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Purification and characterization of functional human paraoxonase-1 expressed in *Trichoplusia ni* larvae

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ABSTRACT

Human serum paraoxonase-1 (HuPON1) is difficult to either purify from plasma or functionally express in high yield from recombinant sources. Here, we describe the characterization of functional HuPON1 expressed and purified from *Trichoplusia ni* (*T. ni*) larvae infected with an orally active form of baculovirus. SDS-PAGE and anti-HuPON1 Western blot analyses yielded only three bands of approximately 41, 42, and 44 kDa. MALDI-TOF confirmed the identity of each of these bands as HuPON1 with greater than 95% confidence. These isoforms result from differential glycosylation of the enzyme as indicated by peptide mapping, mass analysis, and PNGase F deglycosylation experiments. Recombinant insect-produced HuPON1 hydrolyzed phenyl acetate, paraoxon, and the nerve agents GF, VX, and VR. The enzyme had dramatic stereoselectivity for the P+ isomers of VX and VR. *T. ni* larvae expressing HuPON1 were remarkably resistant to the pesticide chlorpyrifos. Together, these results demonstrate that the caterpillar of the *T. ni* moth can be used as an expression system to produce large quantities of functional recombinant HuPON1. Insect production of HuPON1 may provide a source for both *in vitro* enzymatic and crystallographic studies and *in vivo* stability and anti-nerve agent efficacy testing.

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1. Introduction

Organophosphorus (OP) nerve agents readily bind covalently to acetylcholinesterase (AChE) at the active site serine and inhibit the ability of AChE to terminate cholinergic neural transmissions, resulting in a cholinergic crisis [1]. Drugs such as atropine, oximes, and diazepam, are administered post-exposure in an attempt to either reactivate AChE or mitigate the toxic symptoms of nerve agent exposure. While these treatments are effective in averting lethality if administered soon after nerve agent intoxication, they do not prevent performance deficits, behavioral incapacitation, loss of consciousness, or possible permanent brain damage [2]. Current research efforts in our laboratory have been directed at identifying an enzyme that could act as a catalytic bioscavenger of nerve agents *in vivo*.

Human serum paraoxonase-1 (HuPON1) is a Ca^{2+}-dependent and HDL-associated enzyme capable of utilizing multiple substrates such as aryl esters, lactones, oxidized phospholipids, and OP compounds [3–7]. Although the rate at which wild type HuPON1 catalyzes the hydrolysis of nerve agents is likely too slow to protect against a lethal dose of nerve agent, protein engineering could be used to improve this rate and make HuPON1 an effective scavenger of nerve agents. HuPON1 has been notoriously difficult to either purify from plasma or functionally express in high yield from recombinant sources, thereby making it difficult to conduct pharmacologic studies with the enzyme. Expression of HuPON1 from *Escherichia coli* (*E. coli*) has been successful, but only in low yields [8]. Although PON1 variants, generated by directed evolution, have been expressed in high yields in *E. coli* [9], these chimeric recombinant versions of HuPON1 differ from wild type by at least 51 amino acid substitutions, potentially increasing their immunogenicity in humans and thus reducing their functional utility as nerve agent bioscavengers. Hence, there is a need to identify an appropriate expression system for production of large quantities of fully functional HuPON1.

The aim of the present study was to assess the capacity of the caterpillar (4th to 5th instar larvae) from the *Trichoplusia ni*

Abbreviations: OP, organophosphorus; HuPON1, human serum paraoxonase-1; *T. ni*, *Trichoplusia ni*; AChE, acetylcholinesterase; DFP, diisopropyl fluorophosphate.

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(*T. ni*) moth to functionally express recombinant HuPON1, using the Chesapeake-PERL PERLXpress protein expression platform [10]. Here, we describe the characterization of HuPON1 expressed and purified from *T. ni* larvae infected with an orally active pre-occluded virus. This baculovirus encodes the complete amino acid sequence of HuPON1 with a C-terminal 6×-histidine tag. The results demonstrate that *T. ni* larvae are capable of producing high quantities of functionally active recombinant HuPON1, and larvae expressing HuPON1 display remarkable protection from the pesticide chlorpyrifos. Together, these results demonstrate that *T. ni* larvae are a viable alternative for expression of large quantities of functional HuPON1 that can be used for future *in vitro* and *in vivo* studies.

