Human Paraoxonase-1 Variants Affect Stability and Activity

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Abstract

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Paraoxonase-1 (PON1) is a high-density lipoprotein-associated enzyme that has been extensively studied due to its ability to hydrolyze and inactivate toxic compounds such as organophosphate (OP) pesticide metabolites and its anti-atherogenic properties. However, the physiological substrate of PON1 is still unknown. Protein therapeutics have been proposed as a treatment for OP intoxication and PON1 is a major candidate. Production in bacterial cells would allow for expression of an unglycosylated enzyme which would reduce the possibility of an immunogenic response. It would also allow for easy mutagenesis of PON1 and scale-up production. We developed a system for expression of recombinant untagged native and variant human PON1s (rHuPON1s) in *E. coli* cells. The proteins were purified using multiple column chromatographic steps. This was the first expression, purification, and characterization of active native human PON1 from bacterial cells. A variant PON1, rHuPON1$_{K192}$, had increased catalytic efficiency of hydrolysis for the OPs diazoxon (DZO), chlorpyrifos oxon, and paraoxon. Catalytic efficiency is an important characteristic as previous experiments demonstrated that it determines whether PON1 will protect against a specific OP *in vivo*. The rHuPON1$_{K192}$ variant was injected in *PON1*

knockout (*PON1*$^{-/-}$) mice and was found to be long-lasting in plasma, nontoxic, and protect against a 1 LD_{50} dose of dermal DZO and prevent the lethality of high doses (2-3 LD_{50}) of DZO. Other rHuPON1 variants were also expressed, purified, and characterized. Some variants with a single amino acid change had increased protein stability and calcium binding. On the other hand, the disease-associated variant rHuPON1$_{V109I}$ had decreased protein stability. While PON1's physiological substrate is still unclear, homocysteine thiolactone (HCTL), a toxic, reactive metabolite of the amino acid homologue homocysteine, has been proposed as the physiological substrate of PON1. Bleomycin hydrolase (Blmh), another enzyme, was described as the HCTLase in tissues. We purified another HCTLase from liver and identified it by MS as biphenyl hydrolase-like protein (BPHL). Recombinant BPHL (rBPHL) was cloned, expressed, and characterized. BPHL's high catalytic efficiency for HCTL, which is 7700 times greater than that of PON1, total HCTLase activity calculations, and liver HCTLase inhibition assays suggest that BPHL is the most physiologically-relevant HCTLase isolated to date.

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Chapter One: Introduction and Background

The World Health Organization estimates that there are approximately 3 million pesticide poisonings worldwide each year, resulting in 220,000 deaths[1, 2]. Organophosphorus compounds (OPs) are some of the most commonly-used pesticides worldwide. OPs are toxic compounds that are the basis of many insecticides, herbicides, and chemical warfare agents such as sarin. There is the potential of accidental, occupational, or intentional release of insecticides or highly toxic nerve agents. OPs inhibit acetylcholinesterase (AChE), the enzyme responsible for terminating acetylcholine signaling, which causes acetylcholine to accumulate to excessive levels resulting in cholinergic crisis[3]. OPs can also inhibit other serine hydrolases, such as butyrylcholinesterase (BChE) or acylpeptide hydrolase (APH). BChE also hydrolyzes acetylcholine, although its physiological function is not clear. APH unblocks *N*-acetyl peptides and is believed to have a role in cognition[4 5, 6]. Cholinergic symptoms include salivation, lacrimation, pinpoint pupils, dizziness and confusion, muscle dysfunction and, if severe, respiratory difficulties. In addition to respiratory failure, OP exposures can result in temporary or permanent damage to neurological, cognitive and motor functions (reviewed in[7]).

Current treatments for OP intoxication consist of anticholinergic drugs such as atropine, a cholinergic antagonist, and an oxime, which can reactivate AChE, provided that the adducted OP has not undergone an "aging reaction" where one acyl group is lost[8]. These treatments are often supplemented with an anticonvulsant[9]; however, there are often negative side effects associated with these treatment protocols[7]. In addition, atropine, anticonvulsants, and oximes only treat the main symptoms of OP intoxication and do not prevent the inhibition of other proteins. To avoid

these problems, investigators have examined the possibility of using proteins capable of detoxifying OPs as a treatment for OP intoxication.

Stoichiometric bioscavengers such as BChE have been shown to provide some protection against OP intoxication[10, 11]; however, the fact that each very large protein molecule can only inactivate a single OP molecule limits their usefulness due to the large quantities of enzyme that would be required (reviewed by[12]). Catalytic scavengers will function as much more efficient therapeutic agents, since each molecule of enzyme is able to inactivate many thousands or more OP molecules per injected protein molecule. Such treatments would not only be useful in cases of deliberate OP poisoning and large-scale chemical terrorism, but also for treating the many individuals accidentally exposed to commonly-used insecticides. One of the most promising catalytic scavenger candidates is paraoxonase-1 (PON1), a high-density lipoprotein (HDL)-associated enzyme with a wide range of substrates that can hydrolyze and inactivate OPs.

The *PON1* gene is located on chromosome 7q21-q22[13] in tandem with the related genes *PON2* and *PON3*. PON proteins have been found in all animals tested and even more distantly related species of bacteria and fungi[13]. Although PON2 is the oldest member in terms of evolution, PON1 was the first protein in the family to be discovered and characterized due to its OP-hydrolyzing ability. PON1 shares greater than 60% homology with the PON2 and PON3 cDNAs and proteins[13]. PON1 is a 28 kb gene with 9 exons and 8 introns[14]; the cDNA is about ≈1 kb in length and the protein is 355 amino acids long with only the initial methionine residue cleaved in the mature protein. PON1 is an atypical protein in that it retains its signal sequence in the mature protein and activity is reduced, but not inactivated, when it is removed[15 16, 17]. However, the retained signal sequence is necessary for interaction of PON1 with HDL[18]. The

protein has a disulfide at residues Cys42 and Cys353 and a free sulfhydryl at Cys284. While replacement of Cys284 with alanine has no effect on the activity of the protein, the C42A or C353A mutants were inactive and failed to secrete from cells[19, 20]. PON1 is a glycoprotein, with sugar residues at residues Asn253 and Asn323. Work by several groups[21, 22] suggested that PON1 has high-mannose type carbohydrates as well as more complex sugar moieties. Abnormalities in PON1 glycosylation may be associated with disease[23-25]. Although Brushia et al.[21] found that PON1 lost activity when it was deglycosylated, other studies[20, 22] noted that deglycosylation did not affect secretion, activity, or substrate specificity. In addition, active proteins have been expressed from bacterial systems[26, 27 17, 28] indicating that glycosylation is not necessary for the activity of PON1.

Another important characteristic of PON1 is its calcium dependence. Two calcium ions are present in PON1's structure – one is important for structural stability, the other necessary for enzymatic activity[29, 30]. Metal chelating agents such as EDTA inhibit PON1, and the loss of calcium irreversibly inhibits the activity of human PON1. A chimeric PON1 protein has been crystallized and the structure solved; however, the structure was not determined for a native PON1 protein but rather a directly-evolved chimeric protein (rePON1) with a high similarity (91%) to rabbit PON1[26, 29]. The crystal structure is a six-bladed beta sheet propeller (showing similarities to another OP-hydrolyzing protein, squid DFPase[31]) with a deep hydrophobic active-site cavity and a unique active-site "lid" that helps to determine substrate specificity and mediate HDL binding. The team that crystallized the rePON1 also suggested a catalytic mechanism – a His-His dyad involving histidine residues 115 and 134[29]. However, more recent studies have proposed a second catalytic mechanism as well, possibly a catalytic dyad or triad involving residues E53, D269, the histidines at positions 115 and 134 and the calcium ion[32, 33].

PON1 is expressed in liver and is secreted to associate with HDL in circulation. The retained hydrophobic N-terminus has been shown in several *in vitro* studies to be essential for insertion of the PON1 protein in the plasma membrane of hepatocytes. The signal sequence is also necessary for interaction with HDL, and it is believed to bind to phospholipids instead of specific HDL proteins such as Apolipoprotein AI (ApoAI)[18]. The current model was developed using *in vitro* cell culture studies and has enzymatically active PON1 inserted in the external membrane of liver cells via the hydrophobic signal sequence and only secreted when an appropriate acceptor such as HDL is present. Although low levels of PON1 can also be taken up by VLDL, LDL cannot act as an acceptor[34,35]. The interaction between HDL and the plasma membrane is mediated by the HDL scavenger receptor B1 (SR-B1), and PON1 is incorporated into the HDL particle by desorption[34, 36]. HDL is the optimal acceptor and both HDL proteins and phospholipids are needed for PON1 secretion and stabilization of PON1's enzymatic activity[34, 36, 37] although in the absence of ApoAI, a major HDL structural protein, PON1 is still able to interact with the lipoprotein particle. Particle size, HDL protein composition, and oxidative stress affect the ability of PON1 to be incorporated into HDLs[37, 38]. When the N-terminal sequence was mutated and subsequently cleaved, the PON1 protein was freely secreted into the medium with no HDL present[16]. After the crystal structure was solved, it was suggested that the signal sequence has an amphipathic character, with part forming a helical structure (H1). Along with an adjacent helix, H2, H1 is believed to interact with HDL as both helices have what appears to be hydrophobic solvent-facing residues. Harel et al.[29] hypothesized that this hydrophobic patch – along with several hydrophobic residues on adjoining structural loops – is the region of interaction with HDL.

For many years, it was believed that PON1 protein was only present in the liver and in plasma HDL, with smaller quantities in the kidney and intestine. However, more recent reports have suggest PON1 is more widespread than was initially believed. Using immunohistochemistry to localize PON1 in normal mouse tissues, Marsillach et al.[39] found widespread PON1 staining in all tissues tested, including skin, stomach, brain, pancreas, skeletal muscle, lung, and heart. Recent reports confirmed that the mRNA distribution of PON1 is limited to liver, kidney, and colon[40], suggesting that distribution of PON1 may occur through transfer from HDL, as has been proposed[41]. In the report by Deakin et al.[41], *in vitro* cell culture models demonstrated that HDL could transfer enzymatically active PON1 to the membranes of cells not expressing PON1 and that the newly acquired PON1 protected the cells from oxidative stress. As described below, PON1 has also a very important antioxidant function.

Initial interest in PON1 was due to its ability to hydrolyze and inactivate toxic pesticide OP metabolites. Aldridge described an OP hydrolase in plasma as far back as 1953[3]. He termed enzymes that could metabolize OPs and were not inhibited by them "A-esterases" while those that would bind the OP, thus removing it from circulation, but were then irreversibly inhibited were called "B-esterases." PON1 was classified as an A-esterase. PON1 was named for its ability to hydrolyze the OP paraoxon (PO). Paraoxonase activity (POase) was present in the plasma of a number of different species tested, with rabbits having substantially greater activity than the other tested species, which included mice, rats, goat, and humans. In addition, POase activity was measured in various tissues – plasma had the highest levels of OPase activity, followed by liver[42]. Later studies noted that plasma had both OP-hydrolyzing activity and arylesterase activity (activity against aromatic esters such as phenyl acetate) and, after some controversy over whether the activity was due to the same protein or two distinct enzymes, the purification and

cloning of human PON1 attributed both activities to PON1[43, 44]. PON1 activity was characterized in a number of different animal models; some, like rabbits, had very high PON1 activity, even higher than humans, while others such as birds had very low activity[45, 46]. These species differences also generally corresponded to OP sensitivity[45, 47, 48]. Different PON1 substrates were also examined – besides PO, other phosphotriesters that were substrates for PON1 included diazoxon (DZO) and chlorpyrifos oxon (CPO); other substrates included nerve agents such as sarin and soman as well as phenyl acetate[49-51]. In addition, variations in the PON1 activity in populations were studied. Histogram plots of PON1 activity towards different substrates in human populations showed either a bimodal or trimodal distribution, with distinct groups classified as "low metabolizers" or "high metabolizers" (reviewed in[52]), suggesting PON1 is a polymorphic enzyme. The basis for this distribution was determined after the PON1 cDNA was sequenced, showing the presence of two different coding region SNPs – L55M and Q192R, with the Q192R polymorphism[15] defining high vs low metabolism of PO[53, 54].

The Q192R polymorphism is known as the activity polymorphism as it determines PON1's catalytic efficiency towards various substrates[54]. This polymorphism is the basis for the population distributions seen for different substrates – populations are divided into $PON1_{Q192}$ homozygotes, heterozygotes, and $PON1_{R192}$ homozygotes. Gene frequencies vary in the studied populations – while some Northern European populations have a frequency of ≈ 0.7 for the Q allele, some Asian populations are closer to a frequency of ≈0.3. In general, the R allele predominates in Asian and African populations (reviewed in[55]). After the discovery of the PON1 Q192R polymorphism, a two-dimensional assay pioneered by Dr. La Du was developed for separating out the genotypes[56]. An improved version of this two-substrate assay, currently referred to as PON1 status[47], was reported by Davies et al.[51] where POase and diazoxonase

(DZOase) activity were plotted for each individual on a two-dimensional plot[57]. A nontoxic assay that gave the same separation of the $PON1_{192}$ phenotypes was developed[58, 59]. As plots from these assays show, there is a wide range of activity within each of the Q192R phenotypes (QQ; QR; RR)[47]. For any epidemiological study, it is necessary to determine activity as well as genotype, or "PON1 status", as SNP analyses provide no information on activity and have given conflicting results[55, 60, 61]. Interestingly, some substrates such as phenyl acetate are not affected by the 192 polymorphism; however, the Q192R alters the activity of PON1 towards different substrates in different ways – while the $PON1_{Q192}$ protein has a higher catalytic efficiency of hydrolysis for substrates such as the OP nerve agents[51], the $PON1_{R192}$ alloform is a more efficient hydrolyzer of others such as CPO and PO[62].

The L55M polymorphism does not affect PON1 activity; however, there is a small effect on PON1 levels. Initial reports linked the polymorphism to substantial differences in protein levels, but this was mostly due to linkage disequilibrium with a promoter polymorphism, C-108T, with M55 in linkage disequilibrium with the inefficient promoter polymorphism T-108[63, 64]. However, there have been reports that L55M itself affects proteins stability – $PON1_{M55}$ was reported to be more prone to proteolysis than the $PON1_{L55}$[65]. A number of studies have also examined the L55M genotype using SNP analyses to correlate PON1 to disease; these studies have produced mixed results. For example, several studies relating Parkinson's disease (PD) to the L55M polymorphism have proposed a relationship between the $PON1_{M55}$ alloform and PD; however, other studies have reported no correlation and meta-analyses of the relationship between PON1 genetic variation and PD have also provided conflicting results[66]. Besides the C-108T promoter polymorphism, several other noncoding polymorphisms were discovered; these include four others in the promoter and several in the 3' UTR sequences [64]. Promoter

polymorphisms identified are the C-108T and G-126C, A-162G, -832A, and G-909C[63, 64, 67, 68]. Some of these polymorphisms were shown to be linked to protein levels, with the -108 polymorphism having the greatest effect as it is located at an Sp1 binding site. Individuals carrying the -108C allele have on average almost twice the PON1 activity levels of those carrying the -108T allele[63, 64]. The -162 polymorphism had a much smaller effect on PON1 levels.

The discovery that PON1 in association with HDL prevented LDL oxidation[69] and that low serum PON1 levels were associated with diabetes and familial hypercholesterolemia[70] opened up new directions in PON1 research. Following these publications, many groups looked at the correlation between PON1 genotype (either the L55M, Q192R or various promoter SNPs) and an extensive list of disease conditions. The results from these studies were mixed. A number of meta-analyses have called into question the validity of using PON1 genotype only as a marker for disease – weak and conditional correlations were found for coronary heart disease and the *PON1*$_{R192}$ allele[71, 72], no relation was found for Alzheimer's disease[73], conflicting results were reported for PD[66, 74]. As well, genome-wide association studies did not identify the *PON1* gene as a risk factor for vascular disease or myocardial infarction[75, 76]. There is a wide range of PON1 activity within each PON1$_{192}$ genotype so activity must be taken into account along with DNA sequence when correlating PON1 genetic variation to risk of disease or exposure. This is an important issue, as the functionality of the enzyme is more important in disease than the genetic polymorphism[55, 57, 60, 61, 77]. Several studies that have determined PON1 status (which measures PON1 genotype and activity) have found a link between low PON1 activity and disease[60, 61, 77] as well as a meta-analysis[78]. Clearly, activity measurements are more important in PON1 association studies than genotype.

There have been a number of mechanisms suggested for the role of PON1 in cardiovascular disease (CVD). PON1 has been reported to hydrolyze oxidized phospholipids[79], protect HDL[80] and LDL[69] from oxidation, and contribute to cholesterol reverse transport in macrophages (reviewed in[55]). PON1 participates in a number of anti-inflammatory actions – reducing macrophage oxidative stress, inhibiting monocyte to macrophage conversion as well as monocyte chemotaxis and adhesion. Some of the strongest evidence for the role of PON1 in vascular disease has come from mouse studies. Transgenic mice overexpressing PON1 by 2- to 4-fold had decreased atherogenic lesions and their HDL more effectively protected LDL from oxidation[81]. The PON1 knockout mouse (*PON1*$^{-/-}$) showed increased sensitivity to the OP pesticide metabolites DZO[62] and CPO[82] and was more prone to atherosclerosis when fed a high-fat diet[82]. PON1 was essential for the anti-atherogenic effects of HDL as HDLs from knockout mice were pro-atherogenic - HDL taken from these mice could not protect LDL from oxidation in a cell culture model. *PON1*$^{-/-}$ mice also had increased lipoprotein oxidation levels and their LDL was more susceptible to oxidation[82].

The *PON1*$^{-/-}$ mouse also definitively demonstrated the importance of PON1 in protecting against OP exposure. However, although the *PON1*$^{-/-}$ mouse was considerably more sensitive to DZO and CPO, it did not differ from exposed wild-type mice in brain AChE inhibition following dermal exposure to PO[62]. The explanation for this was determined to be the overall low catalytic efficiency of both PON1 alloforms for PO. It was known that the $PON1_{R192}$ alloform had higher activity against PO than the $PON1_{Q192}$, but both $PON1_{192}$ alloforms have an overall very low catalytic efficiency for hydrolysis of PO and thus were unable to protect against PO exposure. These experiments demonstrated the importance of catalytic efficiency of hydrolysis for a specific substrate in determining whether a protein can function as a therapeutic following

exposure to a specific compound. Native human PON1 already has the ability to protect against DZO and CPO. In the case of nerve agents, PON1 is ineffectual at protecting from *in vivo* intoxication, even though the nerve agents are hydrolyzed by PON1 with *in vitro* assays. Numerous efforts have been made to improve PON1's catalytic efficiency. The Tawfik research team developed a system for expressing rePON1, a recombinant chimeric PON1. By solving the crystal structure of this rePON1, the Tawfik group was able to address the mechanism of catalysis and predict which residues should be substituted in order to to affect activity of PON1 for different classes of substrates. Using a rePON1 as a protein backbone, researchers altered a number of residues and determined how these mutations affected activity towards OPs or different substrates. The effect of a number of mutated residues was tested for increased OPase activity[26, 29, 83, 84]. In addition, the publication of the crystal structure has allowed for computational analyses of the importance of individual residues in catalysis and rational approaches for mutagenesis and testing[17, 32, 33, 85]. The PON1 crystal structure has led to many advancements in the PON1 field, including a better understanding of the mechanism of substrate hydrolysis.

Although the native physiological substrate of PON1 is still subject to debate, there have been a number of hypotheses suggesting candidates for the physiological substrate. In 2000, the endogenous substrate was proposed to be a lactone by two separate groups[86, 87]. Lactones are a novel class of chemicals that are metabolized by PON1. Jakubowski[87] suggested that the physiological substrate of PON1 was homocysteine thiolactone (HCTL)[87]. HCTL is generated from homocysteine (Hcy), a non-protein analog of methionine, in error-editing reactions catalyzed by aminoacyl tRNA synthetases. Both Hcy and HCTL levels have been correlated with a number of diseases and conditions such as CVD and Alzheimer's disease[88], although whether

this correlation is causative or symptomatic is still unresolved. Several explanations have been provided for the detrimental effects of Hcy and HCTL, including consumption of energy to generate HCTL from Hcy and homocysteinylation of proteins by HCTL, which can alter or impair function, interfere with protein folding, and generate autoimmune responses.

Jakubowski[87] first discovered a calcium-dependent serum HCTLase that was associated with HDL and hypothesized that it was PON1. PON1 purified from serum hydrolyzed HCTL with a K_m of 23 mM and a specific activity of 76.0 μmol/mg/hr (or 1.27 μmol/mg/min). Although the affinity and efficiency for HCTL by PON1 is very low, there is some physiological evidence suggesting the importance of PON1 in protecting against HCTL toxicity. A number of correlational studies have related low HCTLase to different diseases and conditions such as coronary heart disease and renal failure[89]. The *PON1*$^{-/-}$ mouse model differed significantly in a number of ways from wild-type mice, as it had no plasma HCTLase[87] and was reported to be more susceptible to HCTL toxicity than wild-type mice[90].

Billecke et al.[86] furthered the work done by Jakubowski[87] by characterizing the PON1 activity towards a number of lactones and testing the substrates with both $PON1_{192}$ alloforms, $PON1_{Q192}$ and $PON1_{R192}$. They concluded that PON1 hydrolyzed multiple lactone substrates although its activity towards thiolactones, including HCTL, was very low. Lactone substrates that were metabolized by PON1 differed in lactone ring size and functional groups. Aliphatic and aromatic lactones were both hydrolyzed, with aromatic lactones being better substrates overall. Different functional groups affected the ability of lactones to act as functional PON1 substrates – hydroxyl groups reduced or eliminated PON1 lactonase activity as did sugar groups, but introduction of bromides to the ring increased activity. At the concentrations tested, the

$PON1_{R192}$ alloform hydrolyzed some lactones more efficiently, such as thiolactones, but $PON1_{Q192}$ was better at hydrolyzing other substrates such as 2-coumaranone. Thiolactones were poorer substrates for PON1, and in their comparisons, HCTL had <1% of activity relative to phenyl acetate hydrolysis by both PON1 alloforms. Jakubowski also tested several other lactones in addition to HCTL – L-homoserine lactone was a substrate but lactams were not, a result consistent with the findings of Billecke et al.[86].

PON2 and PON3 also have lactonase activity, further evidence suggesting that lactonase is a physiologically-relevant activity of the PONs. Lactones are substrates that all three PONs hydrolyze and there are naturally existing lactones such as bacterial quorum-sensing factors (acyl-homoserine lactones) and lactones in food products. The discovery of PON2 and PON3 occurred some years after PON1 had been extensively characterized[13]. Draganov et al.[22] characterized a number of substrates for all three recombinant PONs expressed in insect cells. PON2 and PON3 showed little or no activity for OPs but they had activity for various lactones, though few substrates were hydrolyzed by PON2. However, PON2 had the highest activity towards the acyl-homoserine lactones. Serum PON1 degraded 3OC12-HSL, a *Pseudomonas* lactone involved in quorum-sensing and biofilm formation, although *PON1*$^{-/-}$ mice were paradoxically resistant to *P. aeruginosa* virulence due to high levels of PON2 expression. Transgenic *Drosophila* expressing PON1 were resistant to *Pseudomonas* lethality due to PON1 hydrolyzing an acyl-homoserine lactone, demonstrating the importance of PONs in protecting against bacterial infections via their lactonase activity[91, 92]. All three PONs had activity against 5-HETEL and 4-HDoHE, oxidation products of arachidonic acid and docosahexaenoic acid, as well as the lactones 2-coumaranone and dihydrocoumarin although PON2 had the lowest activity for all of these compounds. Draganov et al.[22] supported the suggestion that lactones were the

physiological PON substrates. The authors hypothesized that the biological activity of PON2, the oldest member of the PON family, was against acyl-homoserine lactones and that the other PONs may have biological substrates with structural similarities to 5-HETEL and 4-HDoHE, which include many lipid oxidation products. The studies expressing the chimeric rePON1 in bacterial cells also suggested lactonase was the native activity of the PONs[26]. PON1 and PON3 were directly evolved with gene-shuffling protocols to improve activity against phosphotriesters or esters[26, 29]. The evolved variants generally retained their lactonase activity, indicative of a native function. In addition, structural analyses of the chimeric protein have also supported the idea that lactonase is the physiological PON family function[93]. Although many groups now believe that lactones are the physiological substrate of the PON family, there is still debate over the identity of the specific lactones.

The discovery that PON1 was a lactonase prompted investigation into whether lactone drugs and prodrugs could be PON1 substrates. Billecke et al.[86] found that several lactone drugs were hydrolyzed by PON1. A number of drugs are PON1 substrates or have an effect on PON1 upregulation and activity (reviewed in[94]). More recently, clopidogrel thiolactone, a metabolite of the first-line antiplatelet prodrug clopidogrel, recommended for prevention of ischemic cardiovascular events, was proposed to be a PON1 substrate[95]. Bouman et al.[95] reported that PON1 was the enzyme responsible for hydrolyzing the active thiolactone metabolite of clopidogrel and showed differences in populations depending on the PON1 alloform, with $PON1_{Q192}$ homozygotes having lower efficiency of activation (thiolactone hydrolysis) compared to $PON1_{R192}$ homozygotes. However, association studies published after Bouman et al.[95] failed to replicate their results and other rebuttals have been published[96, 97] suggesting that PON1 only catalyzes the formation of an inactive indothiol metabolite[98].

While PON1 and the PON family have been intensively studied, there are still a number of questions to answer and work to be done. In Chapter 2, the development of an *E. coli* PON1 expression and purification system will be described. Native untagged recombinant PON1 alloforms, as well as variants, were expressed and purified, illustrating the usefulness of a microbial production system for understanding the structure and function of PON1. Chapter 3 describes experiments using the *PON1*$^{-/-}$ mouse model to test the ability of the injected recombinant PON1 to function as a catalytic scavenger following OP exposure. In Chapter 4, the work with the *E. coli* expression system was extended, with both native alloforms and novel variants expressed and characterized. The variants characterized were examined for their increased stability, calcium binding, or association with disease risk. In the final chapter, we initially intended to address the controversy over PON1's ability to hydrolyze clopidogrel; however, the lactonse that was purified was not PON1. Instead, we discovered a new homocysteine thiolactonase activity by purifying HCTLase from human liver. The novel HCTLase turned out to be a previously-described protein called biphenyl hydrolase-like protein (BPHL), which had been characterized for its role in the metabolism of valacyclovir and other drugs. We proposed that BPHL, rather than PON1, is the primary enzyme involved in the detoxication of HCTL.

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Chapter Two: *E. coli* Expression of Untagged Recombinant Human Paraoxonase-1

Abstract

The high density lipoprotein-associated enzyme paraoxonase-1 (PON1) hydrolyzes lactones, aromatic esters, and neurotoxic organophosphorus (OP) compounds including insecticide metabolites and nerve agents. PON1 has been proposed as a prophylactic or therapeutic for treatment of OP exposure. For use as a biotherapeutic, PON1 expression in *E. coli* would fulfill several therapeutic requirements as it has high activity against some OPs and would likely be minimally immunogenic. We have developed a system for expressing and purifying native human PON1 from *E. coli* cells. Previous reports indicated that native active PON1 could not be expressed in bacterial cells; however, this system produces active recombinant PON1 (rHuPON1) in quantities sufficient to be purified and characterized. Purification of the rHuPON1 protein required seven column chromatographic steps, with multiple anion exchange steps and a hydroxyapatite or hydrophobic interaction chromatography column. The two native alloforms of PON1 ($rHuPON1_{R192}$ and $rHuPON1_{Q192}$) were expressed and purified as well as a variant, $rHuPON1_{K192}$, which has increased activity for OP hydrolysis relative to the two native $PON1_{192}$ alloforms. The three rHuPON1 variants were tested with a phenyl ester (phenyl acetate) and three OPs – diazoxon, chlorpyrifos oxon, and paraoxon. Compared to the two native alloforms, $rHuPON1_{K192}$ had higher catalytic efficiency for hydrolysis of the three OP pesticide metabolites. Expression of active rHuPON1 in *E. coli* provides a system in which PON1 can be engineered for increased catalytic efficiencies.

