificity of A-esterases is very complex and not well understood [4,10,11]. In addition, the physiological role of A-esterases has yet not been clarified, since no natural substrates of these enzymes are known [12,13]. At present, paraoxon hydrolase (paraoxonase) has no known function *in vivo*, although serum A-esterase has been reported to be associated with high-density lipoproteins [14–16]. Therefore paraoxonase could be involved in atherogenesis, since this enzyme probably hydrolyses multiple oxygenated forms of polyunsaturated fatty acids at the *sn*-2 position of low-density-lipoprotein-associated phospholipids [17]. Another problem is the distinction between A-esterases and their implications for esterase classification [18–21].

In spite of the toxicological significance of A-esterases, relatively few esterases that hydrolyse foreign compounds have actually been purified, and although numerous studies of organophosphorus hydrolases have been reported, they have mostly focused on the hydrolysis of paraoxon or related phosphate esters by the serum. Mazur [2] was the first to report the partial purification of an A-esterase (di-isopropylphosphorofluoridate hydrolase) from rabbit kidney, obtaining a product that was purified approx. 13-fold. Further studies on this enzyme [22] improved the purification to 65–100-fold. Main [23] purified sheep serum paraoxonase 330–385-fold using ethanol, pH and ionic strength fractionation. Human and rabbit serum A-esterases have been purified and the cDNA characterized [24]. However, compared with serum A-esterase, little is known about rat hepatic A-esterase, perhaps due to the fact that rat liver paraoxon hydrolytic activity (paraoxonase) is essentially a microsomal enzyme associated with vesicles derived from the endoplasmic reticulum [25]. In a previous report [26] we established the first method for the partial purification of rat liver paraoxonase, which consisted of preparation of microsomes, solubilization with Triton X-100, adsorption on hydroxyapatite and chromatography on DEAE-52 cellulose, giving a yield of about 22 % and a purification factor of 77-fold. Huang et al. [27] have purified the A-esterase from mouse hepatic microsomes, with a 1531-fold increase in specific activity and a recovery of 10 %.

The objectives of the present study were: (1) purification of rat liver paraoxonase to homogeneity, (2) determination of the N-terminal amino acid sequence and two internal sequences, (3) comparison of these sequences with previously identified sequences in paraoxonases from human and rabbit serum and mouse liver, and (4) investigation of some biochemical properties of the purified rat liver enzyme and their comparison with those of the microsomal enzyme.

MATERIALS AND METHODS

Chemicals

Hydroxyapatite (Bio-Gel HTP), dithiothreitol, Tris base, SDS, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ammonium persulphate, acrylamide and bisacrylamide were provided by Bio-Rad (Richmond, CA, U.S.A.). DEAE-52 cellulose was from Whatman (Maidstone, U.K.), and DEAE-Sepharose CL-6B and low-M_r standards were from Pharmacia (Uppsala, Sweden).

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Ampholines pH 4–6 were obtained from LKB. Paraoxon, BSA (fraction V) and Cibacron Blue 3GA 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. Poly(vinylidene difluoride) was obtained from Millipore, and ProSpin cartridges from Applied Biosystems. Other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Animals

Male Wistar rats weighing 180–200 g at the time of death were used. Animals were maintained on laboratory chow (Paulab, Barcelona, Spain) and tap water *ad libitum* with a 12 h day/night cycle (light cycle from 07.00 to 19.00 h). Rats were fasted for 16 h before decapitation. Fasting was utilized to increase the yield of the microsomal fraction [25].

Enzyme assay

A-esterase activity towards paraoxon (paraoxonase) was quantified spectrophotometrically by a modification of the method described by Reiner and Radic [28] using 100 mM Tris/HCl buffer, pH 7.4, and 1 mM $CaCl_2$. The reaction was followed for 2 min at 37 °C by monitoring the appearance of *p*-nitrophenol at 405 nm in a Perkin–Elmer Lambda 2 automated recording spectrophotometer. The final substrate concentration during the enzyme assay was 2 mM, and all rates were determined in duplicate and corrected for non-enzymic hydrolysis. For pH values below 7.5, the *p*-nitrophenol formed in the reaction was monitored by HPLC at 315 nm. Briefly, the reaction was stopped by adding an equal volume of 0.35 M perchloric acid/0.36 M sodium acetate, followed by centrifugation (1500 ***g***, 10 min). The clear supernatant was injected on a Perkin–Elmer (5 μm pore size; 12.5 cm) C-18 reverse-phase column, and the *p*-nitrophenol was eluted using a mobile phase of methanol/0.05 M perchlorate buffer adjusted to pH 2.5 (2:3, v/v) at a flow rate of 1 ml/min.

