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Degradation of nerve agents by an organophosphate-degrading agent (OpdA)

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Abstract

Enzyme-catalysed degradation of the nerve agents tabun, sarin, ethyl sarin and soman by three variants of an organophosphate-degrading enzyme was studied at low concentrations of nerve agent. The concentration of nerve agent at a given time was determined by its ability to inhibit the enzyme acetylcholinesterase. Experiments were conducted in 96-well microtitre plates. Values of the ratio of k_{cat} (turnover number) to K_m (Michaelis–Menten constant) were calculated. For tabun, this value (for the most effective OpdA variant) exceeded any value published to date for other enzymes. The value was within an order of magnitude for the highest value reported for sarin, but there appears to be no published value for ethyl sarin for comparison. The OpdA enzymes were relatively inefficient in degrading soman.

Keywords: OpdA; Enzyme; Hydrolysis; Nerve agent; Microtitre plate

1. Introduction

Enzymes have considerable potential for decontamination and detoxification of organophosphorus anticholinesterase compounds, such as pesticides and the nerve agent class of chemical warfare agents [1,2]. They may also be used for destruction of nerve agent stockpiles, in "active" fabrics and filter elements for personal protection, and in sensors for chemical warfare agents [3]. The decontamination solutions currently fielded for use against nerve agents (DS2 and bleach) are corrosive in nature and result in hazardous waste. Enzyme-mediated decontamination is non-toxic, noncorrosive and environmentally compatible [1]. In Australia, an enzyme product known as Landguard™ OP-A is marketed to clean up organophosphate insecticide-contaminated water, in particular sheep dips ([4]; http://www.orica-landguard.com). Many of the enzymes known to hydrolyse organophosphorus esters are known as organophosphorus hydrolase (OPH; EC 3.1.8.1; alternative name phosphotriesterase (PTE)) or organophosphorus acid anhydrolase (OPAA; EC 3.1.8.2). Landguard™ OP-A does not fit neatly into either of these categories, and is known as an organophosphate-degrading agent (OpdA). The first OpdA was described in 2002 [5], and its genetics characterised in 2003 [6]. OpdA enzymes, like OPH/PTE, hydrolyse organophosphates by cleavage of a P–O (or P–S) bond with formation of hydrolysis products that are of low toxicity [7]. The mechanism has been confirmed in numerous studies, e.g. references [8–11]. Recently, mutants of OpdA have been prepared and have shown enhanced activity towards organophosphorus insecticides; the activity is superior to that of OPH in some cases [8,9]. In the present paper, the ability of Landguard™ OP-A to inactivate the G-type nerve agents tabun (commonly abbreviated as GA), sarin (GB), soman (GD) and ethyl sarin (GE) was investigated. In addition to Landguard™ OP-A itself, two mutants of the enzyme that had been designed to enhance its activity against organophosphorus pesticides were also evaluated.

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2. Materials and methods

2.1. Materials

The nerve agents were synthesised in-house (DSTO) and were ≥95% pure. Stock solutions (0.1 mg/ml) were prepared in acetonitrile and were diluted into phosphate-buffered saline pH 7.4 (PBS) just before use. After use, they were decontaminated with 0.1 M NaOH. PBS was prepared by adding the contents of a commercial sachet to 1 l of Milli-Q distilled water. This product, and all other reagents, were obtained from Sigma–Aldrich, Australia. Nunc-Immuno 96-microwell flat bottom plates were obtained from In Vitro Technologies, Melbourne, Australia. Landguard™ OP-A (designated OpdA mark I) and its two mutants (OpdA mark II and mark III) were products of Orica, Australia. All three variants have a molecular weight of approximately 38.6 kDa, and were supplied as a powder. The actual enzyme represented 6% of the powder in the cases of mark I and mark II and 2.5% for mark III. Stock solutions of 50 g/l were stored at 4 °C and diluted in water prior to use.

2.2. Methods

Reactions and assays were performed in 96-well plates. Aliquots (20 μl) of nerve agent (see Table 1 for final concentration) were placed in wells, and 50 μl Landguard™ OP-A in water (designated OpdA from here on) was added per well at time zero and at eight subsequent 1-min intervals. The plate was shaken on a plate-shaker between additions of OpdA. At 10 min, eel acetylcholinesterase (0.6 units per ml; 50 μl) was added to each well and incubated with shaking for 10 min, after which the acetylcholinesterase substrate (acetylthiocholine; final concentration 2.4 mM, with 0.3 mM 5,5′-dithio bis(2-nitrobenzoic acid)) was added. The rate of increase of absorbance at 412 nm was measured with a BioTek Synergy HT plate reader over 10 min [12]. Appropriate controls were run. The rate of increase of absorbance in the presence of acetylcholinesterase and absence of nerve agent was in the range 0.10–0.15 abs/min. A number of wells received nerve agent of different concentrations, but no OpdA, enabling a calibration plot to be obtained (see Section 2.3).