Introduction

Organophosphorus (OP) insecticides are some of the most commonly-used pesticides worldwide. In addition to accidental exposure to insecticides, deliberate exposure to OP nerve agents or toxic industrial OPs remains a concern. The acute toxicity of OPs is due to their inhibition of acetylcholinesterase (AChE) and the standard treatment for OP exposures consists of anticonvulsants, atropine - an anticholinergic drug - and oximes to reactivate the AChE. These approaches, however, treat only the symptoms of OP intoxication and have other drawbacks[1, 2]. Another proposed treatment is injection of proteins that will bind to (stoichiometric scavengers) or metabolize (catalytic scavengers) OPs before they inhibit target proteins. The ability of specific proteins to bind or hydrolyze toxic compounds has led to the idea of bioscavengers as therapeutics for OP exposure (reviewed in[3]).

One approach for treating exposure to OP compounds has been the injection of the stoichiometric scavenger butyrylcholinesterase (BChE)[4-6]. BChE, along with AChE, belongs to the cholinesterase family and acts as a backup for AChE, although it is a plasma protein and is ubiquitously distributed in tissues[7]. The physiological function of BChE remains unclear, but it hydrolyzes a number of substrates including acetylcholine, cocaine, succinylcholine, and butyrylcholine, and stoichiometrically binds chemicals such as OP nerve agents and OP pesticides. BChE acts on its substrates by hydrolyzing them or covalently binding them and removing them from circulation, thus preventing them from inhibiting other proteins such as AChE. BChE protein adducts have been studied as biomarkers of exposure[8-11].

Recombinant BChE has been expressed in a number of systems including transgenic goats[12], plants[13], and stable cell lines[14]. Researchers generated and tested mutants with increased

activity against various substrates[15]. Recombinant BChE protected rodent and primate animal models from succinylcholine, cocaine, and nerve agent toxicity and death[16-19]. One of the advantages of developing BChE as a therapeutic is its broad substrate range. However, there are also disadvantages to using BChE. The primary limitation of stoichiometric scavengers is that they bind only a single OP molecule per large protein molecule thus requiring large quantities of the protein to be injected. In addition, injection of recombinant BChE from cell culture resulted in the production of antibodies, even when the recombinant protein was PEGylated[20-22]. An attractive alternative is the use of catalytic scavengers that hydrolyze many OP molecules per injected protein molecule[3, 23]. Catalytic scavengers would be a more efficient therapy, as each molecule of enzyme is able to inactivate hundreds to thousands of OPs per molecule protein.

There are several important qualities required of a therapeutic catalytic scavenger. It should be active against the required compounds, minimally immunogenic, nontoxic when administered, and should have a long half-life when injected. Human paraoxonase-1 (PON1) is a leading candidate for use as a catalytic scavenger of toxic OP compounds. PON1 is capable of hydrolyzing a wide range of substrates including the OP pesticide metabolites paraoxon (PO), diazoxon (DZO), and chlorpyrifos oxon (CPO) which are manufactured as less toxic organophosphorothioates and bioactivated by the cytochrome P450s to the more toxic oxon forms[3, 24].

An injectable protein therapeutic needs to be nontoxic when administered and would ideally result in a minimal or no immune reaction. The most promising approach to achieve this goal will be the use of recombinant proteins of human origin that are not glycosylated. Other enzymes, such as bacterial phosphotriesterase and squid DFPase, are capable of inactivating OP

toxins and efficiently metabolize them, but these are not of human origin (reviewed in[25]). As recombinant PON1 produced in *E. coli* is a native human protein minus glycosylation, it will be less likely to elicit an immune response compared to bacterial OP hydrolases or BChE produced in different eukaryotic systems. BChE has been shown to be nontoxic in a number of animal models, but a decrease was seen in the half-life of PEGylated BChE upon repeat injection, suggesting it did elicit immune responses[20]. There are also reports of PON1 functioning as a nontoxic and long-lasting catalytic scavenger when administered as a therapeutic. Injections of purified plasma PON1 protein from rabbits and humans were tested in rodent models in a series of experiments based on the early work of Main[26]. Both rats and mice, including *PON1* knockout mice (*PON1*$^{-/-}$), tolerated PON1 injections and elevated PON1 activity was observed in plasma hours or days following injection[27-30]. PON1's activity against multiple substrates, its localization in plasma, and previous work in animal models suggest that injectable rHuPON1 has potential therapeutic applications for preventing OP intoxication. A possibility exists for other potential uses for therapeutic PON1 such as prevention of complications due to vascular disease. PON1's anti-atherogenic effects and the association of low PON1 activity with disease have been intensively studied[31-34].

While native human PON1 has sufficient activity to protect against several OPs, for certain OPs the catalytic efficiency is low. An effective catalytic scavenger must have a high catalytic efficiency in order to detoxify and protect against specific compounds[29]. For many years, it was thought that high levels of PON1 would protect against exposure to PO, from which PON1 derives its name. However, after *PON1*$^{-/-}$ mouse model was developed, this was shown to be incorrect. The *PON1*$^{-/-}$ mice were significantly more susceptible to CPO[35] and DZO exposure[29], but there was no significant difference between wild-type mice and *PON1*$^{-/-}$ mice in

susceptibility to PO as determined by AChE inhibition and symptomology. Similar results were found when mice were injected with purified human PON1 and tested *in vivo* protection. Brain AChE activity was protected when the *PON1* $^{-/-}$ mice were administered purified PON_{R192} or PON_{Q192} followed by exposure to either CPO or DZO. Neither purified PON1 alloform could protect mice against exposure to PO. The *in vitro* catalytic efficiency was determined for hydrolysis of CPO, DZO, and PO for both $PON1_{R192}$ and $PON1_{Q192}$ proteins (measured as V_{max}/K_m). Both PON1s had nearly equivalent catalytic efficiency for DZO hydrolysis, which was reflected in the equal protection provided when injected. PON_{R192} had a higher catalytic efficiency for hydrolysis of CPO and provided significantly more protection than $PON1_{Q192}$ against CPO exposure (although both alloforms provided protection against AChE inhibition). The $PON1_{R192}$ alloform was nearly nine times more efficient than PON_{Q192} at hydrolyzing PO, but the overall catalytic efficiency was so low for both alloforms that neither could protect against a PO exposure *in vivo*. These experiments provided convincing evidence that engineered increases in catalytic efficiency will be required for using recombinant PON1 to protect against PO (as well as nerve agents).

Based on this previous work, the four important requirements for the development of a catalytic scavenger are nontoxicity and minimal immunogenicity, a catalytic efficiency sufficient to protect against the specific compound, a significant half-life following injection, and development of a scalable production process. With PON1, the development of rapid screening protocols for identifying variants with improved catalytic efficiency of hydrolysis would be helpful since this was not achieved. To prevent decoration of the recombinant PON1 with immunogenic carbohydrate chains, PON1 will be best expressed in a non-eukaryotic system. If active recombinant human PON1 (rHuPON1) could be expressed in an *E. coli* system, two of

these goals would be realized. This system would also provide a more convenient system for scale-up production and for rapid screening of variants with increased catalytic efficiencies.

The difficulty of using *E. coli* as a host for producing PON1 has been noted previously[36]. In attempts to produce a more soluble version of PON1, Aharoni et al.[37] used a gene-shuffling protocol to express a soluble variant of PON1 that was used to determine the first crystal structure of a PON1 enzyme[38]. Human, rabbit, rat, and mouse PON1s were cloned and digested, followed by domain-shuffling procedures and selection using varying substrates. Multiple rounds of shuffling and selection were performed for directed evolution of PON1. Early on, it became clear that sequences tended to converge on the rabbit PON1 sequence and the PON1 clone chosen for analysis was closest to rabbit PON1, with 91% identity to wild-type rabbit PON1. In all tested variants, the 192 position was a lysine as in rabbit PON1. The bacterially-produced recombinant PON1 (rePON1) variants had similar activity to wild-type PON1s and produced high protein yields; however, wild-type rabbit, rat, and mouse PON1s required protein tags for soluble expression and therefore the shuffling protocols were determined to be necessary. In addition, later work[39] also found some activity and substrate specificity differences between human plasma PON1 and rePON1, despite the fact that many of the amino acid changes in the rePON1 were far from the active site. Importantly, these studies demonstrated that glycosylation was unnecessary for PON1 activity. To avoid immunological complications it would be desirable to have a protein therapeutic that is as close as possible to the native human sequence, with minimal changes necessary for increasing catalytic efficiency. However, this protein was the first soluble and active PON1 produced in bacterial cells to be reported in the literature. The development of this system allowed researchers to crystallize the rePON1, pinpoint a number of important residues, and suggest the first catalytic mechanism for PON1[38, 40, 41].

The purification of PON1, a highly hydrophobic protein, has also proved to be an obstacle in the development of a catalytic scavenger. Much of the PON1 purification efforts used human serum as starting material, and these efforts produced purified PON1 via several chromatography steps[42, 43]. Purification of serum PON1, however, is not ideal for large-scale production of PON1 as would be required for use as a catalytic scavenger especially since native human serum PON1 has an inadequate catalytic efficiency of hydrolysis for many OP substrates including nerve agents.

To establish conditions for producing active human PON1 in the *E. coli* expression system, a GST-tagged rHuPON1 protein was generated, demonstrating that active, soluble rHuPON1 fusion protein could be expressed and purified from *E. coli*. This expression system also allowed for examination of the effects of amino acid substitutions on the catalytic efficiency of the rHuPON1. Because the $PON1_{192}$ polymorphism has little effect on the activity of the enzyme towards phenyl acetate, the ratio of OP hydrolytic substrate activity to phenyl acetate hydrolytic activity was used to characterize all four constructs. As expected, the $rHuPON1_{K192}$ alloform showed an increased activity against the three OPs tested (CPO, PO and DZO) relative to phenyl acetate (Figure 2.1). While the AREase activity of all four variants was nearly equivalent, $rHuPON1_{K192}$ had a greater ratio of CPOase/AREase compared to $rHuPON1_{R192}$, the best of the other three variants. The $rHuPON1_{K192}$ also had the highest ratio of DZOase/AREase, with the other three variants having similar ratios. The native variant $rHuPON1_{Q192}$ and the $rHuPON1_{N192}$ have similar POase activity ratios, with $rHuPON1_{R192}$ much higher; the $rHuPON1_{K192}$ has the highest activity ratio.

We reported the first expression and purification of native and variant recombinant human PON1s (rHuPON1) from an *E. coli* system. The rHuPON1$_{K192}$ variant, analogous to rabbit PON1 at this position, was assayed for its hydrolytic activity for the OP compounds DZO, CPO and PO, and compared with rHuPON1$_{R192}$, the more efficient of the two natural human PON1$_{192}$ alloforms (PON1$_{Q192}$ and PON1$_{R192}$) against some pesticide OP exposures.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. DZO, PO, and CPO were purchased from Chem Service (West Chester, PA).

Engineering and expression of rHuPON1s

Expression of active native untagged rHuPON1 in *E. coli* was achieved through the use of the Staby plasmid system (Eurogentec, San Diego, CA) where the *E. coli* genome carries the *ccdB* gene that encodes a stable topoisomerase inhibitor and the plasmid carries the *ccdA* gene that encodes a labile inhibitor of *ccdB* expression, thus forcing the cells to retain the plasmid even at high cell densities. The PON1 gene was cloned into the pStaby plasmid and the expression levels were enhanced two- to seven-fold by the addition of a second plasmid (pRARE; Invitrogen) that encodes tRNAs for codons more commonly found in mammalian transcripts.

The plasmid pStaby1.2 (Eurogentec) was modified to generate pStaby-NcoI by replacing an *NdeI* restriction endonuclease site downstream of the T7 promoter with an *NcoI* RE site using PCR primers (5'-CATGCTTGCCATGGCTAGCATGACTGGTGGACAG-3' and 5'-

CTAGCTTGCCATGGGTATATCTCCTTCTTAAAGTTAAAC-3') followed by *NcoI* restriction (NEB, Ipswich, MA) digestion, 0.8% agarose gel purification, ligation using T4 DNA ligase (NEB) followed by transformation into XL1-Blue (Agilent, Santa Clara, CA) *E. coli* cells. Human $PON1_{Q192}$ gene coding sequence with an *NcoI* site at the translational start codon and a *SacI* site at the translation stop codon was cloned into *NcoI-SacI* cut pET32a (Novagen). Site-directed mutagenesis was used to change the Q192 position to encode either R192 or K192. The resulting plasmids contained *NcoI-NotI* fragments encoding $rHuPON1_{R192}$ or $rHuPON1_{K192}$ that were introduced into the pStaby-NcoI plasmid by ligating 0.8% agarose gel-purified *NcoI* to *NotI* DNA fragments of PON1 to *NcoI-NotI* digested pStaby-NcoI vector using T4 DNA ligase. Expression of rHuPON1s in bacteria was carried out using the Staby system expression strain, SE1 (Eurogentec). SE1 cells were transformed with the pStaby-*PON1* plasmids as well as the plasmid pRARE (encoding genes for tRNAs rare in *E. coli*; Invitrogen) and selected on LB-broth (Teknova, Hollister, CA) agar plates containing 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol. A 14 liter MF-114 Microferm Fermentor (New Brunswick Scientific, New Brunswick, NJ) with 6 L/min air flow and 500 rpm stirring was used for production of rHuPON1-expressing *E. coli*. A culture of 200 ml SE1 cells containing pRARE and pStaby-*PON1* exponentially growing at room temperature in LB supplemented with antibiotics was added to 12 liters LB broth containing antibiotics, 10 mM $CaCl_2$, 2% glycerol and trace elements (sodium chloride 5 g/L, zinc sulfate heptahydrate 1 g/L, manganese chloride tetrahydrate 4 g/L, ferric chloride hexahydrate 4.75 g/L, cupric sulfate pentahydrate 0.4 g/L, boric acid 0.575 g/L, sodium molybdate dihydrate 0.5 g/L and 6N sulfuric acid 12.5 ml/L) added at 1:1000 at 23°C. When the A_{600} reached ≈0.8, isopropyl-beta-D-thiogalactopyranoside was added to a final

concentration of 0.25 mM. After 16 h, cells expressing rHuPON1s were harvested by centrifugation and stored at -80°C.

Purification of rHuPON1

Arylesterase (phenyl acetate hydrolysis; AREase) activity of PON1 was measured since the expression strain lacks endogenous AREase activity. Column fractions (5 µl) were assayed for AREase in a SPECTRAmax® PLUS Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA)). Five µl from the fractions were added to wells of a 96-well UV microplate and the reaction was initiated by addition of 200 µl of 3.26 mM phenyl acetate in 9 mM Tris pH 8.0, 0.9 mM $CaCl_2$ buffer. The reaction was followed for 4 min at 270 nm and at room temperature (25^0C). Only the initial linear rates were used for calculations.

E. coli cells (200 g of rHuPON1$_{K192}$ or 167 g of rHuPON1$_{R192}$) were combined with 100 ml of 0.1 mm glass beads (Biospec Products, Bartlesville, OK), 100 µl protease inhibitor cocktail (EMD Millipore, Billerica, MA; EDTA-free protease inhibitors), 125 units benzonase (Novagen) and lysis buffer (20 mM Tris, pH 8.0, 1 mM $CaCl_2$, 5 mM dithiothreitol) to a final volume of 350 ml. Cells containing soluble active rHuPON1 were disrupted using a BeadBeater (Biospec Products) 10 x 1 minute alternating with 1 minute cooling on ice. Extracts were centrifuged for 20 minutes at 17,000 x g and the supernatant was decanted. Cell pellet fractions were pooled and resuspended in lysis buffer containing 1% Tergitol to a final volume of 350 ml, then re-extracted with the BeadBeater as described above. Following centrifugation at 17,000 x g centrifugation, the second supernatant was combined with the initial lysis supernatant prior to purification.

Recombinant rHuPON1$_{K192}$ was purified using 7 column chromatography steps. Cell extracts were loaded onto a 70 ml resin bed volume (rbv) diethylaminoethyl (DEAE) Sepharose Fast Flow column (1.5 cm x 39 cm) (GE Healthcare Biosciences AB, Uppsala, Sweden) equilibrated with DEAE I buffer. After loading of the lysate, the column was washed with DEAE buffer I (20 mM Tris, pH 8.0, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1% Tween-20 detergent) prior to eluting bound proteins with a 40 rbv gradient of 0 M to 0.5 M NaCl in DEAE buffer I. Fractions containing AREase activity were pooled and desalted on a 900 ml Sephadex G-25 desalting column (7.6 cm x 20 cm) (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated and eluted with 20 mM Tris, pH 8.0 and 1 mM $CaCl_2$ buffer. The resulting pooled, active fractions were loaded onto a 40 ml rbv DEAE Sepharose Fast Flow column (1.5 cm x 23 cm) equilibrated with DEAE buffer I and eluted with a 40 rbv NaCl gradient in DEAE buffer I as above. Arylesterase-containing fractions were pooled and loaded onto a 30 ml ceramic hydroxyapatite (HA) (type II, 40μm, BioRad, Hercules, CA) column (1.5 cm x 10 cm). The HA column was washed with HA buffer (2 mM potassium phosphate, pH 6.8, 0.1 mM $CaCl_2$, 0.1% Tween-20), then eluted with a 40 rbv HA buffer gradient from 2 mM to 100 mM potassium phosphate, pH 6.8. AREase-containing fractions were pooled and loaded onto an 11 ml DEAE Sepharose Fast Flow column (0.7 cm x 29 cm), washed with DEAE buffer II (20 mM Tris, pH 7.1 and 1 mM $CaCl_2$) and eluted with a 40 rbv 0 M to 0.75 M NaCl gradient. AREase containing fractions were pooled and NaCl was added to a final concentration of 3 M. The 3 M NaCl pool was loaded onto a 6 ml butyl Sepharose Fast Flow hydrophobic interaction column (HIC) (0.7 cm x 15 cm) (GE Healthcare), washed with HIC buffer (10 mM Tris, pH 8.0, 1 mM $CaCl_2$) containing 3 M NaCl and eluted with a reverse salt gradient of 40 rbv, 3 M to 0 M NaCl in HIC buffer. The butyl HIC column was then washed with HIC buffer containing 0.1% Tween-20 to

elute the AREase-activity. AREase-containing fractions were pooled, concentrated to under 1 ml and loaded onto a 400 ml gel filtration Superdex 200 column (2.6 cm x 64 cm) (GE Healthcare) equilibrated with Superdex buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1 mM $CaCl_2$). The rHuPON1 was further purified by pooling the AREase-containing fractions and loaded onto a 1 ml DEAE column (0.5 cm x 5 cm) equilibrated with DEAE buffer III (20 mM Tris, pH 7.7, 1 mM $CaCl_2$). The column was eluted with 50 rbv of a 0 M to 0.75 M NaCl gradient. The $rHuPON1_{R192}$ and $rHuPON1_{Q192}$ variants were purified as described above; however, the HA column was replaced with a second Sephadex G-25 column due to low yields and the requirement for phosphate buffer in an HA column. The HA column requires a phosphate buffer. PON1 is a calcium-dependent protein, therefore, it was desirable to eliminate that column from the chromatography protocol. The altered protocol also resulted in reasonably pure protein.

Kinetic analysis of rHuPON1 variants

Measurement of PON1 hydrolysis of PO, DZO and CPO was carried out. PON1 activity was measured in 0.1 M Tris-HCl, pH 8.5, 2 M NaCl, and 2 mM $CaCl_2$ with varying concentrations of PO, DZO, or CPO. The reaction was initiated by mixing 10 µl of purified rHuPON1 with 200 µl substrate in assay buffer and the absorbance was monitored continuously for 4 min at 25^0C (DZO, CPO) or 37°C (PO). The reactions were carried out in a Molecular Devices SPECTRAmax® PLUS Microplate spectrophotometer. The amount of hydrolysis product was calculated from initial linear rates of hydrolysis using extinction coefficients of 18 $mM^{-1}cm^{-1}$ at A_{405} for p-nitrophenol (PO hydrolysis); 3 $mM^{-1}cm^{-1}$ at A_{270} for 2-isopropyl-4-methyl-6-hydroxy pyrimidine (DZO hydrolysis); 5.56 $mM^{-1}cm^{-1}$ at A_{310} for 3,5,6-tricholoro-2-pyridinol (CPO hydrolysis). All kinetic analyses were determined with at least 3 substrate

concentrations below and 3 above the K_m values with catalytic constants determined by plotting substrate concentration/velocity vs. substrate concentration[44]. Units of activity were expressed as μmol of hydrolysis product formed per min.

Results

Cloning and expression of untagged rHuPON1

We reasoned that immunogenicity of recombinant PON1 could be minimized by producing rHuPON1 that has neither purification tags fused to the rHuPON1 protein nor any carbohydrate side chains as *E. coli* does not glycosylate proteins. The expression system developed provided active rHuPON1 from *E. coli* without tag domains or carbohydrate chains. Three rHuPON1 variants - the two native human PON1 alloforms, $rHuPON1_{Q192}$ and $rHuPON1_{R192}$, and the variant, $rHuPON1_{K192}$ were generated and used for expression and further kinetic analysis. This variant was chosen based on the GST-PON1 results as well as the prior knowledge that rabbit PON1, which has lysine at amino acid position 192, has the highest OP hydrolysis of multiple PON1 variants tested (reviewed in[45]). In addition, previous work expressing the chimeric rePON1[37, 38] also generated a protein with lysine at amino acid 192.

Column chromatographic purification of untagged rHuPON1

The rHuPON1s were purified from the cell extracts via a series of column chromatographic steps. Figure 2.2 shows the elution profiles of each of the column fractionation steps in the purification of $rHuPON1_{K192}$. The series of columns utilized multiple ion exchange DEAE columns with and without detergent at different pH values and a hydroxyapatite column or hydrophobic interaction column with a final detergent-containing buffer elution. Table 2.1 summarizes the purification of the $rHuPON1_{K192}$ variant using AREase activity to calculate yield

and fold purification. The rHuPON1$_{K192}$ was purified 90-fold with a yield of 17%. SDS-PAGE analysis of each step of the purification of the rHuPON1 variants is shown in Figure 2.3, indicating a final high degree of purity for each protein. The rHuPON1s appear as 39 kDa proteins. As noted in Methods, a slightly modified protocol was used to purify rHuPON1$_{R192}$ 88-fold with a 19% yield. Figure 2.3 shows an SDS-PAGE analysis of each step of the purification for rHuPON1$_{K192}$ and rHuPON1$_{R192}$. The rHuPON1$_{Q192}$ was purified using the same protocol.

Kinetic analysis of rHuPON1 variants

The kinetics of substrate hydrolysis for PO, DZO, CPO, and phenyl acetate were determined for the variants rHuPON1$_{K192}$ and rHuPON1$_{R192}$. The kinetic data are summarized in Table 2.2. Hydrolysis of OP substrates was examined for the rHuPON1 variants rHuPON1$_{K192}$ and rHuPON1$_{R192}$ as the rHuPON1$_{R192}$ alloform has a higher catalytic activity of hydrolysis for the OP pesticide metabolites compared with rHuPON1$_{Q192}$. Kinetic data measuring K_m, V_{max}, and catalytic efficiency (V_{max} /K_m) were based on previous work from Li et al.[29] and are summarized in Table 2.2. The rHuPON1$_{K192}$ variant showed higher K_m and especially V_{max} values for all substrates examined. When calculating catalytic efficiencies, the rHuPON1$_{K192}$ variant of rHuPON1 showed significantly higher catalytic efficiencies for the OP substrates examined when compared to the rHuPON1$_{R192}$ variant, but both had similar values for phenyl acetate. As previously described and therefore expected, catalytic efficiencies for PO hydrolysis were much lower compared to catalytic efficiencies for DZO and CPO hydrolysis of the rHuPON1s[29].

Discussion

PON1 is a leading candidate as a protein therapeutic to treat toxic OP pesticide exposures as well as possible nerve agent exposures. Agricultural workers, their families, and communities are one notable population of concern; the population at risk for deliberate exposures is considerably larger. The possibility also exists for the use of therapeutic recombinant PON1 in treating carotid artery disease and other vascular diseases as its anti-atherogenic effects and cardioprotective effects have long been known and have been intensively studied[31-34, 46, 47]. The experiments described here demonstrate the first successful expression and purification of active, soluble untagged native and engineered rHuPON1 from *E. coli*. Based on previous reports that chimeric rePON1s with lysine in amino acid position 192 such s rabbit had high activity and the work with GST-tagged native PON1, we tested this variant in an untagged rHuPON1 system. The naturally-occurring PON1 alloforms $PON1_{R192}$ and $PON1_{Q192}$ as well as the engineered variant, $PON1_{K192}$, were expressed, purified and characterized. When tested for catalytic efficiency, the native PON1s ($rHuPON1_{Q192}$ and $rHuPON1_{R192}$) showed similar activity to purified human plasma $PON1_{192}$ alloforms. The $rHuPON1_{K192}$ variant had increased activity against all the substrates tested, including PO with a nearly twofold increase in catalytic efficiency compared with $rHuPON1_{R192}$. These experiments demonstrate the feasibility of using *E. coli* as an expression system for both engineering native rHuPON1 and scaling up the production of active rHuPON1 variants.

We expressed and purified to homogeneity the two native untagged human alloforms of PON1, $rHuPON1_{R192}$ and $rHuPON1_{Q192}$, and the variant, $rHuPON1_{K192}$. For many years, it was believed that it was not possible to express active native human PON1 in bacterial cells. After

the development of the hybrid rePON1 expression system, the authors remarked that "Human PON1 is rather unstable, and tends to aggregate in the absence of detergents…Nor is it amenable to functional expression in bacteria or yeast, and thus to mutagenesis, library selection, and protein engineering"[48]. However, the system described here produced active, soluble, human recombinant PON1 with a fully native amino acid sequence from *E. coli* cells. We were able to detect protein by both activity and Western blots even in small induced cultures. The rHuPON1$_{K192}$ variant does not occur in human populations, but was chosen to engineer based on several lines of evidence suggesting it would have higher efficiency of OP hydrolysis, primarily the very high OPase activity of rabbit PON1 which has lysine at position 192. The results with the GST-tagged PON1 showed that this variant did have increased activity towards the oxon metabolites of OP pesticides of interest, especially PO, for which native human PON1 has very low activity. The rePON1 derived from domain-shuffling procedures developed by the Tawfik research team also had lysine at position 192.

The purification of rHuPON1 was achieved with several column chromatography steps. HIC and DEAE chromatography have previously proven useful in purifying PON1 from plasma[49, 50] and indeed, the most productive purification steps for the rHuPON1 were based on a series of DEAE anion exchange chromatography columns using varying buffer and detergent conditions as well as butyl HIC which was run with a reverse salt gradient and a final detergent elution at the end of the gradient. HA was also a highly efficacious step, however, the yield was very low. Both HA and HIC were effective steps, providing increases in purity of the rHuPON1s when analyzed by SDS-PAGE. Purification of rHuPON1$_{R192}$ and rHuPON1$_{Q192}$ and the variant, rHuPON1$_{K192}$ produced highly purified recombinant protein. However, the yield was rather low

and the process was laborious. Future efforts simplifying the chromatography steps and increasing overall yield will be beneficial.

