Baculovirus-mediated expression and purification of human serum paraoxonase 1A

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Abstract **Human paraoxonase 1 (hPON1) is a lipid-associated enzyme transported on HDL. There is considerable interest in hPON1 because of its putative antioxidative/antiatherogenic properties. We have created a recombinant baculovirus (BV) to generate hPON1A in large quantities for structure-function studies and here describe the method for production and isolation of the enzyme. A high level of recombinant hPON1 type A (rPON1A) was produced by Hi-5 insect cells (40 mg/l); a fraction (~10 mg/l) was secreted into the cell culture medium, but the majority (~30 mg/l) remained associated with the host insect cells. Cell-associated rPON1A was purified by detergent extraction (Tergitol NP-10) followed by three simple chromatography steps (DEAE-Sepharose, Sephacryl S-200, and concanavalin A). The purified enzyme bound to concanavalin A and was converted to a lower molecular mass by endoglycosidase H digestion, suggesting that rPON1A contained high-mannose *N*-glycan chains. There was a significant decrease in arylesterase activity (>99%) concomitant with enzymatic deglycosylation. rPON1A was dependent on Ca^{2+} for arylesterase activity, exhibiting kinetic parameters similar to native hPON1A (K_m = 3.8 ± 2.1 vs. 3.7 ± 2.0 mM and V_{max} = 1,305 ± 668 vs. 1,361 ± 591 U/mg protein, rPON1A and hPON1A, respectively). Both rPON1A and hPON1A efficiently inhibited lipoxygenase-mediated peroxidation of phospholipid. In contrast to the arylesterase activity, which was sensitive to endoglycosidase H treatment, enzymatic deglycosylation did not inhibit the antioxidant activity of rPON1A. In conclusion, our BV-mediated PON1A expression system appears ideally suited for the production of relatively large quantities of rPON1A for structure-function studies.—** Brushia, R. J., T. M. Forte, M. N. Oda, B. N. La Du, and J. K. Bielicki. **Baculovirus-mediated expression and purification of human serum paraoxonase 1A. *J. Lipid Res.* 2001. 42: 951–958.**

Supplementary key words recombinant paraoxonase • HDL • lipid peroxidation • antioxidant • atherogenesis

Paraoxonase 1 (PON1; EC 3.1.8.1) is a putative antiatherogenic enzyme that is tightly associated with circulating HDL and apolipoprotein A-I (apoA-I) in mammals (1–5). Experimental evidence suggests that PON1 may function as an antioxidative enzyme and inhibit the oxidation of LDL. It has been demonstrated, in vitro, that PON1 can degrade proinflammatory mediators (i.e., peroxidized phospholipids) that arise from the oxidation of LDL, thereby inhibiting early atherogenic processes (6–11). Indeed, targeted disruption of the PON1A gene renders mice susceptible to diet-induced atherosclerosis; a response attributed, in part, to increased lipid peroxidation (8). Thus, it appears that PON may act as an important antioxidative enzyme in vivo, thereby explaining, in part, the ability of HDL to protect against the development of atherosclerosis.

Human PON1 (hPON1) is a 354-amino acid glycoprotein with a molecular mass of ~45 kDa (2). The enzyme has three potential N-linked glycosylation sites and carbohydrate accounts for approximately 16% of its molecular mass (1, 11). The apparent mass of serum hPON1A varies as a result of its heterogeneous glycosylation pattern. The hydrophobic N-terminal secretory signal is retained in the mature protein and appears to provide the structural basis for its interaction with phospholipids and HDL (12, 13). The primary structure of PON1 is highly conserved across species (5). PON1 requires Ca^{2+} for arylesterase and paraoxonase activities, but its lipid peroxidase activity is Ca^{2+} independent (14–16). There are two PON1 isoforms in humans that are of special interest, because they differ in only one amino acid but exhibit different catalytic properties. Isoform A (hPON1A) has a glutamine residue at amino acid position 191 whereas isoform B (hPON1B) contains an arginine residue at this position (1, 2, 17, 18). These two isoforms have similar arylesterase activities, but the B isoform has significantly higher paraoxonase activity, suggesting that amino acid 191 plays a role in determining substrate specificity. Considerable effort has been directed at identifying specific amino acid residues in PON1 that are involved in catalytic function, but their identity remains elusive (19–23).

Abbreviations: apoA-I, apolipoprotein A-I; hPON1, human serum arylesterase/paraoxonase type 1; MOI, multiplicity of infection.

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An efficient PON1 expression system that generates large quantities of enzyme would greatly facilitate further structure-function studies. Such a system would also provide PON1 in the absence of apoA-I, HDL, and potentially confounding factors in human serum. Here we describe the creation of such a system and show that it can be used to produce large quantities of recombinant human PON1A (rPON1A) that is functionally similar to PON1A purified from human serum. Moreover, functional studies revealed that carbohydrate was not required for antioxidant activity but is essential for arylesterase activity.

MATERIALS AND METHODS

Materials

The Baculogold® recombinant baculovirus kit, baculovirus transfer vector pVL1393, Sf9 insect cells, and Hink's complete TNMFH medium were obtained from PharMingen (San Diego, CA). Hi-5 insect cells were obtained from InVitrogen (Carlsbad, CA). Express Five® SFM medium and antibiotic-antimycotic solution were obtained from GIBCO-BRL/Life Technologies (Rockville, MD). DEAE Fast-Flow Sepharose and Sephacryl S-200 HR chromatography resins were obtained from Sigma (St. Louis, MO). Concanavalin A (ConA)-agarose was obtained from Amersham Pharmacia (Piscataway, NJ). Oligonucleotide primers for polymerase chain reactions (PCR) were obtained from Operon (Berkeley, CA). Details regarding PON1 cDNA were previously described (15, 23).

