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An OPAA enzyme mutant with increased catalytic efficiency on the nerve agents sarin, soman, and GP

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ABSTRACT

The wild-type OPAA enzyme has relatively high levels of catalytic activity against several organophosphate G-type nerve agents. A series of mutants containing replacement amino acids at the OPAA Y212, V342, and I215 sites showed several fold enhanced catalytic efficiency on sarin, soman, and GP. One mutant, Y212F/V342L, showed enhanced stereospecificity on sarin and that enzyme along with a phosphotriesterase mutant, GWT, which had the opposite stereospecificity, were used to generate enriched preparations of each sarin enantiomer. Inhibition of acetylcholinesterase by the respective enantioenriched sarin solutions subsequently provided identification of the sarin enantiomers as separated by normal phase enantioselective liquid chromatography coupled with atmospheric pressure chemical ionization–mass spectrometry.

1. Introduction

Sarin, soman (GD and, GP (Fig. 1) are all G-type chemical nerve agents; members of a class of organophosphates that exert their toxic effect by phosphorylating the catalytically active serine in the active site of acetylcholinesterase and preventing it from catalyzing the breakdown of acetylcholine in the neuromuscular junction. These, and other compounds are included in the Chemical Weapons Convention which prohibits their development, production, stockpiling, and use in the 192 (as of 17 Oct, 2015) signatory nations [1]. However, not all countries have signed the Convention and reports of the use of chemical weapons have continued to appear [2–4]. Two websites provide regularly updated lists of reports of chemical weapons use, many of which have been verified by the Organization for the Prohibition of Chemical Weapons [5,6].

Catalytic enzymes are currently being investigated for their potential as *in vivo* medical countermeasures for nerve agent poisoning [7]. Current therapies comprise symptomatic treatment such as atropine (a muscarinic antagonist) and benzodiazepines (to control seizures), in addition to etiologic treatment of the poisoned acetylcholinesterase with 2-PAM (an oxime reactivator). Given the high toxicity of nerve agents and the fact that existing treatments typically confer protection against only a few lethal doses, treatments can easily be overwhelmed by agent exposure [8,9]. This concern has led to the effort to develop catalytic antidotes which might confer greater protection than would be possible with stoichiometric approaches. The particular advantage of circulating enzymes is that they can detoxify organophosphates in the blood before they enter the tissue where they bind to acetylcholinesterase [10]. For that reason, they have the potential to function either alone or synergistically with existing treatments [7].

Organophosphorus Acid Anhydrolase (OPAA; EC 3.1.8.2) is one of the enzymes described for the catalytic defluorination of G-agents. The wild-type (WT) OPAA enzyme has relatively high levels of activity against soman and cyclosarin [11,12]. WT OPAA has slight activity against Russian VX; some mutants have greater activity and, in the case of one mutant, enhanced stereospecificity such that both enantiomers of Russian VX are catalyzed at similar rates [13]. WT OPAA activity against sarin is significantly less than seen with soman and cyclosarin [15]. In this study we initially used site-directed mutagenesis in an effort to increase the catalytic efficiency of OPAA against sarin. One of those mutants, FL, proved particularly promising. Therefore, we studied its kinetics against soman and a more recently described nerve agent, GP [16]. Finally, enzymatic reactions were used to generate enantioenriched preparations of sarin which were used along with

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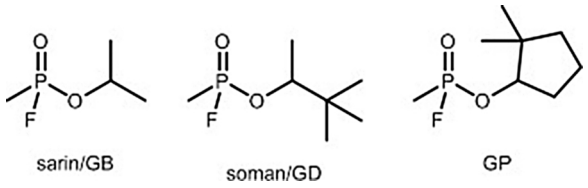

Fig. 1. Structures of sarin/GB (propan-2-yl methylphosphonofluoridate), soman/GD (3-methylbutan-2-yl methylphosphonofluoridate), and GP (2, 2-dimethylcyclopentyl methylphosphonofluoridate).

acetylcholinesterase inhibition assays to assign the respective sarin enantiomers separated and identified by chiral liquid chromatography coupled with atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS).

Table 1
Enzyme genotypes.

Source	Genotype	Abbreviation
OPAA	Y212F/V342L	FL
OPAA	Y212F/V342I	FI
OPAA	Y212F/V342Y	FY
Phosphotriesterase	H254G/H259W/L303T	GWT