Buffers used in the purification of rat liver paraoxonase

Buffer A: 5 mM Tris/HCl (pH 7.4), 0.25 M sucrose. Buffer B: 5 mM Tris/HCl (pH 7.4). Buffer C: 20 mM potassium phosphate (pH 7.5), 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 $CaCl_2$, 0.1 % (v/v) Triton X-100 containing 20 mM NaCl. Buffer F: 20 mM Tris/HCl (pH 7.7), 2.5 mM $CaCl_2$, 0.1 % (v/v) Triton X-100 containing 50 mM NaCl. Buffer G: 20 mM Tris/HCl (pH 7.7), 2.5 mM $CaCl_2$, 0.1 % (v/v) Triton X-100 containing 500 mM NaCl. Buffer H: 20 mM Tris/HCl (pH 7.7), 2.5 mM $CaCl_2$, 0.1 % (v/v) Triton X-100 containing 2 M NaCl. Buffer I: 50 mM Tris/HCl (pH 8.0), 100 μM $CaCl_2$, 0.1 % (v/v) Triton X-100. Buffer J: 50 mM Tris/HCl (pH 8.0), 100 μM $CaCl_2$, 0.1 % (v/v) Triton X-100 containing 1 M NaCl. Buffer K: 100 mM Tris/HCl (pH 8.5), 0.5 M NaCl. Buffer L: 100 mM sodium acetate (pH 4.5), 0.5 M NaCl.

Purification of rat liver paraoxonase

All purification procedures were carried out at 0–4 °C unless otherwise stated.

Preparation of the microsomal fraction

Microsomal fractions were prepared essentially as described previously [25]. 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) with 1.02 mm clearance. The homogenate was transferred to a power-driven close-fitting (0.045 mm clearance) Perspex/glass homogenizer and homogenized as before. After diluting the homogenate to 10 % (w/v) with buffer A, nuclei and mitochondria were removed by successive centrifugation at 460 ***g*** for 10 min and at 12500 ***g*** for 10 min in a Beckman J2-21 refrigerated centrifuge. The post-mitochondrial supernatant fraction was then centrifuged at 105000 ***g*** for 60 min in a Beckman 55.2 Ti rotor operated in a Beckman L8-55 refrigerated centrifuge. The microsomal pellet derived from 20 g of liver tissue was suspended in 20 ml of buffer B.

Solubilization of microsomal membranes

Paraoxonase was extracted by the addition of Triton X-100 [26]. The microsomal fraction was adjusted to 0.75 % Triton X-100, vortexed, stored at 4 °C for 30 min and then centrifuged at 105000 ***g*** for 60 min.

Batchwise hydroxyapatite adsorption

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 room temperature. The exchanger was recovered by centrifugation at 1080 ***g*** for 1 min. The matrix was washed twice with 1 vol. of buffer C and finally with 0.5 vol of the same buffer. After washing, each supernatant was centrifuged as above, and the four supernatants were pooled and centrifuged again at 4300 ***g*** for 2 min to eliminate residual particles of exchanger [26]. The pooled supernatants were concentrated to 20 ml using a Filtron ultrafiltration system (Filtron Technology Corp., Clinton, MA, U.S.A.) fitted with an Omega® Serie Membrane of 30 K. After use, hydroxyapatite was regenerated by washing thoroughly with buffer D and then re-equilibrated in the starting buffer (buffer C).

DEAE-Sepharose CL-6B chromatography/ion-exchange chromatography

The concentrated hydroxyapatite eluate was loaded at a flow rate of 0.5 ml/min on to a column of DEAE-Sepharose CL-6B (column bed 1.6 cm × 22 cm) pre-equilibrated in buffer E. The column was connected to a peristaltic pump P-3 (Pharmacia), and then washed with buffer E until the A_{280} decreased to approx. 0.1 (about 100 ml). The bound enzyme was eluted from the column by using a linear salt gradient of 50–500 mM NaCl in the same buffer (buffers F and G contain 50 and 500 mM NaCl respectively). Fractions of 4 and 2 ml were collected during the washing procedure and gradient elution respectively. Fractions with peak activity were pooled and concentrated to about 10 ml using a Filtron ultrafilter.

