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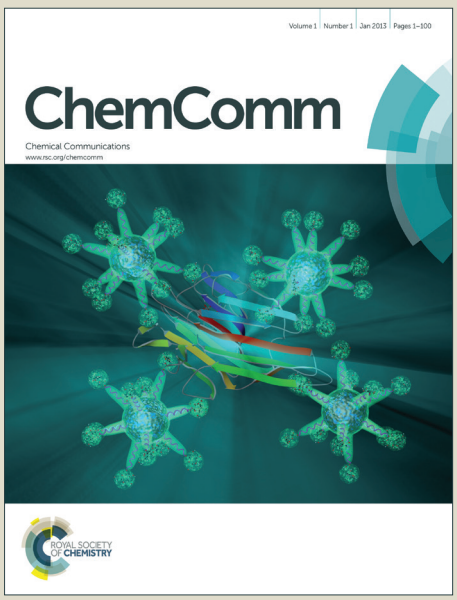

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Rational design of paraoxonase 1 (PON1) for the efficient hydrolysis of organophosphates

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Rational design of paraoxonase 1 based on molecular docking discovered H115W/T332S and I74F/H115W/T332S mutants exhibited 40-fold increased in catalytic efficiency (k_{cat}/K_m) toward hydrolysis of two toxic and popular organophosphates (diethyl-paraoxon and dimethyl-paraoxon). Moreover, the conversions of the paraoxons (741.3~825.6 mg/L) by the evolved mutants were 42~60-fold faster than that by the wild type.

Organophosphates (OPs), which include OP pesticides and nerve agents, inhibit acetylcholinesterase,[1] that is essential for hydrolyzing neurotransmitter acetylcholine, leading to the accumulation of access levels of acetyl-choline and resulting in the cholinergic crisis.[2] Paraoxonase 1 (PON1), a mammalian enzyme that can catalyze the hydrolysis of various OPs (i.e., pesticide metabolites diethyl-paraoxon (EPO), diazinon-oxon (DZO), and chloropyrifos-oxon (CPO) as well as nerve agents sarin, soman, and VX)[3-4] and showed its compatibility with mammals (i.e., non-toxic and minimal immune reaction),[2,5] is a leading candidate for *in-vivo* treatment of individuals accidentally exposure to OPs,[2,5,6] and the removal of these compounds from the environment. However, the catalytic efficiency of PON1wild type for most OPs is low.[7] Attempts to use PON1 as a therapeutic catalytic scavenger have involved the directed evolution of PON1 via DNA shuffling of mammalian PON1 genes, resulting in the generation of G3C9 PON1 enzyme. This enzyme was functionally expressed with a high yield in *Escherichia coli* and exhibited a slightly increased catalytic efficiency for EPO hydrolysis compared to human PON1 (huPON1) wild type R192 (2-fold).[8] Additionally, site-directed mutagenesis of huPON1 at residue 192 based on the alignment with rabbit PON1[2] or at and around putative active residues of PON1 based on homology modelling with squid diisopropyl fluorophosphatase[9] identified HuPON1 mutants (R192K and H115W) with slightly enhanced catalytic activity toward hydrolysis of OPs (i.e., HuPON1 R192K and H115W had 1.3~2.9- and 2-fold higher catalytic activity for hydrolysis of OPs (EPO, DZO, and CPO) and VX compared to its wild type, respectively.[2-3]). Further engineering of PON1 to discover additional PON1 mutants with higher catalytic efficiency is a prerequisite for the detoxification of OPs in practical application.

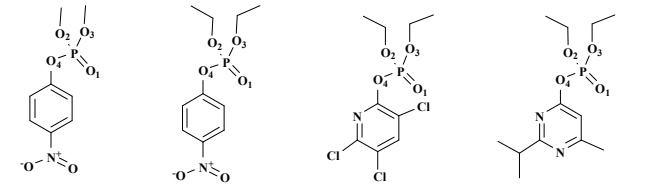

Fig. 1 Structures of popular OP pesticide metabolites

Recently, the rational protein design has been increasingly used as an effectiveness to improve selectivity, enantioselectivity, and thermal stability of several enzymes.[10-13] In this study, we present the first rational design based on molecular docking to engineer G3C9 rePON1 toward the hydrolysis of OPs shown in Fig. 1. Additionally, EPO, a model ligand for directed evolution of lactonase from *Geobacillus kaustophilus* HTA426 for OP hydrolysis (i.e., the evolved mutants for the hydrolysis of EPO also

correlatively improved for the hydrolysis of other OPs (dimethyl-paraoxon (MPO), diazinon, chloropyrifos)[14] due to its structural similarity with other OPs (MPO, DZO, and CPO), was selected as a model ligand for engineering of G3C9 rePON1.