DNA preparation

PON1A cDNA was prepared for insertion into pVL1393 by PCR amplification with a sense primer (5′-CCT TCT AGA *TAT AAA* **TATG** GCG AAG CTG ATT GCG CTC AC-3′, *Xba*I restriction site underlined, PON1A translation start in boldface, and a baculovirus translation initiation sequence in italics) and an antisense primer (5′-GAG ACC CTG CAG **CTA CTA** GAG CTC ACA GTA AAG AGC TTT GTG-3′, *Pst*I site underlined, tandem translation stop codons in boldface). The cDNA was subjected to 30 cycles of amplification (94°C, 30 s, 55°C, 1 min, 72°C, 2 min) with Expand *Taq*® high-fidelity DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The amplified PON1A cDNA was digested with *Xba*I and *Pst*I and inserted into the *Xba*I-*Pst*I sites in the multiple cloning site of vector pVL1393 to create the pVL-PON1A recombinant baculovirus transfer vector. The PON1A cDNA in vector pVL-PON1A was subjected to sequence analysis at the Lawrence Berkeley National Laboratory DNA sequencing facility (Berkeley, CA).

Cell culture and baculovirus preparation

Sf9 insect cells were grown as monolayer cultures at 28°C in complete Hink's TNMFH medium supplemented with antibiotic-antimycotic reagent (GIBCO-BRL, Grand Island, NY). Recombinant PON1A baculoviruses (BV-PON1A) were created in Sf9 cells using the pVL-PON1A vector and the Baculogold® kit. Putative recombinants were screened for rPON1A expression on the basis of arylesterase activity secreted into culture medium and by SDS-PAGE and Western blot analysis. Hi-5 cells were grown as both monolayer and suspension cultures at 28°C in Express Five® SFM serum-free medium supplemented with antibiotic-antimycotic reagent. Hi-5 suspension cultures were maintained at $\sim 1 \times 10^6$ cells/ml in 250- or 500-ml Erlenmeyer flasks in a shaking water bath.

Recombinant PON1A expression and purification

BV-mediated expression of rPON1A was examined in Hi-5 and Sf9 insect cells. Hi-5 cells produced ~2-fold more secreted rPON1A arylesterase activity than Sf9 cells (data not shown). Therefore, Hi-5 cells in suspension cultures were used to express rPON1A for purification. Cultures at a density of $\sim 1 \times 10^6$ cells/ml were infected with recombinant BV-PON1A at a multiplicity of infection (MOI) of ~5. Preliminary studies indicated that maximum expression of secreted rPON1A arylesterase activity occurred between 48 and 72 h postinfection. For purification, cells were collected (72 h postinfection) by centrifugation (800 *g* for 15 min at 4°C). The cell pellet was resuspended in 10 volumes of ice-cold buffer A [20 mM Tris (pH 8.0), 1 mM $CaCl_2$, 0.1 mM DTT, 0.1% Tergitol type NP-10, 1 mM benzamidine, and a low concentration (5 μM) of EDTA]. The use of a low concentration of EDTA was described earlier by Gan et al. (11) and does not adversely affect the arylesterase and paraoxonase activities of hPON1A. The cells were given 10 strokes by hand in a glass-Teflon homogenizer. The resulting crude lysate was centrifuged for 1 h at 20,000 *g* at 4°C. The supernatant (soluble cell extract) was recovered and used to purify PON1A via three simple chromatographic steps as follows.

Step 1. DEAE-Sepharose (buffer A): The soluble cell extract was loaded onto a DEAE-Sepharose Fast Flow ion-exchange column (2.5 × 30 cm Econo-column; Bio-Rad, Hercules, CA) equilibrated in buffer A. The column was washed with ~5 bed volumes of buffer A and eluted with a linear 0–200 mM NaCl gradient in buffer A (total elution volume, 600 ml). Fractions were assayed for arylesterase activity and protein concentrations, and pooled. The DEAE pool was concentrated in a Pall Gelman (Ann Arbor, MI) Jumbosep® centrifugal ultrafiltration device with a 10K molecular weight cutoff membrane. Recoveries shown in **Table 1** were based on arylesterase activity measurements using concentrated samples.

Step 2. Sephacryl S-200 gel filtration (buffer B, buffer A plus 200 mM NaCl): The concentrated DEAE pool was chromatographed on a high-resolution Sephacryl S-200 gel filtration column (2.6 × 100 cm column; Amersham Pharmacia), equilibrated in buffer B. S-200 column fractions were assayed for arylesterase activity and pooled.

Step 3. ConA (buffer C, buffer A plus 500 mM NaCl, 1 mM $MgCl_2$, and 1 mM $MnCl_2$; DTT omitted): The S-200 pool was

TABLE 1. Summary of recombinant PON1A purification from insect cells

Step	Volume	Activity[a]	Protein	Specific Activity	Purification	Recovery[b]
	ml	*Units*	*mg/ml*	*U/mg*	*fold*	*%*
Soluble cell extract	47	118	15.6	7.56		100
DEAE-Sepharose	21	100	1.24	80.6	10.6	38
Sephacryl S-200	15	120	1.07	112.1	14.8	32
ConA–agarose	6	291	0.40	732.0	96.8	31

[a] Units, Micromoles per minute per milliliter of arylesterase activity.
[b] Percentage of total activity.

chromatographed on a ConA-agarose column (2.6 × 5 cm Econo-column; Bio-Rad) as described but with minor changes (15, 24). The ConA column was equilibrated in buffer C. The S-200 pool was pumped onto the column at a flow rate of 0.5 ml/min and the column was washed with ~5 volumes of buffer C. The rPON1A was eluted with 0.2 M α-D-galactomannopyranoside in buffer C. Fractions were assayed for arylesterase activity and pooled. The ConA pool was dialyzed overnight at 4°C against 20 mM Tris (pH 8.0), 10 mM NaCl, 1 mM $CaCl_2$, and 0.1% Tergitol NP-10. The dialysate was concentrated to ~500 U/ml arylesterase activity and stored at 4°C. All chromatography steps were performed at 4°C.