The final specific activity of rHuPON1 was comparable to that of PON1 purified from human serum[42, 43]. As predicted based on previous work, the rHuPON1$_{K192}$ variant demonstrated higher catalytic efficiencies for the OP pesticide oxon metabolites compared to the natural human rHuPON1$_{R192}$ variant and much higher catalytic efficiency than the rHuPON1$_{Q192}$ alloform. Interestingly, the rHuPON1$_{K192}$ variant had a lower affinity for substrates (higher K_m value) than for the rHuPON1$_{R192}$ alloform for all three OPs tested, however, the maximal velocity for the rHuPON1$_{K192}$ variant was also significantly higher, resulting in an overall higher catalytic efficiency for the rHuPON1$_{K192}$ variant as expected.

Since our publication of the untagged native rHuPON1 purification and characterization, further work has been done to optimize bacterial expression of PON1. A publication from the Tawfik research team utilized random insertions, revealing that PON1 is very flexible in accommodating novel amino acids[51]. With the publication of the PON1 chimeric crystal structure, computational analysis for designing rational variants of PON1 has become possible. Along with a more thorough knowledge of the catalytic mechanism, computational analyses have provided suggestions for variants with improved activity[52]. Our efforts have taken an opposite approach – developing a system to express a fully native human PON1, followed by introducing minimal amino acid changes to the protein, using a rational approach based on what was previously known about native rabbit PON1, the chimeric model, and our own tagged rHuPON1 protein expression. These efforts are complimentary and inform each other. There have also been other rational approaches for modulating native human PON1. Sarkar et al.[53]

tested increased solubility of PON1 by altering several amino acids corresponding to the residues in the Tawfik chimeric model. They also removed the retained hydrophobic N-terminal signal sequence of PON1[53]. In addition, many of the Tawfik experiments examined hydrolysis of various nerve agents or agent analogs while the results presented here examined toxic OP pesticide metabolites. However, variants that are highly active against the OP nerve agents and those with high activity against the OP pesticides may not be entirely overlapping, suggesting that testing of multiple variants, as well as multiple strategies, will be required. Indeed, recent work has suggested that these populations will be somewhat divergent and that variants that are protective against one or several OPs many not be broadly applicable to all OPs[52]. An example of this is seen in the greater rates of agent hydrolysis by $PON1_{Q192}$ compared to $PON1_{R192}$ – the opposite of what is observed with some OP pesticide metabolites such as CPO and PO[24, 29].

We and other groups have previously proposed the use of PON1 as a catalytic scavenger for treating OP exposures based on experiments[28-30] demonstrating that injected purified plasma PON1 provided protection against OP exposure in wild-type mice and *PON1*$^{-/-}$. The experiments detailed in the present study have provided evidence that the expression and purification of untagged native and variant human PON1s from bacterial cells is feasible. This *E. coli* expression system will allow for high-throughput screening for mutants with improved catalytic efficiency and for scale-up production of therapeutic PON1.

Acknowledgements

This chapter is based in part on Stevens et al.[54] and Suzuki et al.[55] Clement E. Furlong, Richard C. Stevens, and Stephanie M. Suzuki (author) carried out the planning of cloning, assays and purification. Richard C. Stevens and Stephanie M. Suzuki carried out the cloning, assays and purifications. Leslie Everett and Rachel Jampsa carried out the GST-rHuPON1 cloning, expression, and analysis.

Figure 2.1

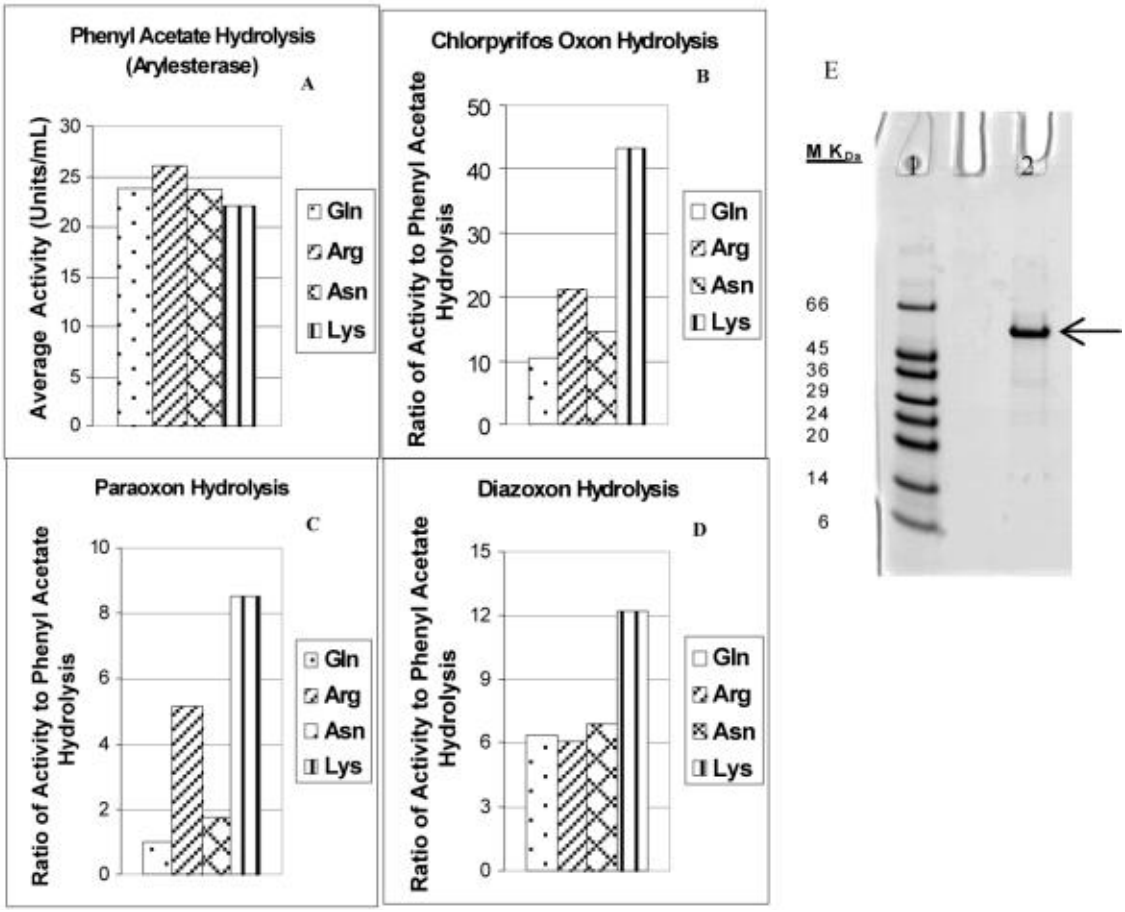

Figure 2.1. GST-PON1 produced in *E. coli*. (**A**) Equivalent rates of arylesterase activity, (**B, C, D**) ratios of rates of hydrolysis of chlorpyrifos oxon, paraoxon and diazoxon, respectively, to rates of phenyl acetate hydrolysis in rHuPON1 variants with Gln, Arg, Asn or Lys at position 192

Figure 2.2

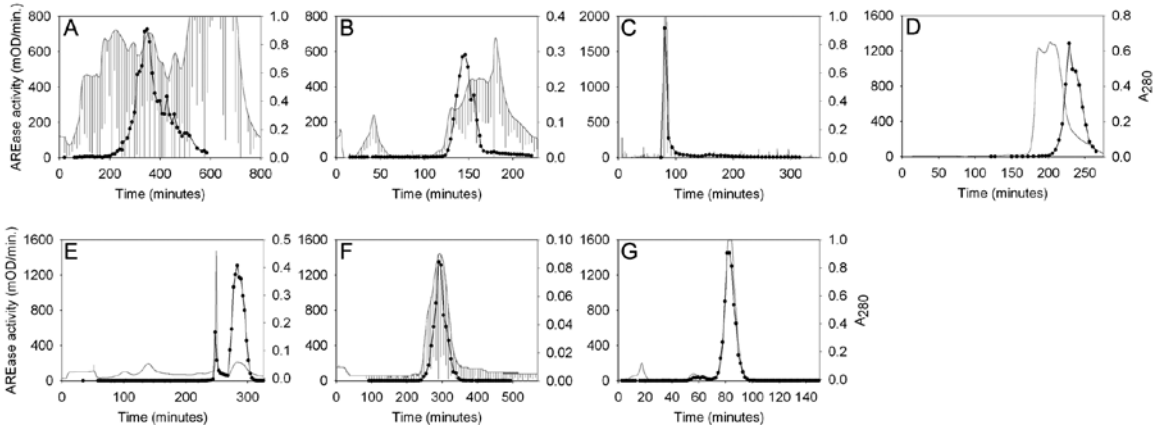

Figure 2.2. Protein (A_{280}) and activity monitoring during chromatographic purification of rHuPON1$_{K192}$. Absorbance measurements at 280 nm (grey line) allowed for continuous monitoring of protein elution from chromatography media while AREase activity (black data points and line) measurements of column fractions allowed monitoring of rHuPON1$_{K192}$. **A**) DEAE I column; **B)** DEAE II column; **C)** Hydroxyapatite column; **D)** DEAE III column; **E)** Hydrophobic interaction column; **F)** Gel filtration column; **G)** DEAE IV column.

Figure 2.3

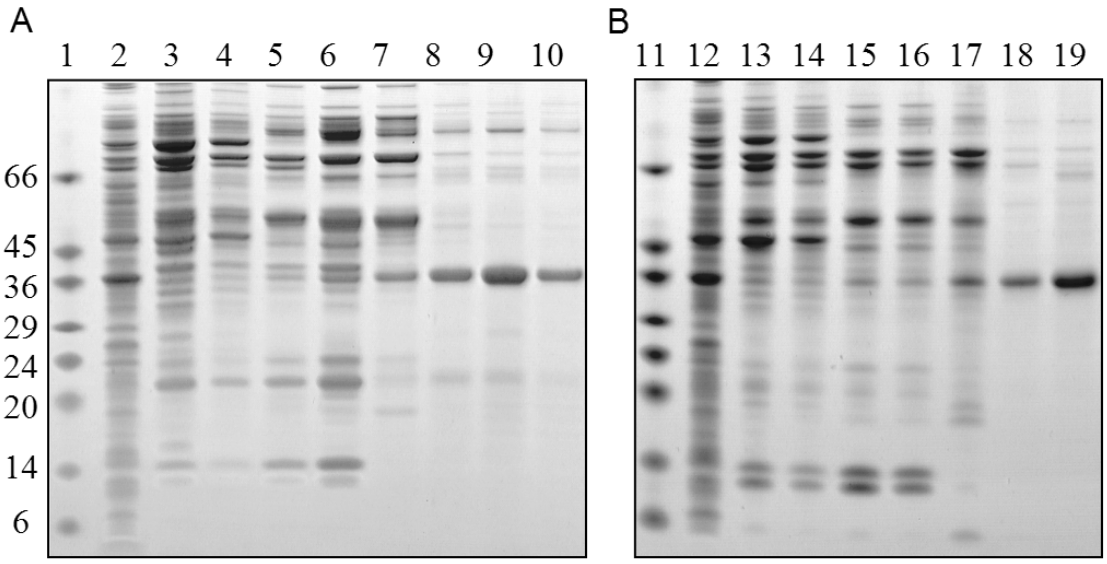

Figure 2.3. SDS-PAGE analysis of the pooled column fractions from the purification of rHuPON1s. **A)** rHuPON1$_{K192}$ variant. Lane 1, molecular weight markers (kDa); lane 2, starting extract; lane 3, DEAE I; lane 4, G-25 desalting column; lane 5, DEAE II; lane 6, hydroxyapatite; lane 7, DEAE III; lane 8, HIC; lane 9, gel filtration; and lane 10, DEAE IV. **B)** rHuPON1$_{R192}$ alloform. Lane 11, molecular weight markers; lane 12, starting extract; lane 13, DEAE I; lane 14, G-25 desalting column; lane 15, DEAE II; lane 16, G-25 desalting column; lane 17, DEAE III; lane 18, HIC; lane 19, DEAE IV.

Table 2.1. Summary of rHuPON1$_{K192}$ purification

Purification Step[a]	Volume (ml)	Total Protein (mg)	Total AREase activity (Units[b])	Specific activity (Units/mg)	Fold enrichment	yield (%)
Cell Extract	900	2880	14835	5.15	-	100
DEAE I	190	850	7673	9.00	1.8	52
Desalting	390	700	6944	9.92	2.0	48
DEAE II	146	175	5627	32.2	6.3	38
HA	62	27	4282	159	31	29
DEAE III	54	9.4	3325	354	69	22
HIC	36	6	2762	460	89	19
Gel filtration	42	6	2762	460	89	19
DEAE IV	10	5.5	2558	465	90	17

[a] abbreviations: DEAE (diethylaminoethyl), HA (hydroxyapatite), HIC (Hydrophobic Interaction Chromatography)

[b] Units of arylesterase activity are μmol phenol produced per minute.

Table 2.2. Kinetic analysis of substrate hydrolysis by rHuPON1 variants

Substrate	rHuPON1 variant	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
Paraoxon	K192	0.925 ± 0.029	11.66 ± 0.06	12.61 ± 0.32
	R192	0.868 ± 0.016	6.61 ± 0.18	7.62 ± 0.23
Diazoxon	K192	2.57 ± 0.62	301 ± 51	118 ± 10
	R192	1.33 ± 0.08	119 ± 5	89.8 ± 2.3
Chlopyrifos-oxon	K192	0.317 ± 0.02	245 ± 3	777 ± 66
	R192	0.131 ± 0.005	34.7 ± 0.3	266 ± 12
Phenyl acetate	K192	3.22 ± 0.79	3020 ± 300	966 ± 166
	R192	0.957 ± 0.02	680 ± 0	711 ± 16

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Chapter Three: Assessment of rHuPON1 as a Catalytic Bioscavenger in a Knockout Mouse Model

Abstract

The HDL-associated plasma enzyme paraoxonase-1 (PON1) hydrolyzes and inactivates toxic organophosphate (OP) pesticides *in vitro*. Experiments using PON1 knockout mice (*PON1*$^{-/-}$ mice) have established that plasma PON1 protects against the pesticide metabolites chlorpyrifos oxon (CPO) and diazoxon (DZO) but does not protect against paraoxon (PO), the toxic metabolite of the insecticide parathion. The aim of this study was to determine if *E. coli*-produced recombinant human PON1 (rHuPON1) was nontoxic and protective against OPs in *PON1*$^{-/-}$ mice. The rHuPON1 had been expressed and characterized previously; a variant, rHuPON1$_{K192}$, had increased catalytic activity against DZO, CPO, and PO. To test the therapeutic potential of the recombinant protein, rHuPON1$_{K192}$ was injected into *PON1*$^{-/-}$ mice in several experiments. Initial experiments determined that rHuPON1$_{K192}$ was nontoxic in mice and that the protein was present in the plasma compartment. The *PON1*$^{-/-}$ mice have no measurable DZO hydrolytic activity (DZOase) in their plasma; therefore, any measureable DZOase is attributable to the injected rHuPON1$_{K192}$ protein. The rHuPON1$_{K192}$ was detectable 48 h after injection. A second experiment assessed the prophylactic potential of the rHuPON1$_{K192}$ variant. In this experiment, increased concentrations of the rHuPON1$_{K192}$ were administered, and, 48 h following injection, mice were exposed to 1 mg/kg or 1 LD_{50} DZO. Brain acetylcholinesterase (AChE) was inhibited nearly 50% in *PON1*$^{-/-}$ mice that had not previously received the protein; however, *PON1*$^{-/-}$ mice that were injected with rHuPON1$_{K192}$ prior to the DZO challenge had almost no inhibition of brain AChE. A following experiment was carried out to determine whether rHuPON1$_{K192}$ protected mice when injected following a high-dose OP exposure. The

injected rHuPON1$_{K192}$ protected mice from what should have been lethal doses of DZO (doses greater than two- to three-times the LD_{50}). Plasma PON1 activity was followed over time and DZOase was detectable in plasma of the *PON1*$^{-/-}$ mice. The results of our experiments indicate that injected rHuPON1 is nontoxic, persists in serum for at least two days following injection, acts as a prophylactic as well as a therapeutic when given following an exposure, protects almost completely against 1 LD_{50} of DZO, and provides protection when given after high-dose exposures of DZO of two- to three- times the LD_{50} value for DZO.

Introduction

Paraoxonse-1 (PON1) has been intensively researched due to its ability to hydrolyze and inactivate toxic organophosphates (OPs) *in vitro* which include the toxic oxon metabolites of commonly-used pesticides and also nerve agents. PON1 has been proposed as a protein therapeutic that could not only prevent inhibition of acetylcholinesterase (AChE), which is responsible for the cholinergic symptoms of intoxication, but also neutralize OPs by hydrolysis before they inhibit other critical serine active-site protease, lipase, and esterase targets (reviewed in[1-3]).

To assess the ability of PON1 to prevent intoxication, animal models (most often rodents) have been used since the earliest days of PON1 research[4]. Previous studies in rats and primarily in mice indicated that injected rabbit or human PON1 proteins were nontoxic, long-lasting *in vivo*, and protective against OPs provided that the catalytic efficiency was sufficient[5-8]. Early work by Main[4] found that rats injected with partially-purified rabbit plasma PON1 with high OPase activity provided protection against the toxicity of paraoxon (PO). This work was extended with a series of experiments using rats and wild-type (*PON1*$^{+/+}$) mice[5, 6, 8]. Costa et al.[5] also used partially-purified rabbit PON1 and injected the PON1 into rats 30 min prior to

exposures to PO or chlorpyrifos oxon (CPO). Pretreatment with the PON1 protein provided mild protection against brain and diaphragm AChE inhibition by dermal exposure to PO and substantially greater protection against dermally-applied CPO. Increases in rates of serum PO hydrolysis (POase) and CPO hydrolysis (CPOase) were observed up to 24 h following injection of the PON1. Using *PON1*$^{+/+}$ mice and rabbit plasma PON1, Li et al.[6] demonstrated that injected rabbit PON1 protected mice from CPO and chlorpyrifos (CPS) dermal exposure. This study also compared various routes of protein administration – previous work had exclusively used intravenous (i.v.) methods to deliver the PON1. Combining i.v. delivery with either intramuscular (i.m.) or intraperitoneal (i.p.) injections resulted in increased persistence of the injected PON1. *PON1*$^{+/+}$ mice pretreated with rabbit PON1 30 min or 24 h prior to exposure to CPO or CPS had only slight inhibition of brain AChE compared to non-treated controls, which displayed significantly higher AChE inhibition. Further experiments determined that exogenous rabbit PON1 was also an effective therapeutic when given following an exposure. Injection of rabbit PON1 protected *PON1*$^{+/+}$ mice against the toxicity of CPS, even relatively high doses, when administered following CPS exposure, although the protective effect when administered at 3 h post-exposure was reduced compared to PON1 injected 30 min following exposure[8].

The PON1 knockout mouse model (*PON1*$^{-/-}$) was developed by Shih et al.[9] in order to study the role of PON1 in cardiovascular disease. We examined the sensitivity of these mice to several OPs[7, 9]. Initial testing was performed with CPO and the mouse response was predictable. *PON1*$^{-/-}$ mice had almost no plasma CPOase, with activity levels about 2% of those of wild-type (WT) mice. The *PON1*$^{-/-}$ mice were dramatically more susceptible to CPO; WT mice exposed to 1.5 mg/kg CPO had no inhibition of brain AChE, but *PON1*$^{-/-}$ mice exposed to the same dose showed severe brain AChE inhibition, with activity reduced 74 to 80%. Higher doses (3 mg/kg

and 6 mg/kg) that minimally inhibited WT mouse AChE were lethal to the *PON1* null mice[9]. The *PON1*$^{-/-}$ mice were also more susceptible to the development of atherosclerotic markers when fed with a high-fat diet, which had been predicted by previous reports linking vascular disease and PON1[10, 11].

Further characterization of the *PON1*$^{-/-}$ mice determined their susceptibility to DZO and PO[7]. The mice had no detectable plasma or liver DZOase or POase activity. The lowest dose of DZO tested, 1 mg/kg, resulted in substantial inhibition of brain AChE activity (80%) in the *PON1*$^{-/-}$ mice but not the WT mice. Higher doses of DZO (2 mg/kg and 4 mg/kg) resulted in lethality for a high percentage of the *PON1*$^{-/-}$ mice tested; however, even at these higher doses, no significant AChE inhibition was observed in the WT mice. The LD_{50} for *PON1*$^{-/-}$ mice in this experiment was determined to be between 1-2 mg/kg DZO. When mice were exposed to PO, however, no significant difference was observed between the WT mice and *PON1*$^{-/-}$ mice in the inhibition of brain AChE, even at the highest doses. The *PON1*$^{-/-}$ mice did not display increased sensitivity to PO, despite their lack of POase plasma activity and their increased sensitivity to the other OPs tested. The explanation for this anomalous result was discovered by determining the *in vitro* catalytic efficacy of the human PON1 alloforms for hydrolysis of PO, CPO, and DZO.

Using the knockout mouse model, purified plasma PON1 (rabbit and the human alloforms $PON1_{R192}$ and $PON1_{Q192}$) were injected to test their protective capabilities against PO. Neither purified rabbit PON1 nor the two human alloforms protected *PON1*$^{-/-}$ mice from dermal exposure to PO, although high levels of PON1 activity were measured in the plasma compartment following injection. Mice were injected i.p. with the PON1 proteins, followed by PO challenge 4 h post-injection. Four hours following the OP challenge, the mice were

sacrificed. The appearance of PON1 in the plasma compartment was generally at maximum levels between 4 and 8 h after administration of the PON1 protein. No protection from PO exposure was observed by any of the PON1s tested – brain AChE inhibition was not significantly different between *PON1*$^{-/-}$ control mice and *PON1*$^{-/-}$ mice that received the prior PON1 injection. These results also contrasted with earlier work[4, 5] which determined that purified, high-activity rabbit PON1 protected rats against exposure to PO. To confirm this result, transgenic mice expressing human $PON1_{R192}$ were also exposed to PO; the high PON1 levels measured in plasma did not protect them from brain AChE inhibition due to PO. The two purified human PON1 alloforms were tested in the mouse model to determine if they could prevent intoxication by CPO and DZO. $PON1_{R192}$ and $PON1_{Q192}$ provided nearly equivalent levels of protection against DZO, but $PON1_{R192}$ was significantly better at protecting against AChE inhibition due to CPO. Kinetic analyses also demonstrated that the catalytic efficiencies of $PON1_{R192}$ and $PON1_{Q192}$ were about equivalent for DZO, while $PON1_{R192}$ had nearly twice the efficiency of $PON1_{Q192}$ for CPO hydrolysis. Although $PON1_{R192}$ had almost nine-times the catalytic efficiency as $PON1_{Q192}$ towards PO, overall the activities of both alloforms were so low that they did not protect against a PO exposure. These experiments demonstrated the utility of the *PON1*$^{-/-}$ mice for testing the efficiency and protective abilities of PON1 alloforms.

As purification of rabbit or human PON1 are time consuming and require large quantities of plasma, our goal with the experiments detailed below was to express and test the utility of recombinant human PON1 (rHuPON1) in protecting against OP exposures in the *PON1*$^{-/-}$ mouse model. These were the first experiments testing *E. coli*-produced PON1 in a mouse model. The $rHuPON1_{K192}$ variant was tested with *PON1*$^{-/-}$ mice in several experiments. First, $rHuPON1_{K192}$ was injected in *PON1* null mice to observe whether it was tolerated by the mice and to measure

the appearance of the protein in the plasma compartment. Protection experiments involving challenge of the mice with OPs either before or after administration of the rHuPON1$_{K192}$ were also carried out.

Materials and Methods

Chemicals

Diazoxon was purchased from Chem Service (West Chester, PA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

PON1 knockout (*PON1*$^{-/-}$) mice

PON1$^{-/-}$ mice were previously generated by Shih et al.[9]. Generation and testing of the *PON1*$^{-/-}$ mice with OPs has already been described[7, 9]. Briefly, the *PON1*$^{-/-}$ mice were generated on a 96% C57BL/6J and 4% 129/SvJ background by targeted disruption of the *PON1* exon 1. The nonfunctional allele was transmitted in Mendelian fashion and *PON1*$^{-/-}$ homozygotes were identified and confirmed by PCR and plasma activity assays. There was no detectable PON1 protein or POase activity and highly reduced phenyl acetate activity in *PON1*$^{-/-}$ mouse plasma. The remaining phenyl acetate activity could be attributed to plasma carboxylesterase which is found in mouse, but not human, plasma. Physically and behaviorally, the mice appeared normal. In our facilities, mice were housed in either barrier or modified SPF (specific pathogen free) facilities with 12 h dark-light cycles and food and water *ad libitum*. All experiments were carried out based on the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health. Animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Purification of rHuPON1

Purification of the rHuPON1 protein used in these experiments was described in Chapter 2 of this manuscript and Stevens et al[12].

Injection of mice with rHuPON1$_{K192}$ protein

The purified rHuPON1$_{K192}$ protein was used to inject *PON1*$^{-/-}$ mice. It was administered to mice by either i.p. injection or by a combination of i.m. and i.p. injection as indicated below. For i.p. injections, a 23G needle was used to inject 200 µL of rHuPON1$_{K192}$ or vehicle (sterile normal saline) into the peritoneal cavity. For i.m. injections, a 30G needle was used to inject 100 µL of rHuPON1$_{K192}$ or sterile saline into the lateral quadriceps (50 µL into each quadriceps muscle). DZOase was used to standardize the concentrations of rHuPON1$_{K192}$ protein prior to injection. Either 1.12 or 3.91 units of DZOase were injected as indicated below. To assess toxicity and longevity, some of the mice injected with low levels of rHuPON1$_{K192}$ were not sacrificed and were monitored for longer than 1 yr to determine if any long-lasting negative side effects were observable.

In the initial, low-level protein injection, the rHuPON1$_{K192}$ was administered to three *PON1*$^{-/-}$ mice by i.p. injection. Mice were administered 1.12 DZOase units of protein. Blood samples were taken from the saphenous vein at 4, 8, 12, 24 and 48 h following the injections (Figure 3.1) and DZOase activity of the plasma was measured. These mice were not sacrificed and were maintained in animal housing following the experiments.

The first DZO challenge experiment involved injection of larger quantities of the rHuPON1$_{K192}$ protein (3.91 DZOase units; 192 µg) than the previous injections, and therefore the injections were combined i.p. and i.m. injections which were administered as described above.

The rHuPON1$_{K192}$ protein was injected into each of two *PON1*$^{-/-}$ mice and blood samples were taken 4, 8, 12, 24 and 48 h followed by monitoring plasma DZOase activity (Figure 3.2). After 48 h, mice were challenged with 1 mg/kg dermal DZO exposure, or about 1 LD_{50}. Six hours following DZO challenge, the mice were sacrificed and tissue taken to assess brain AChE activity.

For a high level DZO exposure, *PON1*$^{-/-}$ mice were exposed dermally to either 3 mg/kg or 7 mg/kg DZO (2 to 3 LD_{50} levels). The DZO challenge is described below. Ten minutes following exposure, the mice were injected with rHuPON1$_{K192}$ (3.91 units; 192 µg; equivalent levels to our previous challenge experiment) by the combination of i.p. and i.m. injections as described above. As the DZO exposures used in our testing group would have been lethal to *PON1*$^{-/-}$ mice, the control *PON1*$^{-/-}$ mice that received i.p./i.m. injections of saline were exposed to lower doses of DZO (1 mg/kg, 1.5 mg/kg, or 2 mg/kg) since high doses would have killed them. Blood samples were taken at 1, 2, 3, 4, and 6 h and tested for DZOase activity, after which the mice were sacrificed.