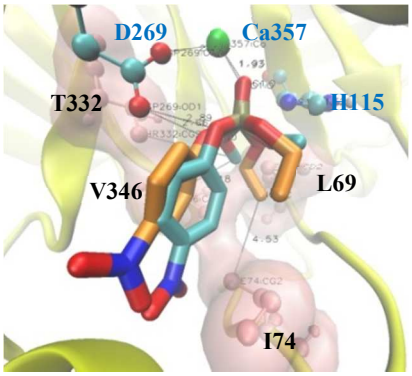

Fig. 2 Molecular docking of PON1 (3srg.pdb)[15] with two paraoxons. EPO (as licoride, orange); MPO (as licoride, cyan); H115 and D269 (as CPK); L69, I74, T332, and V346 (as CPK, pink).

To determine a selective pocket toward different paroxons, molecular docking between PON1 with two paraoxons (EPO and MPO), which possess different size of *O,O*-dialkyl groups, was performed. The binding mode and binding affinity of the two paraoxons in active site of PON1 are seen in Fig. 2 and Table S2 in ESI†. Both paraoxons localized similarly in the active site of PON1 with the exception of their *O,O*- dialkyl group. Two residues (T332 and V346) on the same side of residue D269, which functions as a stabilizing factor for hydroxide group of pentavalent oxy-anionic intermediate of paraoxon,[15] closely contacted *O*-methyl of MPO (i.e., the distances from $C_{\gamma 1}$ (V346), $C_{\gamma 2}$ (V346), and $C_{\gamma 2}$ (T332) to *O*-methyl carbon of MPO were 4.18, 3.5, and 4.85 Å, respectively). EPO possesses a larger *O,O*- dialkyl compared to MPO and couldn't fit well into the pocket composed of T332 and V346. Therefore, EPO was pushed and rotated counter-clockwise further from T332 and V346 compared to the *O*-methyl of MPO. This shift also brought O_4 (EPO) closer to OD_1 (D269), resulting in steric hindrance in van der Waals (VDW) interaction because the 2.66 Å distance between them is shorter than oxygen atom's VDW diameter of 2.95Å.[16] These steric hindrances may be the reason for the large value of the calculated inhibitor constant (which is equal to Michaelis-Menten constant (K_m)) between PON1 and EPO compared to PON1 and MPO (4.49 and 0.801 mM, respectively) (Table S2, ESI†). We hypothesized that site-directed mutagenesis to substitute T332 and V346 by smaller residues may create some space to accommodate the *O*-ethyl of EPO, and therefore would be expected to improve PON1 toward hydrolysis of EPO. Moreover, two residues (I74 and L69) that were in close contact with the respective *O*-ethyl of EPO and *O*-methyl of MPO were also targets for site-directed mutagenesis (i.e., respective distances from $C_{\gamma 1}$ and $C_{\gamma 2}$ of I74 to the O-ethyl carbon of EPO were 4.5 and 4.53 Å, while the distance between $C_{\delta 2}$ (L69) and the O-methyl carbon of MPO was 2.85 Å).

Table 1 Kinetic parameters of G3C9 rePON1 variants for the hydrolysis of EPO at 25 °C

G3C9 rePON1s	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}.s^{-1}$)
Wild type	1.818	0.700	2.60×10^3
I74F	3.162	0.349	9.06×10^3 (**3.49**)
T332S	5.906	0.312	1.89×10^4 (**7.29**)
V346A	3.402	0.598	5.69×10^3 (**2.19**)
I74F/H115W	7.671	0.479	1.60×10^4 (**6.16**)
I74F/T332S	4.191	0.298	1.41×10^4 (**5.42**)
I74F/V346A	10.437	0.676	1.54×10^4 (**5.94**)
H115W/T332S	12.146	0.120	1.01×10^5 (**39.02**)
H115W/V346A	10.464	1.406	7.44×10^3 (**2.86**)
T332S/V346A	2.945	0.473	6.23×10^3 (**2.40**)
I74F/H115W/T332S	12.909	0.137	9.39×10^4 (**36.13**)
I74F/H115W/V346A	22.927	1.290	1.78×10^4 (**6.84**)

(*) The numbers in parentheses present fold enhancement relative to the G3C9 rePON1 wild type

