COMPARATIVE STUDIES OF DIVERGED MEMBERS OF THE PHOSPHOTRIESTERASE FAMILY

FOR UNDERGRADUATE

RESEARCH FELLOWS

A Senior Honors Thesis

by

CRISTINA GALE ARRIENS

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
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April 2002

Group: Life Sciences 1

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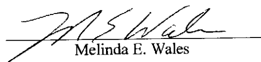
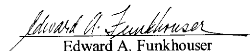

Melinda E. Wales


Edward A. Funkhouser

April 2002

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ABSTRACT

Comparative Studies Of Diverged Members of the

Phosphotriesterase Family. (April 2002)

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Some genes direct the synthesis of specific proteins called enzymes that catalyze specific types of chemical reactions. The class of enzyme of interest in this research, the hydrolases, catalyzes the conversion of functional groups to water. The phosphotriesterase (PTE) family is a subgroup of hydrolases that breaks down organophosphate compounds (OPs). Many organophosphate compounds are potent cholinesterase inhibitors, accounting for their widespread use as insecticides and nerve agents. Enzymes have been found in bacteria and higher organisms that specifically breakdown OPs. One of these enzymes, human serum paraoxonase/arylesterase (PON1), is a calcium-dependent, HDL (High Density Lipoprotein) -associated protein that appears to have multiple roles *in vivo.* In one guise, PON1 hydrolyses organophosphate insecticides and nerve gases and is responsible for determining the selective toxicity of these compounds in mammals. Using the sequence available in NCBI Genebank,

primers flanking the coding region of PON1 were designed and used to screen a human liver cDNA library using polymerase chain reaction (PCR). A single band corresponding to the approximate size of PON1, 1kb, was amplified and subsequently cloned. The identity of the cloned region as PON1 was verified by sequencing using PON1 specific primers, and then cloned into a plasmid named pTYB1 from the IMPACT-CN expression system. Expression was obtained using the BL21-star bacterial strain. The enzyme was found within an inclusion body, and solubilized with urea and DTT. Preliminary activity studies were unsuccessful, so further manipulation of the insoluble fraction is necessary to obtain proper folding.

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INTRODUCTION

Some genes direct the synthesis of specific proteins called enzymes. Enzymes catalyze chemical reactions, and one of their most impressive characteristics is the specificity of their action. The specificity enzymes exhibit is two-fold, they are typically specific to the type of chemical reaction as well as to the physical nature of the substrate subjected to that reaction. With this in mind, the International Commission on Enzymes in 1956 devised six broad classes of enzymes based on the reaction catalyzed: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (5).

The class of enzyme of interest in this research, the hydrolases, catalyzes the conversion of functional groups to water. The phosphotriesterase (PTE) family is a subgroup of hydrolases that breaks down organophosphate compounds (OPs). Many organophosphate compounds are potent cholinesterase inhibitors, accounting for their widespread use as insecticides and nerve agents. In addition to the nearly 3 million cases of pesticide poisonings world-wide each year attributed to OP pesticides, there are risks associated with the international effort to destroy the approximately 25,000 tons of chemical agents (5). Additionally, the widespread repeated exposure to chemical agents (including organophosphate pesticides and nerve gases, DEET, and pyridostigmine) which occurred during the Gulf War could be associated with the neurological symptoms of some Gulf War Veterans (6).

This thesis follows the style and format of the Journal of Bacteriology.

The most extensively characterized member of the PTE family is organophosphorus hydrolase (OPH), a bacterial enzyme that hydrolyzes a broad variety of organophosphate neurotoxins. The substrate range of OPH includes several insecticides, the neurotoxic chemical warfare agents and their analogs. All of the phosphate triesters found to be substrates of OPH are synthetic compounds, and the identity of any naturally occurring substrate for the enzyme is unknown.

Since the initial characterization of OPH, other enzymes have been identified which accomplish this hydrolysis for specific classes of OP compounds. One of these enzymes, human serum paraoxonase/arylesterase (PON1) is a calcium-dependent, HDL (High Density Lipoprotein) -associated protein that appears to have multiple roles *in vivo* (5). In one guise, PON1 hydrolyses organophosphate insecticides and nerve gases and is responsible for determining the selective toxicity of these compounds in mammals. At this time, human PON1 is known to utilize a broad group of substrates including: OP insecticides such as paraoxon, chlorpyrifos oxon, and diazoxon; nerve agents such as soman and sarin (4); and lactones (1).

Although *PON1* shows no homology to any of the proteins with which it shares functional similarity (the bacterial PTEs, including OPH), it is a member of a multigene family composed of three distinct, closely linked genes (*PON1, PON2* and *PON3*). PON1 is believed to be expressed in the liver, brain, and lung and its encoding gene is located at q21-q22 on the long arm of chromosome 7 (7). Sequencing of the human PON1 gene identified two genetic polymorphisms distinguished by amino acid substitutions at positions 55 and 192 (5). The arginine 192 isoform ($PON1_{R192}$)

hydrolyzes paraoxon about six times as fast as the glutamine 192 isoform ($PON1_{Q192}$) (4). However, $PON1_{Q192}$ hydrolyzes diazoxon, soman, and sarin at a faster rate than $PON1_{R192}$ (3). The other polymorphism gives either a leucine or a methionine at amino acid 55, with leucine having a higher paraoxon hydrolysis activity (7). The widely debated Gulf War Syndrome could be connected with a decreased capacity to detoxify OP insecticides resulting from low serum PON1 activity in both the $PON1_{R192}$ and $PON1_{Q192}$ isoforms (8).