DZOase assays

Blood (< 50 µL; no more than 1% body weight total) was drawn from the saphenous vein at 4, 8, 12, 24 and 48 h following the injections. Assays were conducted in a SpectraMax Plus®384 spectrophotometer (Molecular Devices, Sunnyvale, CA) with 96-well UV wavelength microplates. DZOase activity was measured in 0.1 M Tris-HCl, pH 8.5, 150 mM NaCl, and 2 mM $CaCl_2$ with 1 mM DZO. The reaction was initiated by addition of 200 µl substrate in assay buffer to various sample concentrations and the assay was monitored at 270 nm for 4 min at 25^{0}C. The amount of hydrolysis product was calculated from initial linear rates of hydrolysis

using extinction coefficients of 3 $mM^{-1}cm^{-1}$ at A_{270} for 2-isopropyl-4-methyl-6-hydroxy pyrimidine (DZO hydrolysis); DZOase activity was expressed as units (µmol of hydrolysis product formed per minute) and plotted using the three mice. Levels from these mice were also compared to either WT mice or *PON1*$^{-/-}$ mice that had not received the $rHuPON1_{K192}$ protein and whose plasma DZOase activity levels were essentially null.

Challenge with DZO

DZO was dissolved in acetone and applied on the shaved (4 cm^2) back of 12-wk-old mice at 5 µL/g body weight, either 48 h after or 10 min before injection of $rHuPON1_{K192}$. Concentrations of DZO used to challenge the mice are indicated above. Control animals received acetone application only. Following sacrifice by cervical dislocation, organs were dissected, placed immediately on dry ice, and stored at -80^{o}C until assay.

Brain AChE assays

Brain AChE levels were measured to determine inhibition by DZO. Brain AChE assays were performed using a modified method based on Ellman[13]. Brain tissue was homogenized in 9 volumes ice cold 0.1 M sodium phosphate buffer, pH 8.0, and diluted in the buffer to obtain concentrations of 4 mg/ml brain tissue. The tissue homogenate was diluted 1:10 in the same sodium phosphate buffer and 100 µl of this diluted tissue was added to wells in a 96-well visible microplates in triplicate. The kinetic assay was initiated by addition of 100 µl freshly-prepared acetylthiocholine substrate mix which consisted of 2.0 mM acetylthiocholine and 0.64 mM 5,5'-dithio-bis-nitrobenzoic acid (DTNB) in the 0.1 M sodium phosphate buffer, pH 8.0. Appearance of the hydrolysis product 5-thio-2-nitrobenzoate was monitored in a SpectraMax Plus® spectrophotometer (Molecular Devices). The reaction was monitored for 10 min at 412 nm. The

initial linear rates of 5-thio-2-nitrobenzoate formed during the assay were calculated using an extinction coefficient of 13.6 mM^{-1} cm^{-1} and AChE activity was expressed as U/g of wet tissue (U=µmol of acetylthiocholine hydrolyzed per minute).

Results

Injection of rHuPON1$_{K192}$ to assess toxicity and appearance in the plasma compartment

Our initial experiments were carried out to assess the potential toxicity of the injected rHuPON1$_{K192}$, its persistence when injected into the *PON1*$^{-/-}$ mouse, whether the rHuPON1$_{K192}$ entered the plasma compartment, and whether it could be detected by measuring DZOase activity. While *PON1*$^{-/-}$ mice have detectable levels of arylesterase activity (phenyl acetate hydrolysis) in their plasma due to the activity of plasma carboxylesterase, there is no measurable DZOase activity in their plasma. Thus, plasma DZOase was measured to detect the activity of the injected rHuPON1$_{K192}$. As *PON1*$^{-/-}$ mice lack any DZOase activity in their plasma (Figure 3.1), all of the plasma DZOase activity is attributable to the injected rHuPON1. The rHuPON1$_{K192}$ variant protein was used in our experiments as it had the highest catalytic efficiency of hydrolysis for DZO as well as other OPs as measured with *in vitro* assays[12]. The mice injected with rHuPON1$_{K192}$ protein showed no symptoms of illness during or after the experiment. Plasma from mice that received i.p. injection of rHuPON1$_{K192}$ had detectable DZOase activity for at least 48 h post-injection (Figure 3.1). Plasma PON1 levels (DZOase) were maximal at about 8 hours following injection and, at maximum levels, were about half those of WT mice (Figure 3.1). There were no obvious adverse reactions following rHuPON1$_{K192}$ injections or during 1 year following the experiment.

Injection of rHuPON1$_{K192}$ as a prophylactic to protect against DZO exposure

To test the protective capabilities of the rHuPON1$_{K192}$ variant against OP exposures, we carried out an initial experiment in which injection of the rHuPON1$_{K192}$ protein was followed by challenge with DZO 48 h post-injection. The mice that had received rHuPON1$_{K192}$ had detectable plasma DZOase levels – as in our previous experiment, levels were maximal at about 8 hours. However, as considerably more protein had been injected, the maximum DZOase activity was about twice that of the PON1 DZOase activity of WT mice (Figure 3.2). Also, as in our previous experiment, PON1 activity was still detectable at 48 h post-injection when the mice were challenged with DZO. The rHuPON1$_{K192}$ protein was injected into each of two *PON1*$^{-/-}$ mice; *PON1*$^{-/-}$ control mice were injected with saline. Mice were challenged with 1 mg/kg dermal DZO exposure, or about 1 LD_{50}, at 48 h post-injection (Figure 3.2). Brain AChE in *PON1*$^{-/-}$ mice injected with vehicle showed ≈50% inhibition, but mice receiving injected rHuPON1$_{K192}$ were completely protected (no AChE inhibition was detected) (Figure 3.3). Even with the declining PON1 levels present at 48 h (Figure 3.2), the injected rHuPON1$_{K192}$ remaining in the plasma compartment was sufficient to protect brain AChE activity in *PON1*$^{-/-}$ mice from inhibition by DZO. The persistence of activity in the serum is important since it indicates the injected protein will be long-lasting in plasma, an important requirement for a therapeutic. In addition, OPs can accumulate in the fat and can enter the circulation over time. Further, the injected rHuPON1$_{K192}$ protein may distribute to tissues, remain active, but not be measurable in plasma samples[14].

Injection of rHuPON1$_{K192}$ as a therapeutic for a high level exposure

A final experiment addressed the questions of whether injection of rHuPON1$_{K192}$ could protect when given following an OP exposure and also whether it would protect against high

levels of DZO (>2X LD_{50} dermal exposure of DZO), mimicking real-life high-dose exposures. Figure 3.4 shows the appearance of $rHuPON1_{K192}$ in the plasma compartment of the injected mice. DZOase activity was completely absent in the plasma of the saline-injected *PON1*$^{-/-}$ mice (Figure 3.4). PON1 levels in the plasma of the two $rHuPON1_{K192}$-injected mice were maximal at 4 h after injection (Fig. 3.4). Lower plasma DZOase levels were seen in the mouse that was challenged with 7 mg/kg compared to the mouse that received 3 mg/kg. Both of the $rHuPON1_{K192}$-injected mice survived the high level DZO exposures, which would have killed un-injected *PON1*$^{-/-}$ mice. The exposed mice did however show some mild cholinergic symptoms. Toxicity associated with the high level DZO exposures, as measured by AChE inhibition, was similar to that seen in control mice receiving doses as low as 1 to 1.5 mg/kg. While 3 mg/kg or 7 mg/kg of DZO would normally be lethal to *PON1*$^{-/-}$ mice, the mice injected with $rHuPON1_{K192}$ survived these exposures, demonstrating the protective capability of the $rHuPON1_{K192}$ protein, even when administered post-exposure. In addition, the mouse exposed to 3.0 mg/kg DZO had higher brain AChE levels compared to the saline-injected mice exposed to 1.5 mg/kg DZO (70% inhibition versus 82 ± 3% inhibition, respectively). Brain AChE inhibition in the $rHuPON1_{K192}$-injected mouse exposed to 7 mg/kg DZO (89% inhibition) was similar to that seen in the saline-injected mice exposed to 1.5 mg/kg DZO. Injection of $rHuPON1_{K192}$ following exposure protected the *PON1*$^{-/-}$ mice against these high doses of DZO. Normally-lethal doses did not kill the mice that received the $rHuPON1_{K192}$. Although their brain AChE showed significant inhibition, mice injected with the $rHuPON1_{K192}$ had activity levels comparable to the levels of the mice that had been challenged with considerably lower levels of DZO.

Discussion

Having expressed, purified, and characterized several variants of rHuPON1 (in[12] and Chapter 2), we then carried out experiments testing the potential of the rHuPON1 to act as a protein therapeutic and prophylactic. The $rHuPON1_{K192}$ protein that was injected into the *PON1*$^{-/-}$ mice has a single amino acid change differentiating it from the two native human PON1 alloforms (Q192 and R192). This single amino acid change resulted in a rHuPON1 variant with increased OPase efficiency. The experiments described here are the first instance of *E. coli*-produced near-native PON1 recombinant protein tested in a mouse model. The lack of symptoms displayed by the *PON1*$^{-/-}$ mice following injection indicated that the protein, which differed from native human PON1 by only a single amino acid, was tolerated and demonstrated that the PON1 purification protocols were sufficient to remove contaminants such as endotoxin that can cause toxicity in animal models. Another reported system for bacterial expression of PON1 is the chimeric recombinant PON1 (rePON1) generated by directed evolution to engineer a more soluble protein[15, 16]. This work has led to a number of important discoveries in the PON1 field. However, here we have taken an opposite approach – instead of utilizing domain-shuffling and selection procedures, we altered a single amino acid in the protein with the goal of making minimal changes to reduce the possibility of immunogenicity when used with *in vivo* models. Both approaches are valid and are somewhat complimentary with each effort informing the other.

The increase in serum PON1 DZOase levels in the initial experiment was about half that of WT levels and activity peaked about 8 hours following injection. This agrees with findings of Li et al.[7] who found activity of plasma PON1 peaked 4-8 h following i.p. injection of purified

$PON1_{192}$ alloforms into *PON1*$^{-/-}$ mice. In the studies reported here, we extended measurement of the plasma PON1 activity out to 48 h following injection of the rHuPON1 $_{K192}$ protein. At the 48 h time point, DZOase was still detectable, demonstrating that the injected rHuPON1 remained in the plasma for an extended period of time and could still protect against exposures. This aligns with previous findings by Li et al.[6, 8] and Costa et al.[5] The plasma compartment is one of the main physiological reservoirs of PON1, which associates with HDL. It is unknown whether $rHuPON1_{K192}$ associates with HDL when injected, but another, later report indicated that exogenous PON1 does associate with HDL in plasma[17]. The discoveries of Marsillach et al.[14] changed the standard view of PON1 localization when they detected PON1 protein in a wide range of normal mouse tissues; this and subsequent reports have suggested that HDL acts as a delivery method for PON1 to the tissues[18]. It remains to be seen whether the injected $rHuPON1_{K192}$ is also distributed to tissues. This initial experiment examining toxicity and activity of the injected $rHuPON1_{K192}$ protein indicates that the $rHuPON1_{K192}$ fulfills several qualities needed in a catalytic scavenger. It can be injected without causing physical symptoms and remains in the system for a lengthy period following administration.

The next question was to assess if injected rHuPON1 $_{K192}$ could protect *PON1*$^{-/-}$ mice from OP exposure. Our aim was to determine whether we could increase plasma DZOase levels higher than those of WT mice. This aim was achieved – at maximum levels, again observed 8 h post-injection, *PON1*$^{-/-}$ mice had DZOase activity that was twice the level of WT mouse plasma DZOase (Figure 3.2). Since DZOase was still present at 48 h in our earlier experiment, mice were challenged 48 h following injection of a larger quantity of $rHuPON1_{K192}$ with 1 mg/kg DZO. This dosage was chosen because the LD_{50} for *PON1*$^{-/-}$ mice reported by Li et al.[7] was between 1-2 mg/kg and they had also challenged mice with 1 mg/kg. DZO-exposed *PON1*$^{-/-}$

mice that received rHuPON1$_{K192}$ had no observable inhibition of AChE while those that did not receive rHuPON1$_{K192}$ had an approximate 50% reduction in brain AChE (Figure 3.3). The results demonstrated that the rHuPON1$_{K192}$ could provide pre-emptive protection even when given 2 days prior to an exposure.

To simulate a realistic high-level OP exposure, we exposed *PON1*$^{-/-}$ mice to high doses of DZO then injected the rHuPON1$_{K192}$ protein 10 minutes following exposure to test its ability to prevent intoxication when given as a therapeutic to a known exposure. One mouse was exposed to 3 mg/kg and the other to 7 mg/kg, both >2x LD_{50} doses. Unprotected *PON1*$^{-/-}$ mice exposed to 3 or 7 mg/kg DZO should have died in less than 2 h[7], but, as they were treated with rHuPON1$_{K192}$ post-exposure, they survived and showed milder signs of OP toxicity than the control mice exposed to sub-lethal levels of DZO (1.5 mg/kg). These experiments more closely approximate a real-life scenario involving a high-dose exposure event. In this type of event, a therapeutic protein would by necessity be administered post-exposure, instead of pre-exposure as a prophylactic. Both challenge experiments provided further evidence that rHuPON1 meets the requirements for a catalytic scavenger.

Since these experiments were carried out, other reports of testing recombinant PON1 in rodent models have appeared. Recombinant human PON1 was tested in mice via injection of an adenoviral vector. The protein associated with mouse HDL and was protective against high LD_{50} doses of DZO[17]. PON1 expressed from adenovirus was also tested by Hodgins et al.[19] as well as purified recombinant PON1 from insect larvae (iPON1); the iPON1 protected a majority of exposed guinea pigs from CPO toxicity and death but did not protect against nerve agents. The virally-expressed PON1 similarly protected mice against 2 LD_{50} doses of DZO but not high

doses of nerve agents. The chimeric rePON1 was also tested in a mouse model. Evolved variants of the rePON1 provided some protection from lethality of nerve agents[20] compared to standard atropine and oxime treatments which represented a significant improvement from the traditional treatments. The range of animal models, PON1 sources, and PON1 delivery systems has been extended; however, PON1 has not been shown to be highly protective against nerve agents. PON1 will require further engineering for use as a potential therapeutic for exposure to some pesticides such as PO as well as nerve agents. The catalytic efficiency needs to be sufficiently increased in order to protect against PO and nerve agents. It will also be beneficial to develop alternative methods of purifying *E. coli*-produced engineered PON1.

Our experiments with injected rHuPON1$_{K192}$ were the first demonstration that recombinant PON1 produced from bacterial cells was nontoxic, long-lasting and protected brain AChE when injected into *PON1*$^{-/-}$ mice. The rHuPON1$_{K192}$ protected *PON1*$^{-/-}$ mice from lethal and sub-lethal OP exposures when injected either pre- or post-exposure. However, to protect against an exposure to PO, the efficiency would need to be further increased. In addition, a number of other studies utilizing animal models found that protection against nerve agents was not substantial or was only partially effected. Currently, protection against pesticide oxon metabolites such as DZO and CPO has already been achieved in animal models. However, with new insights about the PON1 crystal structure and catalytic site, research has suggested that PON1 variants with high activity against OP pesticide metabolites may not have equally high activity against the nerve agents[21]. Native variants have already demonstrated this principle, with PON1$_{R192}$ having the highest activity against several pesticides and PON1$_{Q192}$ showing higher rates of hydrolysis of nerve agents.

Acknowledgements

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Figure 3.1.

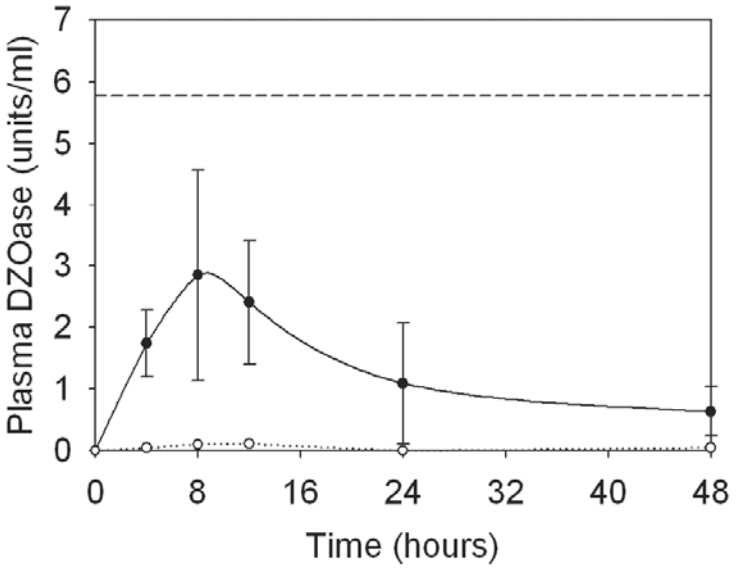

Figure 3.1. Plasma DZOase activity of mice (n=3) injected with 1.12 units of the rHuPON1$_{K192}$ protein as measured with DZOase activity. DZOase activity was monitored following injection of the mice at time = 0 and activity is expressed as units/ml. Blood samples were taken at the indicated time points. The dotted line indicates levels of DZOase activity found in wild-type mouse plasma. Open circles indicate levels of DZOase activity in *PON1*$^{-/-}$ mice.

Figure 3.2

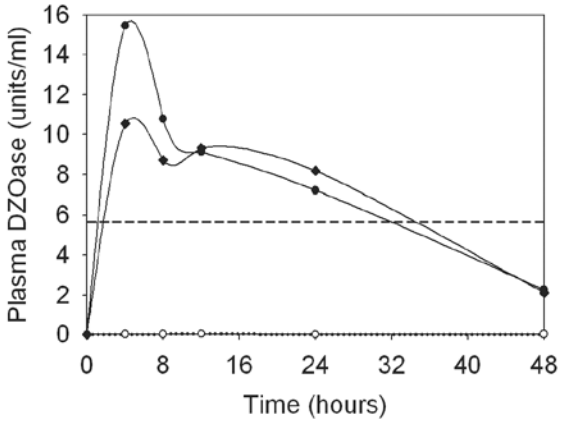

Figure 3.2. Plasma DZOase activity of two mice injected with 3.92 units of the rHuPON1$_{K192}$ protein as measured with DZOase activity. DZOase activity was monitored following injection of the mice at time = 0 and activity is expressed as units/ml. Forty-eight hours post-injection, mice were challenged with a dermal exposure to DZO. Blood samples were taken at the indicated time points. The dotted line indicates plasma levels of DZOase of wild-type mice. Open circles indicate levels of DZOase activity in *PON1*$^{-/-}$ mice.

Figure 3.3

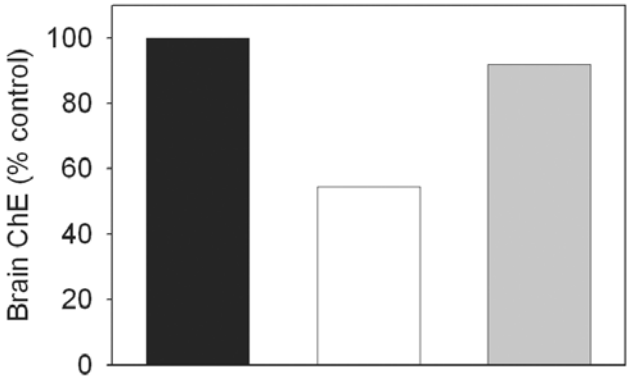

Figure 3.3. Residual brain AChE activity of *PON1*$^{-/-}$ mice. Control mice (black) received a saline injection. Brain AChE activity is expressed as a percentage of control. DZO-exposed mice (white) received a 1 mg/kg dermal DZO exposure. Mice receiving rHuPON1$_{K192}$ (grey) were injected with the 3.92 units rHuPON1$_{K192}$ and 48 h following injection were exposed to a dermal dose of 1 mg/kg DZO. Mice were sacrificed 6 h following DZO challenge.

Figure 3.4

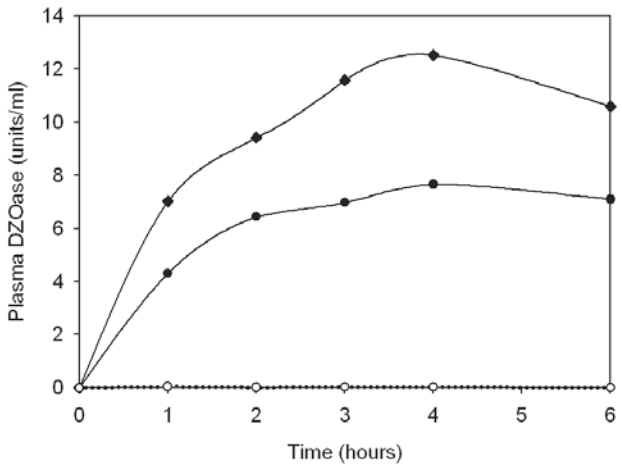

Figure 3.4. Plasma DZOase activity of two mice injected with 3.92 units of the rHuPON1$_{K192}$ protein prior to receiving a 2-3 LD_{50} dose of DZO. Plasma DZOase activity of two mice exposed dermally to either 3 mg/kg (◆—◆) or 7 mg/kg (●—●) ten min prior to rHuPON1$_{K192}$ injection. Blood samples were taken at the indicated time points. Open circles indicate levels of DZOase activity in *PON1*$^{-/-}$ mice.

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Chapter Four: Paraoxonase-1 Variants Affect Protein Stability and Calcium Binding

Abstract

Paraoxonase-1 (PON1) is of interest due to its wide range of substrates and association with disease. A number of efforts have been made to determine the importance of different amino acids and assess the activity of PON1 variants. A crystal structure based on an *E. coli*-produced recombinant hybrid PON1 protein was previously reported in the literature, which provided insight into important residues and potential catalytic mechanisms. We have developed a bacterial expression system and characterized native and variant recombinant PON1s (rHuPON1s). We expressed and purified multiple rHuPON1 variants and characterized them using an *E. coli* expression system for producing untagged PON1. The variants were homologous to rabbit PON1 at the specific modified residues, which is more stable and has greater activity towards OPs than human PON1. Mutants that were computationally-predicted to have increased heat stability and calcium binding were generated and assayed using EDTA and heat inhibition assays. Several of the single mutants had significantly greater resistance to heat and EDTA. Human PON1 has two common coding polymorphisms, the R192Q and the L55M. We expressed and tested the two 55 polymorphisms on a $rHuPON1_{R192}$ backbone and observed a significant difference in heat resistance, with the $rHuPON1_{R192\text{-}L55}$ retaining more of its activity, in accordance with a previous study. In addition, a rare disease-associated rHuPON1 variant (V109I) was expressed and compared with a native rHuPON1 control. The variant had decreased heat resistance.

Introduction

Paraoxonase-1 (PON1) is a multifunctional protein that is of interest due to its varied, multiple substrates and diverse array of activities. Although native human PON1 has a high catalytic efficacy for specific organophosphorus (OP) pesticides metabolites such as chlorpyrifos oxon (CPO) and diazoxon (DZO)[1, 2], it has low activity for other pesticides metabolites such as paraoxon (PO) and OP nerve agents and cannot protect animal models from exposure to these compounds[2]. PON1 has been proposed as a therapeutic or prophylactic for OP poisoning which, instead of merely treating symptoms of intoxication, would prevent inhibition of acetylcholinesterase as well as other proteins[3, 4]. PON1 has also been linked to a number of diseases due to its anti-atherogenic and antioxidative properties[5, 6]. Multiple studies and meta-analyses have demonstrated the importance of PON1 activity as well as phenotype, but publications linking the common PON1 single nucleotide polymorphisms to disease have provided conflicting results.

The human *PON1* gene has two coding polymorphisms, L55M and Q192R, as well as several promoter polymorphisms. The L55M polymorphism has been associated with increased PON1 concentration although this is mainly due to its linkage with the C-108T promoter polymorphism, which is located at an Sp1 binding site[7-11]. However, Leviev et al. reported increased stability of the $PON1_{L55}$ alloform due to increased resistance to proteolysis[12]. The R192Q polymorphism affects activity of the PON1 enzyme towards different substrates[1, 13]. For example, the $PON1_{R192}$ alloform has increased efficiency towards CPO and nine-times the catalytic efficiency towards PO[2]. Both polymorphisms have been studied in a number of disease conditions although PON1 status, which takes into account both the genotype and protein level, is a better predictor than genotype alone since there can be a wide inter-individual range of

plasma PON1 activity towards different substrates within each *PON1*$_{192}$ genotype[14, 15]. However, rare PON1 variants have been linked to a number of diseases such as Parkinson's and amyotrophic lateral sclerosis[16]. These rare polymorphisms have not been as extensively studied as the common polymorphisms and mixed results have been reported – while some studies found an association between rare *PON1* variants and disease, others did not[16, 17].

Prior to the development of the PON1 chimeric crystal structure, a number of PON1 residues had been studied in relation to PON1 activity, structure, and the catalytic mechanism. Josse et al.[18] utilized group-selective reagents to chemically modify PON1 and site-directed mutagenesis to test the effects of amino acid changes at a number of positions. Based on the group-selective labelling, researchers determined that specific His and Asp/Glu residues were critical for PON1 activity and identified a number of important residues including H115, H134, H285, D54, D269, and E53. Yeung et al.[19] proposed that PON1 had a similar structure to *L. vulgaris* diisopropylfluorophosphatase (DFPase) based on amino acid homology. The structure of DFPase is a six-bladed propeller in which each blade-like structure is made up of a series of pleated β-sheets arranged around a central tunnel. Based on this theoretical structure, the researchers used site-directed mutagenesis to mutate various PON1 residues and assessed the effect they had on activity. Mutations to residues in the N-terminal hydrophobic region had no effect on activity which fit with predictions that this region was involved in HDL binding[20, 21]; nor did changes to surface residues 313 or 314 affect activity. Conservative changes to residues predicted to be involve in calcium binding (D54, N168, N224, D269) strongly reduced activity as did alterations in residues suspected to be involved in the catalytic mechanism (L69, H115, H134, H285). However, it was noted that some mutations differentially affected activity towards

different substrates; alterations to H115 eliminated arylesterase (AREase) activity but not paraoxonase activity; changes to F222 abolished paraoxonase but not AREase activity.

The chimeric PON1 crystal structure was solved using gene-shuffling protocols to generate a more soluble hybrid recombinant PON1 protein (rePON1) that could be expressed in bacterial cells[22]. The crystallized rePON1 structure is a six-bladed propeller with similarities to the *L. vulgaris* DFPase[19, 23]. The blades of the propeller are made up of four strands. There are two calcium ions, one located at the top of the central channel denoted Ca1 and assigned to catalysis, while the other, Ca2, is believed to be the structural calcium, loss of which causes irreversible PON1 loss of activity. Ca1 interacts with N168, N224, N270, D269, and E53, and Ca2 is the higher-affinity calcium. The chimeric structure has three helices, with H2 and H3 forming an active site lid which differentiates PON1 from DFPase. As a catalytic mechanism, a His-His dyad with H115 and H134 was proposed, wherein H115 acts as a general base and deprotonates a water molecule to act as an attacking hydroxide while H134 increases the basicity of H115.

The chimeric rePON1 is highly similar to rabbit PON1. PON1 is a calcium-dependent enzyme - removal of calcium leads to irreversible loss of activity for human PON1 – and the rePON1 structure confirmed that PON1 had two calcium ions present, one a higher-affinity calcium ion, and only one that was necessary for protein stability, with the other likely involved in catalysis[24]. Compared to rabbit PON1, human PON1 is significantly more sensitive to EDTA inhibition of activity[25]; it was determined that rabbit PON1 has 25.7 times higher calcium-binding affinity for the calcium ion necessary for protein stability[24]. Rabbit PON1 also has a lysine at position 192 and has higher activity towards OPs when compared to human PON1[26]. The evolved rePON1 variants had lysine at amino acid position 192[22, 23] and generating the

single amino acid change on a native human PON1 protein backbone demonstrated that this variant had increased catalytic activity towards PO, CPO, and DZO[27].

Following the publication of the crystal structure, a number of mutants were tested utilizing the hybrid rePON1 bacterial expression system or other PON1 expression systems. Many studies examined amino acid changes that affected PON1 activity, but other characteristics, such as solubility, were also examined. The rePON1 variants were generated on the hybrid PON1 backbone and several had increased activity towards OPs and other classes of PON1 substrates, including other esters and lactones[22, 28, 29]. PON1 is a flexible protein, tolerant of insertions and deletions[30]. Other research teams have examined PON1 polymorphisms as well, often using a protein backbone that is closer to the native human PON1 than the rePON1[31].

