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Efficient hydrolysis of the chemical warfare nerve agent tabun by recombinant and purified human and rabbit serum paraoxonase 1

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ABSTRACT

Paraoxonase 1 (PON1) has been described as an efficient catalytic bioscavenger due to its ability to hydrolyze organophosphates (OPs) and chemical warfare nerve agents (CWNAs). It is the future most promising candidate as prophylactic medical countermeasure against highly toxic OPs and CWNAs. Most of the studies conducted so far have been focused on the hydrolyzing potential of PON1 against nerve agents, sarin, soman, and VX. Here, we investigated the hydrolysis of tabun by PON1 with the objective of comparing the hydrolysis potential of human and rabbit serum purified and recombinant human PON1. The hydrolysis potential of PON1 against tabun, sarin, and soman was evaluated by using an acetylcholinesterase (AChE) back-titration Ellman method. Efficient hydrolysis of tabun (100 nM) was observed with ~25–40 mU of PON1, while higher concentration (80–250 mU) of the enzyme was required for the complete hydrolysis of sarin (11 nM) and soman (3 nM). Our data indicate that tabun hydrolysis with PON1 was ~30–60 times and ~200–260 times more efficient than that with sarin and soman, respectively. Moreover, the catalytic activity of PON1 varies from source to source, which also reflects their efficiency of hydrolyzing different types of nerve agents. Thus, efficient hydrolysis of tabun by PON1 suggests its promising potential as a prophylactic treatment against tabun exposure.

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1. Introduction

Chemical warfare nerve agents (CWNAs) are of two main classes, G- and V-type [1]. The G-type nerve agents include tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF). These are highly toxic due to their high volatility at room temperature and represent a great inhalation and dermal threat upon exposure. Nerve agents and related compounds irreversibly inhibit acetylcholinesterase (AChE), thereby causing a rapid, life-threatening accumulation of acetylcholine in the cholinergic nervous system [2]. While numerous oximes, including the currently fielded nerve agent antidote, 2-pralidoxime, have been shown to reverse AChE inhibition by some OPs, there is currently no universally effective antidote [3,4]. Reactivation of tabun inhibited AChE is particularly challenging due to the existence of a lone pair of electron on the dimethylamide group of tabun and the structural changes of the gorge of AChE make nucleophilic attack almost impossible [5,6]. An alternative approach for treatment against CWNA poisoning has focused on identification of proteins that can remain stable in circulation for long periods of time while acting as biological scavengers for OPs/CWNAs. For example, prophylactic countermeasures against CWNA exposure using butyrylcholinesterase (BChE) as a bioscavenger to neutralize CWNAs before they reach the target organs have been widely accepted [7–9]. Usually, a stoichiometric bioscavenger, such as BChE, sequesters the OP/CWNAs in a 1:1 ratio, itself representing a limitation. However, catalytic bioscavengers can hydrolyze several OP/CWNA molecules and have broad substrate specificity.

One of the recently discovered catalytic bioscavengers is paraoxonase 1 (PON1), which has the potential to hydrolyze nerve agents [10–12]. It is a 45 kDa arylesterase, synthesized mainly in the liver and secreted in the bloodstream to associate with high density lipoprotein molecules [11,13,14]. PON1 has been described as an efficient catalytic bioscavenger due to its ability to hydrolyze

Abbreviations: PON1, paraoxonase 1; HPON1, human PON1; RPON1, rabbit PON1; rePON1, recombinant PON1; OP, organophosphate; CWNA, chemical warfare nerve agent; DFP, diisopropylfluorophosphate; CPO, chloropyrifos oxon; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidenedifluoride; PBST, phosphate buffered saline/0.1% Tween-20; *p*-NPA, *p*-nitrophenyl acetate; DTNB, dithionitrobenzoic acid; 2-HQ, 2-hydroxyquinoline.

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OPs and CWNAs [10–12,15–17]. Significant hydrolysis of diisopropylfluorophosphate (DFP), paraoxon, diazoxon, and chloropyrifos oxon (CPO) was reported with recombinant as well as human and rabbit serum PON1 [10,12]. These results were further confirmed *in vivo* and in PON1 knock-out mice, which showed dramatic increase in the sensitivity to CPO toxicity [17]. PON1 has been also reported to hydrolyze sarin, soman, and VX [16,18]. The additional cardioprotective nature and the broad substrate specificity of PON1 make it a candidate of choice for the development of a therapeutic catalytic bioscavenger [13,14].