Cibacron Blue 3GA chromatography/non-specific affinity chromatography

The concentrated eluate, previously dialysed against deionized water (two changes of 2 litres each per 4 h) to remove salts, was adsorbed/pumped 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 was eluted with buffer J (containing 1 M NaCl). Fractions (2 ml) were collected, and fractions showing activity were pooled and concentrated by ultrafiltration (Filtron) to a final volume of 1 ml. Only the fractions that contained the highest enzyme activity

(fractions 27–42) were used for Mono Q HR 5/5 chromatography.

Mono Q HR 5/5 Chromatography/anion-exchange chromatography

The concentrated pooled fractions from Cibacron Blue chromatography were dialysed overnight against deionized water (two changes of 2 litres each per 4 h) and applied at 0.1 ml/min on to a column (1 cm × 10 cm) of Mono Q HR 5/5 that had been attached to the FPLC system (Pharmacia) and pre-equilibrated with buffer E. The column was first washed with the same buffer, and then the enzyme was eluted with a linear NaCl gradient from 50 to 500 mM (buffers F and G respectively) at 0.5 ml/min. Fractions of 1 ml were collected.

Protein determination

The protein content of fractions from the column chromatographic separations were monitored by measuring the absorbance at 280 nm; protein concentration was estimated by the method of Lowry et al. [29], with BSA (fraction V) as a reference standard. A modification of this procedure [30] was used to assay protein in the presence of Triton X-100.

SDS/PAGE analysis

Protein samples obtained during the different purification steps were analysed by SDS/PAGE as described by Laemmli [31], using a Bio-Rad Miniprotean II electrophoresis unit. The final monomer concentration in the 0.75 mm-thick slab gels was 15% (w/v) for the separating gel and 4% (w/v) for the stacking gel. Prior to loading, all samples were incubated in the presence of 1% (w/v) SDS and 100 mM dithiothreitol for 2 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.

Isoelectric focusing

Electrofocusing was carried out with ampholine polyacrylamide gel plates (LKB 2297) using 6% acrylamide gels and an ampholyte gradient with a pH range of 4–6 [26]. Aliquots (15 μl) of sample were focused at 1800 V and 7.5 W for 2 h 30 min at 8 °C. After focusing, the gel was washed with water and then incubated in 100 ml of substrate solution (100 mM Tris/HCl, pH 7.4, 1 mM $CaCl_2$, 2 mM paraoxon) at 37 °C for 60 min. The paraoxonase produced a yellow band of enzymic product (*p*-nitrophenol). Protein bands were stained with Coomassie Blue.

Amino acid sequence analysis

For N-terminal sequencing, 1 ml of the purified protein (0.25 mg/ml) in 50 mM Tris/HCl (pH 7.5), 1 mM $CaCl_2$ and 0.1% (v/v) Triton X-100 was applied on to a poly(vinylidene difluoride) membrane using a ProSpin cartridge. After thorough washing, the protein on the membrane was directly sequenced using an ABI 473A pulsed-liquid-phase Protein Sequencer (Applied Biosystems), employing the modified cycles according to manufacturer's instructions.

To obtain internal sequences, 50 μg of purified protein in 600 μl of 50 mM Tris/HCl (pH 7.5), 1 mM $CaCl_2$ and 0.1% (v/v) Triton X-100 was concentrated to 200 μl in a Speedvac Centrifuge, Model RC 10.22 (Jouan), and the pH of the solution was adjusted to 8.5. Trypsin was then added at a 1:20 molar ratio (protease/protein) and the solution was incubated overnight at 30 °C with gentle agitation. Trifluoroacetic acid (0.1 vol.) was added, the mixture was cleared by centrifugation at 12000 ***g*** and 140 μl of the supernatant was injected into a μRPC C2/C18 SC (2.1 mm × 10 mm) reverse-phase column connected to a SMART micro-HPLC system (Pharmacia). Peptides were separated at 100 μl/min using the chromatographic conditions described previously [32], and fractions were collected. Automatic integration of the peaks was utilized. Selected fractions were then applied on to polyprene-coated glass-fibre filters and subjected to sequencing.