Like the PON1 gene, PON2 and PON3 are located on chromosome 7, and although the protein products of human PON2 and PON3 have not been isolated, tissue distribution of PON2 transcripts were observed to be highest in liver, brain, and heart tissue (5). The similarity within the PON genes strongly suggests that these related genes arose by gene duplication. Similar to PON1, PON2 contains a common polymorphism at codon 311 and some population studies indicate that the PON2 S allele may contribute to an increased risk for coronary heart disease (5).

MATERIALS AND METHODS

CHEMICAL AND BIOLOGICAL REAGENTS

The λTriplEx human liver cDNA library was purchased from Clontech (Palo Alto, CA), and the manufacturer's protocol for titering and amplification was followed. PCR reactions were carried out with Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer Corporation, Norwalk, CT), using standard PCR conditions. Cloning and expression vectors (Table 1) were purchased from Clontech and New England Biolabs.

TABLE 1. Plasmids used in this study

Plasmid	Description
pT-Adv (Clontech)	AT Linear cloning vector, Ap^r
pCRT7-TOPO (Clontech)	AT Linear cloning vector, Ap^r, Zeo^r
pTYB1 (New England Biolabs)	Expression vector, Ap^r, lacZ'

Plasmid templates were purified for use in PCR and sequencing reactions using Qiagen Plasmid Mini and Midi Kits (Qiagen, Inc., Valencia, CA). Primers (Table 2) were synthesized by the Gene Technologies Laboratory, using an Applied Biosystems 394T DNA/RNA Synthesizer. DNA sequences were determined using the BigDye DNA Sequencing Kit (Applied Biosystems, Foster City, CA) following the instructions of the manufacturer.

TABLE 2. Primers, sequence indicated is written 5′ – 3′

Purpose	Primer	Sequence
Library screening	3PON5'	CCGACCATGGCGAAGCTG
	PON3'	ATGGCATGGGTGCAAATCGGTCTGTT
	2PON3int	TTGGGTTTAGCGTGGTCGTATGTTGTC
	PON3int	ATGCCATGTACCTCCTGGTGGTGAACC
	3PON3	GCATGGGTGCAAATCGGTCTG
	2PON5int	CTTGCCATCGGGTGAAATGTTGATTCC
	PON5int	TGCCATAAAAGTGCTCAGGTCCCACAG
	3PON5int	CATAAAAGTGCTCAGGTCCCA
Sequencing	M13 Forward	CCCAGTCACGACGTTGTAAAACG
	M13 Reverse	AGCGGATAACAATTTCACACAGG
	PON1-201a	TTCATTTAACCCTCATGGGA
	PON1-201	GAGCCAGCAACTCAGCTATA
	Intein Reverse	ACCCATGACCTTATTACCAACCTC
	T7 Forward	TAATACGACTCACTATAGGG
	T7 Reverse	TATGCTAGTTATTGCTCAG
	V5 Forward	ACCGAGGAGAGGGTTAGGGAT
Restriction site insertion	pon1-nde1	CGGCTTCCGCATATGGCGAAGCTGATTGCG
	pon1-sap	GCTCTTCCGCATTAGAGCTCACAGTAAAGAGC
	pon1-Gsap	GGTGGTTGCTCTTCCGCATTAGAGCTCACAGT AAAGAGC
	pon1-sap*	GCTCTTCTCTGTTAGAGACAGTAAAGAGC
	pon1-sap+	CGGCTTATGGCATGGGTGCGCTCTTCTCTGTTA GAGACAGTAAAGAGC

Reactions were run on Applied Biosystems ABI 373XL and 377XL Automated Sequencers at the Gene Technologies Laboratory at Texas A&M University. Restriction enzymes were purchased from Promega Corporation (Madison, WI) or New England

Biolabs, Inc. (Beverly, MA). SDS-PAGE molecular weight standards and all other chemicals, including IPTG, were obtained from Sigma Chemical Co. (St. Louis, MO). Bacterial strains (Table 3) were purchased from Invitrogen (Carlsbad, CA) and New England Biolabs, Inc. (Beverly, MA). All strains were grown in Luria-Bertani medium (1% Bacto tryptone (Difco, Detroit, MI), 0.5% Bacto yeast extract, 0.5% NaCl, supplemented with 1.5% Bacto agar, when necessary) with vigorous shaking at 37 °C. Bug Buster Protein Extraction Reagent (Novagen, Inc., Madison, WI) was used to lyse cells after expression.