Limited information is available in the literature about the nature of any molecules which can hydrolyze tabun. The presence of an enzyme in squid nerve has been reported to hydrolyze tabun, DFP, sarin, and soman, with possible implications in disposal and detoxification of nerve gases in the ocean [19]. It has also been reported that soluble fraction of rat and human liver homogenates contains an active enzyme which could hydrolyze several OPs including tabun [20–22]. Since PON1 is mainly synthesized in the liver and secreted in plasma, it was tempting to determine the hydrolysis of different nerve agents by purified PON1. Here, we studied the hydrolysis of tabun, sarin, and soman by purified human and rabbit serum and recombinant human PON1, using an AChE back-titration Ellman method. We also evaluated its catalytic nature to eventually develop as a catalytic bioscavenger for protection against CWNAs.

2. Materials and methods

2.1. Materials

Acetylcholinesterase, acetylthiocholine, dithionitrobenzoic acid, *p*-nitrophenylacetate, Cibacron Blue 3GA agarose, DEAE-Sepharose, Sepharose CL-6B, Concanavalin A-Sepharose, 2-hydroxyquinoline, and PON1 polyclonal antibody were purchased from Sigma (St. Louis, MO). Tris–glycine gradient gels (4–20%), protein molecular weight markers, SDS–PAGE running and transfer buffer were purchased from Invitrogen (Carlsbad, CA). BCA assay kit for protein estimation was purchased from Pierce (Rockford, IL). Centricon 30 microconcentrator and immobilon PVDF membrane (polyvinylidene difluoride) were purchased from Millipore (Billerica, MA). ECL reagent for the development of Western blot was purchased from GE Healthcare (Piscataway, NJ). CWNA experiments were carried out at USAMRICD, Aberdeen Proving Ground, MD.

2.2. Purification of human and rabbit serum and recombinant PON1

PON1 was purified from human and rabbit serum as described earlier [23,24]. Briefly, human or rabbit serum (Innovative Research Inc., Novi, MI) was mixed with Cibacron Blue 3GA-agarose, and the bound PON1 was eluted with 0.1% deoxycholate. The fractions were then loaded on DEAE-Sepharose ion-exchange columns followed by elution with NaCl gradient. Further purification of PON1 was done by Concanavalin A-Sepharose affinity chromatography. The enzyme was eluted with α-methyl mannopyranoside gradient. The final purification of the enzyme was performed by using a Sepharose CL-6B gel filtration column. Human recombinant PON1 was expressed in *Trichoplusia ni* larvae (cabbage looper-worm) and purified by His-tag affinity chromatography in collaboration with Chesapeake PERL (Savage, MD).

2.3. SDS–PAGE

The purity of PON1 from human and rabbit serum and recombinant PON1 was analyzed by SDS–PAGE. Briefly, ~2 μg of the protein samples was incubated with equal volume of 2× Laemmli sample buffer containing reducing agent at 100 °C for 5 min [25]. The samples were resolved on 4–20% gradient Tris–glycine gels at a constant voltage of 100 V. The separated proteins were stained using Coomassie Blue stain and were photographed by using AlphaImager (Cell Biosciences, Santa Clara, CA).

2.4. Immunoblotting

PON1 preparations (~0.1 μg) resolved by SDS–PAGE were transferred onto PVDF membrane. Membranes were blocked with 2% nonfat dry milk in phosphate buffered saline/0.1% Tween-20 (PBST) overnight at 4 °C and incubated with PON1 polyclonal primary antibody (1:1000) for 2 h at room temperature. Membrane was washed three times with PBST followed by incubation with peroxidase-labeled goat anti-rabbit secondary antibody (1:5000) for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence method (ECL detection reagent; GE Healthcare) and visualized using FluorChem HD2 system (Cell Biosciences, Santa Clara, CA).