Characterization of the purified enzyme

The effect of pH on rat liver paraoxonase activity was measured at 37 °C over the pH range 7–11 in 50 mM buffer (Tris/HCl, pH 7.0–8.0; glycine/NaOH, pH 9.0–11.0). Spontaneous hydrolysis 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 100 mM buffer (sodium acetate/acetic acid, pH 5.0; phosphate, pH 6.0; Tris/HCl, pH 7.0–8.0; glycine/NaOH, pH 9.0–12.0) 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. Enzyme activity was then measured at pH 7.4 as described under 'Enzyme assay'.

Heat-inactivation studies were performed at 35.0, 40.0, 42.5, 45.0, 47.5, 50.0, 52.5 and 55.0 °C in a constant-temperature water bath for a set time interval in the range 0–60 min. The tubes were then placed on ice and assayed for residual paraoxonase activity using the standard procedure. The first-order rate constants for enzyme inactivation against pH and temperature were calculated by plotting the logarithm of the percentage of the original activity remaining against preincubation time using the Enzfitter program (Biosoft).

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

RESULTS

Purification of rat liver paraoxonase

The purification procedure involved five sequential steps: solubilization of the microsomal fraction, hydroxyapatite ad-

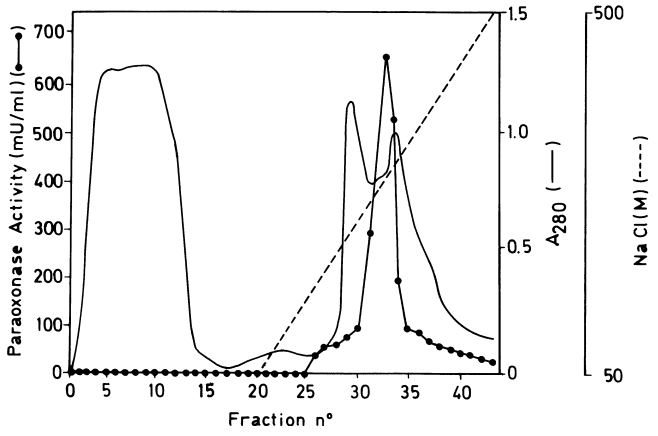

Figure 1 DEAE-Sepharose CL-6B chromatography of rat liver paraoxonase

Pooled fractions from the batchwise hydroxyapatite adsorption were applied to DEAE-Sepharose CL-6B chromatography. The active material was eluted by increasing the NaCl concentration. A_{280} was monitored, and fractions were collected and assayed for paraoxonase activity (m-units/ml) using paraoxon as substrate.

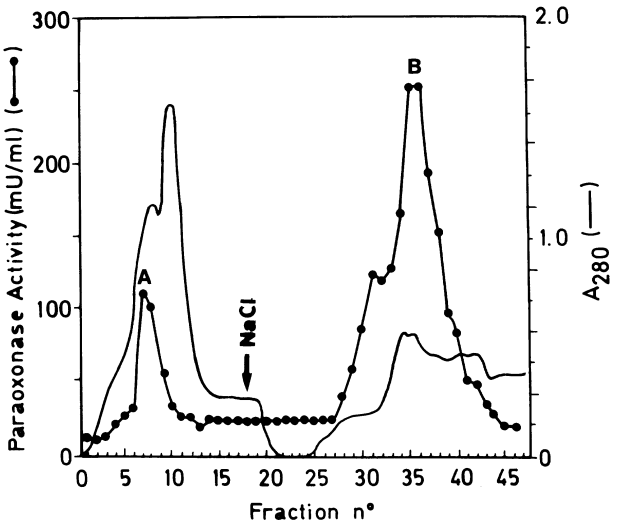

Figure 2 Non-specific affinity chromatography of rat liver paraoxonase on Cibacron Blue 3GA

Fractions 30–35 from the DEAE-Sepharose CL-6B chromatography were pooled, desalted and finally concentrated by ultrafiltration and applied to a Cibacron Blue 3GA column. The material was eluted with 50 mM Tris/HCl (pH 8.0), 100 μM $CaCl_2$ and 0.1% (v/v) Triton X-100 containing 1 M NaCl. Two peaks of paraoxonase activity (A and B) were identified. A_{280} was monitored, and fractions were collected and assayed for paraoxonase activity (m-units/ml) using paraoxon as substrate.

sorption, chromatography on DEAE-Sepharose CL-6B, non-specific affinity chromatography on Cibacron Blue 3GA and anion-exchange on Mono Q HR 5/5.