2.3. Analysis

Nerve agents inhibit acetylcholinesterase irreversibly (in the time frame of the experiment [13]). A plot of abs/min vs. log (nerve agent concentration) for the 10-min acetylcholinesterase–nerve agent incubation was found to be linear over most of the inhibition range (see Fig. 1 for an example). This enabled the concentration of non-degraded nerve agent to be determined after a given time of incubation with OpdA (1–9 min). The limit of detection of the nerve agents was found to be approximately 2 pmol for GA (tabun) and 40, 7.5 and 120 fmol for GB (sarin), GD (soman) and GE (ethylsarin), respectively. The initial concentration of nerve agent was chosen such that approximately 90% inhibition of acetylcholinesterase occurred over 10 min in the absence of OpdA. This concentration is shown in Table 1. Degradation of nerve agent by OpdA was measured for three concentrations of OpdA, these concentrations being selected such that significant degradation (>50%) occurred during the 10-min incubation in each case. It was possible to study each of the three concentrations of OpdA in duplicate on the same 96-well plate (using six of the eight rows), together with the calibration plot in duplicate (the remaining two rows). The first-order rate constant for degradation of nerve agent (see below) was found to be directly proportional to the concentration of OpdA.

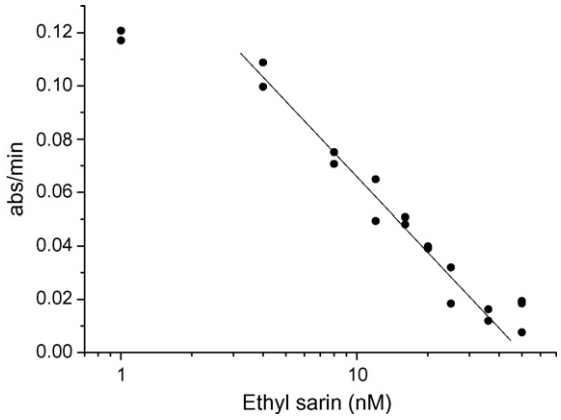

Fig. 1. Calibration plot: acetylcholinesterase activity (rate of increase of absorbance at 412 nm) vs. log (concentration of nerve agent (ethyl sarin)).

Because substantial depletion of the OpdA substrate (nerve agent) occurs during the experiment, analysis of the Michaelis–Menten kinetics by initial rates is not applicable. The kinetics for a progress curve were therefore applied. The equation [14] is

$$V_{\max}t = K_{\mathrm{m}} \ln\left(\frac{S_0}{S}\right) + (S_0 - S) = K_{\mathrm{m}} \ln\left(\frac{S_0}{S}\right) + P \qquad (1)$$

where S_0 and S are the concentration of substrate at time zero and time t, respectively, P is the concentration of product (degraded nerve agent) at time t, and K_{m} and $V_{\max}$ are the Michaelis–Menten kinetic constants.

$$\text{Rearranging,} \quad \frac{V_{\max}}{K_{\mathrm{m}}}t = \ln\left(\frac{S_0}{S}\right) + \frac{P}{K_{\mathrm{m}}} \qquad (2)$$

If the assumption is made that $P \ll K_{\mathrm{m}}$ (see Section 4), the equation reduces (after rearrangement) to

$$\ln S \approx \ln S_0 - \left(\frac{V_{\max}}{K_{\mathrm{m}}}\right) t \qquad (3)$$

A plot of $\ln S$ vs. t is therefore linear with a slope of $V_{\max}/K_{\mathrm{m}}$. Designating the slope as the first-order rate constant k_{obs}, and recalling that $V_{\max} = k_{\mathrm{cat}}[\mathrm{E}]$, where k_{cat} is the turnover number and [E] is the concentration of enzyme (OpdA), enables the ratio of k_{cat} to K_{m} to be determined [3]:

$$\frac{k_{\mathrm{cat}}}{K_{\mathrm{m}}} = \frac{k_{\mathrm{obs}}}{[\mathrm{E}]} \qquad (4)$$

