Electronic Supplementary Material (ESI) for ChemComm.

Electronic supplementary information

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

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Experiment

Materials

G3C9 rePON1 gene was synthesized from Genescript (New York, USA). Vector pET22B (+) and *Escherichia coli* Origami B (DE3) were purchased from Novagen (Darmstadt, Germany). The restrictive enzymes (*NdeI* and *EcoRI*) were purchased from Takara (Shiga, Japan). Diethyl-paraoxon (EPO), dimethyl-paraoxon (MPO), and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, United States of America)

Methods

Experimental Methods

Site-directed mutagenesis of G3C9 rePON1 gene was conducted using one-step Polymerase Chain Reaction (PCR). [1] All primers used for site-directed mutagenesis were listed in supplementary material (Table S1). The sequence of mutants was confirmed at Cosmogentech (Seoul, Korea).

Table S1 Primers for site-directed mutagenesis of G3C9 rePON1 gene by one-step PCR. For all primers, mutagenized are underlined. Reverse primers were designed complimentarily with their forward primers

PRIMER NAME	SEQUENCE (FROM 5' TO 3')
MF_L69A	AGT TCC GGC GCA AAA TAT CCG GGT ATT ATG TCC
MR_ L69A	CGG ATA TTT TGC GCC GGA ACT GAT AAA CGC CAG
MF_ L69V	AGT TCC GGC GTT AAA TAT CCG GGT ATT ATG TCC
MR _L69V	CGG ATA TTT AAC GCC GGA ACT GAT AAA CGC CAG
MF_L69I	AGT TCC GGC ATT AAA TAT CCG GGT ATT ATG TCC
MR_L69I	CGG ATA TTT AAT GCC GGA ACT GAT AAA CGC CAG
MF_I74A	TAT CCG GGT GCT ATG TCC TTC GAT CCG GAC AAA
MR_I74A	GAA GGA CAT AGC ACC CGG ATA TTT CAG GCC GGA
MF_I74V	TAT CCG GGT GTT ATG TCC TTC GAT CCG GAC AAA
MR_I74V	GAA GGA CAT AAC ACC CGG ATA TTT CAG GCC GGA
MF_I74L	TAT CCG GGT CTT ATG TCC TTC GAT CCG GAC AAA
MR_I74L	GAA GGA CAT AAG ACC CGG ATA TTT CAG GCC GGA
MF_I74F	TAT CCG GGT TTC ATG TCC TTC GAT CCG GAC AAA TCA GGC
MR_I74F	GAA GGA CAT GAA ACC CGG ATA TTT CAG GCC GGA ACT GAT
MF_I74W	TAT CCG GGT TGG ATG TCC TTC GAT CCG GAC AAA TCA GGC
MR_I74W	GAA GGA CAT CCA ACC CGG ATA TTT CAG GCC GGA ACT GAT
MF_H115W	TTT AAT CCG TGG GGC ATT AGC ACC TTC ACG GAT G
MR_H115W	GCT AAT GCC CCA CGG ATT AAA CGA TGA GAT ATC
MF_T332A	CAA GGT TCC GCT GTG GCA GCA GTT TAT AAA GGT
MF_T332A	TGC TGC CAC AGC GGA ACC TTG CAG GAC GGT ACC
MF_T332S	CAA GGT TCC TCT GTG GCA GCA GTT TAT AAA GGT AAA CTG
MR_T332S	TGC TGC CAC AGA GGA ACC TTG CAG GAC GGT ACC ATT TTC
MF_T332V	CAA GGT TCC GTA GTG GCA GCA GTT TAT AAA GGT
MR_T332V	TGC TGC CAC TAC GGA ACC TTG CAG GAC GGT ACC
MF_V346A	ATT GGC ACC GCT TTC CAC AAA GCT CTG TAC TGT
MR_V346A	TTT GTG GAA AGC GGT GCC AAT CAG CAG TTT ACC
MF_V346L:	ATT GGC ACC CTT TTC CAC AAA GCT CTG TAC TGT
MR_V346L	TTT GTG GAA AAG GGT GCC AAT CAG CAG TTT ACC