2.5. PON1 activity assay

Aliquots of PON1 samples were incubated with 1 mM of *p*-nitrophenylacetate (*p*-NPA) as substrate in 20 mM Tris–HCl buffer (pH 7.4), containing 1 mM $CaCl_2$. The release of *p*-nitrophenol was measured at 405 nm using Spectramax M5 Spectrophotometer (Molecular Devices, Sunnyvale, CA). One unit of PON1 is defined as the amount of enzyme that will liberate 1 μmol of *p*-nitrophenol per minute at 37 °C.

2.6. Ellman assay

AChE activity was measured spectrophotometrically (Spectramax M5 Spectrophotometer) with a modified Ellman method at 412 nm [26]. The assay mixture consisted of a final volume of 1.0 mM ATCh as substrate and 1.0 mM DTNB as chromogen in 50 mM sodium phosphate buffer (pH 7.4).

2.7. AChE back-titration assay

To evaluate the hydrolytic activity of PON1 against nerve agents, a back-titration of AChE Ellman assay was employed [26]. Briefly, various concentrations of nerve agents (tabun, sarin, and soman) were incubated with 5 ng of AChE for 15 min at room temperature. Residual AChE activity was measured by Ellman assay at 412 nM with a master mix solution containing 1.0 mM ATCh and 1.0 mM DTNB in sodium phosphate buffer (pH 7.4). After determining the concentration of nerve agents required for ~95% inhibition of AChE, various concentrations of human and rabbit serum purified and human recombinant PON1 were incubated with the same concentration of nerve agents (the concentration that inhibit 95% AChE) for 30 min at room temperature. Residual nerve agent in the reaction mixture was determined by incubating with 5 ng of AChE for another 15 min followed by the Ellman assay. Moreover, to determine the catalytic/hydrolytic potential of PON1, a 3-fold concentrated nerve agent than the one used above was added to the reaction mixture and the residual AChE activity was determined. The retention of activity of PON1 after incubation with the nerve agent was determined using *p*-NPA substrate. All the assays were performed 3–5 times in triplicates.

2.8. PON1 inhibition assay

The specificity of PON1 hydrolysis of nerve agents was confirmed by inhibition of the enzymes with a specific PON1 inhibitor, 2-hydroxyquinoline (2-HQ, 10 μM–5 mM), for 15 min at room

temperature [27], followed by AChE back-titration assay with the respective nerve agents as described above.

3. Results

3.1. Analysis of purified recombinant and human and rabbit serum PON1

The purity of PON1 preparation from human and rabbit serum and human recombinant PON1 was analyzed by SDS–PAGE and the data are shown in Fig. 1A. Purified PON1 was able to hydrolyze *p*-NPA, paraoxon, and DFP (data not shown). The human serum purified enzyme showed a single band where as purified rabbit serum purified and human recombinant PON1 enzymes separated as three bands with an approximate molecular weight of 40–45 kDa. The specific activity of purified human and rabbit serum PON1 was ~3.5 U/mg protein, while recombinant PON1 showed a specific activity of ~8.5 U/mg protein.

The purity of PON1 preparations was evaluated by immunoblotting with a polyclonal antibody against PON1. As observed in the immunoblot, human serum PON1 showed a single upper band (~45 kDa), while both rabbit serum PON1 and human recombinant PON1 showed three bands of approximately 40–45 kDa molecular weight in the enzyme preparations at varying intensities (Fig. 1B).

3.2. Hydrolysis of chemical warfare nerve agents with PON1

Hydrolysis potential of purified human and rabbit serum PON 1 and recombinant PON1 against tabun, sarin, and soman was determined using the AChE back-titration Ellman method as described above. The amount of tabun required to inhibit 95% of the AChE activity was found to be ~100 nM (Fig. 1C). Incubation of rabbit serum PON1 (25 mU), human serum PON1 (30 mU) and recombinant PON1 (40 mU) with tabun resulted in its complete hydrolysis, and it was found to be dose-dependent (Fig. 1D–F).