The ratio $k_{\mathrm{cat}}/K_{\mathrm{m}}$ is a measure of the efficacy of OpdA, since the higher the turnover number (k_{cat}) and the higher the affinity of

Table 1
Rate constants for degradation of nerve agents by OpdA variants

Nerve agent		OpdA		$k_{cat}/K_m \pm$ S.E.M. (n)[a] (M^{-1} min^{-1})
Name	Concentration	Variant	Concentration	
Tabun	1.23 μM	I	11–44 nM	$(1.03 \pm 0.07) \times 10^7$ (6)
Tabun	1.23 μM	II	22–89 nM	$(4.44 \pm 0.12) \times 10^6$ (11)
Tabun	1.23 μM	III	2.5–7.4 nM	$(3.88 \pm 0.12) \times 10^7$ (10)
Sarin	29 nM	I	0.17–0.69 μM	$(4.16 \pm 0.20) \times 10^5$ (12)
Sarin	29 nM	II	0.35–1.39 μM	$(1.54 \pm 0.08) \times 10^5$ (11)
Sarin	29 nM	III	0.07–0.29 μM	$(6.37 \pm 0.20) \times 10^5$ (12)
Soman	2.5 nM	I	13 μM	$(4.62 \pm 0.76) \times 10^3$ (3)
Soman	2.5 nM	II	13 μM	$(3.03 \pm 0.64) \times 10^3$ (3)
Soman	2.5 nM	III	5.4 μM	$(1.82 \pm 0.11) \times 10^4$ (3)
Ethyl sarin	86 nM	I	0.35–1.39 μM	$(4.14 \pm 0.24) \times 10^5$ (9)
Ethyl sarin	86 nM	II	0.69–2.78 μM	$(1.16 \pm 0.05) \times 10^5$ (9)
Ethyl sarin	86 nM	III	0.07–0.29 μM	$(1.30 \pm 0.04) \times 10^6$ (9)

n = number of replicate determinations.
[a] S.E.M. = standard error of the mean.

the enzyme for its substrate (nerve agent), as indicated by a low K_m, the higher is this ratio. An example of a plot of ln S vs. t is shown in Fig. 2.

In most enzyme-catalysed reactions, the assumption is made that the initial concentration of enzyme is much less than that of the substrate, and this is the basis of the equations above. In some cases in the present work, the enzyme concentration exceeded the substrate concentration (Table 1). Although the linear relationship predicted by Eq. (3) above was found to occur (Fig. 2) in all cases, the values of k_{cat}/K_m that were determined should be taken as approximations when [E] > [S]. Nevertheless, they provide a good indication of the catalytic efficiency of the OpdA enzymes, and this was the objective of the study.

3. Results

Values of k_{cat}/K_m for the nerve agent–OpdA interaction for tabun, sarin, soman and ethyl sarin are given in Table 1, together with the concentrations of nerve agent and OpdA used. It can be seen that OpdA is most effective in degrading tabun (the least potent nerve agent as an inhibitor of eel acetylcholinesterase), and least effective against soman (the most potent acetylcholinesterase inhibitor). Efficacy is approximately the same for sarin and ethyl sarin, which have intermediate potencies in inhibiting acetylcholinesterase. Of the three variants of OpdA, mark III demonstrates the highest k_{cat}/K_m values for any given nerve agent, followed by mark I and mark II.

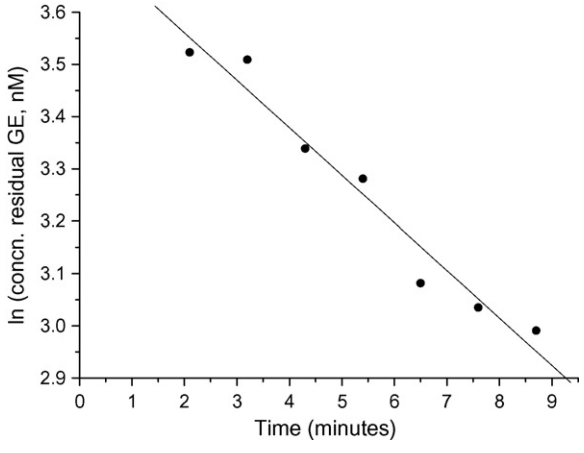

Fig. 2. Degradation of nerve agent (ethyl sarin) by OpdA mark II: concentration of natural log of un-degraded nerve agent vs. time.