2. Materials and methods

2.1. Expression and purification of recombinant HuPON1

T. ni larvae for either colony propagation or baculoviral infection were maintained as previously described [11]. Larvae were infected with an orally active pre-occluded virus [11] that encoded wild type HuPON1 with glutamine at the polymorphic residue 192 as well as a C-terminal 6×-histidine tag. After clarification and filtration, samples of homogenized insect larvae were affinity purified (using immobilized metal-ion affinity chromatography, or IMAC), and protein was eluted using an imidazole gradient. Recombinant HuPON1 was further enriched by ion exchange and hydroxyapatite chromatography. Samples were analyzed by polyacrylamide gel electrophoresis and visualized using Coomassie blue using standard conditions. Western blotting analysis was conducted as described [3]. HuPON1 samples were stored at −80 °C after purification, and kept at 4 °C for short-term storage. Samples remained active at 4 °C for at least 2 weeks.

2.2. Enzyme hydrolysis assays

Hydrolysis of substrates phenyl acetate and paraoxon were conducted in activity buffer (50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.4) with 1.4 μg recombinant HuPON1 as previously described [3]. Kinetic parameters (K_M and k_{cat}) were determined by Michaelis–Menten steady state kinetics. The data were fit to the model using Prism 4.03 software (GraphPad, La Jolla, CA). R^2 values for the non-linear regression were greater than 0.99.

2.3. V-agent hydrolysis assays

Racemic VX (O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothiolate) and VR (O-isobutyl S-(2-diethylaminoethyl) methylphosphonothiolate) were obtained from the U.S. Army Edgewood Chemical Biological Center (ECBC, Aberdeen Proving Ground, MD). Analysis by nuclear magnetic resonance spectroscopy showed them to be 97% pure. Stock solutions of each V-agent were prepared at 0.9 mg mL^{-1} in activity buffer and stored at −70 °C. A modified Ellman-based colorimetric assay was used to measure hydrolysis of VX and VR in activity buffer with 1.4 μg HuPON1 [3]. Kinetic parameters (K_M and k_{cat}) with VX and VR were determined as described above. For stereoselective hydrolysis assays, 6 μg HuPON1 was incubated at room temperature with 3 mM VX or VR in activity buffer. At multiple time points, aliquots were removed, titrated to pH 10.5, and extracted with a 3-fold excess volume of hexane. After extraction, 3 μL samples of organic extract were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described previously [12]. A ChiralCel OD-H column (Chiral Technologies, West Chester, PA) was used to resolve the isomers with detection and quantity analyzed using atmospheric pressure chemical ionization (APCI) and an Agilent 6130 quadrupole MS (Foster City, CA, USA). Elution order of VX isomers was previously determined using a parallel analytical LC method with the P+ isomer eluting first [13]. Assignment of elution order for VR was based on analogy with elution of VX enantiomers. The non-hydrolyzed levels of VX or VR isomers in each sample were estimated by extrapolating the peak area under the curve based on the initial concentration of VX or VR with levels corrected for spontaneous hydrolysis.

2.4. Cyclosarin hydrolysis assay

Racemic cyclosarin (GF) was obtained from ECBC. Analysis by nuclear magnetic resonance spectroscopy showed it to be 95.5% pure. Stock solutions of GF in saline were prepared at 2 mg mL^{-1} and stored at −70 °C. HuPON1 was incubated with increasing concentrations of GF in Dulbecco's Modified Eagle Medium at room temperature. At selected time intervals, 100 μL aliquots were removed and inactivated through extraction with an equal volume of ethyl acetate containing 50 μM diisopropyl fluorophosphate (DFP; internal standard). The organic layer (containing non-hydrolyzed GF) was then removed, dried over molecular sieve beads, and analyzed by gas chromatography/mass spectrometry (GC/MS). The quantity of GF in each sample was determined by comparison to both the DFP internal standard and a standard GF calibration curve. Calibration curves were obtained by using GF at seven different concentrations between 96 nM and 300 μM, containing 50 μM DFP in ethyl acetate. Kinetic parameters (K_M and k_{cat}) were determined as described above.