In this report, we extend our expression and characterization of recombinant human PON1 (rHuPON1) variants. In an effort to improve one characteristic, stability, the PON1 structure was analyzed and amino acid changes homologous to rabbit PON1 were predicted to increase protein stability and calcium binding. These variants were generated and tested using the *E. coli* expression system with a modified purification protocol. In addition to novel variants generated on the rHuPON1 protein backbone, we also tested the effect that a known human polymorphism, L55M, has on protein stability. A rare variant (V109I) associated with disease was also expressed and tested using the protocol.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich unless otherwise noted.

Mutagenesis and expression of variant rHuPON1s

The rHuPON1s were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent), utilizing plasmids containing the previously cloned *PON1* gene[27]. Mutagenic primers were ordered from Fisher Scientific and were designed according to the mutagenesis kit protocol. The mutagenesis protocol was carried out using the pSCodon 2.1 expression vector (Eurogentec) with the cloned *PON1* gene as a template following the manufacturer's protocol.

The rHuPON1 protein was expressed using a protocol based on Stevens et al. (2008) and Chapter 2. The pSCodon plasmid system and SE1 expression cells (Eurogentec) were used for expression of rHuPON1 proteins. Transformants with the *PON1*-containing plasmids were grown in cultures of 5 ml at 37°C overnight and the culture was added at 1:100 to a 100 ml LB starter culture. The starter culture was incubated overnight at 25°C with shaking at 200 rpm; the next day, A_{600} was assessed and 1 or 2 L of LB were inoculated with a volume of starter culture calculated to attain a starting A_{600} of ≈0.1. The LB culture was grown at 25°C with 1% glycerol, 10 mM $CaCl_2$, trace metals added at 1 ml/L (sodium chloride 5 g/L, zinc sulfate heptahydrate 1 g/L, manganese chloride tetrahydrate 4 g/L, ferric chloride hexahydrate 4.75 g/L, cupric sulfate pentahydrate 0.4 g/L, boric acid 0.575 g/L, sodium molybdate dihydrate 0.5 g/L and 6N sulfuric acid 12.5 ml/L), and 50 μg/ml carbenicillin. Cells were grown to ≈0.6 A_{600} and induced with 0.5 mM IPTG. The induced cells were harvested by centrifugation ≈17 h post-induction and stored at -20°C.

Lysis of rHuPON1-expressing cells

Frozen SE1 cells expressing the rHuPON1s were lysed in Tris-calcium buffer with multiple rounds of sonication. Cell masses were generally between 10-20 grams. Frozen cells

were thawed, then diluted with buffer (20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween® 20) containing protease inhibitors (EDTA-free Protease Inhibitor Set III [EMD Millipore],1:10,000 dilution) and 2.5 U/ml Benzonase® nuclease (EMD Millipore). Cell pellets were diluted at ratios of 1:3 cell mass:buffer volume. Thawed, resuspended cell pellets were lysed on ice using an Ultrasonic Processor sonicator. The resuspended cells were sonicated at amplitude 30 with 10 s pulses for 1 min, followed by chilling the lysate on ice and another 1 min sonication. The lysate was centrifuged for 15 min at 7000 rpm at 4°C in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge and the supernatant stored at 4°C. The remaining cell pellet was resuspended again in buffer and the sonication-centrifugation protocol was repeated four times, with a sixth and final sonication-centrifugation using buffer with 1% Tergitol® non-ionic detergent (Type NP-10). Supernatants from multiple centrifugations were pooled, centrifuged again to remove any cell matter, and the decanted supernatant was used for the starting lysate.

Purification of rHuPON1s

The rHuPON1 was purified from clarified lysate using a sequence of column chromatography steps. Multiple diethylaminoethyl (DEAE) anion exchange columns were used in a protocol that was slightly modified from Stevens et al.[27] and Chapter 2 of this work. Clarified lysate from sonication of the frozen cell pellets was loaded onto a 10 ml DEAE (GE Healthcare Biosciences) anion exchange column (0.7 cm diameter x 20 cm height), equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween® 20 buffer, washed with 5 resin bed volumes (rbv) of the same buffer, and eluted with a 40 rbv gradient of 0-500 mM NaCl in equilibration buffer. Fractions from all columns (5 µl of each fraction) were assayed for phenyl acetate (AREase) activity as described below. AREase-containing fractions were pooled and the pool was desalted using Amicon Ultra Centrifugal Filters (30K, EMD Millipore). The PON1

pool was concentrated to ≈2.5 ml in a Beckman J-6B centrifuge at 2500 rpm and was diluted fivefold up to ≈15 ml with 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$ buffer, then concentrated again by centrifugation to 2.5 ml. The fivefold dilution was repeated. Two more concentration-dilutions were performed, with the sample only being diluted twofold. The diluted, desalted rHuPON1-containing pool was then reloaded onto the washed, equilibrated 10 ml DEAE-detergent column. Column running conditions were identical to the first column – the column was equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween® 20 buffer, sample was loaded onto the column and washed with 5 rbv of the same buffer, and the protein was finally eluted with a 40 rbv gradient of 0-500 mM NaCl in equilibration buffer. The AREase-containing fractions were pooled and the pool was again desalted as described above using the Amicon Ultra Centrifugal Filters. A non-detergent DEAE column (5 ml) was equilibrated with 20 mM Tris pH 7.0, 1 mM CaCl2 buffer and the desalted rHuPON1 pool was loaded onto the column, followed by column washing with 5 rbv of equilibration buffer and elution with 40 rbv of a 0-750 mM NaCl gradient. The AREase containing fractions were pooled and loaded on a butyl-hydrophobic interaction column (HIC) (GE Healthcare) (5 ml, 0.9 x 3 cm). The HIC column was initially washed with 5 rbv of a no salt HIC buffer (10 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$), then equilibrated with high salt HIC buffer (10 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 3 M NaCl). NaCl was added to the rHuPON1 pool (from the third DEAE) to obtain a sample concentration of 3 M NaCl to promote binding to the HIC resin. Following sample loading, the column was washed with 5 rbv high salt buffer, and eluted with a 40 rbv reverse salt gradient, 3 M-0 M NaCl in the Tris-calcium buffer. Another elution with no salt HIC buffer followed; protein elution was measured by A_{280} and the elution continued until absorbance returned to the initial levels. The column was finally eluted with 0.1% Tween® 20 in no salt HIC buffer.

Fractions were assayed for AREase activity and the Tween® 20-containing active fractions were pooled. The Tween® 20 rHuPON1 pool was then loaded onto a final DEAE column (1 ml) which was equilibrated with 20 mM Tris pH 7.5, 1 mM CaCl2 buffer, washed with 5 rbv equilibration buffer and eluted with 40 rbv of 0-750 mM NaCl in equilibration buffer.

A modified purification protocol was carried out to purify the disease-associated PON1 mutant V109I and the control rHuPON1$_{R192}$. PON1$_{R192}$ lysate from sonication (300 ml from 22.5 g cells) was loaded on a 40 ml DEAE (1.7 cm diameter x 32 cm height), equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween® 20 buffer, washed with 5 rbv of the same buffer, and eluted with a 200 rbv gradient of 0-500 mM NaCl in equilibration buffer. The AREase-containing fractions were pooled and desalted as described above. The desalted pool was loaded onto a second DEAE column (10 ml, 0.5 x 20 cm) in 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$ buffer, washed until the A_{280} reading reached baseline, then eluted with a 40 rbv 0-500 mM NaCl gradient in equilibration buffer. The pool containing AREase activity was concentrated to 700 µl and loaded on a 75 ml Superdex-200 size exclusion column (1.8 x 45 cm [GE Healthcare]). The column was eluted with 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 150 mM NaCl buffer. AREase containing fractions were pooled and loaded on a HIC resin (3 ml, 0.9 x 3 cm). The HIC column run was carried out as described above – it was initially washed with 5 rbv of a no salt HIC buffer, equilibrated with 3 M high salt HIC buffer, followed by sample loading. NaCl had been added to the rHuPON1 to reach a concentration of 3 M NaCl. Following sample loading, the column was washed with 5 rbv high salt buffer, eluted with a 40 rbv reverse salt gradient (3 M-0 M NaCl), then eluted with no salt HIC buffer until A_{280} reached baseline. A final elution with 0.1% Tween® 20 in no salt HIC buffer was carried out and the Tween® 20-containing active fractions were pooled. The Tween® 20 elution pool was loaded on a final

DEAE column (1.5 ml, 0.5 x 3 cm) equilibrated with 20 mM Tris-HCl pH 7.5,1 mM $CaCl_2$ buffer, washed with the same buffer until absorbance reached baseline, and eluted with a 40 rbv 0-750 mM NaCl gradient in equilibration buffer.

The $PON1_{R192\text{-}V109I}$ lysate from sonication (225 ml from 9.6 g cells) was loaded onto a 30 ml DEAE column (1.7 cm diameter x 24 cm height), equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween® 20 buffer, washed with 4 rbv of equilibration buffer, and eluted with a 40 rbv gradient of 0-500 mM NaCl in equilibration buffer. AREase-containing fractions were pooled and the pool was desalted using Amicon Ultra Centrifugal Filters as described above. The desalted pool was loaded onto another DEAE resin (10 ml, 0.5 x 20 cm) in 20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.1% Tween® 20 buffer, washed with buffer until the A_{280} reached the baseline level, and eluted with a 40 rbv 0-500 mM NaCl gradient in equilibration buffer. The pool was desalted again with Amicon Ultra Centrifugal Filters and loaded onto a 10 ml non-detergent DEAE column (0.5 x 20 cm) equilibrated in 20 mM Tris-HCl pH 7.0, 1 mM $CaCl_2$ buffer, washed with buffer until the A_{280} reading reached zero, and eluted with a 40 rbv gradient of 0-750 mM NaCl in equilibration buffer. AREase containing fractions were pooled and loaded on a butyl-HIC column (3 ml, 0.9 x 3 cm) and purified as described above. The Tween® 20 elution pool was loaded onto a final DEAE column (1.5 ml, 0.5 x 3 cm) in 20 mM Tris-HCl pH 7.5,1 mM $CaCl_2$ buffer, washed with equilibration buffer until the A_{280} reading returned to initial levels, and eluted with a 40 rbv 0-750 mM NaCl gradient in equilibration buffer.

SDS-PAGE and Western blots

After purification of rHuPON1 proteins, pools from each chromatographic step were analyzed with SDS-PAGE (Invitrogen). Protein pools were assayed for protein concentration using a commercial BCA assay kit (Pierce) and 5 µg (or 20 µl maximum sample volume) was

loaded into each well. Gels were stained using Imperial Protein Stain (Pierce) for 1 h following three ddH_2O washes for 5 min. Gels were destained in ddH_2O. Western blot transfer was performed with an iBlot® Dry Blotting System, PVDF membranes, and the anti-rabbit Western Breeze® Chromogenic Western Blot Immunodetection Kit (Invitrogen). Membranes were blotted according to the manufacturer's instructions. The primary antibody used for staining was a Sigma rabbit anti-PON1 antibody (P0123) diluted 1/1,000 in the commercial dilution buffer.

Activity assays

Assays were run in a SpectraMax Plus (Molecular Devices) spectrophotometer. AREase assays used 3.26 mM phenyl acetate in 9 mM Tris-HCl pH 8.0, 0.9 mM $CaCl_2$ buffer according to Richter et al[15]. UV absorbent 96-well plates (Greiner Bio-one) were used for phenyl acetate assays and the assay was run for 4 min at 270 nm with readings taken every 15 s.

AREase of column fractions was assayed using freshly prepared 3.26 mM phenyl acetate in a 9 mM Tris-HCl pH 8.0, 0.9 mM $CaCl_2$ buffer. Five µL of each column fraction was assayed. The reaction was initiated with the addition of 200 µl of phenyl acetate in buffer.

AREase and paraoxonase (POase) activities of the purified PON1s were determined as described previously[15]. Briefly, 20 ul of sample was assayed using the SpectraMax Plus[384] Microplate Reader (Molecular Devices) with transparent UV 96-well plates for 4 min at 270 nm for AREase and transparent 96-well visible plates (Greiner Bio-One) for 4 min at 405 nm for POase, with the assay run at 37°C for the POase assay. Kinetic sample absorbance readings were taken every 15 s for both assays. AREase was assayed using freshly prepared 3.26 mM phenyl acetate in a 9 mM Tris-HCl pH 8.0, 0.9 mM $CaCl_2$ buffer and the POase with freshly prepared 1.2 mM paraoxon in pre-warmed 100 mM Tris-HCl pH 8.5, 2 mM $CaCl_2$, and 2 M NaCl buffer. $PON1_{R192}$ (0.23 mg/ml) was diluted 1:8.75 v:v for the AREase assay and 1:2 v:v for the POase

assay. $PON1_{R192-V109I}$ (0.801 mg/ml) was diluted 1:90 v:v for the AREase assay and 1:10 v:v for the POase assay. Activities were converted to Units/ µg (AREase) or Units/mg (POase) using the molar extinction coefficients of 18 $mM^{-1}cm^{-1}$ or 1.310 $mM^{-1}cm^{-1}$ for AREase and POase, respectively.

Heat resistance assays

Assays measuring purified PON1 resistance to heat were carried out in a GeneMate Mini Dry Bath incubator block. Protein samples were diluted with a Tris-calcium buffer (20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 150 mM NaCl).

Purified protein pools of PON1 variants to be tested for stability were assayed for concentration and 5.89 µg of protein was used for each assay in a total volume of 1 ml. Heat blocks were set at 65°C. Proteins were heated at 65°C and samples were taken every 5 min. Samples were incubated on ice and AREase activity was measured. Samples (20 µl) were assayed in triplicate for each time point, with 6 replicates assayed for the starting zero min time point. Samples were assayed as described above. The average activity at the time zero min reading (no heat) was assigned the 100% value and residual AREase activity was expressed as a percentage of the 100% value.

The $PON1_{R192}$ protein (0.23 mg/ml) was diluted 1:20 v:v and the $PON1_{R192-V109I}$ sample (0.801 mg/ml) was diluted 1:140 v:v to obtain nearly equivalent rates of AREase activity, or about 0.711 Units/ml AREase activity. Total sample (750 µl) was mixed and 125 µl removed prior to heating in the heat block at 55°C for a zero time point sample. Samples were taken at the time points indicated and stored on ice.

EDTA assays

EDTA assays were performed based on the protocols of Kuo and La Du[25]. Equivalent concentrations of the rHuPON1 variant proteins, 1.89 μg of protein, were incubated with increasing concentrations of EDTA for 18 hours at room temperature. AREase assays were performed as described above and the activity was expressed as a percentage of the 0 mM EDTA sample which was used as the 100% value.

Mass spectrometric analysis of rHuPON1$_{R192\text{-}V19I}$

Both PON1$_{R192}$ and PON1$_{R192\text{-}V109I}$ were analyzed by mass spectrometry (MS) to confirm the identity of the mutant PON1$_{R192\text{-}V109I}$. Five μg of each purified PON1 were boiled for 5 min at 90^{0}C in the presence of 0.1% RapiGest^{TM} (Waters Corporation, Milford, MA), reduced with 5 mM dithiothreitol (DTT) for 30 min at 50^{0}C and alkylated with 15 mM iodoacetic acid (IAA) for 30 min at RT in the dark. Then, 1 μg of porcine trypsin (Promega, Madison, WI) was added and samples were incubated at 37^{0}C for 2 h while rotating. Following digestion, RapiGest was hydrolyzed by adding 100 mM hydrochloric acid to each sample and re-incubating them at 37^{0}C for 45 min. The generated peptides were analyzed with an LTQ-Orbitrap instrument (Thermo Fisher Scientific) using nanoflow chromatography (Waters nanoACQUITY UPLC). About 200 ng of each sample were loaded onto a 20 cm long, 75 μm inner diameter (I.D.) silica column packed in-house with Jupiter 4 μm Proteo 90 Å C12 reverse-phase beads (Phenomenex, Torrance, CA). A 5 μm I.D. tip was pulled using a P-2000 CO_2 laser puller (Sutter Instrument Company, Novato, CA). Peptides were separated using a 70 min gradient of 2-32% acetonitrile in 0.1% formic acid, at 250 nL/min flow. The Sequest algorithm (Eng et al. 1994) was used for database searches of the spectral files. All cysteines were monitored as carbamidomethyl

cysteines (57.021464 Da static modification), and the valines as isoleucines (14.01565 Da variable modification).

Results

Expression and purification of rHuPON1 stability mutants

Amino acid changes were made to rHuPON1 that were identical to residues at the same position in rabbit PON1. These variants were predicted to have stronger calcium binding and increased protein stability. The L130M amino acid change was identified as a candidate variant. Other single amino acid changes that were predicted to alter stability and calcium binding were S67A and V206T. The rHuPON1 mutants that were predicted to increase stability and calcium binding were generated and expressed in *E. coli*. All predicted variants were generated on the $rHuPON1_{K192}$ protein backbone which is identical to native human PON1 except for the lysine in the 192 position, which is the amino acid found in rabbit PON1. The predicted mutants were identical to rabbit PON1 residues at three amino acid positions and were predicted to have increased stability and stronger calcium binding using computational models. The purification used was a modification of the protocol in Stevens et al[27]. Hydrophobic interaction chromatography was used in place of the hydroxyapatite column chromatographic step. The purification of one of the mutants, L130M, is shown in Figure 4.1. All variants were recognized by commercial anti-PON1 antibody (Sigma 0123) on a Western blot (data not shown) and showed a high degree of purification. The $rHuPON1_{K192}$ variant was also purified and used as a control for the heat stability and EDTA assays.

Heat stability assays of rHuPON1 variants

General rHuPON1 protein stability was assayed using heat tests based on a protocol by Kuo et al.[25]. Figure 4.2A shows the comparison of the activity vs. temperature curves for the

control rHuPON1$_{K192}$ and three single mutants, rHuPON1$_{K192\text{-}L130M}$, rHuPON1$_{K192\text{-}S67A}$ and rHuPON1$_{K192\text{-}V206T}$, as well as a double mutant (rHuPON1$_{K192\text{-}S67A\text{-}L130M}$) and a triple mutant (rHuPON1$_{K192\text{-}\ S67A\ \text{-}L130M\text{-}V206T}$). Figure 4.2B shows the comparison between rHuPON1$_{K192\text{-}L130M}$ and the rHuPON1$_{K192}$ control.

EDTA inhibition assays of rHuPON1 variants

PON1 is a calcium-dependent enzyme and EDTA, a metal chelator, inhibits activity[32]. EDTA inhibition tests were performed using a protocol from Kuo et al[25]. The rHuPON1 variant proteins were inhibited with increasing concentrations of EDTA and residual activity was assayed. EDTA was added to rHuPON1 proteins at concentrations of 0, 0.5, 1, 2, 4, 8 and 10 mM and the samples were assayed for residual arylesterase activity after overnight (18 h) incubation. Figure 4.3A compares the variant rHuPON1s inhibited by increasing EDTA concentrations. The rHuPON1 mutants were compared to the rHuPON1$_{K192}$ variant which was used as a control. The mutants tested are three single mutants, rHuPON1$_{K192\text{-}L130M}$, rHuPON1$_{K192\text{-}S67A}$ and rHuPON1$_{K192\text{-}V206T}$, a double mutant (rHuPON1$_{K192\text{-}S67A\text{-}L130M}$) and a triple mutant (rHuPON1$_{K192\text{-}\ S67A\ \text{-}L130M\text{-}V206T}$). Only the single mutants L130M and S67A were found to be significantly more resistant to EDTA inhibition although all variants tested showed increased resistance to EDTA when compared to the control. The comparison of EDTA inhibition curves of the rHuPON1$_{K192}$ control and rHuPON1$_{K192\text{-}L130M}$ is shown in Figure 4.3B.

Characterization of native rHuPON1 alloforms

Native human PON1 has two common coding polymorphisms, the activity polymorphism Q192R and the L55M polymorphism. Single amino acid changes, homologous to rabbit PON1, demonstrated increased resistance to heat and EDTA. To test the effect of the L55M polymorphism on protein stability, we expressed the two native variants, rHuPON1$_{L55}$ and

rHuPON1$_{M55}$, which were generated on the rHuPON1$_{R192}$ human protein backbone. The rHuPON1s alloforms were expressed and purified as shown in Figure 4.4. The purified proteins were tested for overall stability by assaying residual AREase activity following heat inhibition. The residual activity is shown in Figure 4.5. Compared to rHuPON1$_{M55}$, rHuPON1$_{L55}$ retained significantly more activity when heated.

Characterization of disease-associated and native rHuPON1 variants

PON1 activity and genotype must both be taken into account for disease-association studies. However, rare PON1 variants have been linked to a number of diseases and conditions. We expressed and purified a recently-discovered variant, V109I, which was linked to ischemic stroke in an African-American population[33]. The mutant was generated on the rHuPON1$_{L55-R192}$ protein backbone, which was identical to the genotype of the disease-associated PON1 alloform, and rHuPON1$_{V109I}$ was expressed and purified along with the rHuPON1$_{R192}$ alloform as a control (Figure 4.6). Protein identity of the rHuPON1$_{V109I}$ variant was confirmed with MS. The rHuPON1$_{V109I}$ was active and could be followed with AREase activity throughout the purification. As expected, it was recognized by an anti-PON1 antibody on a Western blot. Specific activities of the rHuPON1$_{V109I}$ and rHuPON1$_{R192}$ alloform controls were equivalent. We then tested the stability of the proteins by measuring residual AREase activity following heating of the rHuPON1s (Figure 4.7). The rHuPON1$_{V109I}$ variant was significantly more heat-labile compared to the control (rHuPON1$_{L55-R192}$).

Discussion

PON1 is of importance due to its activity against OPs as well as its anti-atherogenic and antioxidative properties and association with multiple diseases. A number of previous studies have examined the effects of single or multiple amino acid changes on the activity and other

characteristics of recombinant PON1 proteins. In this report, we tested mutants computationally-predicted mutants to increase PON1 stability on the rHuPON1$_{K192}$ protein backbone. They turned out to be homologous to rabbit PON1 at the substituted positions. We found that they increased resistance to denaturation and EDTA-induced activity loss. Two common human PON1 variants were also tested for resistance to heat denaturation as a previous report found PON1$_{L55}$ was more resistant to proteolysis; differences were found in overall protein stability with rHuPON1$_{L55}$ the more stable protein. A rare disease-associated variant (V109I) was generated on a native human protein backbone; compared to the rHuPON1$_{R192}$ alloform, it was more susceptible to heat-induced loss of activity.

The predicted changes in the protein were homologous to rabbit PON1. Rabbit PON1 has long been known to have relatively high PON1 activity and higher affinity binding of calcium. Rabbit PON1, unlike human PON1, is reapidly renatured after running samples in an SDS-PAGE[34]. Rabbit PON1 was also the basis of the hybrid rePON1 which was 91% similar to rabbit PON1[23]. In our previous experiments, rHuPON1$_{K192}$, which is identical to rabbit PON1 at position 192, showed increased catalytic activity towards a number of OPs. Here we altered amino acids on the rHuPON1$_{K192}$ protein backbone and observed an effect on the heat and EDTA resistance of the protein. As the amino acid substitutions were homologous to rabbit PON1, it would not be expected that there would be a loss of activity or disruptive structural changes and the specific activity of the variants was not significantly different from the rHuPON1$_{K192}$ control. This supports the idea that small, incremental changes to the native human PON1 protein can be utilized to optimize PON1 characteristics such as activity or stability. A number of publications have examined the effects of varying the chimeric rePON1 sequence, which already has many differences in amino acid sequence from human PON1, to characterize the effect of amino acid

changes on activity of the protein[28, 29]. Our strategy takes the opposite approach, generating and testing as few as possible changes on a native human PON1 backbone to achieve increased catalytic efficiency. Other research teams have also tested the effects of a number of small, rationally-designed amino acid substitutions on the solubility of native human PON1[31]. Both approaches will be important in generating PON1 for possible therapeutic use as well as characterizing the effects of multiple amino acid substitutions on the protein.

The rHuPON1$_{K192\text{-}L130M}$ had only a single amino acid change differentiating it from the rHuPON1$_{K192}$ control which resulted in increased stability against heat and EDTA resistance. The amino acid at position 130 is leucine for native human PON1 and methionine in rabbit PON1. The rePON1s also had methionine at position 130, which was identified in Aharoni et al.[22] as a subtle, conservative change, but they suggested that it may contribute to correct protein folding and oligomeric packing. Our predictions indicated that this change would optimize the β-sheet packing and decrease the distance between the calcium ion and interacting residues, promoting stronger calcium interactions. This was the most resistant variant tested with respect to heat and EDTA inactivation and can be used as a general improved protein backbone for further testing to improve stability, calcium binding, and activity along with the rHuPON1$_{K192}$ variant. The S67A was also a single amino acid substitution that increased stability and EDTA resistance. This variant was predicted to improve overall packing efficiency and improve calcium ion ligation as well and this was observed in the heat and EDTA resistance. While this variant is homologous to rabbit PON1 at position 67, the S67A variant is not present in the Tawfik rePON1. Several other rHuPON1 variants were generated, expressed, and characterized (rHuPON1$_{V206T}$, rHuPON1$_{S67A\ -L130M}$, rHuPON1$_{S67A\ -L130M\text{-}V206A}$); however, the single variants

rHuPON1$_{L130M}$ and rHuPON1$_{S67A}$ demonstrated the greatest increases in calcium binding and heat resistance (Figures 4.2 and 4.3).

We were also interested in naturally occurring variants and whether they would have differences in stability and calcium binding. An optimal place to start was with the L55M polymorphism. This polymorphism has been linked to differences in PON1 mRNA and protein levels; however, the majority of this effect is due to linkage disequilibrium with the C-108T promoter polymorphism believed to be on a Sp1 binding site[7, 8, 10]. Few studies have been done testing the relative properties of the protein; instead, the L55M has been used as a surrogate for PON1 levels in correlational studies. One report compared the two L55M alloforms and found that this change results in increased susceptibility to proteolysis which may be one explanation for the difference in levels[12]. Our results agree with the findings of Leviev et al.[12] as we determined that resistance to heat-induced activity loss is altered between the two variants; activity decreases significantly more for the M55 compared to the L55 variant on a R192 protein backbone. Although an effect was observed between purified bacterially-produced rHuPON1s, the difference is unlikely to have a physiological effect as genotype association studies and meta-analyses of the L55M polymorphism and various disease and conditions have provided conflicting results. In optimizing the native PON1 protein, however, L55 would be the preferred alloform. Native rabbit PON1 and the chimeric rePON1 also have leucine at amino acid position 55 and it is possible that L55 contributes to the overall solubility or stability. In the crystal structure, amino acid 55 is involved in packing efficacy of the central tunnel and its proximity to calcium-ligating residues E53 and D54[23] also suggests an important role in maintaining protein stability.

We additionally tested a rare disease-associated variant, V109I, using the rHuPON1 expression system. While the common polymorphisms have not consistently been associated with disease, rare variants are more promising. The rHuPON1$_{V109I}$ variant has been linked to ischemic stroke in an African-American population[33]. The variant did not show a loss of activity; however, when assayed for resistance to heat, the variant was significantly less stable. This result suggests one possible mechanism for how this variant may contribute to disease conditions although more work needs to be done. Although single amino acid changes associated with disease often affect protein stability rather than function[35], PON1 has multiple substrates and does not have only a single role; it is unknown whether a loss of stability *in vivo* is significant.