Arylesterase, paraoxonase, and protein assays

PON1A arylesterase activity was measured as the hydrolysis of phenylacetate at 270 nm as described (11). Paraoxonase activity was quantified by monitoring the hydrolysis of paraoxon [*o,o*-diethyl-*o*-(*p*-nitrophenyl) phosphate] at 412 nm (11). Reaction mixtures contained 50 mM Tris (pH 8.0) and 1 mM $CaCl_2$. Phenylacetate and paraoxon concentrations were varied for kinetic assays as summarized in **Table 2**. Purified hPON1A used for comparisons was obtained as described (11). Protein concentrations were determined according to the method of Lowry et al. (25).

SDS-PAGE and Western blot analysis

Protein samples were diluted into SDS-PAGE sample buffer [50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 15 mM 2-mercaptoethanol, and 0.25% bromphenol blue] and electrophoretically resolved in Novex (San Diego, CA) 4–20% denaturing polyacrylamide gels according to the method of Laemmli (26). Proteins were visualized with Coomassie Brilliant Blue R-250. Alternatively, proteins were electrophoretically transferred to nitrocellulose membrane as described (27) and processed for immunodetection using a human-specific PON1A antibody (28). The primary anti-PON1A antibody was detected with Alexa 488® fluorophore-conjugated horse antibodies to goat IgG (Molecular Probes, Salem, OR) and visualized directly with a Bio-Rad FX phosphorimager.

Assessment of the antioxidant properties of rPON1A

Lipid peroxidation studies were a modification of that previously described (29, 30). The oxidation of phospholipid was monitored at 234 nm (conjugated dienes) in a basic reaction system: 0.3 mM 1-palmitoyl-2-linoleoyl-phosphatidylcholine dispersed in 3.0 mM deoxycholate and 0.2 M borate buffer (pH 9.0) containing 1.0 mM Ca^{2+} and 20 μM butylated hydroxytoluene. Purified hPON1A and rPON1A enzymes were added to incubation mixtures as described in the figure legends. Soybean lipoxygenase (3 U/μl) was added to initiate lipid peroxidation. In control experiments, we found that calcium was not required for the antioxidant activity of either human or recombinant PON enzyme; moreover, incubations with 1 mM EDTA (inhibits the arylesterase activity of PON) did not alter the ability of these enzymes to inhibit lipid peroxidation (data not shown).

TABLE 2. Summary of kinetic parameters for recombinant PON1A and human PON1A

	Arylesterase Activity[a]		Paraoxonase Activity[a]	
	K_m[b]	V_{max}[c]	K_m[b]	V_{max}[c]
rPON1A	3.8 ± 2.1	1,305 ± 668	3.5 ± 1.0	0.17 ± 0.04
hPON1A	3.7 ± 2.0	1,361 ± 591	1.4 ± 0.1	0.19 ± 0.02

[a] All values represent means ± SD, n = 3. Kinetic parameters were determined by linear regression analysis of Lineweaver-Burk plots. Arylesterase activity was measured with phenylacetate as substrate and paraoxonase activity was measured with paraoxon as described in Materials and Methods.

[b] K_m, millimolar concentration.

[c] V_{max}, units per milligram of protein (1 unit = 1 μmol of substrate hydrolyzed per minute per milliliter).

Enzymatic deglycosylation of rPON1A

Recombinant PON1A was enzymatically deglycosylated with endoglycosidase H (mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase, EC 3.2.1.96; Boehringer Mannheim) for 24 h at 37°C. The reaction mixture contained 100 mU of endoglycosidase H, recombinant PON1A (~330 μg/ml), 100 mM sodium acetate (pH 5.8), 10 mM NaCl, and 1 mM $CaCl_2$. Arylesterase activity was measured at the beginning and end of the 37°C incubation and the reaction products were subjected to SDS-PAGE and antioxidant protection studies.

RESULTS

PON1A expression in insect cells

Hi-5 cells growing in suspension culture for 72 h produced rPON1A as shown in **Fig. 1A**. Recombinant PON1A secreted into the medium (Fig. 1A, lane 2) as well as that which remained cell associated (Fig. 1A, lane 3) exhibited several bands (35–45 kDa), probably owing to heterogeneity in glycosylation. These molecular mass forms were smaller than the native protein isolated from plasma (Fig. 1A, lane 1), where the major band was approximately 47 kDa. As evident in Fig. 1, only a small fraction of rPON1A synthesized by the cells was secreted into the culture medium compared with that which remained cell associated (Fig. 1A, lane 2 vs. lane 3). On the basis of arylesterase activity, 25% (1,800 units) of enzyme activity was in the medium and 75% (5,600 units) was cell associated. A small fraction of the cell-associated protein formed high molecular weight aggregates as well as low molecular weight products (Fig. 1A, lane 3). The high molecular weight forms were efficiently removed by centrifugation (Fig. 1A, lane 4) and likely correspond to denatured and aggregated rPON1A. We estimate that Hi-5 insect cells grown in suspension cultures produced ~40 mg of active rPON1A (secreted plus cell-associated enzyme) per liter of Hi-5 cell suspension culture.

To determine whether the cell-associated rPON1A was membrane associated, Hi-5 cells that had been infected with BV-PON1A were lysed in the presence and absence of a nonionic detergent (Fig. 1B). It was observed that the cell-associated rPON1A was readily solubilized as shown by an increase (10-fold) in arylesterase activity in the presence of 0.1% Tergitol (type NP-10). This suggests that the cell-associated rPON1A likely interacts with membrane phospholipid. A significant fraction of the cell-associated PON1A exhibited the same apparent molecular mass as the secreted rPON1A (Fig. 1A), suggesting that it was glycosylated.