4. Discussion

In measuring the rate of degradation of a substance, a method of determining the concentration of either the product of the degradation or of the un-degraded starting material at various times is obviously required. In the present work, the concentration of residual nerve agent was determined, and an adaptation of the methods of Hammond and Forster [15] and Amitai et al. [16] was chosen. This method is based on the extent of inhibition of acetylcholinesterase activity in a given time period, as measured using the Ellman assay [12]. The experiment is performed using 96-well microtitre plates, and the incubation of nerve agent with OpdA was performed in the same plate. The advantages of using this approach are that many variables and replicates can be accommodated in one 30-min experiment, and very low amounts of nerve agent are used, with the two advantages of (i) conservation of material and (ii) minimal risk to personnel who work with these extremely hazardous chemicals.

The calculation of k_{cat}/K_m values from the results depends on the validity of the assumption that the concentration of degradation product (which is ≤concentration of starting nerve agent) is $\ll K_m$ of the enzymatic reaction. This assumption is based on literature values for degradation of nerve agents by other enzymes. These are listed in Table 2 and represent all values that have been published, to the best knowledge of the current authors. When quoted in these papers, the temperature of the assays was 25 °C in most cases, but 37 °C in the papers by Wang et al. [17], Masson et al. [18] and DeFrank and White [19]. Apart from the study by Amitai et al. [16] on soman (GD) and GF, the

Table 2
Published kinetic constants for degradation of nerve agents by enzymes

Source of enzyme	Method	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,min^{-1}$)	Reference
A. Tabun (GA)					
Rat liver	pH stat	23.4	8.9	2.63×10^3	[22]
Alteromonas undina	NMR		1–10	21.8	[21]
Alteromonas sp. JD6.5	N.A.	5.1×10^3			[23]
Alteromonas haloplanktis	N.A.	6.78×10^3			[23]
A. undina	N.A.	1.75×10^4			[23]
Alteromonas sp. JD6.5	NMR	5.1×10^3			[19]
A. haloplanktis	NMR	1.53×10^4			[19]
A. undina	NMR	2.21×10^4			[19]
B. Sarin (GB)					
Rat liver	pH stat	107.8	1.9	5.67×10^4	[22]
Pseudomonas diminuta	Fluoride electrode	3.36×10^3	0.7	4.8×10^6	[19,24]
A. undina	Fluoride electrode		1–10	30.4	[21]
Human paraoxonase	N.A.			9.1×10^5	[18]
Cloned *A.* sp. JD6.5	Fluoride electrode	9.06×10^3	1.57	5.8×10^6	[25]
Alteromonas sp. JD6.5	N.A.	3.67×10^4			[23]
A. haloplanktis	N.A.	1.54×10^4			[23]
A. undina	N.A.	2.26×10^4			[23]
Alteromonas sp. JD6.5	Fluoride electrode	3.67×10^4			[19]
A. haloplanktis	Fluoride electrode	1.85×10^4			[19]
A. undina	Fluoride electrode	2.56×10^4			[19]
Mutant PTE	N.A.	$>6 \times 10^4$			[26]
C. Soman (GD)					
E. coli	Fluoride electrode	7.65			[27]
NG108-15	pH stat	12.5	5	2.5×10^3	[20]
Thermophilic bacterium	N.A.		10–20		[28]
Rat liver	pH stat	28.7	1.1	2.61×10^4	[22]
A. undina	Fluoride electrode		1–10	1.60×10^2	[21]
Alteromonas sp. JD6.5	NMR		1–10	14.6	[21]
P. diminuta	Fluoride electrode	2.88×10^2	0.5	5.76×10^5	[24]
Human paraoxonase	N.A.			2.8×10^6	[18]
Cloned *Alteromonas* sp. JD6.5	Fluoride electrode	2.5×10^4	2.48	1×10^7	[25]
Alteromonas sp. JD6.5	N.A.	1.89×10^5			[23]
A. haloplanktis	N.A.	8.3×10^4			[23]
A. undina	N.A.	1.50×10^5			[23]
Alteromonas sp. JD6.5	Fluoride electrode	1.89×10^5			[19]
A. haloplanktis	Fluoride electrode	1×10^5			[19]
A. undina	Fluoride electrode	1.7×10^5			[19]
P. diminuta	N.A.	3.0×10^2	0.5	6×10^5	[19]
Human serum PON1, V346A mutant	AChE inhibition			8.67×10^4	[16]
Squid DFPase	AChE inhibition			2.4×10^3	[16]
P. diminuta	AChE inhibition			2.27×10^3	[16]
Human serum PON1	GLC	5.01×10^2–1.03×10^3	0.27–0.91	6.25×10^5–4.13×10^6	[29]
Mutant PTE	N.A.	$>6 \times 10^2$			[25]
D. GF					
A. undina	Fluoride electrode		1–10	1.30×10^2	[21]
P. diminuta	Spectrophotometric	1.8	0.36	5.0×10^3	[30]
Human liver	Spectrophotometric	4.1×10^5			[17]
Cloned *Alteromonas* sp. JD6.5	Fluoride electrode	3.91×10^4	0.63	6.2×10^7	[25]
Alteromonas sp. JD6.5	N.A.	9.9×10^4			[23]
A. haloplanktis	N.A.	1.6×10^4			[23]
A. undina	N.A.	9.5×10^4			[23]
Alteromonas sp. JD6.5	Fluoride electrode	9.9×10^4			[19]
A. haloplanktis	Fluoride electrode	1.94×10^4			[19]
A. undina	Fluoride electrode	1.07×10^5			[19]
Alteromonas sp. JD6.5	Fluoride electrode	1.75×10^3			[31]
A. haloplanktis	Fluoride electrode	3.27×10^3			[31]
E. coli	Fluoride electrode	2.33×10^3			[31]
Human serum PON1, V346A mutant	AChE inhibition			3.6×10^5	[16]