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M N K L A V L Y A E   H I A T L Q K R T R   E I I E R E N L D G   V V F H S G Q A K R   40
Q F L D D M Y Y P F   K V N P Q F K A W L   P V I D N P H C W I   V A N G T D K P K L   80
I F Y R P V D F W H   K V P D E P N E Y W   A D Y F D I E L L V   K P D Q V E K L L P   120
Y D K A R F A Y I G   E Y L E V A Q A L G   F E L M N P E P V M   N F Y H Y H R A Y K   160
T Q Y E L A C M R E   A N K I A V Q G H K   A A R D A F F Q G K   S E F E I Q Q A Y L   200
L A T Q H S E N D T   P Y G N I V A L N E   N C A I L H Y T H F   D R V A P A T H R S   240
F L I D A G A N F N   G Y A A D I T R T Y   D F T G E G E F A E   L V A T M K Q H Q I   280
A L C N Q L A P G K   L Y G E L H L D C H   Q R V A Q T L S D F   N I V N L S A D E I   320
V A K G I T S T F F   P H G L G H H I G L   Q V H D V G G F M A   D E Q G A H Q E P P   360
E G H P F L R C T R   K I E A N Q V F T I   E P G L Y F I D S L   L G D L A A T D N N   400
Q H I N W D K V A E   L K P F G G I R I E   D N I I V H E D S L   E N M T R E L E L D   440
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2. Material and methods

Sarin and soman were chemical agent standard analytical reference material from our stocks. Sarin was 97.4% weight percent pure by acid base titration (traceable to the National Institute of Standards and Technology, or NIST through potassium phthalate). Purity by gas chromatography/thermal conductivity detection (GC/TCD) was 97.9%. Purity by ^{31}P nuclear magnetic resonance (NMR) was 97.7 wt%. Soman was 95.3 wt% pure by ^{31}P NMR relative to a triethylphosphate internal standard and traceable to NIST through the internal standard with purity of 99.87 wt% relative to a NIST traceable standard of dimethylsulfone via quantitative ^{1}H NMR measurements. The purity of the soman by GC/FPD was 98.95%.

GP was synthesized in a two-step procedure from 2,2-dimethylcyclopentanone. First, a reduction of the ketone by $LiAlH_4$ in Et_2O yielded racemic 2,2-dimethylcyclopentanol after acid workup. Then reaction of 2,2-dimethylcyclopentanol with methylphosphonyl difluoride in the presence of trimethylamine in Et_2O yielded the desired 2,2-dimethylcyclopentyl methylphosphonofluoridate after filtration to remove triethylammonium hydrofluoride, removal of the solvent, and distillation under reduced pressure.

Note: GP is extremely toxic and its synthesis is regulated by the Chemical Weapons Convention.

All reagents and solvents were HPLC grade. Hexane and isopropyl alcohol were purchased from Fisher Scientific (Waltham, MA). For the LC–MS analytical analysis, the MS system was operated in total ion chromatogram (TIC) mode at *m/z* 50–300. The analytical separations of the enantiomers were characterized using an Agilent 1200 LC with atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS) performed on a Phenomenex Lux Cellulose-1 column, 250 × 4.6 mm, 5 μm with a mobile phase consisting of *n*-hexane (A) and isopropyl alcohol (B) and a sample volume of 20 μL. The enantiomers were baseline resolved within 15 min with a mobile phase of 95/5 A/B (v/v%) with a flow rate 0.6 mL/min. Samples for analytical separation were prepared at 0.1 mg/mL.

The OPAA enzyme was prepared as described previously [12]. Briefly, the *Escherichia coli* host cell containing the cloned OPAA gene was grown to late log phase in 1 L of Luria Broth in a flask. Cells were harvested and the enzyme was purified by ammonium sulfate fractionation and the 45–65% pellet obtained was redissolved, passed through a size exclusion column and the active fractions were pooled and loaded on a Q Sepharose column followed by a 0.2–0.6 M NaCl gradient elution.

The resulting sample was apparently homogeneous by polyacrylamide gel electrophoresis.

The wild-type opaa amino acid sequence is as follows:

The opaa gene was cloned into the *Nco*I and the *Eco*RI sites of the pSE420 expression vector. The cloned gene lacks the last 77 carboxyl-terminus amino acid residues of the OPAA enzyme; these residues were removed in a previous investigation and found to have no discernible effect on enzymatic activity [15]. The OPAA enzyme with the FL mutations was constructed by DNA 2.0 (Menlo Park, CA) by site-directed mutagenesis [17]. The mutants have the Y212F mutation in combination with L, I, or Y substitutions at the V342 site (Table 1). The GWT mutant of the PTE gene was made by directed mutagenesis performed using the Quick Change (Agilent Technologies) protocol [18].

Enzyme activity was determined with a fluoride electrode connected to an Accumet XL250 ion selective meter (Thermo Fisher Scientific, Inc.) calibrated against authentic standards. Assays were conducted in 2.0 mL of 50 mM bis-tris-propane buffer, pH 8.0, containing 0.1 mM $MnCl_2$ which was added just prior to the assay. Data were logged every 30 s. Sarin was used at a concentration of only 0.5 mM for stereochemistry assays and for enantioenriched preparations since the rate of racemization is at least partially a function of the concentration of fluoride in the sample [19].