Purification of rPON1A expressed in insect cells

To purify and characterize rPON1A, we used the cell-associated form because it represented the major source of rPON1A produced by the insect cells. A summary of the results from the purification procedure is shown in Table

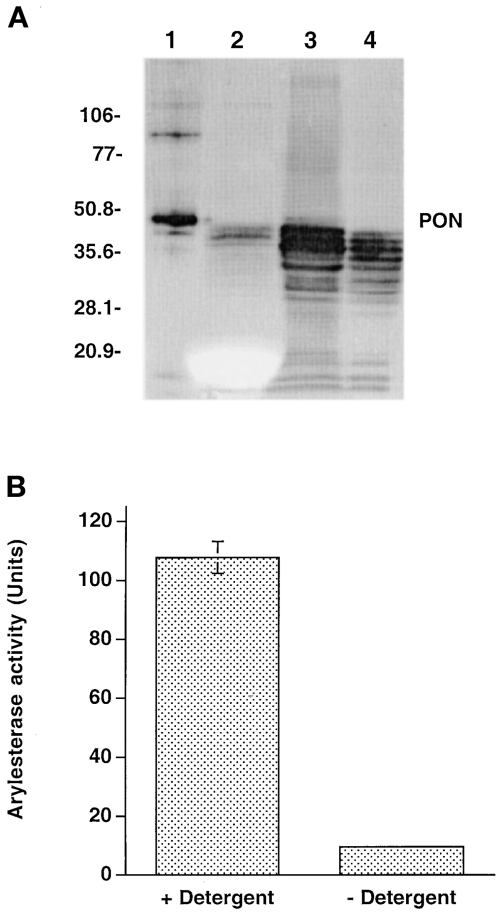

Fig. 1. Expression of rPON1A by Hi-5 insect cells. A: Western blot analysis of rPON1A. Hi-5 cells in suspension culture were infected with BV-PON1A at an MOI of ~5 and harvested 72 h postinfection. The cells and culture medium were separated by centrifugation (800 *g* at 4°C for 10 min). The cell pellet was resuspended in a volume of buffer [20 mM Tris (pH 8.0), 1 mM $CaCl_2$, and 0.1 mM DTT] equivalent to the original volume of culture medium and cells were lysed as described in Materials and Methods. Equal aliquots of cell lysates and culture medium were electrophoresed on denaturing 4–20% SDS-polyacrylamide gels and protein was transferred to nitrocellulose sheets for Western blot analysis. Lane 1, PON1A purified from human serum. The high molecular weight bands persist on treatment with reducing agents and SDS (data not shown) and may represent different glycoforms of PON1A as described [Sorenson et al. (23)]; lane 2, culture medium from infected cells; lane 3, total cell lysate; lane 4, detergent extract of lysed cells [described in (B)]. B: Detergent solubilization of cell-associated arylesterase activity. Infected Hi-5 cells were collected and lysed as described above. The nonionic detergent Tergitol type NP-10 was added to one aliquot of cells to give a final concentration of 0.1% (+ Detergent). Water was added to another aliquot of cells (−Detergent). Cells were homogenized (as described in Materials and Methods) and the resulting lysates were centrifuged at 20,000 *g* for 30 min at 4°C. The supernatants (soluble cell extracts) were recovered and arylesterase activity was quantified.

1 and **Fig. 2**. The starting material for the purification was ~50 ml of soluble insect cell extract, obtained from a 250-ml suspension culture, that had an arylesterase activity of ~118 U/ml (Table 1). DEAE ion-exchange chromatography (Fig. 2A) provided the first purification. The DEAE pool was further fractionated by gel-filtration chromatography (Fig. 2B). We used a ConA-agarose column for the final step (Fig. 2C) of purification as this methodology has been successfully used to isolate the enzyme from human serum (15, 24); this yielded approximately 2.5 mg of highly purified (100-fold) rPONA1 enzyme that represented approximately 30% of the initial arylesterase activity (Table 1). The fact that the cell-associated rPON1A bound to the ConA column suggests that it was indeed glycosylated. The rPON1A pool from the ConA column was dialyzed as described in Materials and Methods and stored at 4°C. Recovery of purified rPON1A after these chromatographic steps was typically 6–10 mg/l of insect cell