With sarin, the concentration required to inhibit ~95% of AChE activity was found to be ~11 nM (Fig. 2A). Incubation of sarin with rabbit serum PON1 (80 mU) and human serum PON1 (~120 mU) showed efficient hydrolysis of 11 nM of sarin (Fig. 2B and C). However, higher concentration of recombinant PON1 (~250 mU) was required for similar level of hydrolysis of sarin (Fig. 2D).

The concentration of soman required to give ~95% inhibition of AChE activity was found to be 3 nM (Fig. 3A). All the three PON1 preparations hydrolyzed 3 nM of soman used in the reaction. The concentration of rabbit serum PON1 (180 mU) and human serum PON1 (240 mU) required for complete hydrolysis of 3 nM soman was higher than that required for the hydrolysis of 11 nM of sarin (Fig. 3B and C). The concentration of recombinant PON1 (240 mU) required for the hydrolysis of soman and sarin that inhibited 95% AChE was very similar (Fig. 3D, compare with Fig. 2D).

3.3. Hydrolysis of nerve agents by PON1 is specific

To analyze the specificity of PON1 activity, a specific inhibitor of PON1 (2-HQ) was used in the assay system. Pre-incubation of PON1 with 2-HQ showed a dose-dependent inhibition of enzyme activity (Fig. 4A). On the other hand, incubation of AChE with 2-HQ did not affect its activity (Fig. 4B). To further investigate that