3. Theory/calculation

Kinetic parameters were calculated using Biosoft EnzFitter© software (Biosoft.com). Activity data were generally collected at substrate concentrations ranging from 1/3 to three times the K_m under conditions that consumed less than 10% of the substrate. At least six, more typically ten, different substrate concentrations were used for each curve. The percent uncertainties of the Vmax and K_m values determined from the software were added together to determine the percent uncertainties of the k_{cat}/K_m values.

Acetylcholinesterase inhibition was determined on a plate reader (BioTek Synergy4) using a modified Ellman Assay, as described in Results

The rationale for selecting the respective OPAA sites for mutation was described previously [13]. Briefly, the OPAA enzyme active site comprises a small pocket, a large pocket and a leaving group pocket.

Table 2
OPAA genotypes and kinetic parameters on sarin.

Enzyme	Genotype	k_{cat} min^{-1}	K_m (μM)	k_{cat}/K_m (min^{-1} M^{-1})	k_{cat}/K_m Mutant/WT
WT	Wild-type	1.20E + 04 ± 4.31E + 02	9.02E + 03 ± 6.32E + 02	1.33E + 06 ± 1.41E + 05	
FL	Y212F/V342L	4.52E + 04 ± 4.62E + 03	5.31E + 03 ± 1.01E + 03	8.50E + 06 ± 2.49E + 06	6
FY	Y212F/V342Y	1.10E + 04 ± 1.80E + 03	5.62E + 03 ± 1.57E + 03	1.96E + 06 ± 8.65E + 05	1
FI	Y212F/V342I	1.63E + 04 ± 1.99E + 03	6.00E + 03 ± 1425.09	2.72E + 06 ± 9.80E + 05	2

Structural analysis revealed Y212 to be the largest amino acid in the small pocket and our initial mutagenesis efforts involved the replacement of all amino acids except isoleucine at the Y212 site. Screening showed most of these substitutions to be deleterious but Y212F and Y212V mutants showed enhanced activity on several nerve agents. Subsequent mutations at other sites in the Y212V background were not productive so were not pursued further. Mutations at the small pocket-forming V342 site in the Y212F background, particularly V342L, showed further activity increases on several nerve agent substrates.

The rationale for the construction of the phosphotriesterase (PTE) GWT mutant was described previously as well [15]. Briefly, a series of mutants were screened on *p*-nitrophenyl derivatives of G-type nerve agents and promising mutants were subsequently tested in several nerve agents. GWT was also shown to have a reversed stereospecificity on cyclosarin compared to the wild type enzyme.

4. Results

A series of OPAA mutants was screened by assaying the specific activity of their crude lysates on 3 mM sarin. Although crude lysate assays do not account for potential differences in enzyme expression levels, they are frequently indicative of catalytic efficiency and as such they can provide a useful screening tool with which to select mutants for subsequent purification and kinetic analysis. Promising mutants FL, FY, and FI from the screening were purified and full kinetic parameters were determined (Table 2 and Fig. 2).

The kinetic data with purified enzymes showed that the catalytic efficiency of FY actually represented only a marginal improvement over WT, and FI was about twice WT. FL however, had a k_{cat}/K_m value on sarin approximately six times that of the WT enzyme.

Following the encouraging results seen with FL on sarin, kinetic parameters for the degradation of soman and GP by FL and WT were determined. The catalytic efficiencies using these two substrates were approximately five times and ten times respectively that of WT (Table 3 and Fig. 3).

Typical of bioactive compounds, G-agents are chiral molecules and the enzymes that catalyze their hydrolysis often show significant stereospecificity. Sarin, soman and GP all have a stereogenic phosphorus atom and soman and GP have an additional stereogenic carbon atom. Benschop et al. in 1984 published the toxicity values for the four soman stereoisomers [19]. Separations or toxicities of the GP isomers have not been reported to our knowledge. Here we sought to separate and identify, by differential enzymatic activity, the respective sarin enantiomers by LC-APCI-MS. Fig. 4a shows the total ion chromatogram (TIC) of the sarin enantiomers eluting at 11.3 min and 12.9 min and Fig. 4b shows extracted ion chromatogram (EIC) of the sarin enantiomers. The mass spectrum of the enantiomer at 11.3 min was identical to that at 12.9, minute as expected (Fig. 4c). The mass spectrum of the enantiomer at 11.3 min was identical to that at 12.9, minute as expected.

With substrates such as sarin, a molecule comprising two enantiomers, the rates of enzymatic degradation can reveal that the process is stereospecific. A single exponential curve would indicate that both enantiomers are degraded at the same rate; whereas a clear midpoint deflection in the curve would indicate that an enzyme has a preference for one enantiomer over the other. Fig. 5 shows such a deflection when sarin is degraded by FL, but no apparent stereospecificity was observed with WT.