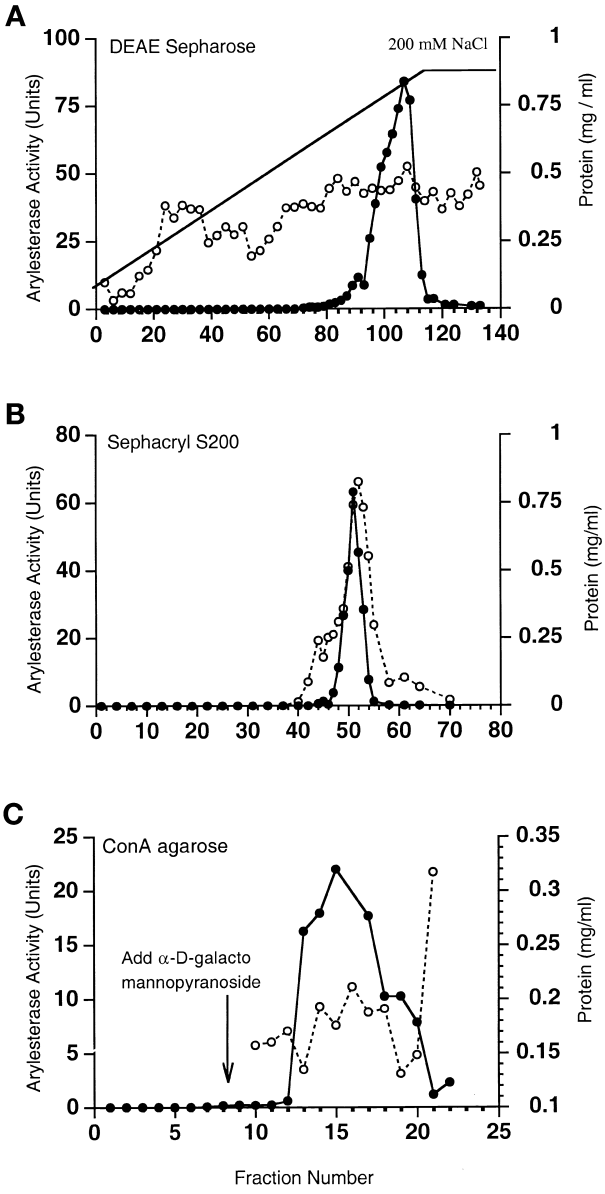

Fig. 2. Purification of rPON1A. PON1A was expressed in Hi-5 cells and purified as described in Materials and Methods. A: DEAE elution profile, fractions 92–112 were pooled. B: S-200 elution profile, fractions 48–54 were pooled. C: ConA elution profile, fractions 13–20 were pooled. The closed circles represent arylesterase activity and the open circles represent total protein (in milligrams per milliliter).

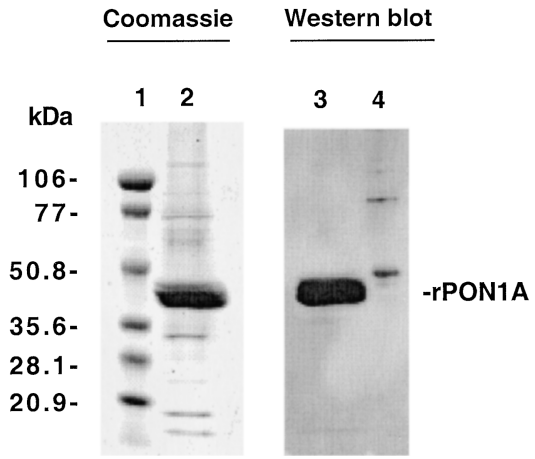

Fig. 3. SDS-PAGE and immunoblot analysis of rPON1A after purification. After the final column chromatography step (ConA), rPON1A was visualized by Coomassie staining and immunoblot analysis. The left lanes show a Coomassie-stained gel of the purified enzyme and right side shows the Western blot. Scanning densitometry of the Coomassie-stained gel indicates >95% of the protein was present as rPON1A. Note that the additional bands seen in lane 2 are not evident in the Western blot (lane 3). Lane 1, molecular weight markers; lane 2, rPON1A; lane 3, rPON1A; lane 4, PON1A purified from human serum.

suspension culture and the protein was >95% pure on the basis of SDS-PAGE and immunoblot analyses (**Fig. 3**). The major contaminants in the protein preparation at this point probably corresponded to ConA protein fragments as previously noted by Kuo and La Du (24). The molecular mass of the purified rPON1A enzyme was approximately 44 kDa as shown in Fig. 3, slightly less than that of the enzyme purified from human plasma (approximately 48 kDa). During the course of these studies, we have used several preparations of purified enzyme from human serum having molecular masses ranging from 45 to 48 kDa. Thus, it appears our recombinant protein is structurally similar to the human enzyme as judged by its apparent molecular mass. The purified rPON1A maintained stability for several months when stored at 4°C in the presence of 0.1% Tergitol NP-10 and 1 mM $CaCl_2$ (data not shown).

Arylesterase and paraoxonase activities

Consistent with previously published studies of human serum PON1A (14, 25), we found that EDTA (1 mM) completely and irreversibly inhibited the arylesterase activity of rPON1A (data not shown). Furthermore, Mg^{2+}, Mn^{2+}, and other divalent metal ions could not substitute for Ca^{2+} and led to a loss of arylesterase activity (data not shown), indicating that rPON1A is strictly dependent on Ca^{2+} for enzymatic activity.

The rates of phenylacetate and paraoxon hydrolysis as a function of substrate concentration were compared for rPON1A purified from insect cells and hPON1A purified from human serum (Table 2). Kinetic parameters of V_{max} and K_m were calculated as described (31). Despite the slight difference in apparent molecular weight (Fig. 3), the two enzymes displayed nearly identical V_{max} and K_m values for phenylacetate hydrolysis. Analysis of the kinetic data obtained using paraoxon as the substrate also showed similar results between the two enzymes; however, the K_m values were somewhat lower for the human enzyme. The data provide compelling evidence that the rPON1A produced by BV-infected insect cells is functionally similar to hPON1A purified from human serum.

Antioxidant capacity of rPON1A versus hPON1A

Figure 4 demonstrates that our rPON1A enzyme behaves like hPON1A with regard to its ability to inhibit lipid peroxidation. In the absence of a protective PON enzyme, lipoxygenase treatment of phospholipid caused a rapid induction in lipid peroxidation as judged by an increase in conjugated dienes (absorbance at 234 nm). The presence (15 μg of protein per ml, i.e., 20 units of arylesterase activity per ml) of hPON1A enzyme inhibited lipid peroxidation by approximately 50% (Fig. 4A). A high degree of protection was also consistently observed when using