2.5. GC/MS analysis of GF samples

Non-chiral gas chromatographic analysis of GF was performed using an Agilent 6890 gas chromatograph (Foster City, CA, USA) fitted with a 30 m × 0.25 mm internal diameter (i.d.) DB-17 column, 0.25 mm film thickness (J&W Scientific, Folsom, CA). Helium was used as the carrier gas at an average linear velocity of 32 cm s^{-1}. The oven temperature was held initially at 50 °C for 1 min, programmed from 50 to 250 °C at 15 °C min^{-1}, and held at 250 °C for 1 min. Splitless injections of 1 μL volume were made using an Agilent 7683 autosampler. The injection port temperature was 250 °C and the split vent delay set at 1 min. The GC was interfaced to an Agilent 5973 mass spectrometer (MS) with an electron impact ion source. The MS operating conditions were as follows: ion source pressure approximately 1.0×10^{-5} Torr; source temperature, 230 °C; quadrupole temperature, 150 °C; electron energy, 70 eV; and transfer line temperature, 265 °C. The MS was operated using selected ion monitoring. Ion pairs m/z 99 and 137 and m/z 101 and 127 were monitored for GF and DFP, respectively. A dwell time of 50 m/s for each ion pair resulted in a scan rate of 8.26 cycles s^{-1}.

2.6. In vivo efficacy with chlorpyrifos

A chlorpyrifos dose response was executed on *T. ni* larvae 73.5 h after inoculation with pre-occluded recombinant baculovirus encoding HuPON1 or empty virus. Chlorpyrifos was added on top of the larval media, providing a continuous percutaneous exposure to the pesticide. Larvae ($n = 28$) were examined for survival 16 h after chlorpyrifos treatment. Larvae were scored as dead if they did not respond to a touch stimulus

3. Results and discussion

3.1. Characterization of HuPON1 Expressed in T. ni larvae

Orally active baculovirus was used to direct expression of rHuPON1 in *T. ni* larvae. Western blot analyses of crude larval

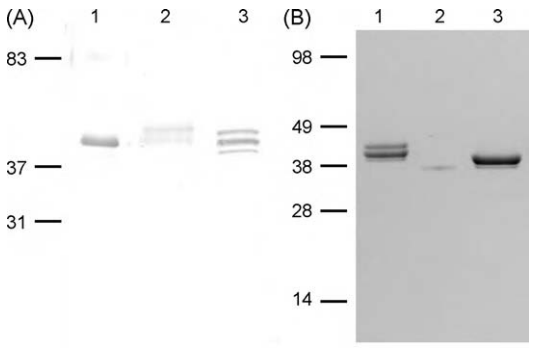

Fig. 1. Western blot and deglycosylation analysis of rHuPON1. (A) Protein was detected with a HuPON1-specific mouse monoclonal antibody (kindly provided by R. James, University Hospital of Geneva, Geneva, Switzerland) and a secondary alkaline-phosphatase conjugated antibody (Sigma–Aldrich, St. Louis, MO, USA). Lanes are as follows: (1) non-functional, bacterially expressed HuPON1 (Randox Life Sciences, Crumlin, UK); (2) functional non-purified 293T-produced HuPON1; (3) rHuPON1. (B) rHuPON1 was incubated with or without PNGase F and analyzed by SDS-PAGE and Coomassie staining. Lanes are as follows: (1) rHuPON1 alone; (2) PNGase F alone; (3) rHuPON1 after PNGase F treatment. Molecular weight markers are in kDa.

lysates indicated that rHuPON1 was present at 0.7% of the total protein. After purification, the final yield of rHuPON1 was ~320 mg per kg of larva. Although the predicted molecular weight of rHuPON1 with a 6×-histidine tag is 40 kDa, three bands with apparent molecular weights of 41, 42, and 44 kDa were observed when rHuPON1was examined by Coomassie blue staining of polyacrylamide gels run under denaturing conditions (data not shown). No other protein bands were detected. The molecular weights of the three bands were subsequently confirmed via mass spectrometry to be 41,000, 42,100, and 43,800 Daltons (data not shown). The three isoforms could result from alternate glycosylation of rHuPON1 or may represent other proteins that co-purified with the enzyme. However, each one of these proteins reacted with a HuPON1-specific monoclonal antibody (Fig. 1A). The multiple bands from rHuPON1 migrated similarly to functional non-purified HuPON1 transiently expressed in human 293T cells, while HuPON1 expressed and purified from *E. coli* (Randox Life Sciences, Crumlin, UK) migrated as a single band, as expected. As an independent method of verification, each band of rHuPON1 was excised from a Coomassie-stained gel and analyzed by peptide mapping and mass analysis. All three bands were identified as HuPON1, with a confidence level greater than 95% (data not shown); no evidence suggested that other proteins from *T. ni* larvae co-purified with the enzyme. We then examined the possibility that the multiple rHuPON1 proteins corresponded to differentially glycosylated versions of the enzyme. After treatment with PNGase F, the 42 and 44 kDa bands of the sample were no longer visible, while the 41 kDa band was still observed. These data suggest that the isoforms represent alternately glycosylated versions of rHuPON1 (Fig. 1B).