In order to identify the respective sarin enantiomer peaks, the phosphotriesterase GWT enzyme (1.6 μg/mL) was added to a solution (15 mL) of 0.5 mM racemic sarin in 50 mM bis-tris-propane, pH 7.2. The release of fluoride was monitored by fluoride ion selective electrode and when approximately 80% of the substrate had reacted the entire contents of the reaction was added to 4 mL of cold ethyl acetate, shaken gently and the organic fraction was subjected to LC-APCI-MS analysis (Fig. 6). Of the two peaks, at 11.6 and 13.3 min, the area of the former was approximately five times that of the latter in the enriched extract, indicating a distinct preference of the GWT enzyme for the enantiomer corresponding to the 13.3 min peak. The total concentration of sarin in the sample was determined by standard curve to be 104.1 μg/mL. A sample of racemic sarin was prepared to the same concentration (104.1 μg/mL), and these two samples, one racemic and one enriched for the 11.6 min peak, were used to inhibit acetylcholinesterase.

EC_{50}s: (±)-sarin: 4.16 ± 0.22 nM

GWT Enriched sarin: 48.1 ± 9.7 nM

Solutions of racemic sarin and enantioenriched sarin (GWT) were prepared in phosphate buffer (0.100 M PO_4^-, pH = 7.5) in a 96-well plate at varying concentrations and human acetylcholinesterase (*h*AChE) (20 μL) was added. The final concentrations of sarin in the

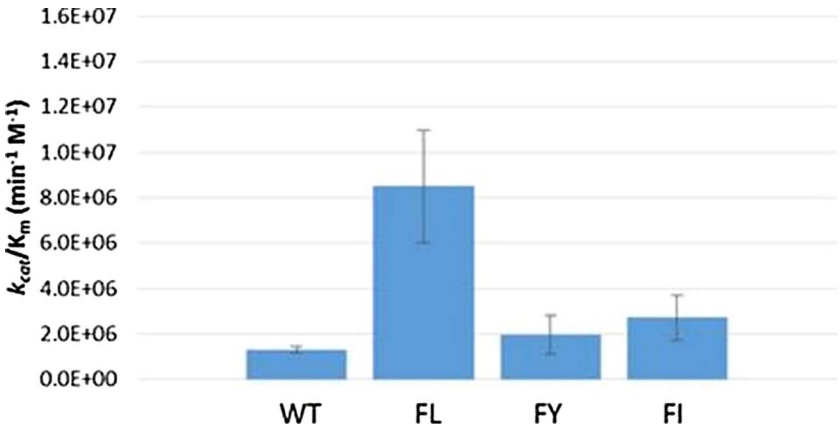

Fig. 2. Comparison of k_{cat}/K_m values for WT and mutant enzymes on sarin (second version of this graph is added in response to reviewer's comments – I don't think we should include it, though).

Table 3
Kinetic parameters of WT and FL enzymes on soman and GP.

Enzyme and Substrate	k_{cat} min^{-1}	K_m (μM)	k_{cat}/K_m (min^{-1} M^{-1})	k_{cat}/K_m FL/WT
WT on soman	2.13E + 05 ± 1.71E + 04	1.32E + 04 ± 1.62E + 03	1.61E + 07 ± 3.26E + 06	
FL on soman	6.60E + 04 ± 3.40E + 03	883.16 ± 156.395	7.48E + 07 ± 1.71E + 07	5
WT on GP	7.91E + 04 ± 7.44E + 03	6.12E + 03 ± 1.21E + 03	1.29E + 07 ± 3.78E + 06	
FL on GP	6.90E + 04 ± 3.08E + 03	534.924 ± 6.78E + 01	1.29E + 08 ± 2.21E + 07	10

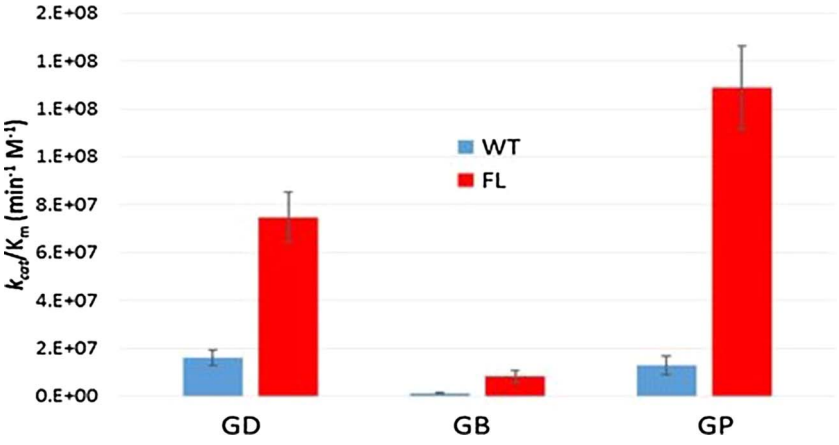

Fig. 3. Comparison of k_{cat}/K_m values for WT and FL enzymes on soman, sarin and GP.