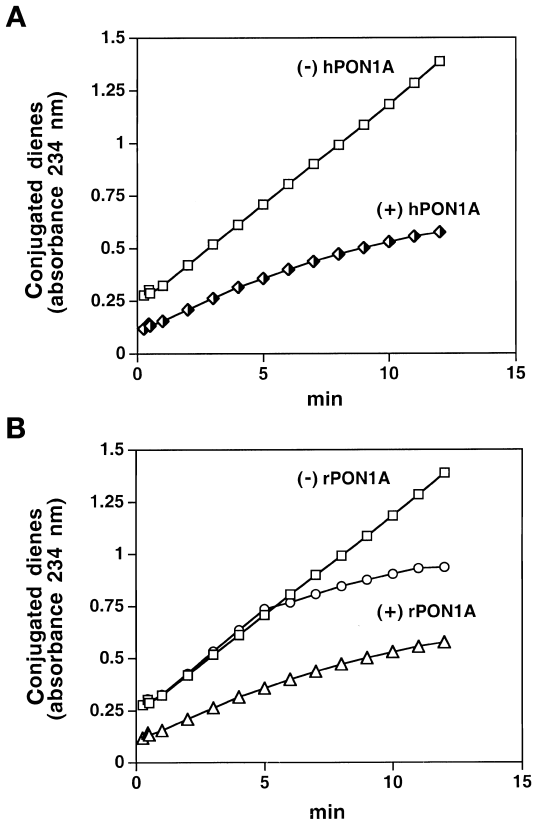

Fig. 4. Comparison of the antioxidant properties of recombinant and human serum-derived PON1A. Peroxidation of phospholipid, shown in (A) and (B) as open squares, was initiated with soybean lipoxygenase (3 U/μl) as described in Materials and Methods. A: The protective effect of hPON1A (diamonds). B: The effect of rPON1A purified from insect cells. The concentration of paraoxonase was 15 μg of protein per ml (20 units of arylesterase activity per ml) for both enzymes. In the experiment described in (B), rPON1A was added at the initiation of lipid peroxidation (triangles), as well as 5 min after addition of the lipoxygenase enzyme (circles); the latter shows that rPON1A can protect well after peroxidation was initiated. Values are from a single experiment that is representative of three separate experiments.

rPON1A regardless of whether the enzyme was added to the system before or after the initiation of lipid peroxidation (Fig. 4B). Indeed, for the experiments described in Fig. 4A and B, addition of PON1A was found to act immediately, preventing the initial, rapid (seconds) induction of lipid peroxidation. Protection from oxidation was found to be dependent on rPON1A concentration, with approximately 90% reductions in lipid peroxidation observed at 30 μg of protein per ml (data not shown).

Enzymatic deglycosylation of expressed rPON1A

Insect cells attach predominantly high-mannose sugars to secreted glycoproteins (32). Because rPON1A was bound by ConA, which preferentially binds high-mannose carbohydrate groups, we subjected rPON1A to digestion with endoglycosidase H and the effects on molecular mass, arylesterase activity, and antioxidant properties were evaluated. SDS-PAGE demonstrated a major rPON1A band of 44 kDa and a minor product of 46 kDa; enzymatic deglycosylation reduced the apparent molecular mass of rPON1A to 42 kDa (**Fig. 5A**); only trace amounts of the originally glycosylated material were present after endoglycosidase H treatment. This shift in molecular weight concomitant with enzymatic deglycosylation was sufficient to completely abolish the arylesterase activity of rPON1A (Fig. 5B). Moreover, quantification of enzymatic activity, using paraoxon as a substrate, yielded similar results (data not shown). Thus, endoglycosidase H treatment was found to inhibit both the arylesterase and paraoxonase activities of rPON1A enzyme. These data suggest that the cell-associated form of rPON1A was indeed glycosylated with high-mannose *N*-glycan chains that are apparently necessary for maintaining arylesterase activity. However, as seen in Fig. 5C, the deglycosylated form of rPON1A was equally effective as the native protein in protecting against the peroxidation of phospholipid.

DISCUSSION

Serum hPON1 is an antioxidative enzyme associated with circulating HDL. PON1 is thought to provide protection against coronary artery disease in vivo by inhibiting early events (i.e., LDL oxidation) in atheromatous lesion development. This antioxidative ability of hPON1 has generated considerable interest in the structure and function of the enzyme. We have created a baculovirus-mediated hPON1A expression system and were able to demonstrate that the system is capable of producing large quantities (approximately 40 mg/l before purification) of rPON1A enzyme. Most of the expressed protein was cell associated but easily isolated by detergent treatment followed by three easy chromatography steps. Because rPON1A was produced in the absence of apoA-I and HDL, we were able to omit the blue agarose chromatography and secondary DEAE chromatography steps required for purifying PON1A from human serum (11). Like hPON1A purified from human plasma (11), rPON1A dissociated from DEAE at a relatively low ionic strength (i.e., <175 mM NaCl), suggesting that it has surface charge properties similar to those of hPON1A.

We found that enzymatic deglycosylation completely abolished the arylesterase activity of rPON1A. This is corroborated by our observation that PON1A expressed in *Escherichia coli* cells, which are unable to glycosylate proteins, does not have arylesterase and paraoxonase activity (R. J. Brushia, T. M. Forte, M. N. Oda, B. N. La Du, and J. K. Bielicki, unpublished observations). It is possible that glycosylation is essential for PON1A structural stability, or alternatively, that carbohydrate plays a direct role in catalysis. Our observation that deglycosylation of PON1A under relatively mild conditions leads to a complete loss of enzymatic activity is in contradiction to the report by Josse et al. (21) that glycosylation is not essential for PON1A activity.