Table 2 (*Continued*)

Source of enzyme	Method	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} min^{-1})	Reference
Squid DFPase	AChE inhibition			5.07×10^3	[16]
P. diminuta	AChE inhibition			3.6×10^3	[16]
E. VX					
P. diminuta	N.A.	18	0.44	4.1×10^4	[19]
Corynebacterium glutamicum	N.A.	~350			[32]

N.A.: not available.

Table 3
Comparison of kinetic constants for OpdA variant III with maximum literature values

Nerve agent	Maximum (k_{cat} and k_{cat}/K_m) or minimum (K_m) published values			(Maximum k_{cat})/(minimum K_m) (M^{-1} min^{-1})	k_{cat}/K_m for OpdA III
	k_{cat} (min^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} min^{-1})		
Tabun	2.21×10^4	$1–10 \times 10^{-3}$	2.63×10^3	2.21×10^7	3.88×10^7
Sarin	$>6 \times 10^4$	7×10^{-4}	5.8×10^6	$>8.57 \times 10^7$	6.37×10^5
Soman	1.89×10^5	5×10^{-4}	1×10^7	3.78×10^8	1.82×10^4

quoted pH was in the range 7.0–7.4. In the present work, the pH was approximately 7.4, and the temperature was room temperature (approximately 20 °C). Most authors did not quote their concentration of nerve agent-degrading enzyme. In the few cases in which they did (or the concentration could be calculated from the data and the quoted molecular weight), the concentration varied from 0.4 to 27 μM, which overlaps our range of OpdA concentration (2.5 nM to 13 μM; Table 1). In these conditions of temperature, pH and enzyme concentration, therefore, our experimental conditions are comparable to most relevant literature reports. The lowest value of K_m found in the literature is 0.36 mM, a value that is 300-fold greater than the highest concentration of nerve agent used in the present study (1.23 μM). The assumption above, that the concentration of degradation product (which is ≤concentration of starting nerve agent) is $\ll K_m$ of the enzymatic reaction, is therefore reasonable. The excellent linearity of the plots of (ln[S]) vs. time (Fig. 2) also supports the validity of the kinetic analysis. It follows from the relationship between nerve agent concentration and K_m that it is not practically possible to determine K_m and k_{cat} individually, since the initial rates of the enzyme-catalysed degradation cover only the smallest initial portion of the v vs. s curve. However, since the rate of an enzymatic reaction increases with high k_{cat} and low K_m, the ratio of these two constants is a valid measure of the efficiency of an enzyme–substrate system. It is planned to study the degradation of millimolar concentrations of nerve agents by OpdA by NMR and infrared spectroscopy to confirm that K_m is in this range of concentration, and this study will be reported separately.

The kinetic analysis also assumes that the degradation occurs via a catalytic reaction, and not by stoichiometric binding (scavenging) of the nerve agent by OpdA or other components of the preparation. The latter possibility is feasible for sarin, ethyl sarin and soman, since [OpdA] > [nerve agent] in these cases. In the case of tabun, however, [OpdA] ≪ [nerve agent] (Table 1), and the fact that most of the nerve agent is degraded during the 10-min incubation points to a catalytic reaction. It is reasonable to infer that the other nerve agents are degraded by enzyme catalysis also.