The activity recovered after solubilization with Triton X-100 was no greater than 80–85%. The initial batchwise hydroxyapatite fractionation step was required to eliminate a high percentage of proteins present in the crude extract. The fraction obtained after this treatment showed a specific activity of 90.0 units/mg and an overall purification of 27.5-fold. In the third step, the extract was chromatographed on DEAE-Sepharose CL-6B (Figure 1), which typically yielded 50% of the initial activity applied. In the fourth step, selective removal of albumin was achieved by non-specific affinity chromatography on Cibacron Blue 3GA. Although two peaks of paraoxonase activity were apparent, only the peak with the highest activity was considered; 60% of the activity applied was recovered with this procedure (Figure 2). Fractions collected from the Cibacron Blue 3GA column were chromatographed on a Mono Q HR 5/5 column. The elution profile (Figure 3) revealed a single peak of paraoxonase activity. At the end of the purification procedure, rat liver paraoxonase was purified 415-fold to a final specific activity of 1370 units/mg, and an overall recovery of about 6% was obtained. Table 1 gives a summary of the purification. Using this procedure, approx. 600 μg of purified paraoxonase can be obtained from 20 g of rat liver within 72 h.

The presence of 2.5 mM Ca^{2+} (as a cofactor) and 0.1% (w/v) Triton X-100 (as a 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 quickly lost activity. The purified enzyme activity was stable over a period of 2 weeks, showing no decrease in specific activity after this period of time if stored at 4 °C.

Molecular mass of rat liver paraoxonase

Figure 4 shows that the purified product separated by SDS/PAGE appears as a single band (lane E) after silver staining, with an apparent M_r of 45000 estimated using a standard curve.

Isoelectric focusing

Electrofocusing showed the presence of one protein yellow band located at an isoelectric point of 4.7–4.8.

Amino acid sequence analysis

The purity of the enzyme was further confirmed by amino acid sequence analysis of the protein band. The N-terminal sequence of 10 amino acid residues of the protein (fragment a) from the reverse-phase column was Ala-Lys-Leu-Leu-Gly-Leu-Thr-Leu-Val-Gly. No evidence for contaminating proteins (with unblocked N-termini) was indicated by the sequence results. The pure protein was submitted to digestion with trypsin and the resulting peptides were separated by reverse-phase HPLC. Two additional peptidic fragments (b and c) were obtained: Gly-Ile-Glu-Ala-Gly-Ala-Glu-Asp-Leu-Glu and Ile-Gln-Ser-Ile-Leu-Asp-Glu-Asp-Pro-Lys respectively. All fragments (a, b and c) are shown in Figure 5, and the sequences are of great similarity to

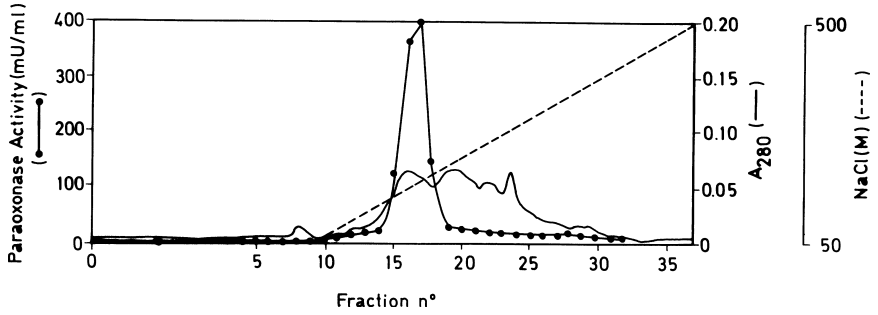

Figure 3 Anion-exchange chromatography of rat liver paraoxonase on Mono Q HR 5/5

Fractions 27–42 from the Cibacron Blue 3GA chromatography were pooled. This material was dialysed and concentrated by ultrafiltration before being applied to a Mono Q HR 5/5 FPLC column. The material was eluted by increasing the NaCl concentration. A_{280} was monitored, and fractions were collected and assayed for paraoxonase activity (m-units/ml) using paraoxon as substrate.

Table 1 Summary of purification and yields of rat liver paraoxonase

The crude extract contained 20 g of rat liver. These data are typical of seven individual purifications performed.