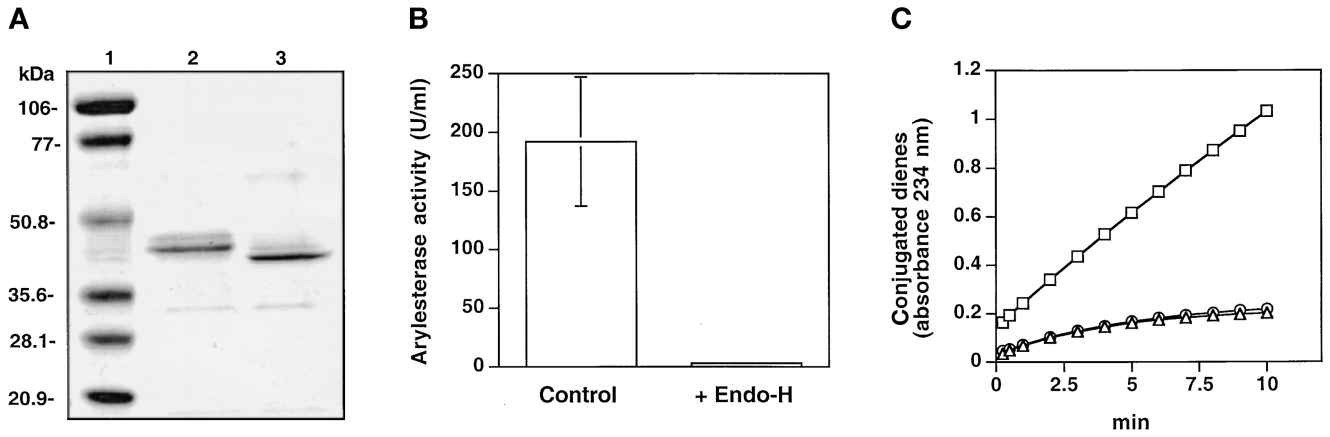

Fig. 5. Enzymatic deglycosylation of rPON1A. Recombinant PON1A purified from Hi-5 insect cells was subjected to deglycosylation with endoglycosidase H and analyzed by SDS-PAGE as described in Materials and Methods. A: A representative SDS-polyacrylamide gel stained with Coomassie R-250. Lane 1, molecular weight markers; lane 2, rPON1A incubated at 37°C in the absence of endoglycosidase H; lane 3, rPON1A after endoglycosidase H digestion (37°C). B: Arylesterase activity measurements for control (no endoglycosidase H) and endoglycosidase H-treated rPON1A. Values of arylesterase activity (U/ml) represent means ± SD, n = 3 separate experiments. C: A representative experiment (from a total of three) of the antioxidant activity of control (triangles) and endoglycosidase H-treated rPON1A (circles); both enzymes were used at a concentration of 30 μg of protein per ml. The squares represent lipoxygenase-mediated oxidation of phospholipid in the absence of rPON1A as measured by an increase in conjugated dienes.

These investigators reported that removal of amino acid residues Asn-252 and Asn-323 by site-directed mutagenesis had no significant effect on the activity of recombinant PON1A produced in cultured mammalian cells (21). However, it has been suggested that PON1A may contain three potential N-linked glycosylation sites instead of only two (11, 24). Our observation that the arylesterase activity of rPON1A is sensitive to deglycosylation could possibly be attributed to the essential role of an alternative N-linked glycosylation site within the PON enzyme. This possibility needs to be further explored.

For our BV-mediated expression system to be useful for PON1 structure-function studies it is essential that the rPON1A be functionally similar to hPON1A. Therefore, we compared various kinetic parameters for rPON1A and hPON1A. The V_{max} and K_m of rPON1A for both arylesterase and paraoxonase activities were similar to those obtained with hPON1A isolated from serum, thus indicating that the recombinant enzyme is functionally similar to the plasma form of the enzyme. The functional similarity between rPON1A and hPON1A is further corroborated by our studies addressing the antioxidant properties of rPON1A. We found that like the human enzyme, rPON1A was effective at preventing lipoxygenase-mediated oxidation of phospholipids. The ability of rPON1A to inhibit lipid peroxidation was dependent on enzyme concentration and did not require calcium, consistent with previously published studies using hPON1A (15). However, in contrast to the arylesterase and paraoxonase activities of rPON1A, which apparently required carbohydrate, we found that the deglycosylated form of rPON1A possessed full antioxidant activity. This suggests that the antioxidant activity of rPON1A, which is thought to involve Cys-283, does not require full glycosylation to stabilize the putative "peroxidase-like" activity of the enzyme. It is now recognized that the paraoxonase/arylesterase activities of PON1A do not require Cys-283, suggesting that the antioxidant function of the enzyme is independent of its other activities (15). Our results dissociating arylesterase activity from antioxidant activity on enzymatic deglycosylation support the notion that PON1A may possess two different active sites: one site responsible for antioxidant activity involving Cys-283 and, a second, unknown site catalyzing the hydrolysis of organophosphates. Specific details regarding the structural determinants of each active site are still unknown. Thus, the availability of our rPON1A expression system is likely to facilitate the elucidation of such mechanisms.

The use of the BV expression system has many advantages over human serum for obtaining purified PON1A. Recombinant BV can be quickly and easily prepared with the Baculogold® kit and can facilitate the creation of site-specific PON1 mutants for structure-function studies. The overall procedure for purifying rPON1A from insect cells is simple. Insect cell culture is easy and inexpensive, and insect cells do not contain apoA-I, HDL, serum antioxidants, or other potentially confounding factors present in human serum. Insect cells routinely yielded 6–10 mg of highly purified PON1A per liter of cultured cells, which is comparable to the quantity of purified PON1A obtained from 1 liter of human serum (11). We have shown that rPON1A is functionally similar to hPON1A and can efficiently inhibit lipid peroxidation. Thus our expression systems appears useful for defining structure-functional relationships of the paraoxonase enzyme.