MF_V346I	ATT GGC ACC ATT TTC CAC AAA GCT CTG TAC TGT
MR_V346I	TTT GTG GAA AAT GGT GCC AAT CAG CAG TTT ACC

The G3C9 rePON1 gene wild type and its mutants were cloned to pET22b (+) through double restrictive sites *NdeI/EcoRI* and then transformed to *E.coli* Origami B (DE3). The positive transformants were inoculated to LB medium with 1 mM $CaCl_2$ and two selective antibiotics (i.e., Kanamycin 15 μg/ml and Ampicilin 100 μg/ml) and grown at 25 °C, 150 rpm for 20 hours and induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation and disrupted by Novagen bug buster (Damstadt, Germany). G3C9 rePON1 in the cleared lysate was further purified by Qiagen Ni-NTA column purification kit (Venlo, Netherlands). The Bradford micro assay using sigma Aldrich reagent was used to determine the purified enzyme concentration.

The hydrolysis of EPO (0.5 mM) was carried out in 50 mM Glycine buffer with 1 mM $CaCl_2$, pH10.5 at 25 °C to search for the improved G3C9 rePON1 toward hydrolysis of EPO. The releasing p-nitrophenol was measured by UV-Vis at wavelength of 412 nm (molar coefficiency of para-phenol at 412 nm is 16,900 $M^{-1}.cm^{-1}$).[2] The pre-test result for hydrolysis of EPO catalyzed by G3C9 rePON1 variants is seen in Fig. S1.

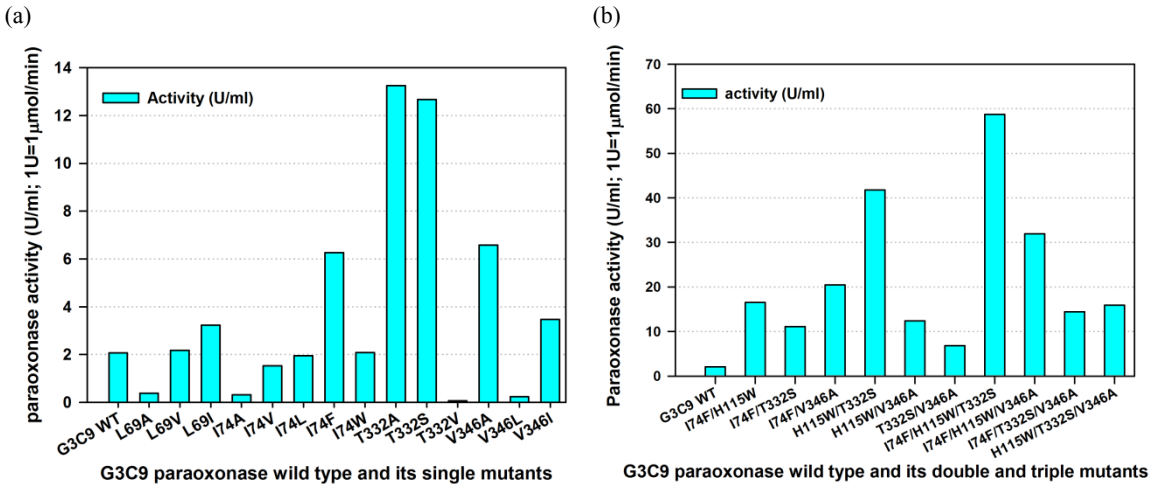
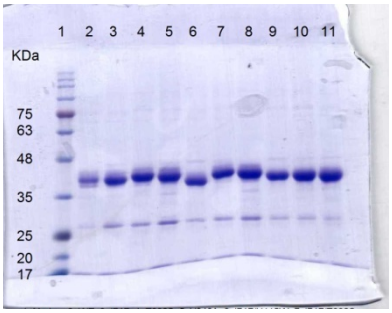


Fig S1 Hydrolysis of EPO (0.5 mM) at pH10.5 catalyzed by (a) G3C9 rePON1 wild type and its single mutants and (b) G3C9 rePON1 wild type and its double and triple mutants.