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Overall purification (fold)	Overall yield (%)
Crude extract	81.0	48.1	3896.1	3.3	12814.2	1	100.0
Solubilization	13.5	28.1	379.1	27.9	10576.6	8.5	82.5
Hydroxyapatite	192.0	0.6	105.6	90.0	9600.0	27.5	74.9
DEAE-Sepharose	15.5	2.3	35.0	122.5	4283.4	37.1	33.4
Cibacron Blue	21.0	0.5	11.2	226.8	2529.5	68.7	19.7
Mono Q	2.0	0.3	0.6	1370.4	780.2	415.3	6.1

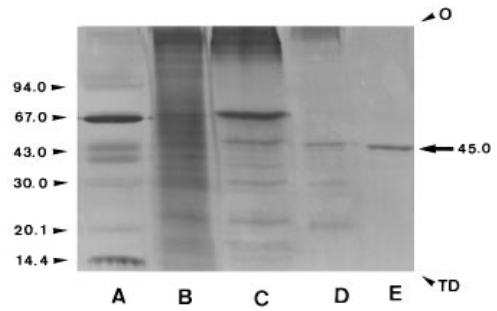

Figure 4 SDS/PAGE of rat liver paraoxonase

Pooled samples from consecutive steps in the purification of rat liver paraoxonase were analysed by SDS/PAGE (15% gel) and revealed by silver staining. Experimental conditions were as described in the text. Lane A contains 3 μg of various M_r standards (shown on the left as $10^{-3} \times M_r$): phosphorylase *b* (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α-lactalbumin (14400). Lanes B, C, D, E contain respectively a 5 μg sample of the solubilized microsomes and 10 μg samples of the DEAE-Sepharose, Cibacron Blue and Mono Q eluates; all contain paraoxonase activity. Purified rat liver paraoxonase (lane E) migrated with a mobility corresponding to an apparent M_r of 45000. Abbreviations: O, origin; TD, tracking dye front.

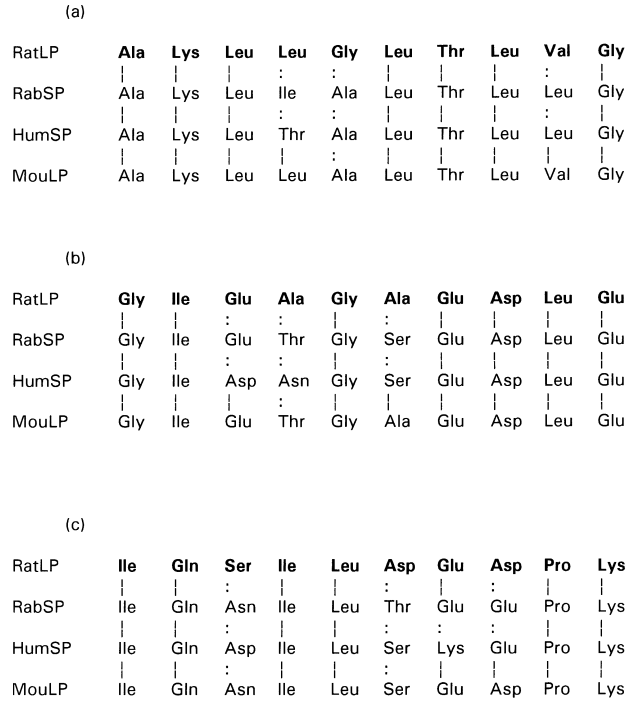

Figure 5 N-terminal and internal amino acid sequences of rat liver paraoxonase (RatLP), compared with rabbit (RabSP) and human (HumSP) serum paraoxonases and mouse liver paraoxonase (MouLP)

(**a**) N-terminal sequences; (**b**) and (**c**) represent two fragments generated after hydrolysis with trypsin. Vertical lines indicate identity of amino acid residues, and dots indicate conserved replacements.

those of paraoxonases from human and rabbit serum and mouse liver.

Enzyme characterization

The pH profile showed optimum activity at 8.5 (Figure 6). Regarding the pH stability, the first-order rate constants for enzyme inactivation at different pH values are shown in Table 2. Maximum stability was achieved between pH 7.0 and 9.0, and enzyme activity declined markedly above pH 9.0 and below pH 6.0. The kinetic constants evaluated for heat inactivation are also listed in Table 2. Rat liver paraoxonase appeared relatively stable at 47.5 °C, with 50% of its activity remaining after 30 min of incubation. However, at 52.5 and 55 °C, rapid inactivation occurred after 15 and 60 min respectively (results not shown). The K_m value was 1.690 ± 0.091 mM and the catalytic rate constant (k_{cat}) was 1031.7 ± 27.8 m-units/ml.