Site-directed mutagenesis at four above-mentioned residues (T332, V346, I74, and L69) significantly impacted the hydrolysis of EPO (Fig. S1, ESI†). The kinetic parameters of G3C9 rePON1 variants for the hydrolysis of EPO were determined in 3 mL reaction solution with increasing concentration of EPO from 0.05 to 0.5 mM. Each highly purified G3C9 rePON1 (molecular weight is ~40 kDa)[17] was added to the reaction solution to a final concentration of 0.021 μM (see detailed information in ESI†). The results showed that G3C9 rePON1 T332S, I74F, and V346A exhibited increased catalytic efficiency (k_{cat}/K_m) by 7.3-, 3.5-, and 2.2-fold for EPO hydrolysis compared to the wild type (Table 1). It was found that H115W mutant of G2E6 rePON1, which was indentified prior to G3C9 rePON1 during the directed evolution of PON1s,[8] had 2-fold higher hydrolysis activity toward EPO compared to its wild type.[18] Better G3C9 rePON1 mutants were generated by combining the evolved mutants (i.e., T332S, I74F, V346A, and H115W) (Table 1

and Fig. S1, ESI†). The two best G3C9 rePON1 mutants (H115W/T332S and I74F/H115W/T332S) dramatically increased the catalytic efficiency toward the hydrolysis of EPO compared to the wild type. The catalytic efficiency (k_{cat}/K_m) of G3C9 rePON1 H115W/T322S and I74F/H115W/T332S for EPO hydrolysis at 25 °C reached 1.01×10^5 and 9.39×10^4 $M^{-1}.s^{-1}$; these values are 39.0- and 36.1-fold higher than that of the wild type (2.6×10^3 $M^{-1}.s^{-1}$). The enhancement of the catalytic efficiency is accounted for 5.1~5.8- and 6.7~7.1-fold increase in binding affinity and k_{cat} value (Table 1). The catalytic efficiency (V_{max}/K_m) of G3C9 rePON1 H115W/T332S for EPO hydrolysis at 25 °C was 20.0- and 12.1-fold higher than the values reported for the reHuPON1 R192 wild type and R192K at 37 °C, respectively (i.e., V_{max}/K_m of 152.5, 7.61, and 12.61 for G3C9 rePON1 H115W/T332S, reHuPON1 wild type, and R192K, respectively).[2] Arylesterase activity assays were also done for G3C9 rePON1 variants toward hydrolysis of phenyl acetate (a preferential substrate of PON1 wild type)[18] (see detailed information in ESI†). Most of the evolved mutants exhibited decreased its specific activity toward hydrolysis of phenyl acetate compared to the wild type; in particular, all mutants from H115W (e.g., H115W/T332S) lost almost the activity (>99.5%) (Fig. S3, ESI†). This implies that the evolved mutants prefer hydrolyzing paraxons to phenyl acetate. The increase in k_{cat}/K_m of the evolved mutants for the paraoxons hydrolysis related to substrate selectivity of the enzyme, and not to the increase in percentage of active enzyme in the purified mutants.

To verify the effect of the mutation on EPO hydrolysis at the molecular level, we performed the molecular docking of EPO into the PON1 mutants. The free binding energies for the improved mutants were -3.6~-5.07 kcal/mol, which are significantly lower than that for the wild type (-3.22 kcal/mol) (Table S3, ESI†). The change in binding affinity between EPO and the PON1 mutants compared to the wild type obtained from molecular docking was well correlated with the results obtained from experiments (i.e., correlation efficient of 0.732) (Fig. S4, ESI†). Additionally, the distance of 4.4~4.5 Å between the O_4 (EPO) and OD_1 (D269) of the two best G3C9 rePON1 mutants was favourable for their van der Waals interactions (Table S3 and Fig. S5, ESI†) and large enough to accommodate one additional reacting water molecule that would be stabilized by the D269 residue during the hydrolysis reaction.

The kinetic parameters of the G3C9 rePON1 wild type and the two best mutants (final concentration of each enzyme in the reaction solution was 0.104 μM) for hydrolysis of MPO (from 0.05 to 0.5 mM) were also determined. The results clearly showed that the two best G3C9 rePON1 mutants remarkably improved in MPO hydrolysis compared to the wild type (Table 2). The catalytic efficiency (k_{cat}/K_m) in MPO hydrolysis for G3C9 rePON1 H115W/T332S and I74F/H115W/T332S at 25 °C reached 2.83×10^4 and 2.01×10^4 $M^{-1}.s^{-1}$, which are 40.0- and 28.5-fold higher the value obtained for the wild type (7.07×10^2 $M^{-1}.s^{-1}$). The improvements in catalytic efficiency were mostly attributed to the increase in k_{cat} values (i.e., k_{cat} of G3C9 rePON1 H115W/T332S, I74F/H115W/T332S for the hydrolysis of MPO were 29.4 and 28.3-fold higher than that of the wild type, respectively, in table 2). Moreover, the best mutants and their wild type behaved similarly following the change of temperature (Fig. S4, ESI†) and had an optimum temperature of approximately 35 °C (i.e., the specific activity at 35 °C was 1.4~1.6-fold higher than at 25 °C). Thus, the mutation in the evolved mutants did not impact their thermal activity.