The purified G3C9 rePON1 (Molecular weight of PON1 is ~40 kDa)[3] with high purity were used for kinetic assay (Fig. S1). Kinetic assay for hydrolysis of EPO and MPO catalyzed by G3C9 rePON1 variants was carried out in 3 mL reaction solution of 50 mM Glycine buffer (pH10.5) and 1 mM $CaCl_2$ with increasing concentration of each paraoxon (i.e., EPO or MPO) from 0.05 to 0.5 mM. The highly purified G3C9 rePON1 enzyme (i.e., 2.5 μg for the hydrolysis of EPO and 12.5 μg for hydrolysis of MPO) was added to the reaction solution (3 mL) to final concentrations of 0.021 and 0.104 μM for the hydrolysis of EPO and MPO, respectively. The reaction was performed at 25 °C for 1~5 minutes. The releasing p-nitrophenol was measured by UV-Vis at wavelength of 412 nm (molar coefficiency of para-phenol at 412nm is 16,900 $M^{-1}.cm^{-1}$).[2] Lineweaver-Burk plots were plotted to determine K_m and V_{max} of the G3C9 rePON1.[4]



Fi g. S2. SDS-PAGE for purified G3C9 rePON1 wild type and its mutants; Lane1 : protein marker; Lanes 2: wild type; Lane 3: I74F mutant;

Lane 4: T332S mutant; Lane 5: V346A mutant; Lane 6: I74F/H115W mutant; Lane 7: I74F/T332S mutant; Lane 8: I74F/V346A mutant; Lane 9: H115W/T332S mutant; Lane 10: I74F/H115W/T332S mutant; Lane 11: I74F/H115W/V346A

The arylesterase activity assay for G3C9 rePON1 variants was carried out by adding the purified enzyme (Fig. S2; ~1 µg of the wild type and the mutants not from H115W (i.e., I74F, T332S, V346A, I74F/T332S, I74F/V346A, and T332S/V346A) and ~20 µg of the mutants from H115W (i.e., I74F/H115W, H115W/T332S, H115W/V346A, I74F/H115W/T332S, and I74F/H115W/V346A)) to 3 mL reaction solution of 50 mM Triz, HCl buffer with 1 mM CaCl2, pH 8.0 containing 1 mM phenyl acetate. The reaction was performed at 25 °C for 1~5 min. The releasing phenol was measured by UV-Vis at 270 nm (molar coefficiency of phenol at 270 nm is 1,310 $M^{-1}.cm^{-1}$).[2] The specific activity of G3C9 rePON1 variants toward phenyl acetate hydrolysis is seen in Fig. S3.

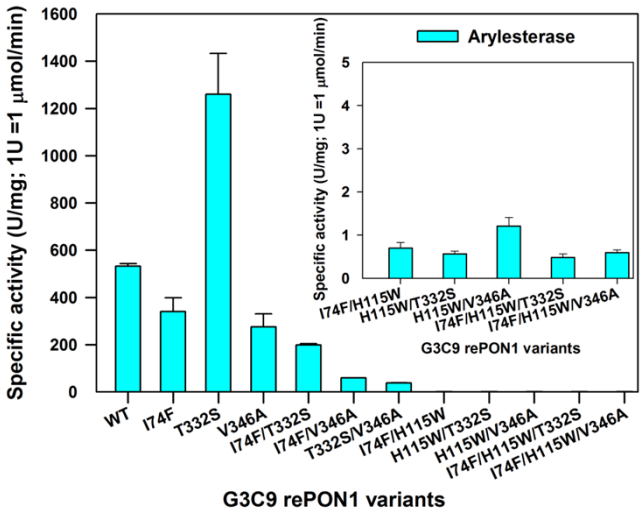


Fig S3. The specific activity of G3C9 rePON1 toward hydrolysis of 1mM phenyl acetate at 25 °C.