DISCUSSION

To our knowledge, there are no previous data on the purification to homogeneity of rat liver paraoxonase. The fact that rat liver paraoxonase is a membrane protein has limited considerably the techniques normally available for purification. However, the purification procedure achieved in the present study is straightforward and efficient.

Our previous report suggested that rat liver paraoxonase requires to be solubilized prior to purification [26]. Loss of activity was observed during the various steps of purification in spite of maintenance of calcium and Triton X-100 throughout the whole process, as previously reported for serum A-esterases [23,33–36]. The loss of activity during purification could explain the low purification indexes obtained in previous reports of attempts to purify A-esterases.

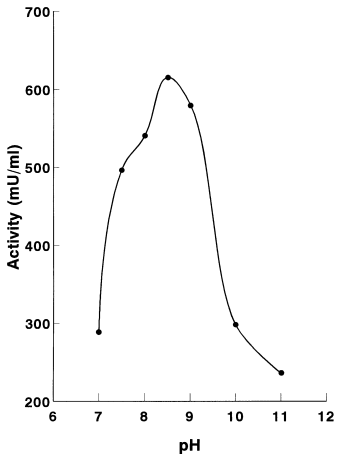

Figure 6 Optimum pH profile of rat liver paraoxonase

The hydrolysis of paraoxon by purified rat liver paraoxonase was determined over the pH range 7.0–11.0. Data are shown from one experiment representative of three experiments performed. The conditions were as described in the Materials and methods section.

Table 2 Kinetic constants for pH and heat inactivation of rat liver paraoxonase

Rat liver paraoxonase was incubated in 0.1 M Tris/HCl buffer, pH 7.4, for fixed periods of time in the ranges 0–30 min and 0–60 min for the indicated pH values and temperatures respectively. Results are mean values obtained from three different experiments assayed in duplicate. Inactivation reactions showed a monophasic pattern, and the apparent first-order rate constants (k_1) were calculated from the slopes of plots of log of the percentage activity remaining against preincubation time.

	k_1 (min^{-1})
pH	
5.0	0.0053
6.0	0.0101
7.0	0.0027
8.0	0.0034
9.0	0.0024
10.0	0.0089
11.0	0.0462
Temperature (°C)	
35.0	0.0019
40.0	0.0041
42.5	0.0076
45.0	0.0078
47.5	0.0238
50.0	0.0274
52.5	0.0541
55.0	0.1330

Our results are an improvement on those obtained using other purification methods proposed for serum paraoxonase [14,23,33]. Huang et al. [27] have reported a solubilization procedure with sodium cholate and Triton N-101 and a method for purification of paraoxonase from mouse hepatic microsomes involving gel-permeation chromatography followed by ion-exchange chromatography and non-specific affinity chromatography; this resulted in a peak of A-esterase activity judged to be homogeneous by SDS/PAGE and a 1531-fold increase in specific activity, with a recovery of 10 %. The overall purification factor in our study may be significantly underestimated, due to a high protein content in the crude extracts. However, our purification procedure yielded excellent results and the final specific activity compared favourably with the values reported for other A-esterases. Nevertheless, the purification factor reported here is not directly comparable with those reported by other workers because of different procedures used for determination of the protein content at various stages of purification.

The purity of the final enzyme preparation was confirmed by the appearance of a single band on SDS/PAGE with an M_r of approx. 45000, and the absence of any secondary sequences in the N-terminal amino-acid sequence analysis. While Gan et al. [37] purified human serum A-esterase to give a single band on SDS/PAGE with an M_r of 43000, Furlong et al. [38] reported two bands of purified rabbit serum A-esterase with M_rs of 35000 and 38000, and two bands of purified human serum A-esterase with M_rs of 44700 and 47900. Huang et al. [27] indicated the presence of one major protein band on SDS/PAGE, with M_r values of 40000 and 39000 with and without an acrylamide gradient respectively, for mouse hepatic A-esterase.