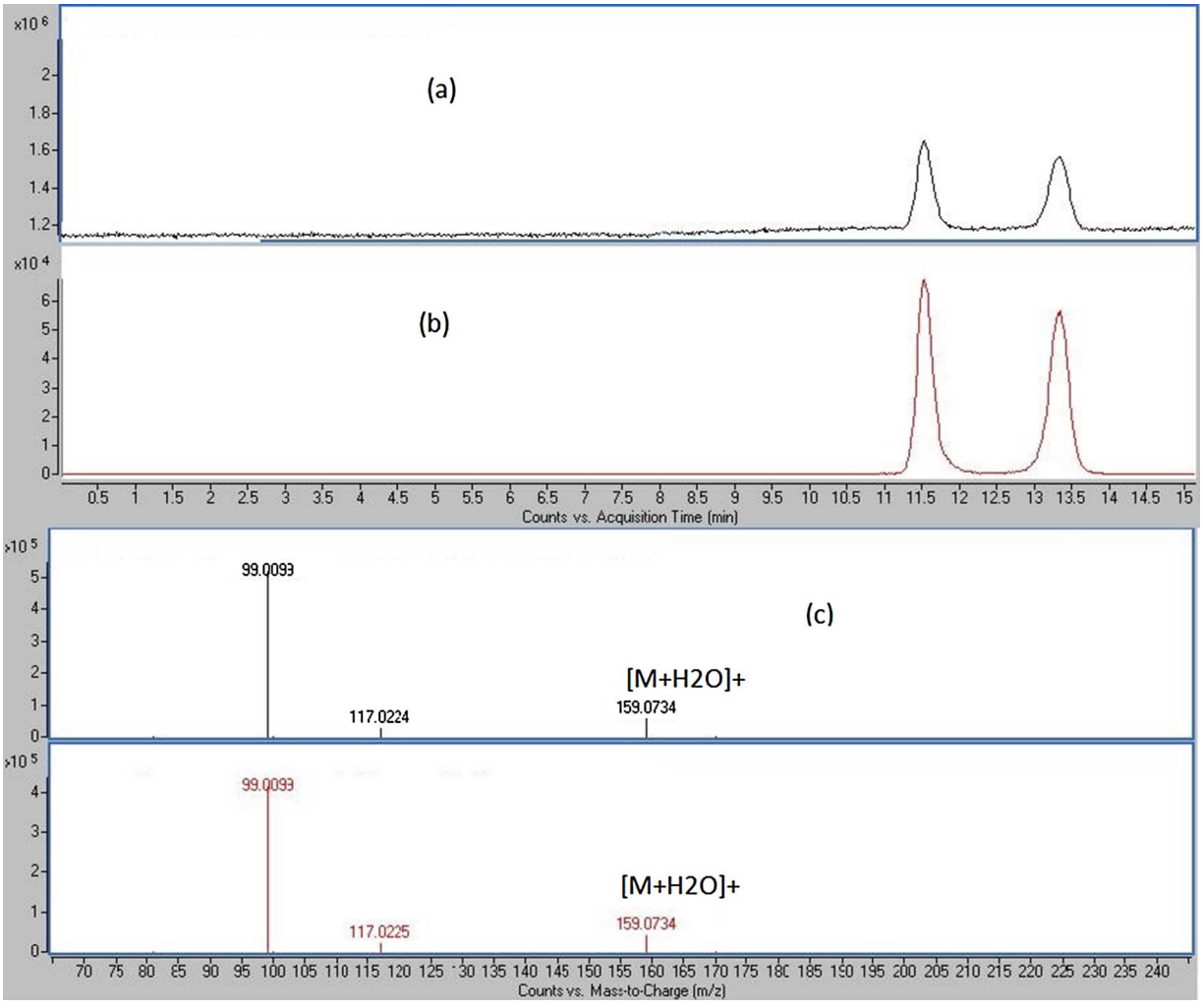

Fig. 4. A representative (a) TIC, (b) EIC, and (c) MS for racemic sarin.

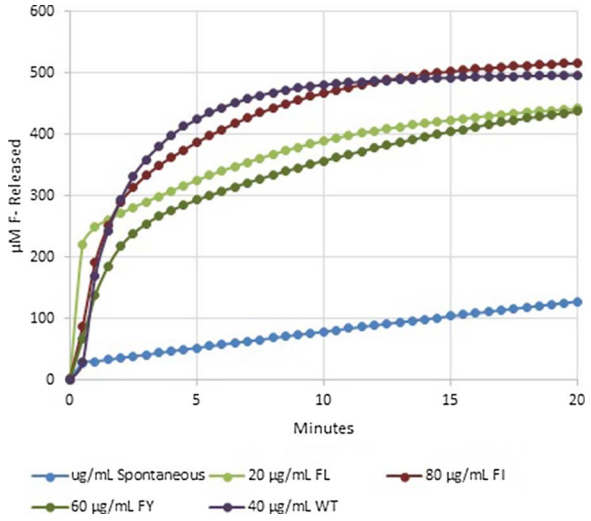

Fig. 5. Enzymatic and spontaneous hydrolysis of 500 µM sarin. Enzyme activities differ, therefore concentrations for each enzyme were chosen to provide similar initial hydrolysis rates.