The effect of temperature on the activity of G3C9 rePON1 variants toward hydrolysis of EPO (0.1 mM) was carried out in 50 mM Glycine buffer with 1 mM $CaCl_2$, pH 10.5, 200rpm with different reaction temperatures (25–45 °C) for 15 minutes. The releasing p-nitrophenol was also measured by UV-Vis at 412 nm mentioned above. The effect of temperature on specific activity toward hydrolysis of EPO is seen in Fig. S4.

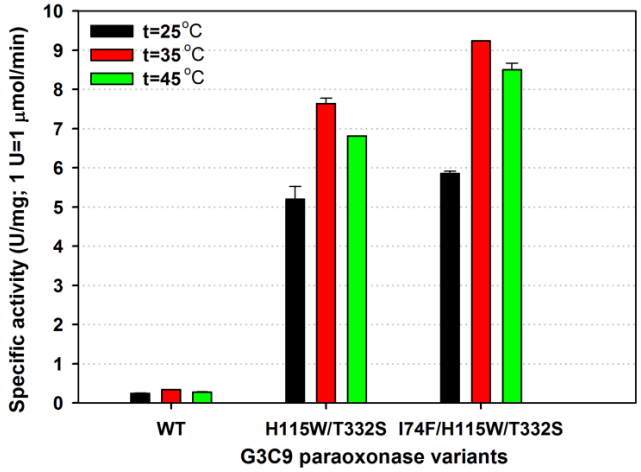


Fig. S4 Effect of reaction temperature on specific activity of G3C9 rePON1 variants toward hydrolysis of EPO (0.1 mM) at pH10.5

The efficiency of the evolved G3C9 rePON1 mutants toward hydrolysis of large quantity of the two paraoxons was performed in 3 mL reaction solution of 20 mM Glycine buffer (pH10.5) containing 1 mM $CaCl_2$ and 3 mM each paraoxon (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). The highly purified G3C9 rePON1 enzyme (i.e., 20.0 µg for the hydrolysis of EPO and 60.0 µg for

hydrolysis of MPO) was added to the reaction solution (3 mL) to final concentrations of 0.167 and 0.500 μM for the hydrolysis of EPO and MPO, respectively. The reaction was performed at 25 °C, 200 rpm and measured at different reaction time intervals. The releasing p-nitro phenol was measured by UV-Vis at 412 nm. The calibration curve of known concentrations of p-nitro phenol (1~30 μM) was firstly done and then used for quantification of the releasing p-nitro phenol from the above reactions.

Computational Methods

The ligands for molecular docking (i.e., dimethyl-paraoxon (MPO) and diethyl-paraoxon (EPO)) were firstly prepared and optimized at Hartree-Fock level with basis set of 6-31 G(d) in Gaussian 03 package.[5] The crystal structure of PON1 (i.e., PDB code 3SRG with resolution 2.19 Å)[6] used for molecular docking study. The mutants of PON1 were firstly prepared by SWISS PDBVIEWER program[7] and further minimized around 5 Å of mutated residues with short steepest descent (SD) [8] and adopted basis Newton Raphson (ABNR) methods[9] to remove nonphysical interactions using CHARMM program.[10] The missing hydrogen was added to PON1 variants by HBUILD module of CHARMM program.[10]The ligands were docked into PON1 variants using Autodock 4.2 program.[11] The docking process with fully flexible ligands was performed in a grid of 37×37×37 points centered at active site of PON1 with a grid spacing of 0.375 Å. The Lamarckian Genetic Algorithm (LGA) was applied for docking process with 100 trials and the maximum number of energy evaluation was set to 2.5×10^8 The charge of calcium of PON1 was set to +2. The default values were used for other parameters during molecular docking.