TABLE 3. Bacterial strains used in this study

Strain	Genotype
XL1-Blue *E.coli*	*endA1, gyrA96, hsdR17, lac⁻, recA1, relA1, supE44, thi-1, [F'lacIqZDM15, proAB, Tn 10]*
ER2566 *E.coli*	*F⁻ λ⁻ fhuA2[lon] ompT lacZ::T7gene1 gal sulA11D(mcrC-mrr)114::IS10R (mcr-73::miniTn10-TetS)2 R(zgb-210::Tn10)(TetS) endA1[dcm]*
Rosetta[1]	*F– ompT hsdS (r^- m^-) gal dcm lacY1 pRARE2 (Cm^R)*
Top 10F' *E.coli*	*F'{$lacI^q$ Tn10(Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZDM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)* 7697 galU galK rpsL endA1 nupG
DH5α *E.coli*	*F' Φ80d lac ΔM15 Δ(lacZYA-argF) U169 endA1 rel A1 hsdR17(r_K-m_K+) deoR thi-1 phoA supE44 λ⁻ gyrA96*
BL21-star	*F⁻ ompT $hsdS_B$ (r_B-m_B-) gal dcm rne131 (DE3)*

[1] pRARE: This is a ColE1, chloramphenicol-resistant plasmid which supplies tRNAs under the control of their native promoters for the codons AUA, AGG, AGA, CUA, CCC and GGA, facilitating expression of genes that encode rare *E. coli* codons.

LIBRARY TITER

Overnight culture of XL1-Blue *E.coli* was centrifuged at 5,000 RPM for 5 minutes to pellet the cells. After decanting the supernatant, the pellet was resuspended in 10mM $MgSO_4$. A 1:10,000 dilution of the library was prepared and mixed with 200 μL of cells in the following amounts; 0 μL, 5 μL, 10 μL, and 20 μL. Each was added to a 4 mL tube of melted top agar and poured over LB agar plates. After hardening, they were incubated at 37°C for 7 hours. Then, the plaques were counted to calculate the titer in pfu/mL by the following equation:

$$(\text{number of plaques} \quad 10^4 \quad 10^3\ \mu\text{L/mL})/\ \mu\text{L of diluted phage}$$

LIBRARY SCREENING

The *pon1* sequence was retrieved from the NCBI GenBank database, entry NM_000446. Polymerase Chain Reaction (PCR) primers were designed to match the sequence at its beginning and end and named 3PON5′ and PON3′. The library was diluted 1:100 and mixed with 500 μl of an overnight culture of XL1-Blue *E. coli* in 15ml LB with 10 mM $MgSO_4$. After a 20 minute room temperature incubation, the mixture was aliquoted into a sterile 96-well plastic plate making an 8 x 8 grid. The plate was incubated at 37°C for 6 hours after which 25 μl from each well in row A was pooled. This was repeated for each horizontal and vertical series, resulting in 16 pools. After boiling 10 minutes, 13 μl of each pool was added to a 0.5mL PCR bead tube. Then 2 μL of a 1:800 dilution of each primer and 8 μL of water were added for a total volume of 25 μL. The samples

were then placed in a thermal cycler for an initial 5 minute 95°C denaturation followed by 40-cycles of a 1 minute 95°C denaturation, 1 minute at 55°C, and a 2 minute 72°C elongation step. Each reaction was run on an agarose gel using electrophoresis. Pool #2 had a band 1kB in length, the size predicted for *pon1*.

CLONING AND SEQUENCING PON 1

To generate the PCR fragment for cloning, PCR was repeated with Pool #2 and a small portion was run on a gel to verify the presence of the 1 kb band. The remaining PCR product was mixed with the pT-Adv vector, T4 DNA ligase, and ligation buffer and allowed to ligate overnight at 14°C. The resulting circular plasmid was then mixed with Top10F′ chemically competent cells and 0.5M β-mercaptoethanol. The mixture sat on ice 30 minutes, was heat shocked at 42°C for 30 seconds, returned to ice 2 minutes, then 250 μl LB was added. After 1 hour at 37°C, the mixture was spread on IPTG/X-gal LB agar plates and incubated at 37°C overnight. Twenty white colonies were picked and used to inoculate a 5 mL tube of LB and an LB agar plate, both containing 100 μg/ml ampicillin. Rapid plasmid preparations utilizing a phenol/chloroform extraction and an ethanol precipitation were used to isolate the plasmid DNA. This was then used in PCR along with the M13 forward and reverse primers which flank the AT cloning region. The PCR products were run on a gel and two of them appeared to have products about 1 kb in size. The primer set originally used to locate the gene was used in PCR with the two samples and confirmed the previous result. Overnight cultures from the two positive colonies (#20 and #22) on the LB agar plate were started in 100mL of LB with

ampicillin. Plasmid was purified using the Qiagen Midi kit according to manufacturers instructions, yielding 140 μg/ml of pT-Adv-Pon1-20 and 190 μg/ml of pT-Adv-Pon1-22. To verify the fragment as PON 1, the fragment was sequenced. Three pmol of M13 forward and reverse primers were used separately with 150 ng of pT-Adv-Pon1-20 or pT-Adv-Pon1-22, and 2 μl of Big Dye. The samples were cleaned using Bio-Rad Micro Bio-Spin P-30 purification columns, according to the manufacturers protocol. The samples were dried and submitted to the Gene Technologies Lab of the Institute of Developmental & Molecular for analysis. The sequencing protocol was repeated using internal primers PON1-201a and PON1-201 to obtain the complete sequence of the gene.