The literature does not always quote both k_{cat} and K_m (Table 2), so their ratio is not always available. Some nerve agents not considered in the present study are included in Table 2 for information only. Table 3 lists the highest published k_{cat} for a given nerve agent (tabun, sarin or soman only) with any enzyme, and the lowest K_m, plus the ratio of the two, regardless of their source (ethyl sarin does not appear in Table 3 since no literature value was found for this nerve agent). This maximum k_{cat}/K_m has not necessarily been, or can be, achieved. Nevertheless, this value, together with the maximum value of k_{cat}/K_m that has been determined and published, enables an assessment of the ranking of the OpdA enzymes reported above within the list of enzymes that have been reported to degrade nerve agents. The k_{cat}/K_m values from the present paper for OpdA mark III (the most efficacious variant) are reproduced in Table 3. It can be seen that for OpdA mark III and tabun, the value of k_{cat}/K_m is four orders of magnitude higher than the maximum published value, and even slightly higher than the hypothetical maximum value that can be achieved with any enzyme studied to date. For sarin, k_{cat}/K_m for OpdA mark III is within one order of magnitude of the best actual value published. The comparison is valid because, as mentioned above, our experimental conditions are similar in several aspects to those of the literature studies in Table 2. The differences that do exist are in the aspects of substrate (nerve agent) concentration, type of enzyme, and method of measuring the rate of degradation. The degradation methods listed in Table 2 are dominated by use of a fluoride electrode or a pH stat apparatus, and the substrate (nerve agent) concentration is therefore of necessity in the millimolar range, the lowest being 0.05 mM [20]. The exception is the paper of Amitai et al. [16] in which the method is inhibition of AChE (the method used in the current paper), but even in this case the concentration of substrate was 10 μM, and the solution of substrate and degrading enzyme was diluted 50–1000-fold before incubation with AChE. By contrast, we used nerve agent con-

centrations of 2.5 nM to 1.23 μM. With respect to the type of enzyme, there is no consensus on the nomenclature of enzymes that degrade organophosphorus anticholinesterases [21]. The enzymes of Table 2 are known as DFPase, organophosphorus acid anhydrolase (OPAA), organophosphorus hydrolase (OPH), phosphotriesterase (PTE), paraoxonase (PON1) or somanase. OpdA is genetically different from these enzymes, although closely similar to some [5,6].

In contrast to degradation of tabun and sarin by OpdA mark III, the soman/OpdA III system is markedly inferior to other enzyme/nerve agent systems in terms of catalytic efficiency. This is probably related to the lack of symmetry of its methyl groups opposite the phosphorus atom on the ester target bond, although the evidence for this is anecdotal. The variation in the ability of OpdA to degrade different nerve agents is consistent with its behaviour with respect to organophosphorus pesticides; for example, Horne et al. [5] demonstrated a 3200-fold range in k_{cat}/K_m ratios of nine pesticide substrates, from 480 (phosmet) to $1.54 \times 10^6\ M^{-1}\ min^{-1}$ (parathion-methyl).

5. Conclusion

OpdA variants, especially mark III, are of greater efficacy in degradation of the nerve agent tabun than any other enzymes reported to date. The efficacy for degradation of sarin by OpdA mark III is only slightly less than that of the best system published in the literature. No literature data is available for ethyl sarin, which has similar kinetic constants in our system to sarin. On the other hand, soman is relatively resistant to degradation by OpdA. These results have implications for the development of safe decontaminants for selected nerve agents. OpdA is the active ingredient of Landguard™ OP-A. Applications have been developed for the use of Landguard™ OP-A in the treatment of water run-off, equipment rinsing and soil decontamination. Whilst these applications are focussed on agricultural and remediation markets, the modifications required for nerve agent detoxification will be relatively minor, particularly as the enzyme can be applied in powder, liquid or matrix-bound forms. Further information on Landguard™ OP-A applications can be found at http://www.orica-landguard.com.

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Glossary

DSTO: Defence Science & Technology Organisation (Australia)
GA: Tabun
GB: Sarin
GD: Soman
GE: Ethyl sarin
k_{cat}*:* Enzyme turnover number
K_m*:* Michaelis–Menten constant
OPAA: Organophosphorus acid anhydrolase
OpdA: Organophosphate-degrading agent
OPH: Organophosphorus hydrolase
PBS: Phosphate-buffered saline