The molecular docking results between PON1 (PDB code: 3SRG) with 2 paraxons (EPO and MPO) is seen in Table S2. In addition, the results for molecular docking between PON1 variants with EPO are in Table S3 and Fig. S4

Table S2 Molecular Docking of PON1 wild type with two paraoxons

Ligands	Interactions (Å)		ΔG_{bind}	K_i
	$O_1(P)\ldots Ca_{357}$	$O_4(P)\ldots OD_1$ (D269)	(kcal/mol)	(mM)
EPO	1.93	2.66	-3.2	4.49
MPO	1.93	2.89	-4.22	0.801

(*) EPO: diethyl-paraoxon; MPO: dimethyl-paraoxon;P is abbreviated for ligand with name in Fig. 1;

(**) ΔG_{bind} and K_i are free binding energy and inhibitor constants, respectively. $K_i = e^{(\frac{\Delta G(binding)}{R*T})}$ (M); R=1.987 cal.mol^{-1}.K^{-1}; T=298.15 K.[11]

Table S3 Molecular Docking of PON1 wild type and its mutants with two paraoxons

PON1 variants	Interactions (Å)		ΔG_{bind}	K_i
	$O_1(P)\ldots Ca_{357}$	$O_4(P)\ldots OD_1$ (D269)	(kcal/mol)	(mM)
Wild type	1.93	2.66	-3.20	4.49
I74F	2.06	2.75	-3.72	1.86
T332S	1.87	2.86	-3.60	2.31
V346A	2.16	3.58	-4.28	0.727
I74F/H115W	1.99	4.42	-4.63	0.404
H115W/T332S	1.99	4.52	-5.01	0.214
I74F/T332S	1.99	2.67	-3.84	1.54
I74F/H115W/T332S	2.01	4.52	-5.07	0.192

(*) P is abbreviated for ligand with name in Fig. 1;

(**) ΔG_{bind} and K_i are free binding energy and inhibitor constants, respectively. $K_i = e^{(\frac{\Delta G(binding)}{R*T})}$ (M); R=1.987 cal.mol^{-1}.K^{-1}; T=298.15 K.[11]

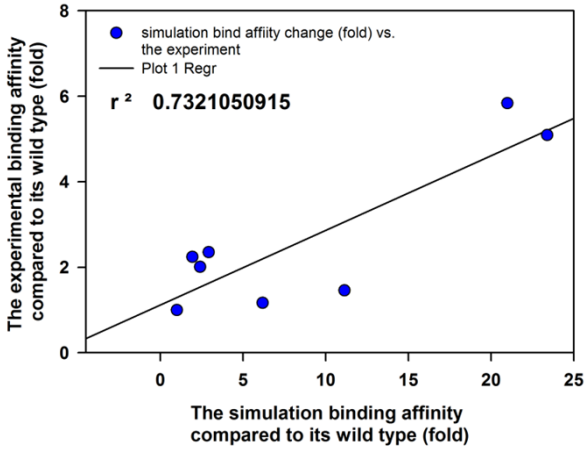


Fig. S4 The change in binding affinity between EPO and PON1 mutants compared to their wild type in experiment and simulation