INTRODUCTION OF RESTRICTION SITES

Primers PON1-sap and PON1-nde were used in PCR with the pT-Adv-Pon 20 plasmid to introduce a *Sap*I site on the 3′ end and a *Nde*I site on the 5′ end. The PCR products were ligated with the pCRT7-TOPO and incubated at room temperature for 10 minutes. The ligation was immediately transformed into Top10F′ competent cells and spread on LB/zeocin agar plates. PON1-sap and PON1-nde were used to screen the colonies, with 11 of 12 colonies giving a 1 kb band on the gel. Two colonies were chosen, 8b and 20b, from which to isolate DNA and sequence using the T7reverse and V5forward primers.

CLONING INTO THE EXPRESSION VECTOR

Single digests were first performed with *Nde*I and *Sap*I on 8b, 20b, and pTYB1 to insure proper cutting. Difficulty achieving complete digestion with *Sap*I was overcome by

incubation at 37°C with 3 units of *Sap*I added 1 unit at a time over a 3 hour interval. Comparison to uncut samples on an agarose gel showed that the digests were successful. *Sap*I digests were performed as before, except when the 3rd unit of *Sap*I was added 1 unit of *Nde*I was also added to the three samples. The enzymes were heat inactivated at 70°C for 20 minutes. CIP (Calf Intestinal Phosphatase) was added to 8b and 20b to dephosphorylate the ends, to prevent self ligation, followed by heat inactivation. The pTYB1 digest was divided in half and added to the 8b and 20b digests, co-precipitated by the addition of ethanol, and resuspended in 15 μL of water. Four microliters of 5X Ligase buffer and 1 μL of DNA ligase was added, and the ligation was incubated at 14°C overnight. The ligation mixtures were transformed into DH5α chemically competent cells and spread on LB/ampicillin plates and incubated overnight at 37°C. Colonies from the transformation plate were picked on an LB/zeocin plate and an LB/ampicillin plate and incubated overnight at 37°C. 9 of 10 grew only on ampicillin, indicating they contained the pTYB1 vector. Colonies 1-6 and 8-10 were screened by PCR using the Intein reverse and T7forward primers. 4 colonies were chosen for plasmid isolation and sequencing using the T7, Intein, PON20-201, and PON20-201a primers. Sequence analysis indicated that PON20-8 was the correct sequence.

PON 1 EXPRESSION

Pon 20-8 DNA was transformed into *E.coli* ER2566, Rosetta, and BL21-star hosts. Colonies were picked to inoculate two 5mL tubes of LB/ampicillin broth and grown at 37°C for 4 hours, or to an OD_{600} of 0.5-0.7. At that time, IPTG was added to half the

tubes to induce expression of PON1 and growth continued either for 2.5 hours at 37°C or 16 hrs. at 15°C. The cells were pelleted and resuspended in 250 μl of Bug Buster. The sample was centrifuged, separating the soluble fraction from the insoluble. The pelleted insoluble proteins were resuspended in 100 μL of Bug Buster. Both fractions were run on an SDS- polyacrylamide gel electrophoresis and stained with comassie blue.

PURIFICATION AND SOLUBILIZATION OF INCLUSION BODIES

The inclusion body fraction was re-solubilized by resuspension in 5 mL 8M urea, 100 mM DTT, 1 mM EDTA, 0.1 M Tris-HC, pH 8 and incubation for 2 hours at 25°C. The pH was lowered to 3 by the dropwise addition of 1 M HCl. To determine the effect of removing DTT from the solubilization buffer, the sample was clarified by centrifugation at 10,000 x g. The supernatant was pipetted into dialysis tubing and dialyzed against 500 mL 6M urea, 10 mM HCl for 2 hours at 25°C and again against 1 liter 6 M urea at 4°C overnight. The sample appeared cloudy the next day indicating the formation of a precipate during dialysis. Centrifugation to separate the soluble and insoluble fractions was followed by SDS-PAGE electrophoresis to determine which fraction contained the PON1 protein.

To evaluate the effect of Urea, the re-solubilized inclusion body fraction was diluted to achieve a urea step gradient of 8 M, 6 M, 4 M, and 2 M, keeping DTT, EDTA, and Tris-HCl levels constant. After a 2 hour incubation at room temperature the samples were

centrifuged and the pellets resuspended in 8 M urea buffer. The soluble samples were run on a gel.

PAROXONASE SPOT ASSAY

Four and one-half milliliters of a 20 mM CHES, pH 9 solution was mixed with 0.5 mL of 10 mM paraoxon. Of this solution, 100 μL was added to a 4 x 3 grid in a 96-well plate. The first row was a buffer control that had the 8 M urea solution, 6 M, 4 M, and 2 M. The next row was a enzyme dilution control that mixed the enzyme in 8M urea solution with enzyme-free 8M urea solution in the following ratios: 50μl:0μl, 37.5μl:12.5μl, 25μl:25μl, and 12.5μl:37.5μl. The next row mixed the enzyme solubilized in 8M urea solution with enzyme-free 0M urea solution in the same ratios, giving final urea concentrations of 8M, 6M, 4M, and 2M. A known active OPH enzyme was added to a final well as a positive control.