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REFERENCES

1. La Du, B. 1992. Human serum paraoxonase/arylesterase. *In* Pharmacogenetics of Drug Metabolism. W. Kalow, editor. Pergamon Press, New York. 51–191.
2. La Du, B. N., S. Adkins, A. K. Chung-Liang, and D. Lipsig. 1993. Studies on human serum paraoxonase/arylesterase. *Chem. Biol. Interact.* **87:** 25–34.
3. Mackness, B., P. N. Durrington, and M. I. Mackness. 1998. Human serum paraoxonase. *Gen. Pharmacol.* **31:** 329–336.
4. Mackness, M. I., B. Mackness, P. N. Durrington, A. M. Fogelman, J. Berliner, A. J. Lusis, M. Navab, D. Shih, and G. C. Fonarow. 1998. Paraoxonase and coronary heart disease. *Curr. Opin. Lipidol.* **9:** 319–324.
5. Hegele, R. 1999. Paraoxonase genes and disease. *Ann. Med.* **31:** 217–224.
6. Mackness, M. I., S. Arrol, and P. N. Durrington. 1991. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett.* **286:** 152–154.
7. Shih, D. M., L. Gu, Y. R. Xia, M. Navab, W. F. Li, S. Hama, L. W. Castellani, C. E. Furlong, L. G. Costa, and A. M. Fogelman. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature.* **394:** 284–287.
8. Aviram, M., M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo-Parmo, and B. N. La Du. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J. Clin. Invest.* **101:** 1581–1590.
9. Mackness, M. I., S. Arrol, C. Abbott, and P. N. Durrington. 1993. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis.* **104:** 129–135.
10. Watson, A. D., J. A. Berliner, S. Y. Hama, B. N. La Du, K. F. Faull, A. M. Fogelman, and M. Navab. 1995. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96:** 2882–2891.
11. Gan, K. N., A. Smolen, H. W. Eckerson, and B. N. La Du. 1991. Purification of human serum paraoxonase/arylesterase. *Drug Metab. Dispos.* **19:** 100–106.
12. Sorenson, R. C., M. Aviram, C. L. Bisgaier, S. Billecke, C. Hsu, and B. N. La Du. 1999. Properties of the retained N-terminal hydrophobic leader sequence in human serum paraoxonase/arylesterase. *Chem. Biol. Interact.* **119–120:** 243–249.
13. Sorenson, R. C., C. L. Bisagaier, M. Aviram, C. Hsu, S. Billecke, and B. N. La Du. 1999. Human serum paraoxonase/arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids: apolipoprotein A-I stabilizes activity. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2214–2225.
14. Mackness, M. 1998. Human serum paraoxonase is inhibited in EDTA plasma. *Biochem. Biophys. Res. Commun.* **242:** 249.
15. Aviram, M., S. Billecke, R. Sorenson, C. Bisgaier, R. Newton, M. Rosenblat, J. Erogul, C. Hsu, C. Dunlop, and B. N. La Du. 1998. Paraoxonase active site required for protection against LDL oxidation involves its free sulfhydryl group and is different from that required for its arylesterase/paraoxonase activities: selective action of human paraoxonase allozymes Q and R. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1617–1624.

16. Cao, H., A. Girard-Globa, F. Berthezene, and P. Moulin. 1999. Paraoxonase protection of LDL against peroxidation is independent of its esterase activity towards paraoxon and is unaffected by the Q→R genetic polymorphism. *J. Lipid Res.* **40:** 133–139.
17. Adkins, S., K. N. Gan, M. Mody, and B. N. La Du. 1993. Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. *Am. J. Hum. Genet.* **52:** 598–608.
18. La Du, B. 1988. The human serum paraoxonase/arylesterase polymorphism. *Am. J. Hum. Genet.* **43:** 227–229.
19. Josse, D., W. Xie, P. Masson, and O. Lockridge. 1999. Human serum paraoxonase (PON1): identification of essential amino acid residues by group-selective labelling and site-directed mutagenesis. *Chem. Biol. Interact.* **119–120:** 71–78.
20. Josse, D., W. Xie, P. Masson, L. M. Schopfer, and O. Lockridge. 1999. Tryptophan residue(s) as major components of the human serum paraoxonase active site. *Chem. Biol. Interact.* **119–120:** 79–84.
21. Josse, D., W. Xie, F. Renault, D. Rochu, L. M. Schopfer, P. Masson, and O. Lockridge. 1999. Identification of residues essential for human paraoxonase (PON1) arylesterase/organophosphatase activities. *Biochemistry.* **38:** 2816–2825.
22. Doorn, J. A., R. C. Sorenson, S. S. Billecke, C. Hsu, and B. N. La Du. 1999. Evidence that several conserved histidine residues are required for hydrolytic activity of human paraoxonase/arylesterase. *Chem. Biol. Interact.* **119–120:** 235–241.
23. Sorenson, R. C., S. L. Primo-Parmo, C. L. Kuo, S. Adkins, O. Lockridge, and B. N. La Du. 1995. Reconsideration of the catalytic center and mechanism of mammalian paraoxonase/arylesterase. *Proc. Natl. Acad. Sci. USA.* **92:** 7187–7191.
24. Kuo, C. L., and B. N. La Du. 1995. Comparison of purified human and rabbit serum paraoxonases. *Drug Metab. Dispos.* **23:** 935–944.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **117:** 265–275.
26. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–675.
27. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedures and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
28. Oda, M. N., J. Bielicki, T. Berger, and T. M. Forte. 2001. Cysteine substitutions in apolipoprotein A-I primary structure modulate paraoxonase activity. *Biochemistry.* **40:** 1710–1718.
29. Bielicki, J. K., and T. M. Forte. 1999. Evidence that lipid hydroperoxides inhibit plasma lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* **40:** 948–954.
30. Zhang, L., M. Maiorino, A. Roveri, and F. Ursini. 1989. Phospholipid hydroperoxide glutathione peroxidase activity in tissues of rats of different age and comparison with other glutathione peroxidase. *Biochim. Biophys. Acta.* **1006:** 140–143.
31. Segel, I. 1975. Enzyme Kinetics. John Wiley & Sons, New York. 46–49.
32. Ailor, E., and M. J. Betenbaugh. 1999. Modifying secretion and posttranslational processing in insect cells. *Curr. Opin. Biotechnol.* **10:** 142–145.