3.2. Kinetic analysis of rHuPON1

These initial studies demonstrated that *T. ni* larvae was an appropriate system to produce soluble recombinant HuPON1 protein. HuPON1 is a promiscuous enzyme with the capacity to hydrolyze aryl esters, pesticides, and OP nerve agents. Therefore, the capacity of rHuPON1 to hydrolyze several classes of substrates was examined (Table 1). rHuPON1 catalyzed the hydrolysis of phenyl acetate with values for both turnover (k_{cat}) and binding affinity (K_M) very similar to those reported for non-purified HuPON1 transiently expressed in 293T cells [3]. Although the k_{cat} value of rHuPON1 for paraoxon (the toxic metabolite of the pesticide parathion) was also similar to that of 293T-produced HuPON1, rHuPON1 had a 2.5-fold lower binding affinity for paraoxon than did HuPON1 expressed in 293T cells ([3], K_Ms of 1.5 and 0.57 mM, respectively). The basis for this difference is unclear, but may be the result of differential glycosylation.

Table 1
Kinetic constants of rHuPON1.

Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
Phenyl acetate	850 ± 50	0.5 ± 0.09	$16.7 \pm 1.7 \times 10^5$
Paraoxon	0.45 ± 0.007	1.5 ± 0.04	300 ± 3.3
VX[a]	ND	ND	200
VR	0.10 ± 0.002	0.5 ± 0.03	200 ± 10
GF[a]	ND	ND	0.83

Values are shown ± standard deviation. Where no deviation is indicated, the error was <1% of the value.

[a] At the maximum regulated nerve agent concentration (1.4 mM for VX; 4 mM for GF), no evidence of saturation was detected, indicating that $K_M \gg [S]$. The Michaelis–Menten equation was thus reduced to $v_0 = k_{cat}[E][S]/K_M$; k_{cat}/K_M is equal to the slope of the [S] versus v_0 plot, divided by the concentration of total enzyme ([E]). ND = not determined.

We also examined the activity of rHuPON1 with the V-agents VX and VR. Restrictions of surety material concentrations established at USAMRICD prevented substrate saturation studies with VX at concentrations higher than 1.4 mM; rHuPON1 did not reach saturation under these conditions, as was observed for 293T-produced HuPON1 [3]. Consequently, the assumption was made that the K_M is much greater than the VX substrate concentration ([S]), and catalytic efficiency (k_{cat}/K_M) was estimated from the slope of the [S] versus rate plot, divided by the concentration of total enzyme ([E]). The apparent k_{cat}/K_M of rHuPON1 for VX was about 3-fold lower than that reported for HuPON1 from 293T cells (Table 1; 200 and 550 $M^{-1}\,s^{-1}$, respectively). rHuPON1 was also examined for its capacity to catalyze the hydrolysis of the nerve agent VR, a structural isomer of VX. Although saturation could be reached with VR, the k_{cat}/K_M of rHuPON1 for VR was identical to the apparent k_{cat}/K_M for VX (Table 1). Interestingly, 293T-produced HuPON1 had a slightly higher k_{cat}/K_M for VR ([3], 700 $M^{-1}\,s^{-1}$), and this difference was reflected in a 2.5-fold lower K_M of rHuPON1 for VR versus 293T-produced HuPON1 ([3], 0.5 and 0.2 mM, respectively).