solutions were 892, 357, 143, 57.1, 22.8, 9.13, 3.65, and 1.46 nM. This solution was incubated at 37 °C for 15 min to allow for complete inhibition, whereupon a solution (210 µL) containing acetylthiocholine iodide (1.2 mM; final concentration 1 mM) and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (1.6 mM; final concentration 1.3 mM) was added to each well. The rate of hydrolysis of acetylthiocholine to thiocholine and acetate was measured by monitoring the change in absorbance of the solution at 412 nm using a plate reader (Synergy4, BioTek). The path length was previously determined to be 0.479 mm, and the extinction coefficient for TNB^{2-}, $\varepsilon_{412} = 14150\ M^{-1}\ cm^{-1}$ [20].

The effective concentration at which 50% of the acetylcholinesterase enzyme was inhibited (EC_{50}) was determined for the racemic and GWT enriched samples. EC_{50}s were determined by fitting the activity data to a sigmoidal dose-response curve model with variable slope using GraphPad Prism version 6.07 for Windows (Fig. 7).

Boter and Van Dijk reported in 1969 that (*S*)-(−)-sarin was a much stronger inhibitor of AChE than (*R*)-(+)-sarin [21]. Against bovine erythrocyte AChE, the ratio of reactivities was $\geq 4.2 \times 10^3$ whereas the ratio for electric eel AChE inhibition was 3.6×10^4. Obviously, in both cases, essentially all the toxicity from sarin derives from the (*S*)-(−) enantiomer. The mouse intravenous LD_{50} values for (*S*)-(−)-sarin (41 µg/kg) and racemic sarin (142 µg/kg) follow this same trend [21,22]. We therefore reasoned that if the GWT enzyme preferentially degraded the (*R*)-(+) enantiomer, the GWT-enriched sample should inhibit AChE somewhat more strongly than racemic sarin, whereas if GWT preferred the (*S*)-(−) enantiomer, the inhibition should be correspondingly reduced. The acetylcholinesterase inhibition data show an approximately 11-fold reduction in inhibition, consistent with the latter case, and indicating that the 11.6 min peak is (*R*)-(+) sarin and the 13.3 min peak (which was reduced in the GWT-enriched preparation) is