In conclusion, using the rHuPON1 *E. coli* expression system, we generated and characterized several rHuPON1 variants. Variants with single amino acid changes that were homologous to rabbit PON1 and which had been analyzed using computational methods had increased protein stability and calcium binding. One variant, rHuPON1$_{K192\text{-}L130M}$, was found to be significantly more stable than the rHuPON1$_{K192}$ control, suggesting that it could be useful as a starting protein backbone for testing other PON1 variants. Two alloforms, rHuPON1$_{L55}$ and rHuPON1$_{M55}$, were also expressed and compared for protein stability. In accordance with an earlier study which found that PON1$_{L55}$ was more stable due to resistance to proteolysis[12], rHuPON1$_{L55}$ was found to be more resistant to heat. A disease-associated variant (V109I) was also characterized and was significantly less resistant to head-induced denaturation, suggesting a possible molecular mechanism for the association of rare variants and disease conditions.

Acknowledgements

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Figure 4.1

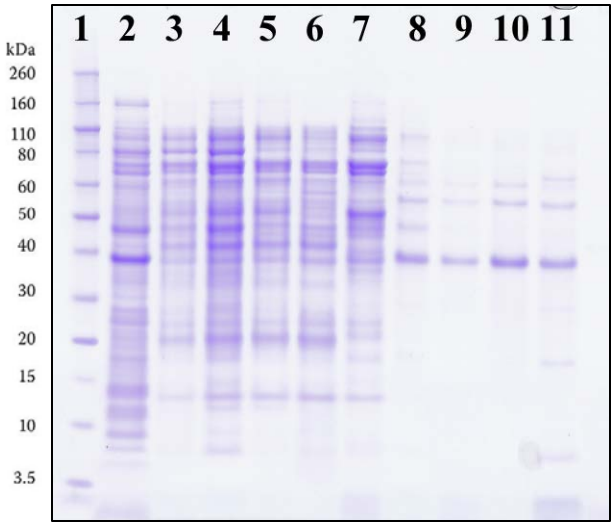


Figure 4.1. SDS-PAGE analysis of pooled column fractions from the purification of rHuPON1$_{L130M}$. **Lane 1**, molecular weight markers (kDa); **lane 2**, *E. coli* lysate; **lane 3**, pool of diethylaminoethyl (DEAE) I fractions; **lane 4**, concentrated DEAE I pool; **lane 5**, DEAE II pool; **lane 6**, concentrated DEAE II pool; **lane 7**, DEAE III pool; **lane 8**, hydrophobic interaction column (HIC) pool; **lane 9**, DEAE IV pool; **lane 10**, concentrated DEAE IV pool; **lane 11**, positive control rHuPON1$_{K192}$. The gel was stained with Imperial Protein Stain (Pierce).

Figure 4.2

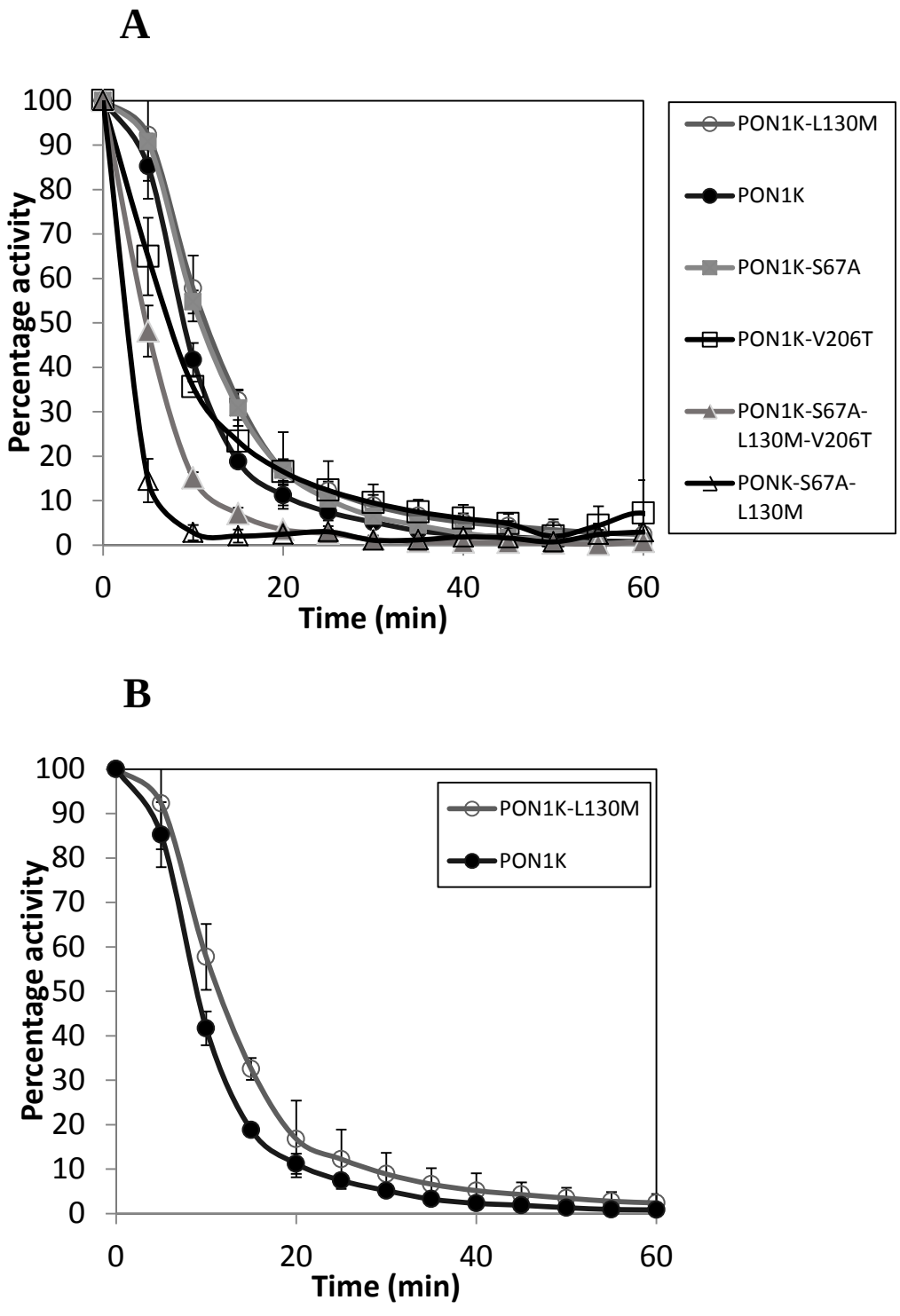
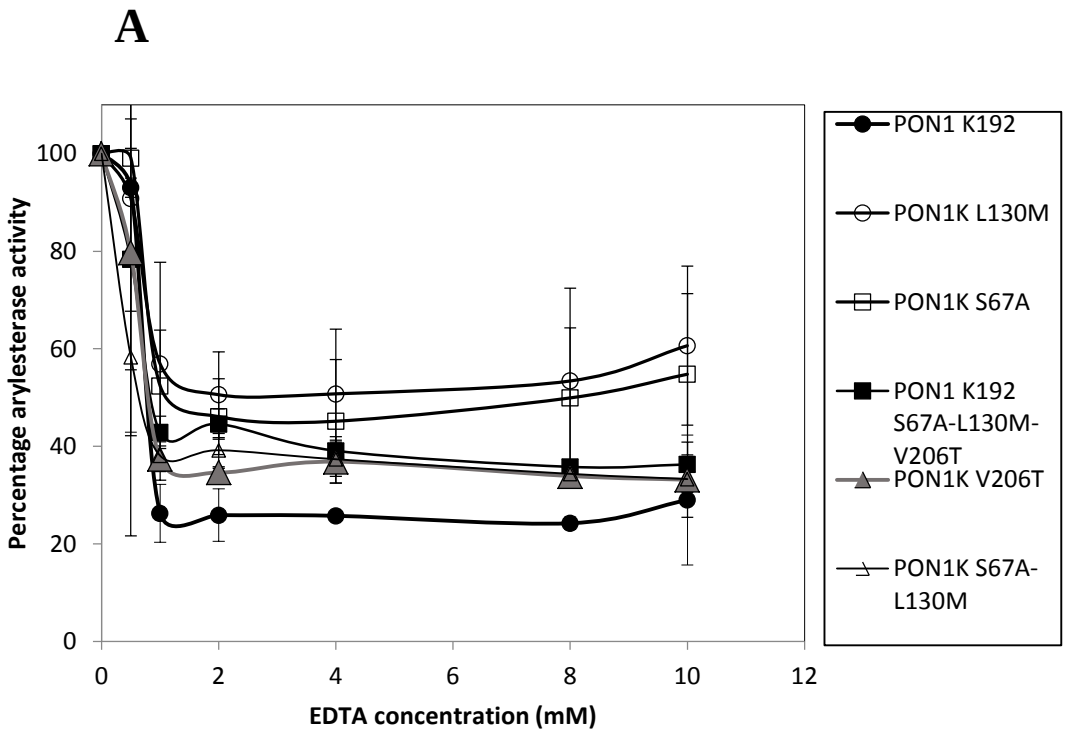

A
Percentage activity
Time (min)
PON1K-L130M
PON1K
PON1K-S67A
PON1K-V206T
PON1K-S67A-L130M-V206T
PONK-S67A-L130M
B
Percentage activity
Time (min)
PON1K-L130M
PON1K

Figure 4.2. Residual arylesterase activity of the individual rHuPON1 variants in a heat denaturation assay. The $rHuPON1_{K192}$ variant was used as a control. Proteins were heated at 65°C and samples taken at the indicated time points. Residual AREase was expressed as a percentage of the 0 min unheated sample. **A)** residual AREase activity of all tested mutants; **B)** activity of $rHuPON1_{L130M}$ and the control $rHuPON1_{K192.}$

Figure 4.3


A
Percentage arylesterase activity
EDTA concentration (mM)
PON1 K192
PON1K L130M
PON1K S67A
PON1 K192 S67A-L130M-V206T
PON1K V206T
PON1K S67A-L130M

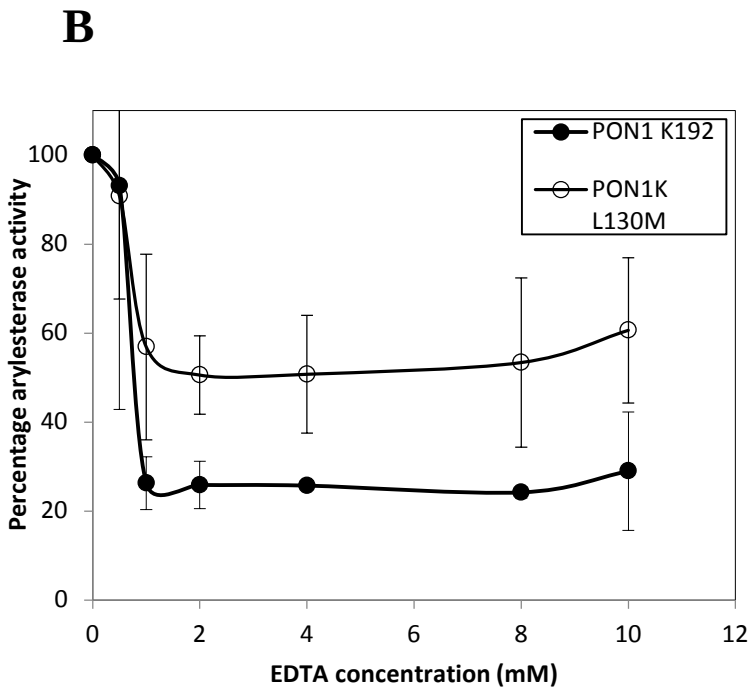

B
Percentage arylesterase activity
EDTA concentration (mM)
PON1 K192
PON1K L130M

Figure 4.3. Residual arylesterase activity of individual rHuPON1 variants in an EDTA inhibition assay. The rHuPON1$_{K192}$ variant was used as a control. Proteins were incubated with increasing concentrations of EDTA for 18 h and AREase was assayed. Residual AREase was expressed as a percentage of the 0 mM EDTA sample. **A**) residual AREase activity of all tested variants; **B**) residual AREase activity of rHuPON1$_{L130M}$ and the control rHuPON1$_{K192}$

Figure 4.4

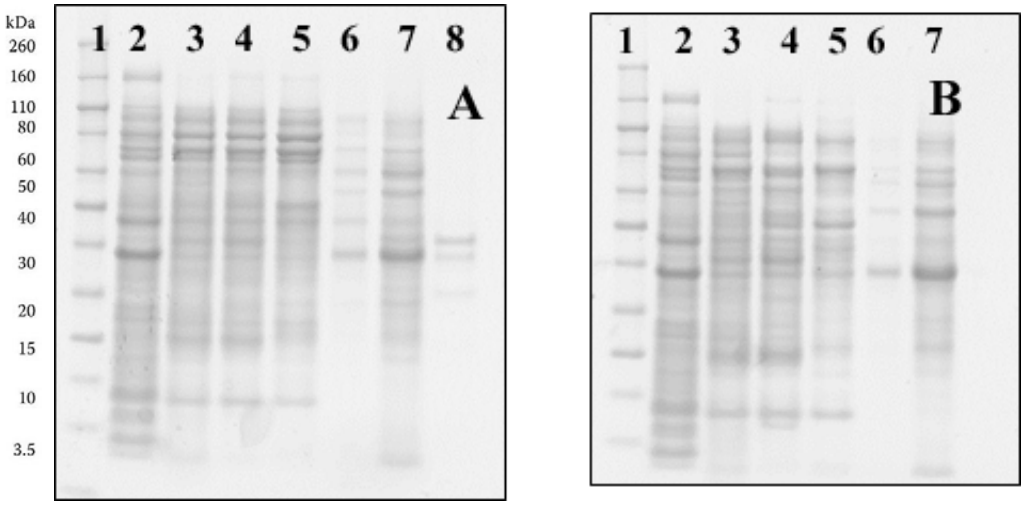


Figure 4.4. SDS/PAGE analysis of pooled column fractions from the purification of **A**, rHuPON1$_{L55}$ and **B**, rHuPON1$_{M55}$. **A**) **Lane 1**, molecular weight markers (kDa); **lane 2**, *E. coli* lysate; **lane 3**, diethylaminoethyl (DEAE) I pool; **lane 4**, DEAE II pool; **lane 5**, DEAE III pool; **lane 6**, HIC pool; **lane 7**, DEAE IV pool; **lane 8**, positive control rHuPON1$_{K192}$. **B**) **Lane 1**, molecular weight markers (kDa); **lane 2**, *E. coli* lysate; **lane 3**, DEAE I pool; **lane 4**, DEAE II pool; **lane 5**, DEAE III pool; **lane 6**, HIC pool; **lane 7**, DEAE IV pool. Gels were stained with Imperial Protein Stain (Pierce).

Figure 4.5

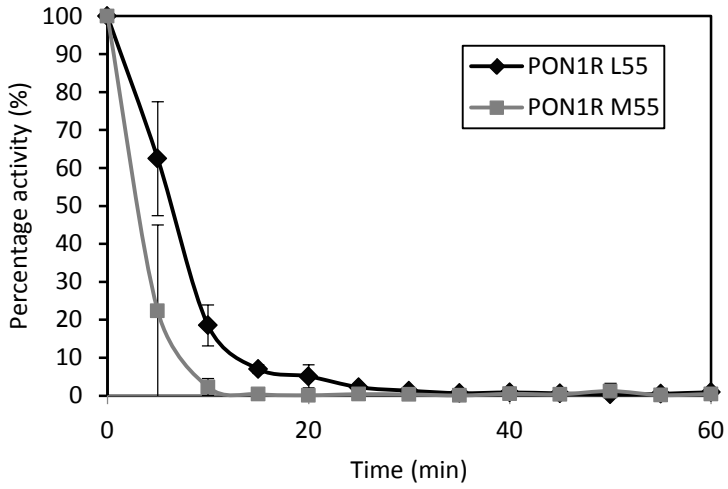


Figure 4.5. Residual arylesterase activity of two native PON1 alloforms, rHuPON1$_{L55}$ and rHuPON1$_{M55}$ following heating at 65°C for the indicated time points. Residual AREase was expressed as a percentage of the 0 min unheated sample.

Figure 4.6

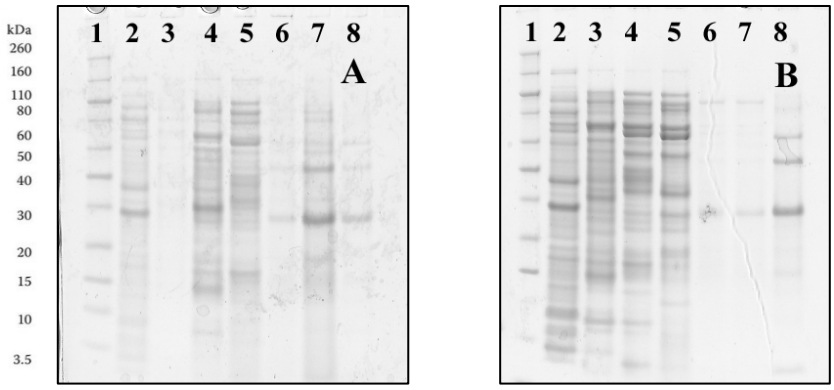


Figure 4.6. SDS/PAGE analysis of pooled column fractions from the purification of rHuPON1$_{V109I}$ and the rHuPON1$_{R192\text{-}L55}$ control. **A**, rHuPON1$_{R192\text{-}L55}$ control, **B**, rHuPON1$_{V109I}$. **A**) **Lane 1**, molecular weight markers (kDa); **lane 2**, *E. coli* lysate; **lane 3**, diethylaminoethyl (DEAE) I pool; **lane 4**, DEAE II pool; **lane 5**, DEAE III pool; **lane 6**, HIC pool; **lane 7**, DEAE IV pool; **lane 8**, positive control rHuPON1$_{K192}$. **B**) **Lane 1**, molecular weight markers (kDa); **lane 2**, *E. coli* lysate; **lane 3**, DEAE I pool; **lane 4**, DEAE II pool; **lane 5**, DEAE III pool; **lane 6**, HIC pool; **lane 7**, DEAE IV pool; **lane 8**, positive control rHuPON1$_{K192}$.Gels were stained with Imperial Protein Stain (Pierce).

Figure 4.7

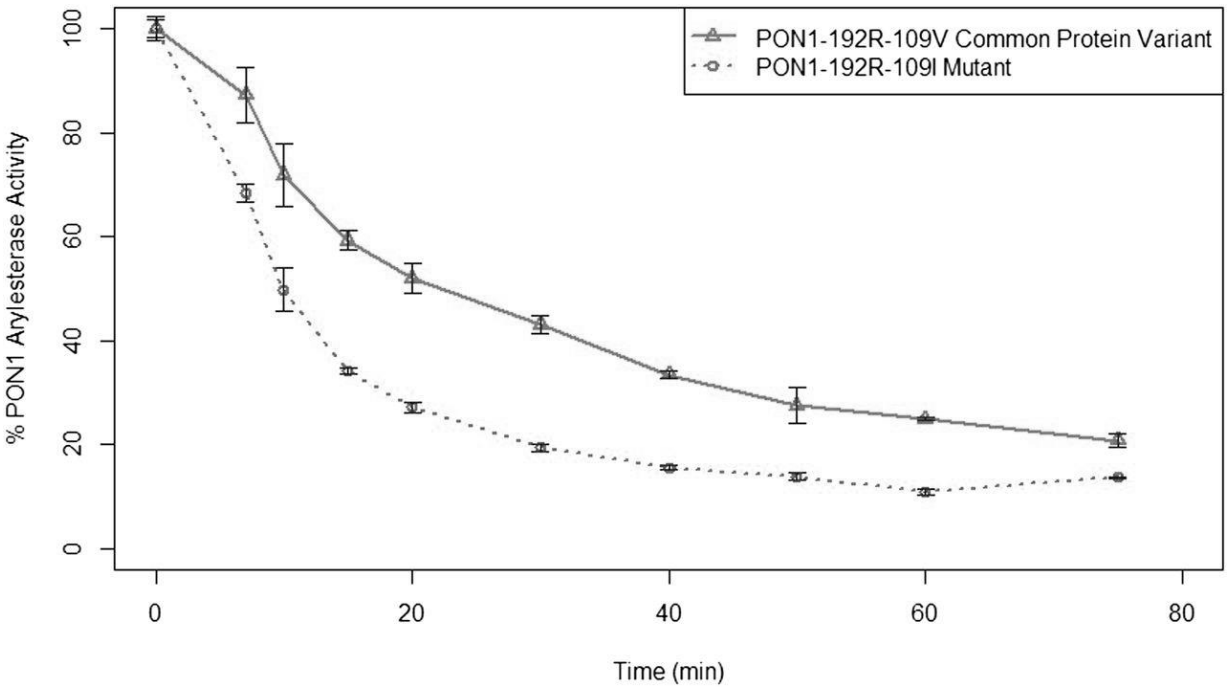


Figure 4.7. Residual arylesterase activity of a disease-associated PON1 variant ($rHuPON1_{R192-V109I}$) and the $rHuPON1_{R192}$ alloform control. Proteins were heated at 55°C and samples were assayed at the indicated time points. Residual AREase was expressed as a percentage of the 0 min unheated sample.

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Chapter Five: Biphenyl Hydrolase-like Protein is a Highly Efficient Homocysteine Thiolactonase

Abstract

Paraoxonase-1 (PON1) metabolizes a number of xenobiotic substrates, but the identity of the physiological substrate of PON1 is still unclear. Following the discovery of PON1's lactonase activity, it was proposed that PON1 is the enzyme physiologically responsible for hydrolyzing homocysteine thiolactone (HCTL), a reactive, toxic homocysteine metabolite. However, rates of HCTL hydrolysis by PON1 are very low. HCTL is a known risk factor for cardiovascular, neurological, and autoimmune disease. Adduction of proteins by HCTL causes them to misfold and aggregate, contributing to disease conditions. Another enzyme, bleomycin hydrolase, was later determined to have a catalytic efficiency for HCTL hydrolysis that is 100-fold that of PON1. We purified a highly-efficient HCTL hydrolase activity (HCTLase) from human liver and identified the protein by mass spectrometry (MS) as a previously-characterized drug-metabolizing protein, human biphenyl hydrolase-like protein (BPHL). *BPHL* cDNA was cloned and expressed in *E. coli*. The recombinant BPHL protein (rBPHL) was purified and characterized. Its hydrolytic activity for HCTL and valacyclovir, a previously-identified anti-herpetic substrate of BPHL, was confirmed by MS. The HCTLase activity of BPHL was nearly two orders of magnitude more efficient than that of bleomycin hydrolase and close to four orders of magnitude more efficient than PON1. The much higher HCTLase activity of BPHL compared with that of PON1 and the calculation of the total HCTLase activity of human liver compared with the plasma HCTlase activity of PON1 indicates that BPHL likely makes a more significant contribution than PON1 in protecting against HCTL-associated risk for vascular disease.

Introduction

Paraoxonase-1 (PON1) is a multifunctional enzyme with a wide range of substrates, but there is debate about its physiological substrate. Jakubowski et al.[1] proposed that homocysteine thiolactone (HCTL) was the physiological PON1 substrate. HCTL is a thiolactone metabolite of homocysteine (Hcy), a sulfur-containing homologue of cysteine and precursor to methionine. Increased levels of Hcy and HCTL have been associated with a number of disease conditions although there is some debate over whether they are causative or merely correlative[2-4]. While severe hyperhomocysteinemia is rare, mild hyperhomocysteinemia is common in the general population[5]. Elevated Hcy and HCTL levels have been associated with cardiovascular disease (CVD), diabetic retinopathy, Alzheimer's disease (AD), and other conditions[2-4, 6-8]. HCTL modifies proteins via formation of an isopeptide bond with the epsilon amino groups of lysine residues[9]; these homocysteinylated proteins have the potential to misfold or lose biological activity[8, 9]. Misfolded proteins aggregate, which can activate the adaptive immune system and increase clot resistance to lysis, leading to atherosclerotic lesion formation. For example, the clots formed from homocysteinylated fibrinogen present show increased resistance to lysis, contributing to a higher risk of thrombosis and vascular disease[8].

Dysfunction in various enzymes can result in increased physiological concentrations of Hcy and HCTL and associated detrimental effects. Hcy is generated from methionine following demethylation and can be converted back to methionine or to cysteine. Cystathionine beta-synthase (CBS) is necessary to convert Hcy to cysteine while methionine synthase converts it to methionine. Methylenetetrahydrofolate reductase (MTHFR) generates a metabolite required for the conversion of Hcy to methionine. Deficiencies in these enzymes can result in increased levels

of Hcy and HCTL (reviewed in [2]). Hcy is converted to HCTL through error-editing reactions catalyzed by methionyl tRNA-synthetase while HCTL hydrolytic activity (HCTLase) converts HCTL to Hcy; this latter reaction is catalyzed by PON1 and other proteins.

The discovery of PON1 as an HCTLase was the first identification of an enzyme with HCTLase activity. Human plasma PON1 hydrolyzes a number of lactones besides HCTL and has been described as a lactonase[1, 10-12]. PON1 has two common human alloforms, $PON1_{R192}$ and $PON1_{Q192}$, which differ in the catalytic efficiency of hydrolysis of some substrates. It has been shown[13, 14] that the PON1's ability to protect against organophosphorus (OP) insecticide intoxication is highly dependent on the catalytic efficiency of hydrolysis of a given OP substrate. The reported low substrate affinity and very low catalytic efficiency for hydrolysis of HCTL by PON1[1] raise doubts about the physiological relevance of this activity[10, 15]. However, there is some evidence for a physiological role of PON1 in protecting against HCTL toxicity. In a small sample, an association between both Q192R and L55M genotype and HCTLase activity was found, but levels of HCTLase were also taken into account and shown to affect the disappearance of HCTL and appearance of homocysteinylated proteins[16]. PON1 knockout mice (*PON1*$^{-/-}$) have no plasma HCTLase activity[1], have higher HCTL levels in the brain, and excrete significantly more HCTL in urine[17]. The *PON1*$^{-/-}$ mice were also more susceptible to HCTL-induced seizures and had a shorter latency period for seizures[17]. For HCTLase measurement, two substrates are commonly used, HCTL and a surrogate lactone, γ-thiobutyrolactone (GTBL), which has been used due to concerns that HCTL is unstable and/or because of albumin interference with the assay[18, 19].

Following the discovery of PON1's HCTLase activity, another more efficient HCTLase activity was identified. Bleomycin hydrolase (Blmh) was purified by isolating the HCTLase activity present in tissues instead of plasma. Blmh has a considerably higher catalytic efficiency for hydrolysis of HCTL compared to PON1 – it was reported to be ≈100-fold greater although it was not saturated at 20 mM HCTL[20]. Before its identification as an HCTLase, Blmh was studied for its ability to metabolize the anti-cancer drug bleomycin and had been linked to AD as it can process β-amyloid peptides[21, 22]. Northern blots detected Blmh in skeletal muscle, pancreas, testis, and at lower levels in small intestine, heart, brain, spleen, and lung[23]; another study found Blmh in all tissues examined[24]. The physiological effects of Blmh were tested in animal models. *Blmh*$^{-/-}$ mice had higher levels of HCTL in the brain and excreted significantly more HCTL in urine. When injected with HCTL, *Blmh*$^{-/-}$ mice had an increased incidence of HCTL-induced seizures compared to wild-type mice. The authors concluded that Blmh provided protection against the neurotoxicity of HCTL by metabolizing HCTL to Hcy[25]. In humans, HCTLase activity attributed to Blmh differed significantly between brains of AD patients and controls[26].

Our initial aim was to discover the enzyme responsible for the second step in the bioactivation of clopidogrel, since a recent report identifying it as a PON1 was contested. A previously undescribed thiolactonase enzyme was identified. Biphenyl hydrolase-like protein (BPHL) was identified as the new HCTLase. Recombinant BPHL protein was expressed and characterized. Liver lysates were incubated with specific inhibitors to determine whether the liver HCTLase was attributable to PON1, Blmh, or BPHL. Plasma and recombinant PON1s were also characterized for HCTLase kinetic properties.