The ability of HuPON1 to hydrolyze GF has not been reported. Using a GC/MS method, we examined the capacity of rHuPON1 to catalyze the hydrolysis of GF. Although saturation was not achieved under surety conditions in place at USAMRICD, rHuPON1 was capable of hydrolyzing this nerve agent with an apparent k_{cat}/K_M of 0.83 $M^{-1}\,s^{-1}$ (Table 1).

3.3. Stereoselective hydrolysis of VX and VR

OP nerve agents contain at least one chiral center at the phosphorus molecule, and the toxicity of different nerve agent stereoisomers can vary dramatically. Therefore, we investigated whether rHuPON1 has detectable stereopreference toward a particular isomer of VX or VR. Under the experimental conditions used here (3 mM V-agent, 0.48 μM rHuPON1), the P+ isomer of VX was completely hydrolyzed after 240 min (Fig. 2A). No enzymatic hydrolysis of the P− isomer was detected after either 360 min, when >97% of the isomer still remained (Fig. 2A), or 24 h (data not shown). With VR, the hydrolysis of Isomer 1 (the first isomer to elute from the ChiralCel OD-H column, and presumed by analogy with VX to be P+) was nearly complete after 420 min (Fig. 2B). In contrast, no detectable hydrolysis of VR Isomer 2 (presumed to be P−) was observed at this time point, with >95% of the initial material remaining. After 24 h, no further hydrolysis of Isomer 2 was seen (data not shown).

rHuPON1 hydrolysis was exclusively preferential for both the P+ isomer of VX and the presumed P+ isomer of VR. The preferential hydrolysis of only one of the isomers is most likely indicative

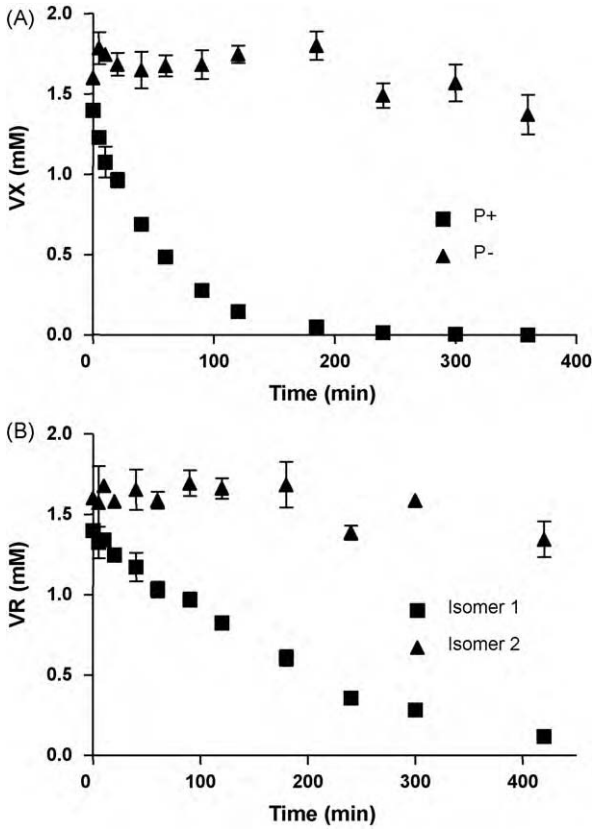

Fig. 2. rHuPON1 stereospecificity determined for VX and VR isomers using LC/MS assay. The hydrolysis of the isomers of VX (A) and VR (B) was followed to determine if a preferential use of the isomers as substrate existed. Data are plotted as concentration of isomer versus time and are shown ± standard deviation. Isomer designation is indicated in each plot.

of restrictions imposed by the active site architecture, wherein binding and/or catalysis is contingent on the three-dimensional arrangement of O-alkyl substituents around the chiral phosphate. The enzyme sample consists of three forms of protein with differing degrees of glycosylation; the homogeneous response of the heterogeneous enzyme mixture suggests that the glycosylation state of rHuPON1 does not affect substrate stereoselectivity. This is the first report that HuPON1 has stereoselectivity for the different V-type OP nerve agent isomers.