RESULTS AND DISCUSSION

LIBRARY TITERING AND SCREENING

The library titering yielded approximately 10^9 pfu/mL. According to the manufacturer's protocol a successfully amplified library will have a very high titer of approximately 10^9 pfu/mL. Various primer combinations were initially used in PCR with the library pools, but only primer set 8, 3PON5′ and PON3′, resulted in an amplified product of the target size with Pool #2 (Fig. 1 A.). PCR with Pool #2 was repeated with primer set 8 and also with set 2, PON3int and PON3′, to verify the initial result (Fig. 1 B.).

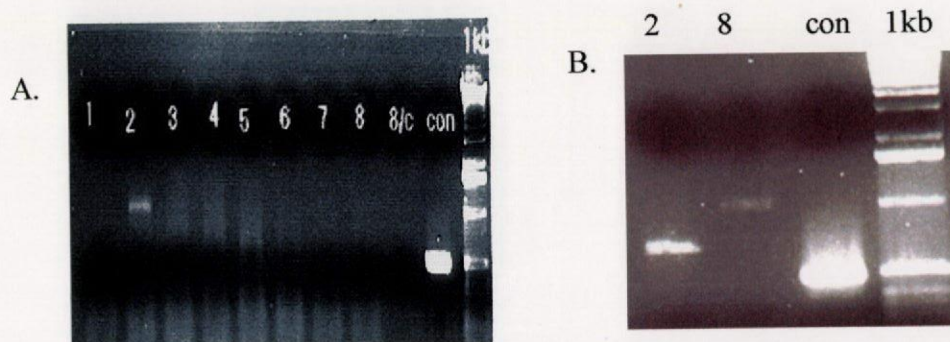

FIG. 1. A. Gel of library screening PCR products of Pools 1-8 with negative (8/c) and positive (con) controls, and 1kb DNA ladder. In lane 2 there is a 1 kb band from Pool #2. B. Gel verifying *pon1* identity using primer set 2 in addition to primer set 8. Set 2 (lane 1) is expected to give a 719 bp product and set 8 (lane 2) is expected to give a 1000 bp product.

CLONING AND SEQUENCING *pon1*

Colonies resulting from the transformation of DH5. with pT$_{Adv}$-*pon*1 were screened using the M13 forward and reverse primers, which flank the gene. Colony #20 and #22 each gave a product approximately 1 kb in size (Fig. 2).

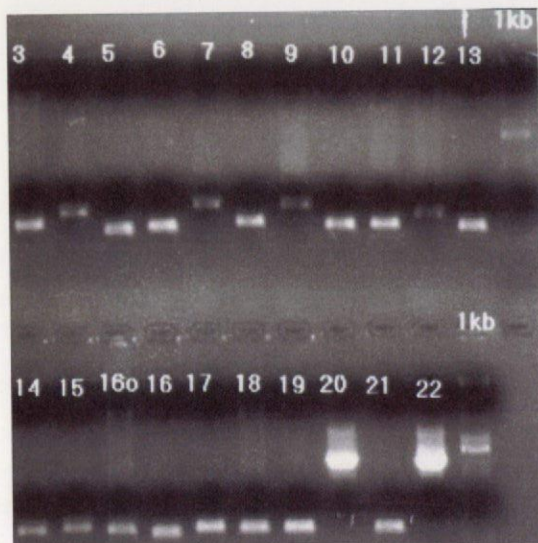

FIG. 2. Gel showing the PCR products from screening of transformants. Colony #20 and #22 (bottom row lanes 8 and 10) gave 1kb products.