Table 2 Kinetic parameters of G3C9 rePON1 variants for the hydrolysis of MPO at 25 °C

G3C9 rePON1s	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}.s^{-1}$)
Wild type	0.223	0.316	7.07×10^2
H115W/T332S	6.572	0.232	2.83×10^4 (**40.01**)
I74F/H115W/T332S	6.322	0.314	2.01×10^4 (**28.48**)

(*) The numbers in parentheses present fold enhancement relative to the G3C9 rePON1 wild type

To ascertain the efficiency of the two best G3C9 rePON1 mutants (H115W/T332S and I74F/H115W/T332S) toward the hydrolysis of a large quantity of the two paraoxons, the highly purified G3C9 rePON1 variants (i.e., final concentration of each enzyme in the reaction solution for hydrolysis of EPO and MPO were 0.167 and 0.500 μM, respectively) were added to 3 ml reaction solution containing 3 mM of each paraxon (i.e., 825.6 mg/L of EPO and 741.3 mg/L of MPO; this concentration is very close to solubility limitation of the paraoxons in tested condition) and incubated at 25 °C, 200 rpm (see detailed information in ESI†). The hydrolysis reactions of the two paraoxons catalyzed by the two best PON1 mutants were dramatically faster than that catalyzed by the wild type (Fig. 3). The conversion of EPO catalyzed by the G3C9 rePON1 I74F/H115W/T332S and H115W/T332S mutants reached almost 100% after approximately 20 and 25 minutes at 25 °C, respectively, which was 42.0- and 33.6-fold faster than that

catalyzed by the wild type (840 minutes or 14 hours) in Fig. 3a. In addition, the conversion of MPO catalyzed by the G3C9 rePON1 wild type and mutants reached 82~85% (Fig 3b). The time for converting almost 82% of MPO was sharply decreased from 600 minutes (10 hours) catalyzed by the wild type to approximately 10 minutes catalyzed by the H115W/T332S and I74F/H115W/T332S mutants. The results clearly showed that the two best G3C9 rePON1 mutants (H115W/T332S and I74F/H115W/T332S) exhibited sharp improvement toward hydrolysis of the large quantity of the two paraoxons compared to the wild type.

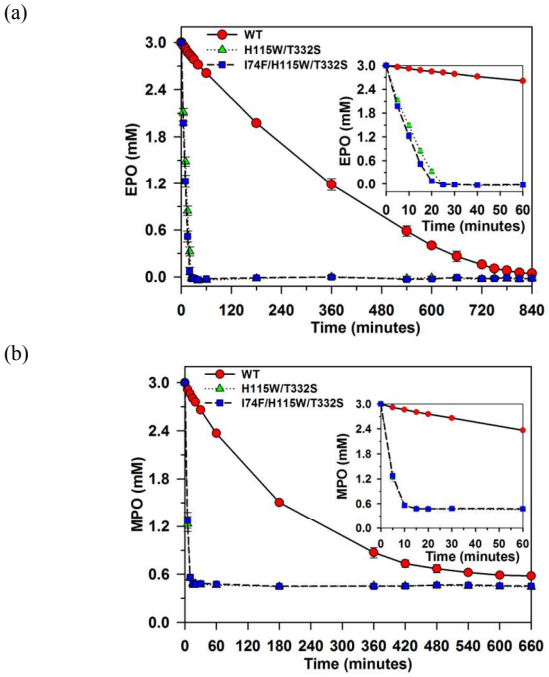

Fig. 3 The effect of reaction time on the hydrolysis of (a) EPO and (b) MPO catalyzed by G3C9 rePON1 variants at 25°C, 200 rpm.

The two best mutants are anticipated to play an important role for *in-vivo* treatment of individuals accidentally exposure to OPs and for the removal of other OPs in the environment. Enzyme immobilization techniques (e.g., immobilization on the porous and multipoint covalent immobilization on glyoxyl agarose or expoxy-activated supports, etc.), which were successfully used to increase operational stability of several enzymes,[19,20] would be beneficial for cost-effective applications of the evolved mutants in detoxification of OPs in the environment.

Conclusion

The rational design of G3C9 rePON1 based on molecular docking identified two G3C9 rePON1 mutants (H115W/T332S and I74F/H115W/T332S) that tremendously the increased catalytic efficiency (k_{cat}/K_m) of EPO hydrolysis while retaining their thermal activity compared to that of the wild type. The change in binding affinity between EPO and the PON1 mutants compared to the wild type obtained from molecular docking correlated well with that obtained from the experiment. The G3C9 rePON1 mutants H115W/T332S and I74F/H115W/T332S also showed remarkable improvement in the hydrolysis of MPO compared to that of the wild type. The evolved mutants are anticipated to significantly increase the hydrolysis of large *O,O*-dialkyl OPs such as DZO and CPO.

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Notes and references

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