Although the *in vivo* toxicity of each stereoisomer of the V-agents is not known, by analogy with soman (where the P– isomers are ~1000-fold more toxic than the P+ isomers), it is unlikely that rHuPON1 in its wild type form will afford any *in vivo* protection against V-agents. Variants of HuPON1 that enhance the preference of the enzyme for the more toxic isomer may need to be identified. The lack of hydrolysis of the P– isomers of VX or VR by rHuPON1 may result either from the enzyme failing to bind the substrates or from binding them in a way that inhibits substrate catalysis. If the non-hydrolyzed isomers do not bind the enzyme, then the K_M value for VR reported in Table 1 as 0.5 mM should be 0.25 mM, because the actual concentration of substrate (P+) is present at roughly 50% in racemic VR. If the non-hydrolyzed P– isomer of VR does bind to rHuPON1 and acts as an inhibitor of hydrolysis of the P+ isomer, then the actual K_M value for VR will be even lower than 0.25 mM. We are currently attempting to distinguish between these two possibilities for the P– isomers of VX and VR with rHuPON1.

Table 2
In vivo efficacy of rHuPON1 with chlorpyrifos.

% chlorpyrifos	% mortality of control larvae	% mortality of HuPON1-expressing larvae
0.00625	0	0
0.0125	3.6	0
0.02	0	7.1
0.025	10.7	3.6
0.05	60.7	0
0.1	92.9	0
0.2	100	0
5[a]	100	0

A chlorpyrifos dose response was executed on *T. ni* larvae at 73.5 h after inoculation with either HuPON1 recombinant baculovirus or empty baculovirus ($n = 28$ for each condition). For all doses except 5%, larvae were scored for survival at 16 h after chlorpyrifos treatment.

[a] A stock concentration of 5% chlorpyrifos was used with larvae 89.6 h post-inoculation, as above; results shown are 7 h after chlorpyrifos treatment.

3.4. *In vivo* efficacy of rHuPON1 with chlorpyrifos

HuPON1 has been shown to catalyze the hydrolysis of the pesticide chlorpyrifos [14]. A bioassay was conducted to investigate the ability of *T. ni* larvae expressing rHuPON1 to survive exposure to chlorpyrifos. In the absence of rHuPON1 expression, the median lethal dose (LD_{50}) of chlorpyrifos was ~0.05% (Table 2). Conversely, larvae expressing HuPON1 tolerated concentrations of chlorpyrifos as high as 0.2%. This result was promising, as our research is targeted at utilizing rHuPON1 as a catalytic bioscavenger of nerve agents. We further tested the ability of these larvae to survive even higher concentrations of chlorpyrifos. With a 5% stock concentration of chlorpyrifos, there were no OP driven symptoms of tissue constriction and paralysis or mortality in the larvae expressing rHuPON1, but immediate symptoms and subsequent mortality by 1.5 h post-treatment were observed in the larvae infected with a negative control virus (Table 2). Larvae expressing rHuPON1 were still viable up to 7 h post-treatment, at which time the experiment was terminated.

4. Conclusions

HuPON1 has the potential to be used as a catalytic bioscavenger of nerve agents. However, the enzyme has been difficult to functionally express and purify from many sources, making it difficult to examine the *in vivo* efficacy of the enzyme for protection against nerve agent exposure. Insect expression systems have been utilized to produce large quantities of recombinant protein inexpensively. In this study, we examined the capacity of *T. ni* to express functionally active rHuPON1 protein.

Preliminary results suggest that the expression level of rHuPON1 protein in baculovirus infected larvae is approximately 7 g kg^{-1} of biomass. While the purification approach utilized in this study yielded ~320 mg of rHuPON1 per kg (4.5% final recovery), it is important to emphasize that little effort was made to optimize yield during any of the purification steps. Refinement of this purification approach is likely to result in improved recovery rates. Analysis of deglycosylated rHuPON1 (Fig. 1B) suggests that the enzyme is either glycosylated at two different sites or is glycosylated by one of two different glycan structures; we are currently examining the location and composition of the glycans on rHuPON1. The data in Table 2 indicate that recombinant expression of HuPON1 affords dramatic protection to *T. ni* larvae from the pesticide chlorpyrifos. This expression system has the potential to produce sufficient quantities of rHuPON1 to test the capacity of this enzyme to act as a nerve agent bioscavenger in mammalian test species.

Conflict of interest statement

E.K., Z.L., G.B., M.T., D.D., and R.B. are employees of ChesapeakePERL, Inc., and have a financial interest in the commercial production of recombinant HuPON1.

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