The present paper also reports the first amino acid sequence of rat liver paraoxonase, and this protein shares 70 % identity in the N-terminal region with both human and rabbit serum paraoxonases [24] and 90 % identity with mouse liver paraoxonase [39]. Internal amino acid sequences gave similar results. A database search for comparable N-terminal and intermediate peptide sequences was performed at the Biology Molecular Center (Madrid) using the SwissProt software. Neither identity nor similarity to other known proteins was found. Therefore we are unable to rationalize the natural substrate of paraoxonase by this means.

The pH optimum determined by us for the purified enzyme (pH 8.5) is identical to that previously reported for rat liver microsomal preparations [40]. Our results are similar to those reported for diazoxonase [41] and paraoxonase [42] from rat liver microsomes. However, optimum pH values of 7.7 and 7.4 have been found by others [42,43]. Similar values to those found by us have been reported for paraoxonases from sheep serum [44] and human serum [45]. pH inactivation studies showed a monophasic pattern, which is in contrast with the biphasic kinetics observed previously [40] for rat liver microsomal paraoxonase at extreme pH values (pH 3.0, 4.0 and 10.5). This finding was reported to be consistent with a membrane effect [40].

The time-dependent heat inactivation of purified rat liver paraoxonase also showed a monophasic pattern, in contrast with the microsomal paraoxonase, which was inactivated in a biphasic manner above 45 °C [40]; again, the biphasic reaction could be due to a membrane effect. A similar pattern has been described by others for rat and human plasma paraoxonases [43,46,47]. The first-order rate constants observed for heat inactivation at 47.5 and 50 °C ($k_1 = 0.0238$ and $k_1 = 0.0274$ min^{-1} respectively) were similar to those described by Traverso et al. [48] for human plasma paraoxonase ($k_1 = 0.024$ min^{-1}) and by Pellin et al. [43] for rat plasma paraoxonase ($k_1 = 0.038$ min^{-1}). At 52.5 and 55.0 °C the rate of inactivation increased ($k_1 = 0.0541$ min^{-1} and 0.1330 min^{-1} respectively), in accordance with data reported by Reiner et al. [47] ($k_1 = 0.097$ min^{-1} at 53 °C) and Gil et al. [40] ($k_1 = 0.061$ min^{-1} at 52.5 °C) for the paraoxonase activity found in rat liver microsomes.

The K_m for purified rat liver paraoxonase was 1.69 mM. Gil et al. [25] found a value of 0.48 mM for the rat liver microsomal enzyme. Wallace and Dargan [49] reported different values to those obtained by us: 0.18 and 0.13 mM in microsomes from rat and mouse respectively. A much higher value (7.5 mM) has been reported by Pellin et al. [43].

Another interesting point is the degree of similarity between the paraoxonases from rat liver and serum. In a previous study [40] based on kinetic properties, we found more differences than analogies between the rat plasma and liver microsomal enzymes, which suggested the presence of two different forms of the same enzyme. From a molecular point of view and based on the data presented in Figure 5, we can reasonably assume a high degree of similarity between rat liver and serum paraoxonases, in view of the 70% identity between the rat liver enzyme and the serum enzymes from other species (human and rabbit). However, to our knowledge no data have been reported on the amino acid sequence of rat serum paraoxonase.

On the other hand, we previously reported the existence of both EDTA-sensitive and EDTA-resistant paraoxonase fractions in rat liver microsomes [40]. In the purification procedure described, chromatography on Cibacrom Blue resulted in two peaks with paraoxonase activity; that with the highest specific activity was used to complete the purification process. The fraction purified to homogeneity was found to be EDTA-sensitive (results not shown), indicating that only one of the two fractions previously detected was obtained. Accordingly, from our results it is not possible to establish whether the purified paraoxonase is the liver-specific form of paraoxonase or the serum paraoxonase destined for secretion. These possibilities are currently being investigated in our laboratory.

In conclusion, our results show an excellent purification protocol for rat liver paraoxonase, and indicate marked similarities between the N-terminal and internal amino acid sequences of rat liver paraoxonase and paraoxonases from human and rabbit serum and mouse liver. The maintenance of such a degree of similarity throughout phylogenetic evolution suggests an important metabolic role for paraoxonase activity, which is as yet unknown. On the other hand, we have found no significant differences in some biochemical properties studied with respect to the rat liver microsomal enzyme, confirming the validity of our previous results [40].

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