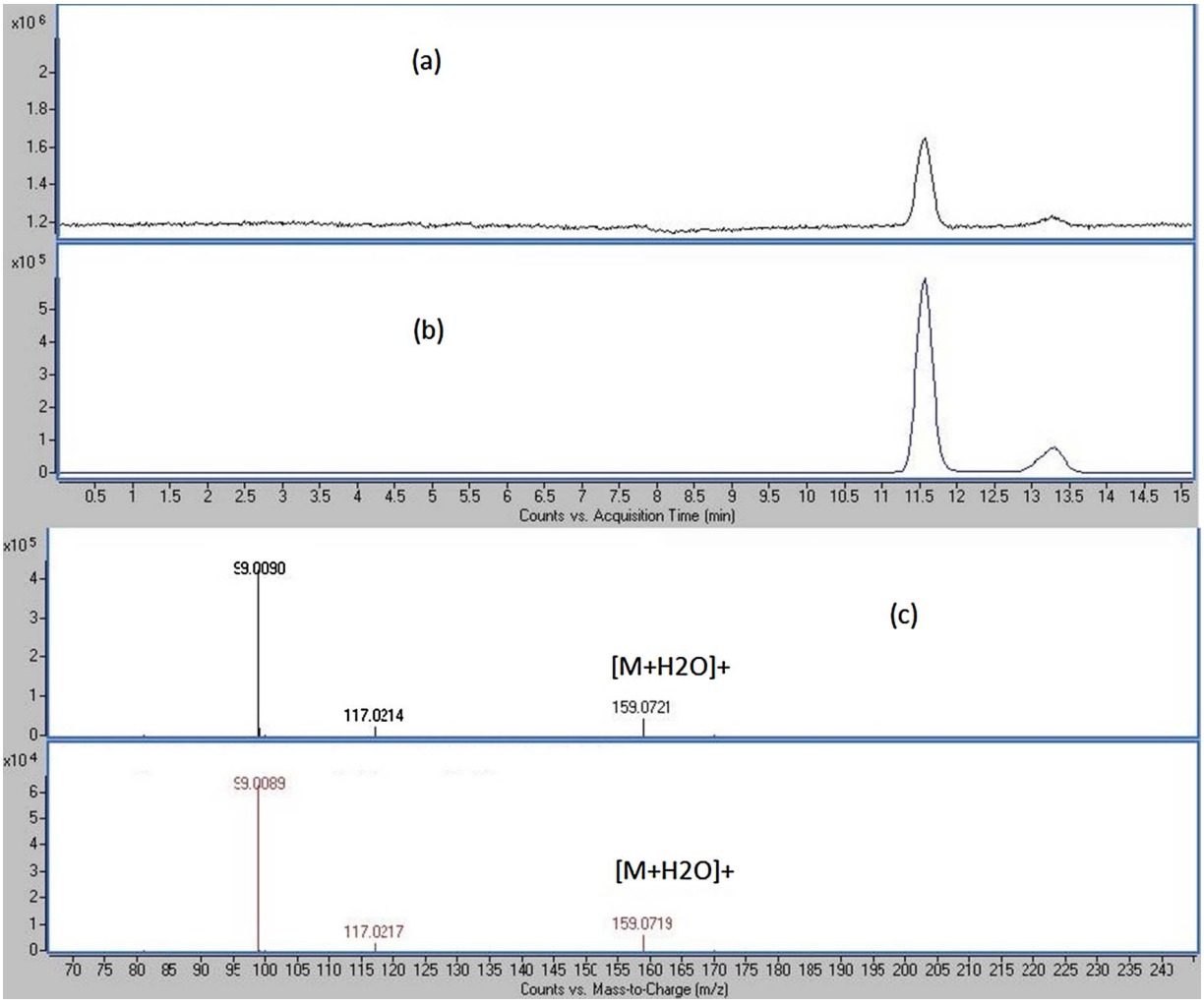

Fig. 6. A representative (a) TIC, (b) EIC (*m/z* 99.0100), and (c) MS at 11.5 min and 13.2 min for phosphotriesterase GWT-degraded sarin.

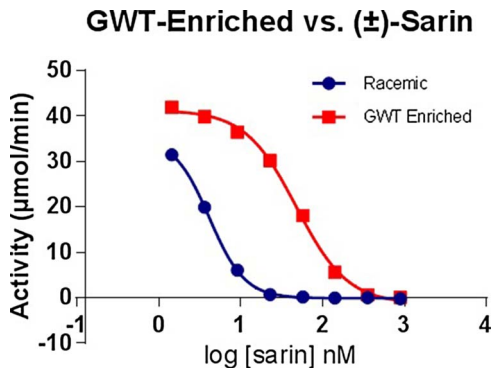

Fig. 7. Acetylcholinesterase inhibition with (±)-sarin and GWT enriched sarin.

(*S*)-(−)-sarin. These results were corroborated by an OPAA-FL catalyzed sample enriched for the 13.3 min peak which showed little change in acetylcholinesterase inhibition, as would be expected for a sample where primarily the (*R*)-(+) sarin was hydrolyzed. We can also conclude on this basis that OPAA-FL exhibits a preference for the (*R*)-(+) enantiomer while PTE GWT prefers (*S*)-(−)-sarin (Fig. 8).

5. Discussion

Regarding the kinetic determinations on sarin, the mutants FY and FI revealed little actual change in catalytic efficiency from the WT enzyme. However, FL had an approximately six-fold increased k_{cat}/K_m value compared to WT. Specifically, the K_m value of FL was about half that of WT and the k_{cat} value was about three times that of the WT enzyme. FY and FI had k_{cat} and K_m values that were all comparable to WT. Clearly, FL was the most promising mutant obtained with respect to sarin activity, and it showed significant increases in soman and GP activity as well.

Interestingly, the increased catalytic efficiencies seen on soman and GP were both caused by a decrease in the K_m with the FL mutant, while the k_{cat} of FL remained essentially unchanged from the WT. Soman and GP are related in that they both possess a stereogenic center adjacent to the P-O bond in addition to the chiral phosphorus atom, giving a total of four stereoisomers.

The stereospecificity of the OPAA FL and PTE GWT mutants proved useful to us for making enriched preparations of each enantiomer which permitted assignment of the respective peaks in the LC chromatogram based on the differential inhibition of acetylcholinesterase, which was correlated with the published toxicity values for the two enantiomers of sarin. Similar mutants might also aid the assignment of unidentified peaks in the chiral separation of soman and/or GP, although the situation is obviously much more complex given the increased stereochemical complexity of these molecules.