Materials and Methods

Enzyme assays

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Clopidogrel thiolactone, prasugrel thiolactone and the exo-thiol metabolites were generous gifts of Eli Lilly.

HCTLase activity was measured using a modification of the Ellman cholinesterase method[27]. A fresh solution of 5, 5' dithio-bis-(2-nitrobenzoic acid) (DTNB) (10.3 mM) was prepared in 100 mM $NaPO_4$, pH 7.0, and was added at a final concentration of 0.32 mM to a solution of 3 mM L -HCTL, in Dulbecco's PBS, with the volume dependent on the number of assays to be carried out. The substrate/DTNB solution (200 µL) was added immediately to enzyme samples (5-20 µL) in 96-well visible wavelength microplates. The assay was monitored at 405 nm for 4 min at 25^0C for column fractions or at 37^0C for kinetic analyses in a SPECTRAmax® PLUS Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Inhibitors were added to the assays at levels indicated in the figures.

GTBLase activity was measured by preparing a 28 mM solution of GTBL in Dulbecco's PBS. Just prior to use, a fresh solution of DTNB (10.3 mM) was prepared and added as described above. The substrate and DTNB solution (200 µL) was added immediately to samples (5-20 µL) in 96-well visible microplates. The assay was monitored at 405 nm for 4 min at 37^0C.

A clopidogrel thiolactone solution was prepared as described above; however, the stock solution of clopidogrel thiolactone was at a concentration of 1 mM in methanol and was diluted to a final concentration of 5 µM. DTNB was prepared and added as described above. The

substrate and DTNB solution (200 µL) were added immediately to samples (5-20 µL) in 96-well visible microplates. The assay was monitored at 405 nm for 4 min at 37^0C.

Dihydrocoumarin activity was measured by diluting a 100 mM stock solution of dihydrocoumarin in methanol into 50 mM Tris-HCl, pH 8.0 buffer for a final concentration of 1 mM dihydrocoumarin. The solution was vigorously mixed and added (200 µL) immediately to samples (5-20 µL) in 96-well UV microplates. The assay was monitored at 270 nm for 4 min at 37^0C.

Phenyl acetate activity (arylesterase) was determined by adding 200 µL of a 3.26 mM mixture of phenyl acetate in 9 mM Tris-HCl, pH 8.0, 0.9 mM $CaCl_2$ immediately to samples (5-20 µL) in 96-well UV microplates. The assay was monitored at 270 nm for 4 min at 25^0C.

The 2-coumaranone activity was measured by addition of 200 µL of 1 mM 2-coumaranone in 50 mM Tris-HCl, pH 8.0 buffer to samples (5-20 µL) in 96-well UV microplates. The assay was monitored at 274 nm for 4 min at 25^0C.

Activities are expressed in Units/mL or Units/mg protein (Units=µmol hydrolyzed/min), based on the molar extinction coefficient of 1.310 $mmol/L^{-1}cm^{-1}$ for the hydrolysis of phenyl acetate, 14.15 $mmol/L^{-1}cm^{-1}$ for the hydrolysis of DTNB, 0.876 $mmol/L^{-1}cm^{-1}$ for the hydrolysis of dihydrocoumarin, and 1.295 $mmol/L^{-1}cm^{-1}$ for the hydrolysis of 2-coumaranone[28]. Only the initial linear rates of hydrolysis were used for calculations.

Purification of human liver HCTLase

Liver tissue was obtained from the Human Liver Bank maintained within the School of Pharmacy at the University of Washington[29]. Samples were obtained from deceased, anonymous

individuals with approval of the Institutional Review Board (IRB) at the University of Washington. A 33 g human liver sample (pooled from five individual donors) was homogenized in 76 mL of 20 mM Tris-HCl (pH 8.0) buffer on ice using a Polytron tissue homogenizer. The sample was centrifuged at 17,000 x g in a Sorvall RC5B centrifuge for 40 min at 4^0C. The supernatant was centrifuged at 424,000 x g in a Beckman TL-100 ultracentrifuge for 30 minutes at 4^0C.

The soluble supernatant fraction (58 mL) was loaded onto a 25 mL DEAE (diethylaminoethyl) Sepharose Fast Flow (GE Healthcare, Piscataway, NJ) column (2.8 x 5.5 cm) equilibrated with 20 mM Tris-HCl, pH 8.0. The HCTLase activity did not bind to the column. The flow-through fractions with HCTLase activity were adjusted to pH 7.0 with 0.1N HCl, then loaded onto a 20 mL column of CM (carboxymethyl) Sepharose Fast Flow (1.8 x 8 cm) (GE Healthcare) that had been equilibrated with 20 mM Tris-HCl, pH 7.0 (CM buffer). The column was washed with 6 resin bed volumes (rbv's) of CM buffer, then eluted with 20 rbv's of a 0-600 mM NaCl linear gradient in CM buffer. Active fractions were pooled and loaded onto a 2 mL ceramic hydroxyapatite (HA) column (1 x 4 cm, HA type II 40 µm, BioRad, Hercules, CA) that was equilibrated with 5 mM KPO_4, pH 6.9. The HA column was washed with equilibration buffer until the A_{280} reached baseline level, then eluted with a 25 rbv gradient from 5-200 mM KPO_4, pH 6.9. HCTLase-containing fractions were pooled and concentrated to 0.5 mL with a 10,000 molecular weight cut off (MWCO) Amicon Ultra centrifugal filter (EMD Millipore, Billerica, MA) at 1,500 x g. The HA pool was then loaded onto a 25 mL Superdex 200 (GE Healthcare) (1.2 x 32 cm) column equilibrated with 20 mM Tris-HCl (pH 7.0), 100 mM NaCl (equilibration buffer) and eluted with the same buffer. HCTLase-containing fractions were pooled, diluted 1:2 with 20 mM Tris-HCl, pH 7.0 and loaded onto a 0.5 mL CM Sepharose

column (0.5 x 3 cm) equilibrated with CM buffer. The column was eluted with a gradient from 0-600 mM NaCl in 20 rbv's of CM buffer. The fractions containing HCTLase activity were pooled and concentrated to 0.2 mL on a 10,000 MWCO Amicon Ultra concentrator at 1,500 x g, then loaded onto a 5 mL Superdex 200 column (0.8 x 22 cm) in 20 mM Tris-HCl, pH 8.0. Fractions containing HCTLase activity were pooled and stored at 4^0C. Protein concentrations were measured with the BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL).

In-gel activity and Coomassie Blue staining

Human liver purified BPHL and rBPHL (described below) were in-gel activity stained using an adapted protocol from Korenovsky et al.[30]. Both enzymes were first buffer-exchanged into deionized water using Zeba™ spin desalting columns (Pierce Chemical, Rockford, IL) and then surface loaded on a 4.5 x 8 cm native IsoGel® agarose isoelectric focusing (IEF) gel with a pH range of 6-10.5 (Lonza, Walkersville, MA). The anode solution used was 10 mM acetic acid (Thermo Fisher Scientific), and Cathode Fluid 10 was used as the cathode solution (SERVA Electrophoresis GmbH, Heidelberg, Germany). The IEF gel was electrophoresed at 4°C up to 60 V/cm over a period of 50 min (250 V maximum). Lysed human red blood cells were used as visible markers to ensure that the focusing of the proteins was completed. Following electrophoresis, half of the gel was used for the HCTLase activity stain and the other half for a Coomassie Blue protein stain. For the activity stain, the gel was washed with deionized water for 5 min and then equilibrated with reaction buffer [0.1 M sodium acetate, 20 mM glycine, 4 mM cupric sulfate, 24% (w/v) sodium sulfate and 3 mM magnesium chloride (J.T. Baker Chemicals-Avantor Performance Materials, Center Valley, PA)] without substrate for 2 min. Then, the gel was incubated with 3.8 mM HCTL in reaction buffer with shaking for 1 h at room temperature.

Following incubation with substrate, the gel was washed in deionized water for 5 min followed by a 3 M ammonium sulfate wash for 5 min. The gel was developed with 0.16% w/v dithio-oxamide in deionized water (Fluka) until appearance of brown activity bands of copper- thio-oxamide was observed. For the Coomassie Blue stain, the gel was fixed for 15 min in a solution of 36% (v/v) methanol, 6% (w/v) trichloroacetic acid and 3.6 % (w/v) sulfosalicylic acid. After rinsing with deionized water, the gel was stained in Coomassie Blue stain solution [0.1%(w/v) Coomassie brilliant blue (Amresco, Solon, OH), 25% (v/v) ethanol and 9% (v/v) acetic acid (both from Thermo Fisher Scientific)] overnight at room temperature and de-stained in 25% (v/v) ethanol with 9% (v/v) acetic acid for 3 h.

Inhibition assays

Human liver homogenates were incubated with specific inhibitors for BPHL, Blmh, or PON1. Briefly, 1 g of frozen human liver was thawed and homogenized on ice using a Tissue-Tearor hand-held homogenizer (Cole-Parmer, Vernon Hills, IL) in 1.5 mL of 20 mM Tris-HCl, pH 8.0 supplemented with 1 mM $CaCl_2$ (to preserve PON1 activity). Liver homogenates were centrifuged as described above. The obtained supernatants were incubated for 10 min with BPHL inhibitors valacyclovir (VACV; 20 mM) or L-proline benzyl ester (10 mM); Blmh inhibitors E-64 (50 μM) or iodoacetamide (2 mM); and PON1 inhibitors EDTA (10 mM) or 2-hydroxyquinoline (200 μM). Then, HCTL hydrolysis was monitored as described above but using 10 mM HCTL as substrate.

Cloning of human liver BPHL

A clone of human liver BPHL (BC106901) was obtained from Source Bioscience (Nottingham, UK). The 770 bp BPHL gene was amplified with primers (Thermo Fisher Scientific) containing an NdeI restriction site [(forward) CAG CAG CCA TGG GCA TGC CCA GGA ATC TGC TT] and NcoI [CAG CAG CAT ATG TCA TTG TAG GAA GTC TTC TGC (reverse)]. The resulting PCR product was cloned using the Invitrogen/Life Technologies (Grand Island, NY) TOPO® TA Cloning Dual Promoter Kit, following the manufacturer's protocol. The pET15 expression vector (EMD Millipore) and the TOPO-BPHL clone were digested with NdeI and NcoI, ligated with T4 Ligase (NEB, Ipswich, MA), then transformed into XL10-Gold competent subcloning cells (Agilent, Santa Clara, CA). The pET15-BPHL insert was sequenced to verify the fidelity of the sub-cloning protocol. The sequence-verified construct was transformed into Rosetta-Gami 2(DE3) expression cells (EMD Millipore).

Expression of rBPHL

Rosetta-Gami 2(DE3) cells transformed with the pET15-BPHL were grown overnight and inoculated into 16 L of LB media containing carbenicillin at 50 µg/mL to obtain an A600 of ≈0.1 followed by growth at 25^{0}C. Cells were induced at an A_{600} of 0.6 with 1 mM IPTG and grown for an additional 12 hours. Cells were harvested by centrifugation at 4,800 x g for 20 min at 4^{0}C. Aliquots (0.1 g) of induced and uninduced cells were solubilized with BPER II Protein Extraction Reagent (Pierce Chemical) for analysis of HCTLase activity. The remaining induced cells (19 g) were aliquoted and frozen at -20^{0}C.

Purification of rBPHL

Frozen, induced *E. coli* cells (9.6 g) were thawed on ice, then lysed and extracted with 2 volumes (w/v) of B-PER II containing 20 units/mL of Benzonase (EMD Millipore) and Protease Inhibitor Cocktail Set III, EDTA-Free (EMD Millipore) diluted 1:200. The lysed, resuspended cells were gently mixed and incubated at room temperature for 30 min. Insoluble particulate was removed by centrifugation at 12,000 x g for 20 min at 4^0C. The soluble supernatant fraction was loaded onto a 10 mL DEAE column (GE Healthcare) (1.5 x 6 cm) equilibrated with 20 mM Tris-HCl (pH 8.0) buffer. The HCTLase activity did not bind to the DEAE column. The pooled flow-through was adjusted to pH 6.9 with 0.1N HCl and loaded onto a 10 mL ceramic HA column (1.2 x 8 cm) then washed with 5 mM KPO_4 (pH 6.9) buffer, and eluted with a 20 rbv 5-225 mM KPO_4 gradient. The active fractions were pooled and concentrated to 1.0 mL with a 10,000 MWCO Amicon Ultra centrifugal filter at 1,500 x g, then loaded onto a 75 mL Superdex 200 (1.8 x 45 cm) column equilibrated with 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and eluted with the same buffer. Fractions containing HCTLase activity were analyzed for purity by SDS-PAGE.

Mass spectrometric analysis of the purified proteins

The purified liver BPHL and rBPHL were sequenced by high-resolution mass spectrometry (MS). Five μg of the purified hepatic protein were loaded to a denaturing NuPAGE® 4-12% Bis-Tris gel (Life Technologies). After staining the gel with Imperial Protein Stain Solution (Thermo Fisher Scientific), three bands were excised and saved for protein sequencing by MS. The excised bands were reduced in-gel with 10 mM dithiothreitol (DTT) and alkylated with 55 mM iodoacetamide (IAA). The carbamidomethylation step was followed by

in-gel digestion with 2 μg of porcine trypsin (Promega, Madison, WI), with shaking at 37^0C overnight. The generated tryptic peptides were extracted with 5% formic acid (v/v): acetonitrile (1:2), evaporated to near dryness (CentriVap vacuum concentrator, Labconco SpeedVac, Kansas City, MO) and reconstituted in 20 μL of 2% acetonitrile, 0.1% formic acid.

Five μg of the purified rBPHL were digested in solution. The surfactant RapiGest^{TM} SF (Waters Corporation, Milford, MA) was added to a final concentration of 0.1% and the sample was reduced with 5 mM DTT and alkylated with 15 mM IAA. Then, the samples were digested with 1 μg of porcine trypsin, shaking at 37^0C for 2 h. Following digestion, hydrochloric acid was added to a final concentration of 100 mM to hydrolyze the surfactant. The white precipitate formed was separated by centrifugation.

Both gel extracted peptides and peptides obtained by in-solution digestion were separated by nanoflow chromatography in a Waters nanoACQUITY UltraPerformance liquid chromatography® (UPLC®) system (Waters Corporation). A 20 cm, 75 μm fused I.D. silica capillary column was used, with a 5 μm I.D. tip pulled using a P-2000 CO_2 laser puller (Sutter Instrument Company, Novato, CA). The column was packed in-house to the stated length with Jupiter 4 μm Proteo 90Å C12 reversed-phase resin (Phenomenex, Torrance, CA). The peptides were eluted using a 50 min gradient of 2-32% acetonitrile gradient in 0.1% formic acid, at 250 nL/min, and analyzed by an LTQ-Orbitrap (Thermo Fisher Scientific). The SEQUEST algorithm[31] was used for database searches of the tandem MS output, with a 57.021464 Da static carbamidomethyl modification on cysteine residues.

Mass spectrometric analysis of the products of HCTL hydrolysis

HCTLase (1 or 10 ng; for VACV or HCTL, respectively) was pre-incubated in PBS buffer at 37^0C and shaken at 70 rpm in a water bath for 2 min prior to addition of substrate (VACV or HCTL at 100 µM final concentrations) in a 200 µL reaction volume. Reactions were quenched at time points of t = 0 and t = 5 min with the addition of 20 µL of a 15% aqueous $ZnSO_4$ solution on ice, then centrifuged to remove protein and buffer salts. Supernatants were analyzed by LC-MS/MS.

Reaction products were analyzed with a Micromass Quattro Micro API, Tandem Quadrupole Mass Spectrometer (Waters Corporation) equipped with a Shimadzu HPLC system, consisting of two LC-10AD pumps, an SCL-10Avp controller, and an SIL-10ADvp autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD). The MS was run in positive electrospray ionization mode at a source temperature of 120^0C and a desolvation temperature of 330^0C. The cone voltage was set at 20 volts and the collision energy was set to 20 eV. The following mass transitions were monitored in separate ion channels: *m/z* 117 > 89 (for HCTL) and *m/z* 136 > 89 (for Hcy) for the thiolactonase assay, and *m/z* 326 > 152 (for VACV) and *m/z* 226 > 152 (for acyclovir) for the VACV assay. Hcy and HCTL were separated on a Zorbax CN 4.6 x 250mm HPLC column (Dupont Instruments, Cincinnati, OH) using an isocratic mix of 10% methanol and 90% aqueous (0.05% formic acid) with a flow rate of 0.7 mL/min. Acyclovir and VACV were separated using the same column, solvents and flow rate, but with an isocratic mix of 20% methanol and 80% aqueous (0.05% formic acid).

Results

Purification of human liver HCTLase

The initial purification followed HCTLase activity of a human liver homogenate through several column chromatography steps. The chromatographic steps for purification of the liver HCTLase are shown in Figure 5.1A-E and the purification is summarized in Table 5.1. Following the final Superdex-200 column step, active fractions were pooled and analyzed with SDS-PAGE (Figure 5.2A). Three major bands were observed with masses of ≈44 kDa, 34 kDa, and 30 kDa. All three were excised and digested in-gel with trypsin for MS analysis. The most probable match for the highest molecular weight band was acetyl-coenzyme A acyltransferase 1, with a sequence coverage of 55.2%. The ≈34 kDa band was identified as hydroxyacyl-coenzyme A dehydrogenase, with a 56.4% sequence coverage. Finally, the low molecular weight band at 30 kDa was identified as BPHL, with a sequence coverage of 56.6%. As the HCTLase activity had eluted at a mass of ≈30 kDa on the calibrated Superdex-200 column, we chose to pursue BPHL as the active protein. In addition, the ≈30 kDa protein band was shown to have HCTLase activity on an IEF activity-stained gel (Figure 5.3A).

Characterization of purified human liver BPHL

The substrate concentration dependence and pH optimum of the HCTLase activity of the partially-purified liver protein identified as BPHL were characterized as shown in Figure 5.4A. Human liver BPHL had a specific activity of 34.6 μmol/min/mg when assayed at 3 mM substrate, a sub-saturating concentration (Table 5.1). The K_m value of a partially-pure BPHL for HCTL hydrolysis was 3.92 mM (Figure 5.4A). However, the protein was one of three main bands visualized after the liver purification. This would not affect the K_m, but likely affected the

specific activity. A previous research team had expressed a bacterially-produced recombinant BPHL (rBPHL)[32-34] and purified the recombinant enzyme to homogeneity without the use of protein tags. To confirm that BPHL was indeed the liver HCTLase and to carry out a more detailed substrate analysis, we also chose to express rBPHL in *E. coli*.

Expression and purification of rBPHL

Following putative identification of BPHL, the most likely candidate responsible for the HCTLase activity, a cDNA clone of BPHL was subcloned, and recombinant BPHL (rBPHL) protein was expressed in *E. coli*. The expressed rBPHL was purified using a series of column chromatographic steps based on our initial liver purification as well as previously-published rBPHL purifications[32-34]. The chromatographic purification steps are shown in Figure 5.1F-G. The purified protein had the same molecular weight as the partially-purified liver HCTLase and rBPHL from the literature[32]. A high degree of protein purity - greater than 90% pure as assessed by gel electrophoresis - was obtained after the three column chromatographic steps (Table 5.2). The purification steps are shown using SDS-PAGE analysis in Figure 5.2B. The major protein after purification was a ≈30 kDa band which was shown to have HCTLase activity and was identical to the liver ≈30 kDa protein band when both were run on an IEF gel and stained with Coomassie Blue protein stain or stained for HCTLase activity (Figure 5.3). To confirm the identity of the purified recombinant protein, we digested it with trypsin and analyzed the peptides by LC-MS/MS. The obtained spectra were searched against the same peptide database used for the purified human liver BPHL. Human BPHL was the most abundant protein, with a 51.9% sequence coverage.

Characterization of rBPHL activity

The HCTLase substrate specificity of highly-purified rBPHL was determined. The rBPHL was assayed with 3 mM substrate (a sub-saturating concentration, performed as previously with the partially-purified liver BPHL) and was determined to have high HCTLase activity. The specific activity was 230 μmol/min/mg and the K_m value for HCTL was 3.18 mM (Figure 5.4B). The rBPHL had a V_{max} of 454 μmol/min/mg and a catalytic efficiency of 7.7 x 10^4 M^{-1} sec^{-1} (k_{cat}/K_m). HCTLase activity as a function of pH was also examined; maximal activity was found at pH 8.0 (Figure 5.4C). As described in previous reports, the rBPHL metabolized both valacyclovir (VACV), an anti-viral drug, and L-proline benzyl ester (LPBE), for which it had very high activity[32, 34]. We confirmed that rBPHL hydrolyzed both HCTL and VACV by MS (Figure 5.5A-D); the rBPHL hydrolytic reaction produced the expected end products of Hcy and acyclovir (Figure 5.5). Besides HCTL, several other potential substrates were tested. GTBL is a commonly-used alternative substrate for assaying PON1 plasma HCTLase activity[18, 19]. Therefore, it was surprising to find that GTBL was not hydrolyzed by the rBPHL. PON1 is a lactonase that hydrolyzes a number of lactone substrates[10, 12]. Since rBPHL has thiolactonase activity, other PON1 lactone substrates such as dihydrocoumarin, 2-coumaranone, and the thiolactone metabolites of the platelet-inhibiting drugs clopidogrel and prasugrel were tested as substrates for rBPHL. The rBPHL had no measurable hydrolytic activity against any of these lactone substrates (Table 5.3).

Several compounds were also tested as inhibitors of HCTL hydrolysis by rBPHL. As reported previously, rBPHL has high activity against VACV and LPBE, with the latter reported as the best substrate for BPHL[34]. We assessed whether these substrates could act as inhibitors of

the HCTLase activity of rBPHL. Both substrates inhibited HCTLase activity, with almost complete inhibition seen by VACV and LPBE concentrations of 5 mM (Figure 5.5). In addition, EDTA, which irreversibly inhibits human PON1, had no effect on BPHL activity at a concentration of 10 mM.

Inhibition of liver HCTLase

Our results confirmed previous reports that BPHL was not inhibited by EDTA[32], which is a known inhibitor of PON1. In addition, the same report found that E-64 failed to inhibit rBPHL activity[32]. To determine whether the HCTLase present in human liver homogenate was mainly attributable to BPHL, PON1, or Blmh, we assayed the HCTLase activity of human liver homogenate after incubation with specific inhibitors for PON1 (EDTA and 2-hydroxyquinoline), Blmh (E-64 and iodoacetamide) and BPHL (VACV and LPBE). PON1 inhibitors EDTA (10 mM) and 2-hydroxyquinoline (200 μM) and the Blmh inhibitor E-64 (50 μM) did not significantly decrease the liver HCTLase activity. The Blmh-specific inhibitor iodoacetamide (2 mM) was found to partially inhibit human liver HCTLase, but rBPHL activity was inhibited as well (by ≈50%). Only the BPHL inhibitors VACV (20 mM) and LPBE (10 mM) significantly inhibited liver homogenate activity, with HCTLase inhibited 74 and 88% respectively (Table 5.4).

BPHL and PON1

To further compare the activities of BPHL and PON1, we assayed the HCTLase activity of purified human plasma PON1 and purified recombinant human PON1 (rHuPON1). Other investigators have reported specific activities for the hydrolysis of HCTL by purified PON1 and for activity in Units/liter for human plasma hydrolyzing HCTL and GTBL (Table 5.5). Both

purified human plasma PON1 and purified rHuPON1 were assayed for HCTLase activity. Human plasma PON1 was purified as previously described[35]. The $rHuPON1_{K192}$ was cloned, expressed, and purified as reported in Stevens et al.[36] and Chapter 2 of this work. This $rHuPON1_{K192}$ contains an amino acid substitution at position 192 and has higher activity towards several PON1 OP substrates when compared to the native human PON1[36]. The initial discovery of PON1 as an HCTLase found that PON1's specific activity for HCTL was 1.3 μmol/min/mg. However, later reports gave much lower estimates, from 0.0014-0.011 μmol/min/mg. The $rHuPON1_{K192}$ had a higher specific activity compared with the reported values of purified human plasma PON1 at 0.0146 μmol/min/mg (Table 5.5). Purified $PON1_{Q192}$ had a specific activity of 0.0045 μmol/min/mg. However, both purified serum and recombinant PON1 had very low HCTLase activity relative to BPHL (Table 5.5).

Albumin and DTNB

To examine the basis of albumin interference of the Ellman assay, we monitored the rate of appearance of 2-nitro-5-thiobenzoic acid (NTB) in the absence of added substrate. Both purified and recombinant serum albumin exhibited a rate of appearance of NTB, characteristic of the enzymatic hydrolysis of thiolactones and thio esters (Figure 5.7), thus, it is important to be cognizant of this background activity when measuring rates of formation of free sulfhydryl groups in samples containing albumin. Albumin with DTNB alone exhibits activity similar to that observed with PON1 in the presence of HCTL, so controls must be carried out to correct for the "albumin interference" when using DTNB as a substrate to measure HCTLase with serum or plasma samples. EDTA at a concentration of 10 mM did not affect the rates of the DTNB/serum assay.

Discussion

Hcy can cyclize to form the more toxic lactone HCTL through error-editing reactions catalyzed by tRNA synthetases. HCTL homocysteinylates proteins, leading to progressive loss of enzymatic activity, denaturation, precipitation, and autoimmune responses[9, 37]. Previous reports have identified PON1, a plasma esterase/lactonase, as an HCTL-metabolizing enzyme; however, the specific activity is very low[1, 10]. Another tissue-specific HCTLase, Blmh, was identified by the same research team[20]. We have purified an HCTLase enzyme from human liver and determined that it is a serine hydrolase previously identified as valacyclovirase (VACVase) or VACV hydrolase, also known as BPHL. Recombinant BPHL was expressed and its HCTLase activity characterized. The rBPHL has a high catalytic efficiency for hydrolysis of HCTL and also differs from PON1 in its substrate specificity. Previously, a physiological role for BPHL was unknown[34, 38] although it was believed to have an important physiological role as it has genomic feature indicative of constitutively expressed genes[34, 39]. Due to its localization in the liver and kidney using Northern blots (with lower levels in intestine, heart, and skeletal muscle), BPHL was suggested to be involved in detoxification and drug metabolism pathways[34, 40]. The discovery of BPHL's role in converting the prodrug VACV to acyclovir identified one such pathway[32]. However, proteins did not evolve to metabolize xenobiotics such as antiviral drugs. The identification of BPHL as the major liver HCTLase and its high catalytic efficiency for HCTLase activity relative to PON1 and Blmh indicates an important physiological role for this protein and should open up new areas of inquiry.

The discovery of the HCTLase activity of BPHL occurred serendipitously. We initially began this study with the aim of identifying the thiolactonase activity responsible for the second

metabolic step in the bioactivation of clopidogrel since it was clear that PON1 was not the physiologically-relevant enzyme for this second step of bioactivation[41, 42]. The identity of the physiological substrate of PON1 has been the matter of some debate. One candidate for a physiological PON1 substrate is HCTL, proposed by Jakubowski et al[1]. PON1 has low activity and low binding efficiency for HCTL - the ranges reported for human plasma or serum (Table 5.5) are from 110-941 Units/ml and the ranges of specific activity for purified plasma PON1 enzyme are from 0.0045-1.3 μmol/min/mg with a K_m value of 23 mM[1]. It was claimed that PON1 was the main HCTLase in plasma.

After an initial report stating that PON1 was the enzyme responsible for the bioactivation of clopidogrel[43], a number of reports were published refuting this claim[41, 44]. PON1 can generate an endothiol from clopidogrel thiolactone; however, it is not the biologically active metabolite[42]. Our initial efforts were aimed at identifying the thiolactonase responsible for the clopidogrel metabolism. We chose HCTL as a convenient thiolactonase substrate. Following purification of the liver HCTLase activity, SDS-PAGE analysis revealed several protein bands of 30-40 kDa (Figure 5.2A). However, these bands were smaller than previously reported HCTLases, PON1 and Blmh.