To confirm the screening result, pT-Adv-*pon1*-20 and pT-Adv-*pon1*-22 were sequenced. The complete sequence was aligned with the known *pon1* sequence and found to have five base pair differences with the published pon1 sequence (Fig. 3). Since these discrepancies were found in both pT-Adv-pon1-20 and pT-Adv-pon1-22, and repeated sequencing of that region gave the same result, these changes are considered allelic difference rather than errors introduced during PCR.

```
pon1-20     GAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCT
NM_000446

pon1-20     GCAGAATTCGGCTTCCGACCATGGCGAAGCTGATTGCGCTCACCCTCTTGGGgATGGGACTGGCACTCTTCAGGAACCAC
NM_000446                        ATGGCGAAGCTGATTGCGCTCACCCTCTTGGGGATGGGACTGGCACTCTTCAGGAACCAC

pon1-20     CAGTCTCCTTACCAAACACGACTTAATGCTCTCCGAGAGGTACAACCCGTAGAACTTCCTAACTGTAATTTAGTTAAAGG
NM_000446   CAGTCTTCTTACCAAACACGACTTAATGCTCTCCGAGAGGTACAACCCGTAGAACTTCCTAACTGTAATTTAGTTAAAGG

pon1-20     AATCGAAACTGGCTCTGAAGACTTGGAGATACTGCCTAATGGACTGGCTTTCATTAGCTCTGGATTAAAGTATCCTGGAA
NM_000446   AATCGAAACTGGCTCTGAAGACATGGAGATACTGCCTAATGGACTGGCTTTCATTAGCTCTGGATTAAAGTATCCTGGAA

pon1-20     TAAAGAGCTTCAACCCCAACAGTCCTGGAAAAATACTTCTGATGGACCTGAATGAAGAAGATCCAACAGTGTTGGAATTG
NM_000446   TAAAGAGCTTCAACCCCAACAGTCCTGGAAAAATACTTCTGATGGACCTGAATGAAGAAGATCCAACAGTGTTGGAATTG

pon1-20     GGGATCACTGGAAGTAAATTTGATGTATCTTCATTTAACCCTCATGGGATTAGCACATTCACAGATGAAGATAATGCCAT
NM_000446   GGGATCACTGGAAGTAAATTTGATGTATCTTCATTTAACCCTCATGGGATTAGCACATTCACAGATGAAGATAATGCCAT

pon1-20     GTaCCTCCTGGTGGTGAACCATCCAGATGCCAAGTCCACAGTGGAGTTGTTTAAATTTCGAGAAGAAGAAAAATCGCTTT
NM_000446   GTACCTCCTGGTGGTGAACCATCCAGATGCCAAGTCCACAGTGGAGTTGTTTAAATTTCAAGAAGAAGAAAAATCGCTTT

pon1-20     TGCATCTAAAAACCATCAGACATAAACTTCTGCCTAATTTGAATGATATTGTTGCTGTGGGACCTGAGCACTTTTATGGC
NM_000446   TGCATCTAAAAACCATCAGACATAAACTTCTGCCTAATTTGAATGATATTGTTGCTGTGGGACCTGAGCACTTTTATGGC

pon1-20     ACAAATGATCACTATTTTCTTGACCCCTACTTAC[illegible]ATCCTGGGAGATGTATTTGGGTTTAGCGTGGTCGTATGTTGTCTA
NM_000446   ACAAATGATCACTATTTTCTTGACCCCTACTTACAATCCTGGGAGATGTATTTGGGTTTAGCGTGGTCGTATGTTGTCTA

pon1-20     CTATAGTCCAAGTGAAGTtCGAGTGGtGGCAGAAGGATTTGATTTTGCTAATGGAATCAACATTTCACCCGATGGCAAGT
NM_000446   CTATAGTCCAAGTGAAGTTCGAGTGGTGGCAGAAGGATTTGATTTTGCTAATGGAATCAACATTTCACCCGATGGCAAGT

pon1-20     ATGTCTATATAGCTGAGTTGCTGGCTCATAAGATTCATGTGTATGAAAAGCATGCTAATTGGACTTTAACTCCATtGAAG
NM_000446   ATGTCTATATAGCTGAGTTGCTGGCTCATAAGATTCATGTGTATGAAAAGCATGCTAATTGGACTTTAACTCCATTGAAG

pon1-20     TCCCTTGACTTTAATACCCTCGTGGATAACATATCTGTGGATCCTGAGACAGGAGACCTTTGGGTTGGATGCCATCCCAA
NM_000446   TCCCTTGACTTTAATACCCTCGTGGATAACATATCTGTGGATCCTGAGACAGGAGACCTTTGGGTTGGATGCCATCCCAA

pon1-20     TGGCATGAAAaTCTTCTTCTATGACTCAGAGaATCCTCCTGCATCAGAGGTGCTTCGAATCCAGAACATTCTAACAGAAG
NM_000446   TGGCATGAAAATCTTCTTCTATGACTCAGAGAATCCTCCTGCATCAGAGGTGCTTCGAATCCAGAACATTCTAACAGAAG

pon1-20     AACCTAAAGTGACACAGGTTTATGCAGAAAATGGCaCAGTGTTGCAgGGCAGTACAGTTGCCTCTGTGTACAAAGGGAAA
NM_000446   AACCTAAAGTGACACAGGTTTATGCAGAAAATGGCACAGTGTTGCAAGGCAGTACAGTTGCCTCTGTGTACAAAGGGAAA

pon1-20     CTGCTGATTGGCACAGTGTTTCACAAAGCTCtTTACTGTgAGCTCTaACAGACCGAtTTGCACCCATgCCATAAGCCGAA
NM_000446   CTGCTGATTGGCACAGTGTTTCACAAAGCTCTTTACTGTGAGCTCTAA

pon1-20     TTCCAGCACACTGGCGGCCGTTACTAG
NM_000446
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FIG. 3. The sequence of PON1-20 was aligned with NM_000446, the known sequence of PON 1 from the NCBI database. There are five differences that are boxed. Two of them result in the R192 and and L55 isoforms when translated, two do not alter the amino acid sequence, and the final difference could be an new isoform.

The sequence was then translated into the amino acid residues (Fig. 4). At position 55 there was a leucine and at position 192 there was an arginine. $PON1_{R192}$ has greater paraoxon hydrolysis and less hydrolysis activity with diazon, soman, and sarin when compared to $PON1_{Q192}$, the other common isoform. $PON1_{L55}$ has not been shown to have an impact on enzyme activity, but results in a higher serum PON1 concentration when compared to $PON1_{M55}$ (2). Mutagenesis will be used to create all four isoform combinations for enzyme activity and structure comparisons. The guanine substituted for adenine resulted in pT-Adv-*pon1*-20 and pT-Adv-*pon1*-22 having an arginine at position 146 rather than a glutamine. Mutagenesis will also be used to change 146 to glutamine for comparison.

GAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCG

GCTTCCGACCATGGCGAAGCTGATTGCGCTCACCCTCTTGGGGATGGGACTGGCACTCTTCAGGAACCACCAGTCTCCTTACCAAACACG

M A K L I A L T L L G M G L A L F R N H Q S P Y Q T R

ACTTAATGCTCTCCGAGAGGTACAACCCGTAGAACTTCCTAACTGTAATTTAGTTAAAGGAATCGAAACTGGCTCTGAAGACTTGGAGAT

L N A L R E V Q P V E L P N C N L V K G I E T G S E D L E I

ACTGCCTAATGGACTGGCTTTCATTAGCTCTGGATTAAAGTATCCTGGAATAAAGAGCTTCAACCCCAACAGTCCTGGAAAAATACTTCT

L P N G L A F I S S G L K Y P G I K S F N P N S P G K I L I

GATGGACCTGAATGAAGAAGATCCAACAGTGTTGGAATTGGGGATCACTGGAAGTAAATTTGATGTATCTTCATTTAACCCTCATGGGAT

M D L N E E D P T V L E L G I T G S K F D V S S F N P H G I

TAGCACATTCACAGATGAAGATAATGCCATGTACCTCCTGGTGGTGAACCATCCAGATGCCAAGTCCACAGTGGAGTTGTTTAAATTTCG

S T F T D E D N A M Y L L V V N H P D A K S T V E L F K F R

AGAAGAAGAAAAATCGCTTTTGCATCTAAAAACCATCAGACATAAACTTCTGCCTAATTTGAATGATATTGTTGCTGTGGGACCTGAGCA

E E E K S L L H L K T I R H K L L P N L N D I V A V G P E H

CTTTTATGGCACAAATGATCACTATTTTCTTGACCCCTACTTACGATCCTGGGAGATGTATTTGGGTTTAGCGTGGTCGTATGTTGTCTA

F Y G T N D H Y F L D P Y L R S W E M Y L G L A W S Y V V Y

CTATAGTCCAAGTGAAGTTCGAGTGGTGGCAGAAGGATTTGATTTTGCTAATGGAATCAACATTTCACCCGATGGCAAGTATGTCTATAT

Y S P S E V R V V A E G F D F A N G I N I S P D G K Y V Y I

AGCTGAGTTGCTGGCTCATAAGATTCATGTGTATGAAAAGCATGCTAATTGGACTTTAACTCCATTGAAGTCCCTTGACTTTAATACCCT

A E L L A H K I H V Y E K H A N W T L T P L K S L D F N T L

CGTGGATAACATATCTGTGGATCCTGAGACAGGAGACCTTTGGGTTGGATGCCATCCCAATGGCATGAAAATCTTCTTCTATGACTCAGA

V D N I S V D P E T G D L W V G C H P N G M K I F F Y D S E

GAATCCTCCTGCATCAGAGGTGCTTCGAATCCAGAACATTCTAACAGAAGAACCTAAAGTGACACAGGTTTATGCAGAAAATGGCACAGT

N P P A S E V L R I Q N I L T E E P K V T Q V Y A E N G T V

GTTGCAGGGCAGTACAGTTGCCTCTGTGTACAAAGGGAAACTGCTGATTGGCACAGTGTTTCACAAAGCTCTTTACTGTGAGCTCTAACA

L Q G S T V A S V Y K G K L L I G T V F H K A L Y C E L !

GACCGATTTGCACCCATGCCATAAGCCGAATTCCAGCACACTGGCGGCCGTTACTA

FIG. 4. Amino acid sequence of PON 1 aligned with the DNA sequence.

PON 1 EXPRESSION

Two PCR products, pon1-20^{8b} and pon1-20^{20b}, resulting from amplification with the pon1-nde and pon1-sap primers were ligated into pCRT7-TOPO and sequenced. The sequencing results verified that the restriction sites had been successfully introduced without mutation. Enzyme digests using *Sap*I and *Nde*I cut pTYB1 and pCRT7-*pon1*-20^{8b} and pCRT7-*pon1*-20^{20b} as expected.(Fig. 5).

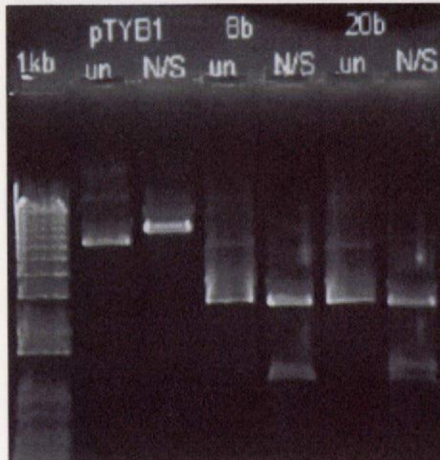

FIG. 5. Gel showing successful digests with *Sap*I and *Nde*I. pTYB1 is shown uncut (lane 2) and cut with both SapI and NdeI (lane 3). Lanes 4 and 5 include uncut and cut pon1-20^{8b}, and lanes 6 and 7 show pon1-20^{20b} uncut and cut. The double digests of pon1-20^{8b} and pon1-20^{20b} released the expected 1 kb fragment.

The 1 kb fragment released by *NdeI/SapI* digestion was mixed and ligated with similarly digested pTYB1. Screening and sequencing were performed on the resulting colonies to verify the presence and proper insertion of the gene. This construct, pTYB1-pon1-20, was transformed into a variety of host strains for expression. Expression studies were then begun in ER2566 and Rosetta, but lack of overexpression led to the use of a similar strain BL21-star. Expression was found in the insoluble fraction at 37°C, so induction was repeated at 15°C (Fig. 6). To improve solubility researchers sometimes grow cells at a lower temperature, co-express the protein with chaperones and foldases, or use solubilizing fusion partners (2). A thick band of about 41kD, the size of PON 1, was seen in the induced insoluble fraction from BL21-star. The temperature lowering was unsuccessful at improving the protein's solubility.

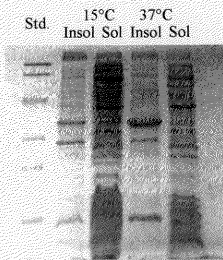