6. Conclusions

Beyond the laboratory, the practical significance of stereospecificity

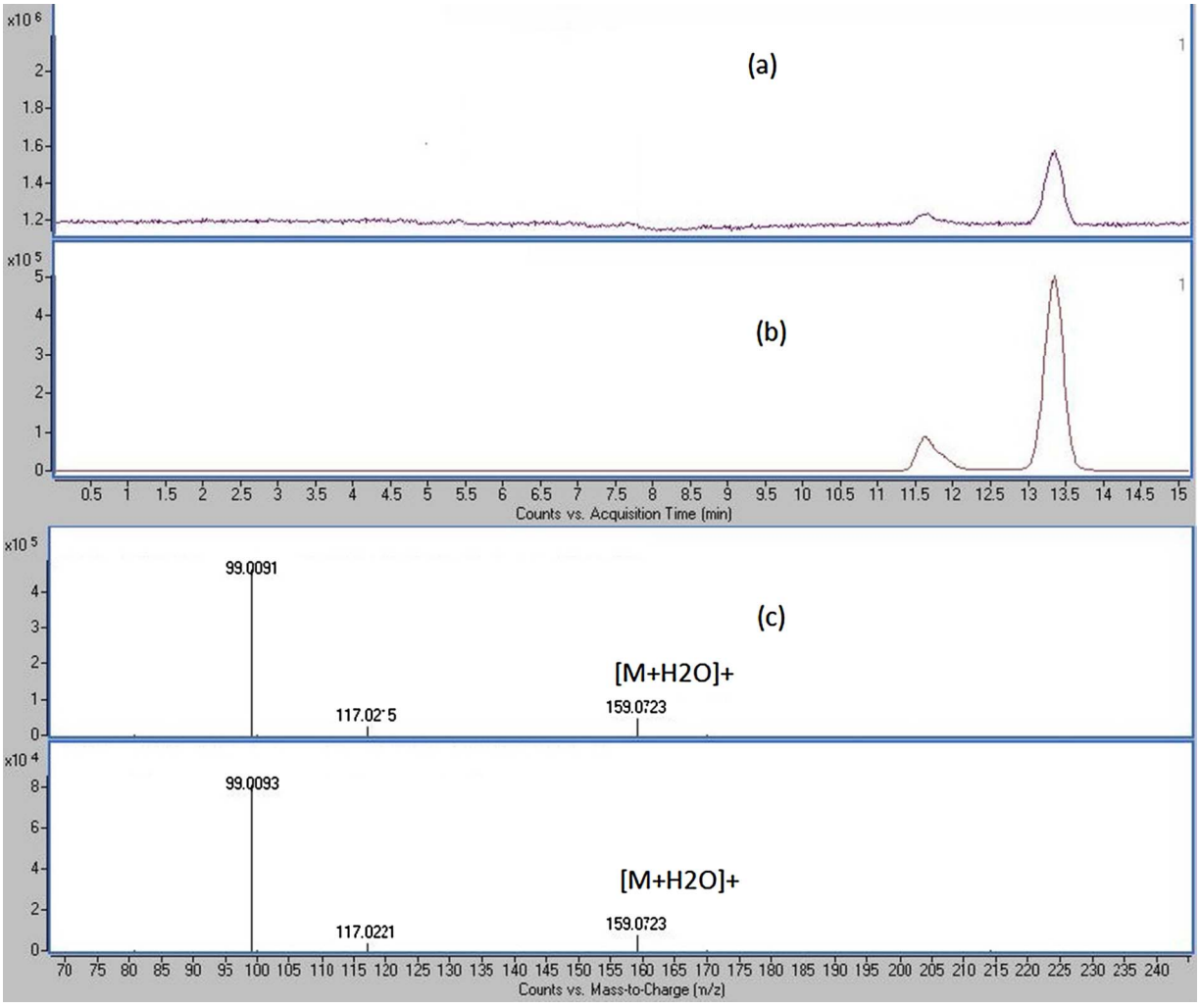

Fig. 8. A representative (a) TIC, (b) EIC, and (c) MS for OPAA FL-degraded sarin.

depends on the purpose for which the enzymes might be used. If the enzymes are intended for use as a medical countermeasure where they must act in blood with agent concentrations likely in the micromolar range or below, stereospecificity is a critical consideration since an enzyme with strong stereospecificity might degrade one enantiomer while leaving the other present at toxic concentrations, thereby conferring little protection. However, the situation is considerably different if the enzymes are intended for use as a surface decontaminant. Owing to Le Chatlier's Principle, decontamination could potentially be performed using enzymes preferring either enantiomer, as G-agents are known to racemize rapidly in the presence of concentrations of F^- above the low mM range [23]. If the enzymes are to be used as part of a decontamination solution, small amounts of fluoride can easily be added to the solution to ensure racemization even at very low agent concentrations. On that basis, it might be concluded that the enhanced catalytic efficiency observed with the FL mutant on sarin could be more beneficial for decontamination than for *in vivo* treatment, since it comes with an increased preference for the less toxic (*R*)-(+) sarin enantiomer.

To our knowledge these OPAA FL kinetic data represent the highest catalytic efficiencies reported with soman. And while the FL mutant represents a significant improvement over the WT OPAA enzyme in regards to GP activity, we cannot compare it to other enzymes yet since these are the first enzymatic data reported with that compound.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2017.11.001.

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