The SDS-PAGE bands identified from the liver HCTLase purification were too small to be either PON1 or Blmh, the known HCTLases. Although PON1 is expressed in the liver, the bands were not PON1, as they were around ≈30 kDa and PON1 is a 45 kDa enzyme. As the bands were excised and identified using MS, we excluded identification of PON1 as well as Blmh, which is a 48 kDa protein. MS analysis identified BPHL as the most likely candidate; production and characterization of rBPHL confirmed this.

Before this discovery of the HCTLase activity of BPHL, previous studies had mainly focused on its drug metabolizing activities. BPHL is also called VACV hydrolase or VACVase due to its bioactivation of the anti-herpetic prodrug VACV; other prodrugs such as valganciclovir are also metabolized[32, 33]. BPHL is a serine hydrolase named for its similarity to bacterial enzymes such as *Pseudomonas bphD* - genes involved in the degradation of biphenyl compounds which are persistent organic environmental contaminants. Although BPHL cannot degrade the biphenyls, it has some similarities to the bacterial hydrolases – an active-site serine and catalytic triad with a GXSXG motif around the serine[40]. The BPHL gene, comprised of 8 exons and 7 introns, is a 30 kb gene on chromosome 6p25 and has a similar organization to some other hydrolytic enzymes such as conserved exon-intron distributions[39]. This protein is also distantly related to other serine hydrolases[39, 40]. The rBPHL was about 30 kDa, the same size as purified human liver BPHL, indicating a lack of post-translational modification. BPHL has activity against *p*-nitrophenyl butyrate, a standard serine hydrolase substrate, but no activity against biphenyl compounds. Although BPHL does not degrade biphenyl compounds, it was suggested that the enzyme could potentially be involved in drug metabolism and detoxification due to its localization in the liver and kidneys[40].

Although no physiological substrates were identified previously, the substrate specificity of BPHL has been extensively examined. A number of amino acid prodrugs are converted by BPHL into the therapeutic compounds – acyclovir, ganciclovir, floxuridine, zidovudine[32, 33] - and a variety of other amino acid esters were substrates as well. L-isomers of the prodrugs were preferentially hydrolyzed[33]. In previous studies, typical esterase (*p*-nitrophenyl acetate, *p*-nitrophenyl butyrate) and peptidase substrates (amides such as Lys-*p*-nitroanilide) were tested. BPHL had almost no activity for any of the substrates except *p*-nitrophenyl butyrate, for which it

had only limited activity[32, 40]. In this study, we found that the L-HCTL isomer was also preferentially hydrolyzed. Although BPHL hydrolyzed the lactone HCTL, it failed to hydrolyze other lactones hydrolyzed by PON1 such as dihydrocoumarin and 2-coumaranone. Kim et al.[33] reported that BPHL substrate range was distinct from those of porcine kidney carboxylesterase and porcine kidney leucine aminopeptidase. In this report, BPHL's specificity and catalytic efficiency are shown to differ from that of PON1. BPHL also had no activity against clopidogrel or prasugrel (another platelet inhibitor) thiolactones (Table 5.3).

In this study, we also tested BPHL activity against GTBL, a commonly-used surrogate substrate for HCTL. Determining HCTLase activity and levels is of importance due to the toxicity of HCTL and its association with disease. However, researchers cite the problems of albumin interference and degradation of HCTL at alkaline pH and have used GTBL to assess lactonase activity of plasma and purified PON1[7, 18, 19, 45]. BPHL and Blmh catalyze the HCTL hydrolysis reaction but not GTBL[20]; measurements of GTBL to determine HCTLase activity would exclude the activity of these two much more catalytically efficient enzymes. In addition, the results with DTNB and albumin indicate that albumin interference is an issue of concern with the GTBL assay (Figure 5.7). This study (and previous publications describing Blmh) point to the limitations of using GTBL as the substrate for measuring the HCTLase activities of BPHL and Blmh as they would not be detectable using GTBL as an alternate substrate[20].

The behavior and activity of the HCTLase BPHL can be compared and contrasted to PON1 and Blmh. PON1 and Blmh also have HCTLase activity; as seen with regards to size and substrate and inhibitor range, they differ substantially from BPHL. In previous reports, HCTLase activity has been attributed to PON1, then to Blmh[1, 20]; however, the data reported here strongly

suggest that the previously unrecognized HCTLase activity of BPHL is physiologically more relevant than PON1 for hydrolyzing HCTL and reducing the risk of vascular disease. Comparing the total HCTLase capacity of PON1 and BPHL supports this. With an average individual plasma volume of ≈3 L with ≈0.0057 μmol HCTL hydrolyzed/min/mL plasma (at 1 mM HCTL)[1], the HCTL detoxication capacity for plasma PON1 would be ≈17.1 μmol/min/individual. Our experiments measured liver HCTLase which was attributed to BPHL. In our purification, 33 g of human liver yielded 118.3 units of HCTLase (measured at 3 mM HCTL), which would equate to ≈1.195 U/g liver tissue at 1 mM HCTL (Table 5.1). Based on an average weight of a human liver of 1,414 g[46], the total HCTLase units in a liver would be ≈1,690 μmol/min/liver or 98.8-times the detoxication capacity of plasma PON1. This approximation does not take into account the levels of liver PON1 (which in mice represent a small percentage of total PON1), or BPHL levels in kidney and other tissues[40]. It is also useful to compare the catalytic efficiencies of HCTL hydrolysis by Blmh (1×10^3 M^{-1} s^{-1})[20] and PON1 with that of BPHL (7.7×10^4 M^{-1} s^{-1}) (this study). The reported catalytic efficiency of HCTL hydrolysis by Blmh was ≈100-fold higher than that of PON1 indicating that the catalytic efficiency of HCTL hydrolysis by BPHL is almost four orders of magnitude higher than that of PON1 and nearly two orders of magnitude higher than that of Blmh.

Expression and localization of the three HCTLases also differs. PON1 is expressed in the liver and associated with HDL in plasma although some results have indicated that its distribution is more widespread than previously believed[47]. Blmh is a tissue-specific HCTLase; initially PON1 was identified as the plasma HCTLase and Blmh as the tissue HCTlase[20]. The results in this report indicate that Blmh is not the only tissue HCTLase. BPHL is believed to be a membrane-bound and mitochondrial protein[32, 48] and is not present in the plasma. However,

BPHL is highly expressed in the liver, kidney, and intestine with lower levels found in the heart and skeletal muscle. Blmh is also expressed in a tissue-dependent manner. The highest levels are found in the skeletal muscle, testes, and pancreas with low levels in liver, kidney, and colon. Therefore, the expression patterns of the two are not identical and indicate that the importance of each enzyme with regard to HCTLase may vary between tissue types. It is also possible that Blmh and BPHL are equally important in eliminating HCTL from tissues. An alternative explanation is that BPHL is more physiologically-relevant as it has a higher catalytic efficiency compared to Blmh. To determine which enzyme predominates in liver, human liver homogenates were incubated with specific inhibitors for PON1, Blmh, and BPHL and assayed for residual HCTLase activity. Only BPHL inhibitors substantially reduced the liver HCTLase, and indeed a Blmh-specific inhibitor, iodoacetamide, which reduced liver HCTLase by ≈50% was also found to inhibit rBPHL to a similar extent. This strongly indicates that BPHL is the major liver HCTLase. However, the physiological role of BPHL and whether it protects against HCTL toxicity *in vivo* has yet to be determined.

We report here the purification of a human hepatic protein that demonstrates high HCTLase activity and was identified as BPHL by MS. When expressed in *E. coli*, the purified rBPHL also had high catalytic efficiency for HCTL. Both native and recombinant purified BPHLs have a much higher catalytic efficiency for hydrolysis of HCTL than purified plasma PON1, making BPHL a much more likely candidate for protecting against HCTL toxicity under physiological conditions. BPHL also has a higher efficiency towards HCTL than Blmh and is likely the predominant HCTLase in the liver. Patterns of expression of Blmh and BPHL appear to be complementary. Inhibition of liver extracts with specific inhibitors found that only BPHL-specific inhibitors significantly reduced liver HCTLase activity. BPHL has a different substrate

and inhibitor specificity from the other two HCTLases. This is the first report of a potential physiological substrate for BPHL and suggests a possible important physiological role for the protein. As Hcy and HCTL have been linked to CVD and a number of other disease conditions, BPHL may play an important role in human health and disease.

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This chapter was taken in large part from Marsillach et al.[49]. A number of contributors are responsible for the work described. Judit Marsillach, Rebecca J. Richter and Clement E. Furlong were responsible for planning the liver purifications. Judit Marsillach and Rebecca J. Richter carried out the liver homogenizations and purifications. Rebecca J. Richter performed the majority of the assays. Stephanie M. Suzuki (the author), Rebecca J. Richter and Clement E. Furlong were responsible for planning the cloning experiments. Stephanie M. Suzuki and Rebecca J. Richter carried out the cloning experiments and expression of the recombinant BPHL. Characterization of the enzymes was planned by Clement E. Furlong, Judit Marsillach, Rebecca J. Richter and Stephanie M. Suzuki and carried out by Rebecca J. Richter. Inhibition experiments were planned by Clement E. Furlong, Judit Marsillach, Rebecca J. Richter and Stephanie M. Suzuki and carried out by Judit Marsillach. The IEF gel experiments were planned by Judit Marsillach, Rebecca J. Richter and Clement E. Furlong and carried out by Tom Bukowski. Mass spectrometric identification of the protein and analysis of enzymatic activity was planned and carried out by Matthew G. McDonald, Peter M. Rademacher, Judit Marsillach, Edward J. Hsieh, Michael J. MacCoss, and Allan E. Rettie.

Figure 5.1

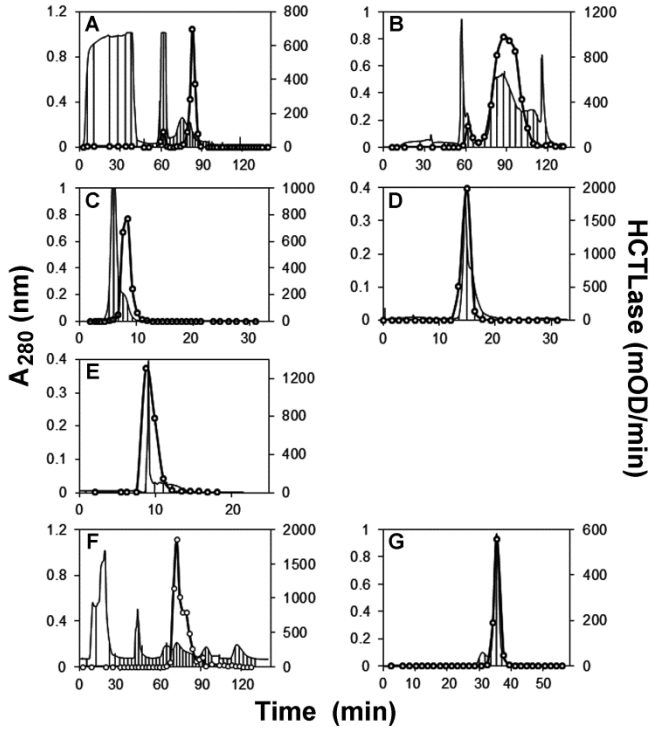

Figure 5.1. Protein absorbance (A_{280}) and HCTLase activity of column fractions from the chromatographic purification of human liver HCTLase (**A-E**) and recombinant rBPHL (**F, G**). A_{280} nm, solid lines; HCTL activity, solid lines with open circles. **A**, carboxymethyl (CM) column, **B,** ceramic hydroxyapatite (HA) column, **C**, Superdex 200 column, **D,** second CM column, **E**, Superdex 200 column, **F**, HA column, **G**, Superdex 200 column. Ticks represent fraction changes.

Figure 5.2

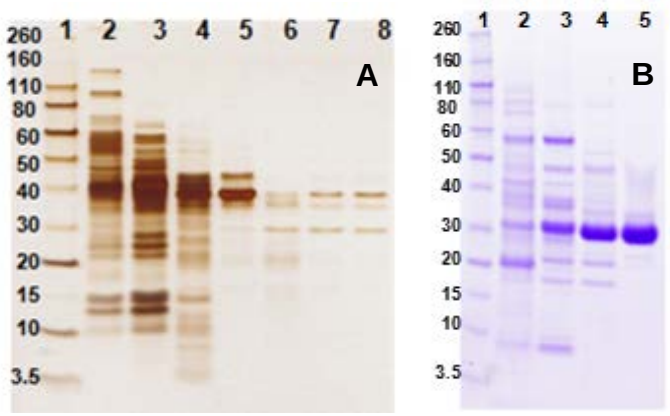

Figure 5.2. **A**, SDS/PAGE analysis of pooled column fractions from the purification of human liver HCTLase. **Lane 1**, molecular weight markers (kDa); **lane 2**, liver extract; **lane 3**, diethylaminoethyl (DEAE) pool; **lane 4**, CM-1 pool; **lane 5**, ceramic hydroxyapatite (HA) pool; **lane 6**, Superdex 200-1 pool; **lane 7**, CM-2 pool; **lane 8**, Superdex 200-2 pool. Gel was stained with a silver stain kit (Pierce). **B**, SDS/PAGE of pooled column fractions from the purification of recombinant human BPHL. **Lane 1**, molecular weight markers (kDa); **lane 2**, *E. coli* extract; **lane 3**, DEAE pool; **lane 4**, HA pool; **lane 5**, Superdex 200 pool. Gel was stained with Imperial Protein Stain (Pierce).

Figure 5.3

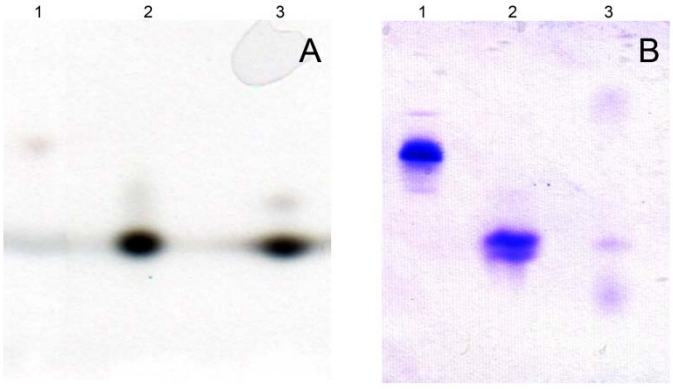

Figure 5.3. IEF activity and Coomassie stain of liver BPHL and rBPHL. (**A**) HCTLase activity stain of rBHPL (lane 2) and human liver BPHL (lane 3). Hemoglobin control shows a faint band as well (lane 1). (**B**) Coomassie Blue stain of hemoglobin control (lane 1), rBPHL (lane 2), and human liver BPHL (lane 3).

Figure 5.4

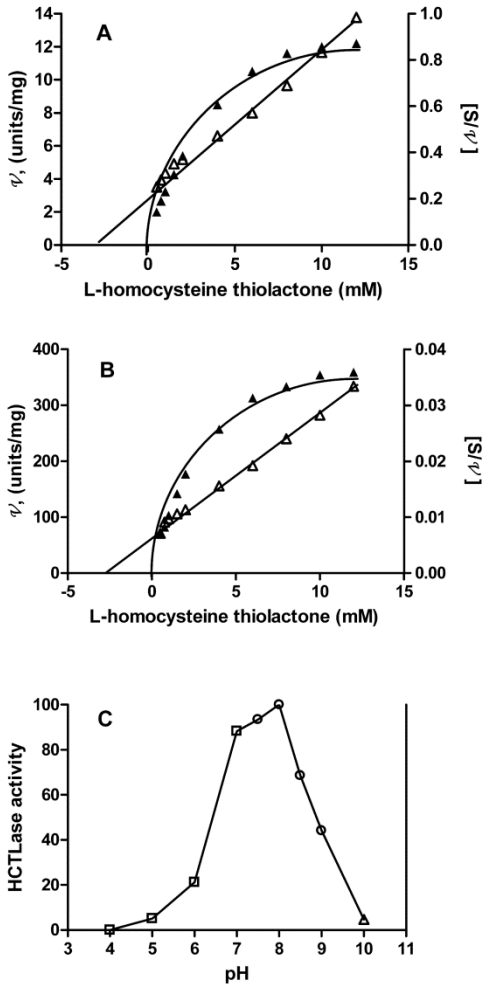

Figure 5.4. Substrate dependence of HCTL hydrolysis by purified human liver HCTLase (BPHL) (**A**) and recombinant rBPHL (**B**). Closed symbols, velocity (v) versus substrate concentration [S]; open symbols, [S]/v versus [S]. The K_m for HCTL hydrolysis by purified liver BPHL= 3.92 mM and for rBPHL K_m = 3.18 mM. **(C)** HCTLase activity of rBPHL (% maximal activity) as a function of pH. Open squares, □—□ 50 mM citrate/Na_2HPO_4; open circles ○—○, 50 mM Tris-HCl; open triangle,△—△ 50 mM Na_2PO_4.

Figure 5.5

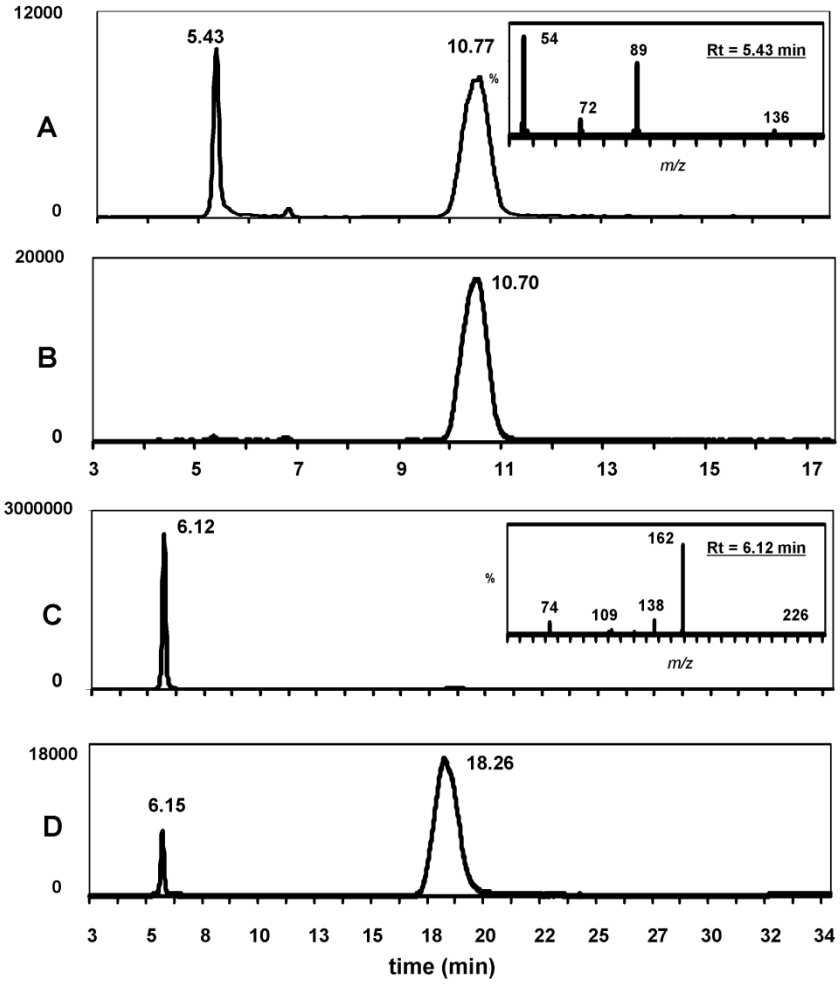

Figure 5.5. LC-MS chromatograms showing rBPHL-mediated HCTL cleavage at t = 5 (**A**) and t = 0 min (**B**) of reaction time and valacyclovir ester cleavage at t = 5 (**C**) and t = 0 min (**D**). Chromatograms A and B represent the sum of two MRM channels monitoring the following transitions: *m/z* 117 > 89 (for HCTL, Rt = 10.7 min) and *m/z* 136 > 89 (for Hcy, Rt = 5.4 min). The inset shows the daughter ion spectrum (for *m/z* 136) of the product peak at Rt = 5.4 min, and

is consistent with the spectrum obtained from commercial Hcy. Chromatograms C and D also represent the sum of two MRM channels monitoring the following transitions: *m/z* 326 > 152 (for VACV, Rt = 18.3 min) and *m/z* 226 > 152 (for acyclovir, Rt = 6.1 min). The inset shows the daughter ion spectrum (for *m/z* 226) of the product peak at Rt = 6.1 min, and is consistent with the spectrum obtained from commercial acyclovir. The small product peak at t = 0 in chromatogram D reflects the extremely rapid metabolism of VACV by human rBPHL, which generated a detectable amount of acyclovir in even the very short period of time (≈10s) between initiation and quenching of the enzymatic reaction.

Figure 5.6

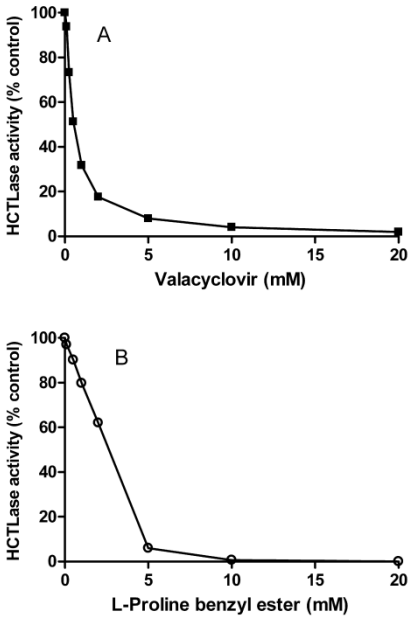

Figure 5.6. Concentration-dependent inhibition of rBPHL HCTLase by VACV (**A**) and L-proline benzyl ester (**B**).

Figure 5.7

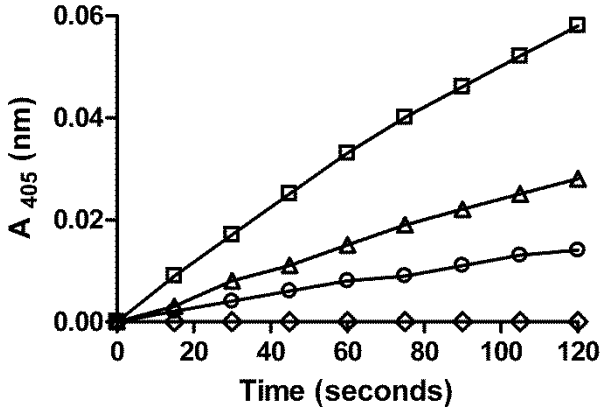

Figure 5.7. Reaction of human serum albumin (HSA) with Ellman's reagent (DTNB). Recombinant HSA (500 µg from *Pichia pastoris*) □—□; Human plasma (10 µL) Δ—Δ; Antibody purified HSA (200 µg) ○—○; No protein control ◇—◇.

Table 5.1. Purification of Human Liver HCTLase.

Purification Step	Volume mL	Total Protein mg	Total HCTLase activity Units*	Specific activity Units/mg	Fold Purification	Yield %
Liver extract	58.0	435.0	118.3	0.27	-----	100
DEAE	58.0	336.4	93.1	0.28	1.0	77.7
CM I	32.0	13.4	73.1	5.50	20.0	62.0
HA	8.0	8.3	48.5	5.80	21.4	41.0
Superdex I	3.5	1.5	41.7	27.90	102.7	34.7
CM II	2.5	0.8	27.6	34.10	125.5	23.4
Superdex II	2.2	0.7	23.6	34.60	127.0	20.0

DEAE indicates diethylaminoethyl; CM, carboxymethyl; and HA, hydroxyapatite.

*Units of HCTLase activity are μmol NTB produced per minute measured at ≈K_m concentration of substrate.

Table 5.2. Purification of Recombinant Human HCTLase.

Purification Step	Volume mL	Total Protein mg	Total HCTLase activity, Units*	Specific activity, Units/mg	Fold Purification	Yield %
Bacterial extract	18.0	410.0	3802	10.4	-----	100
DEAE	50.0	64.5	1284	19.9	1.9	34
HA	30.0	10.1	911	90.1	8.7	24
Superdex	6.5	3.0	857	230.4	22.1	23

DEAE indicates diethylaminoethyl; and HA, hydroxyapatite.

*Units of HCTLase activity are μmol NTB produced per minute measured at ≈K_m concentration of substrate.

Table 5.3. BPHL substrates

Compound	Specific Activity, Units/mg
L-homocysteine thiolactone	454.0 †
γ–thiobutyrolactone	< 0.001 †
Dihydrocoumarin	< 0.001 †
2-coumaranone	< 0.001 †
Phenyl acetate	< 0.001 †
DL-N-acetyl homocysteine thiolactone	< 0.001 †
Clopidogrel thiolactone	< 0.02 †
Prasugrel thiolactone	< 0.02 †
Valacylovir	103.7 ‡
L-Phe benzyl ester	358.3 ‡
L-Proline benzyl ester	5148.4 ‡
3-Amino-3-phenylpropionic acid ethyl ester	0.6 ‡
L-Ala benzyl ester	1367.7 ‡
ß-Ala benzyl ester	1.8 ‡
L-Gly benzyl ester	849.4 ‡
L-Leu benzyl ester	635.0 ‡
L-Val benzyl ester	156.3 ‡
L-Met methyl ester	156.2 ‡
L-Phe benzyl ester	358.3 ‡
L-Tyr benzyl ester	41.4 ‡
L-Trp methyl ester	35.3 ‡
L-Ser methyl ester	484.3 ‡
L-Phe ethyl ester	75.3 ‡

† from this study

‡ from Lai et al.[34]

Table 5.4. Inhibition of human liver HCTL activity

Inhibitor name	% HCTL activity*
No inhibitor	100
BPHL inhibitors	
Valacyclovir (20 mM)	25.9
L-Proline benzyl ester (10 mM)	12.0
Blmh inhibitors	
E-64 (50 μM)	97.5
Iodoacetamide (2 mM)	57.9 †
PON1 inhibitors	
EDTA (10 mM)	141.5
2-hydroxyquinoline (200 μM)	107.0

* HCTLase activity was measured at 10 mM concentration of substrate

† Iodoacetamide inhibited rBPHL to the same extent

Table 5.5. Activities of Thiolactonases.

Author	Year	Thiolactonase Substrate	Enzyme Source	umol/min/mg	umol/min/liter
Jakubowski[1]	2000a	S^{35} HCTL	purified HDL	0.01020 *	---
Jakubowski[1]	2000a	S^{35} HCTL	purified serum HCTLase	1.3	---
Stevens[36]	2008	HCTL/DTNB	purified recombinant PONK	0.01460	---
Bayrak[11]	2011	HCTL/DTNB	purified PON/HCTLase	0.0045 - 0.011	---
Results		HCTL/DTNB	purified Q192 PON1	0.0045	---
Togawa[50]	2005	HCTL/OPA	plasma	0.0014	---
Results	2013	HCTL/DTNB	purified liver BPHL	35	---
Results	2013	HCTL/DTNB	purified recombinant BPHL	230	---
Kosaka[18]	2005	HCTL/DTNB	whole human plasma	---	110 - 590
Sonoki[51]	2009	γTBL/DTNB	whole human plasma	---	276 - 355
Kosaka[18]	2005	γTBL/DTNB	whole human plasma	---	175 - 941
Sztanek[19]	2012	γTBL/DTNB	whole human plasma	---	166 - 332
Koubaa[45]	2008	γTBL/DTNB	whole human plasma	---	387 - 503

* converted from data reported in nmol/mg protein/hour

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