FIG. 6. SDS-PAGE of expression at 15°C and 37°C. The insoluble (insol) and soluble (sol) fractions are shown for both expression temperatures. PON 1 is a thick band at 41kD in the insoluble fractions.

The inclusion body was isolated by centrifugation and solubilized using 8M urea as a denaturant. High concentration denaturants completely disrupt the protein structure, also known as unfolding (2). The buffer also included Tris-HCl, a buffer, EDTA, and dithithreitol (DTT). DTT is a reducing agent used to maintain cysteine residues and prevent non-native intra- and inter-disulfide bond formation (2). An attempted removal of the DTT led to the protein becoming insoluble again (gel not shown). PON 1 is known to have three- cysteine residues in positions 41, 283, and 352 capable of forming disulfide bonds (5). The next attempt at renaturation involved removal of excess denaturant (urea) by dilution (Fig. 7).

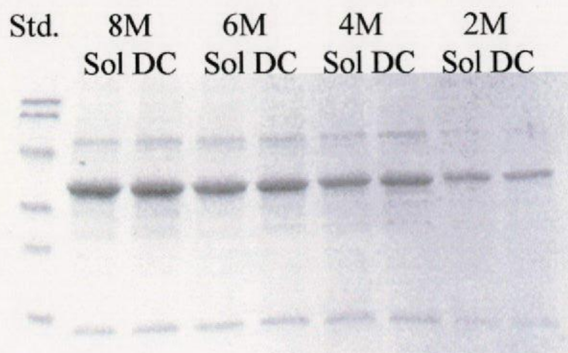

FIG. 7. SDS-PAGE showing urea step gradient dilution. The soluble fractions (sol) with their corresponding dilution control (DC) are shown. Bands of matching intensity between sol and DC indicate that the protein remained soluble at all 4 concentrations of urea.

The urea dilution results indicated that the urea concentration could be decreased while the protein remained soluble. Each concentration was used in a paraoxonase spot assay, but after a 2-hour incubation no activity was observed. The lack of activity indicates that the protein is not folded properly. The presence of DTT could cause a problem because Cys 41 and Cys 352 form a disulfide-bond essential for paraoxonase activity of the enzyme (5). Other methods of maintaining solubility should be attempted. A metal catalyst and reducing agent might be necessary to facilitate disulfide-bond reshuffling to permit proper folding and low molecular weight additives, such as arginine, might improve refolding. Insertion of a eukaryotic gene into a bacterial expression system often leads to inclusion body formation. A eukaryotic expression system, such as Pichia or Baculovirus, might produce soluble PON 1 enzyme.

CONCLUSION

In this study *pon1* was initially located in a human liver cDNA library. The gene was cloned and sequenced, then compared to the known *pon1* sequence. Restriction enzyme sites were added to the ends of *pon1*, then by a digest and a ligation *pon1* was inserted into pTYB1, the expression vector. Protein overexpression was obtained in the BL21-star host strain, but it was insoluble. Inclusion body isolation and purification steps were performed. Refolding of the protein did not result in recovery of activity, as determined by a paraoxonase spot assay. Further studies will include use of different expression systems and other refolding techniques. Mutagenesis will be used to create the four known isoform combinations (R192L55, R192M55, Q192L55, and Q192M55) and to change the arginine at 146 to the glutamine present in the known sequence. The mutants will be compared in activity assays and be crystalized to compare structural characteristics.

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I am a senior genetics major at Texas A&M University. I was a recipient of the President's Endowed Scholarship (1999-2002). I have also been on the Dean's List for the College of Agriculture and Life Sciences (1999-2001). I was inducted into the Golden Key National Honor Society in the spring of 2001, and have since been an active member. Currently I hold the office of Vice President in the Aggie Speleological Society. I have also held officer positions in Phi Eta Sigma, Bioethics Forum, and Genetics Society. I have been a research assistant/ student lab worker in Dr. Wild's laboratory since the summer of 1999. In the spring of 2001I studied abroad in Denmark, taking part in the Medical Practice and Policy program at Denmark's International Studies Program. Currently, I volunteer in the surgical ward at St. Joseph's Hospital. I hope to enter medical school in the fall of 2003.

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