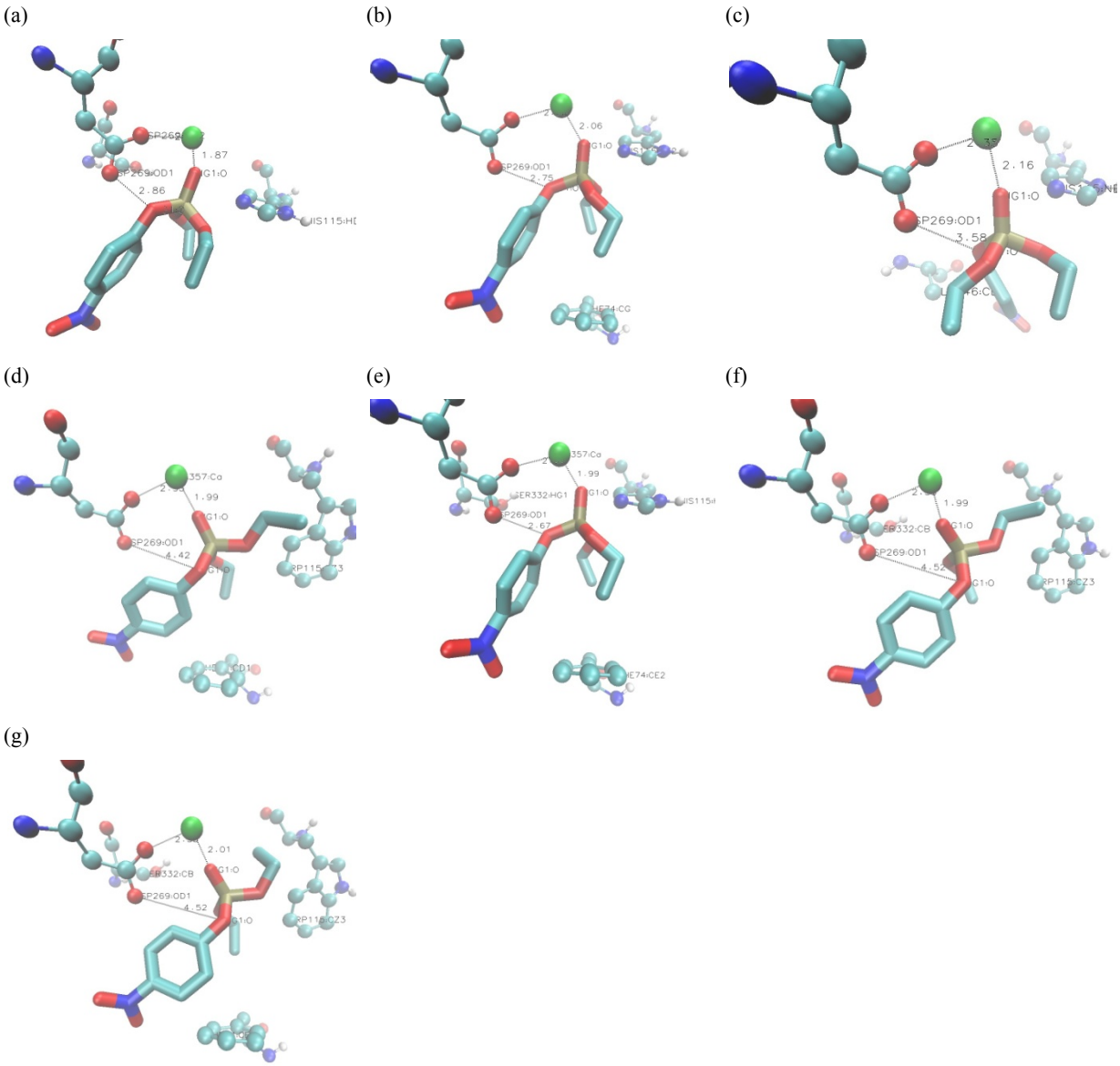


Fig. S5 The docked structure of PON1 mutants with EPO: (a) T332S mutant with EPO, (b) I74F mutant with EPO, (c) V346A mutant with EPO, (d) I74F/H115W mutant with EPO, (e) I74F/T332S mutant with EPO, (f) H115W/T332S mutant with EPO, and (g) I74F/H115W/T332S with EPO. EPO (as licoride), catalytic calcium ion (as CPK, green), and amino acid residues (as CPK).

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