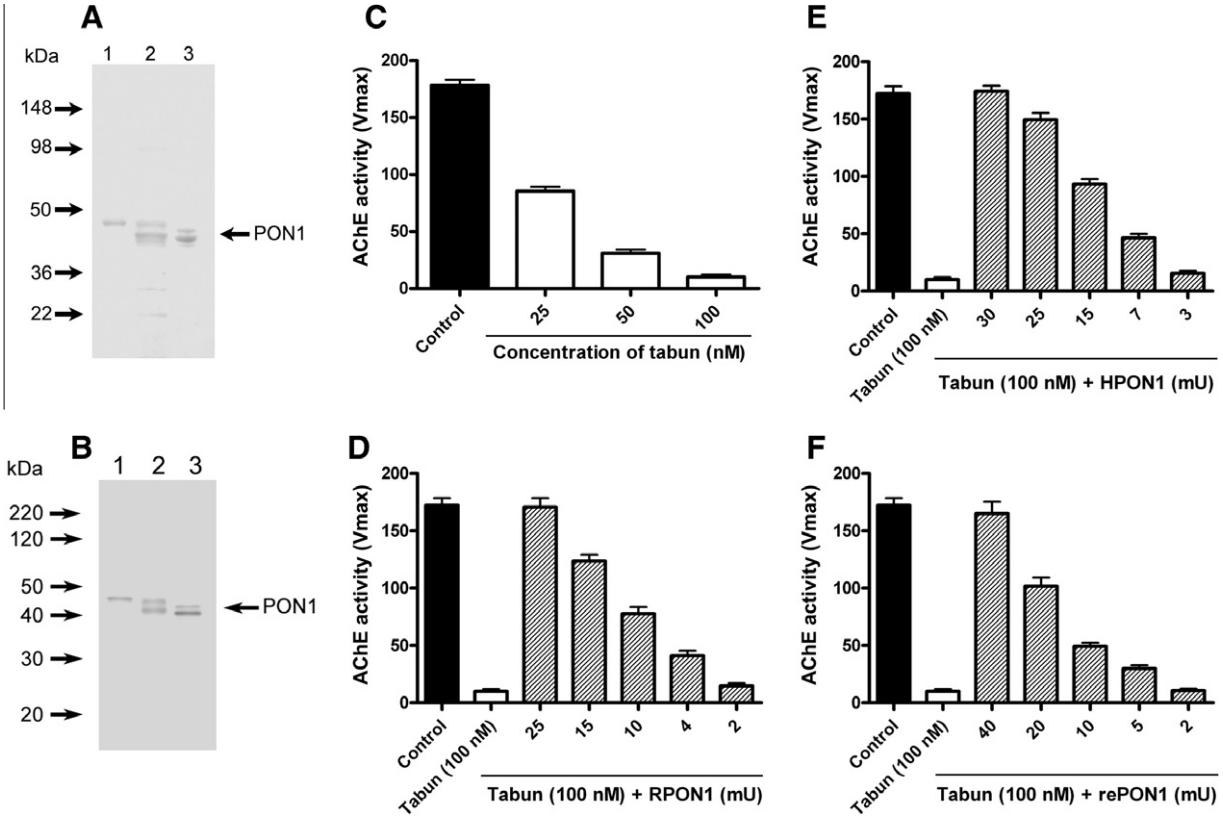

Fig. 1. Hydrolysis of chemical warfare nerve agent tabun with PON1. (A) Two micrograms of PON1 preparations were separated on 4–20% gradient gel and stained with Coomassie Blue (lane 1: HPON1, lane 2: RPON1, and lane 3: rePON1). (B) 0.1 μg of PON1 preparations were separated on 4–20% gradient gel, transferred to PVDF membrane and immunoblotted with polyclonal antibody against PON1 (lane 1: HPON1, lane 2: RPON1, and lane 3: rePON1). Molecular weight markers are shown on the left. (C) Indicated concentrations of tabun were pre-incubated with 5 ng of AChE for 15 min, followed by incubation with 1 mM of ATCh and DTNB and the absorbance was measured at 412 nm for 5 min in a kinetic mode using Spectramax M5 spectrophotometer. (D–F) Indicated concentrations of RPON1 (D), HPON1 (E) and rePON1 (F) were incubated with 100 nM of tabun for 30 min followed by incubation with 5 ng of AChE for 15 min. The samples were further incubated with 1 mM of ATCh and DTNB and the reaction was monitored at 412 nm for 5 min in a kinetic mode.

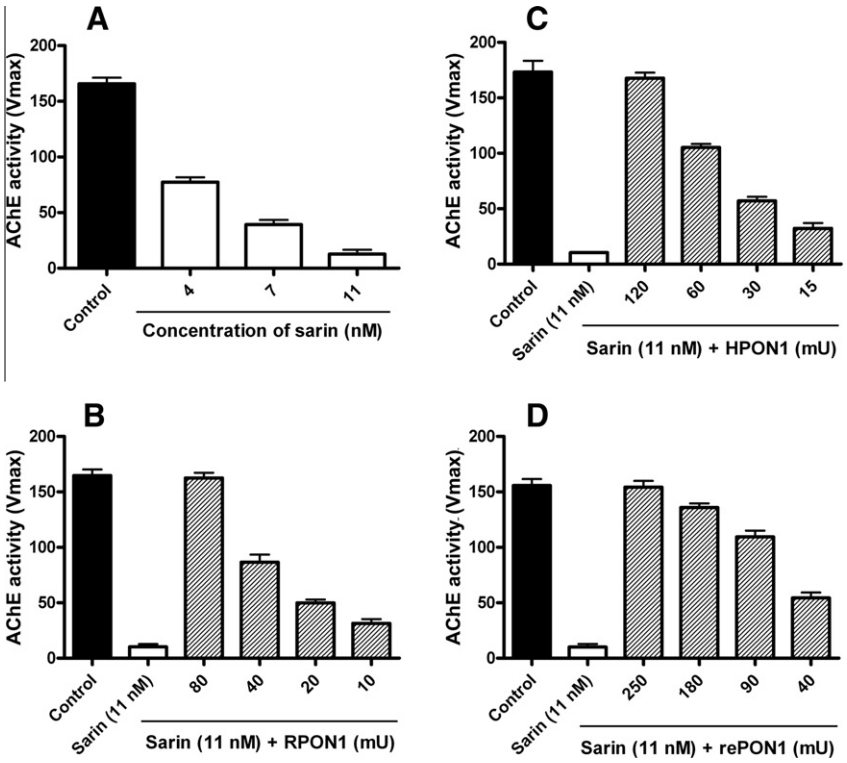

Fig. 2. Sarin hydrolysis with PON1. (A) Indicated concentrations of sarin were pre-incubated with 5 ng of AChE for 15 min, followed by Ellman assay as described in Fig. 1. (B–D) Indicated concentrations of RPON1 (B), HPON1 (C) and rePON1 (D) were incubated with 11 nM of sarin for 30 min followed by Ellman assay.

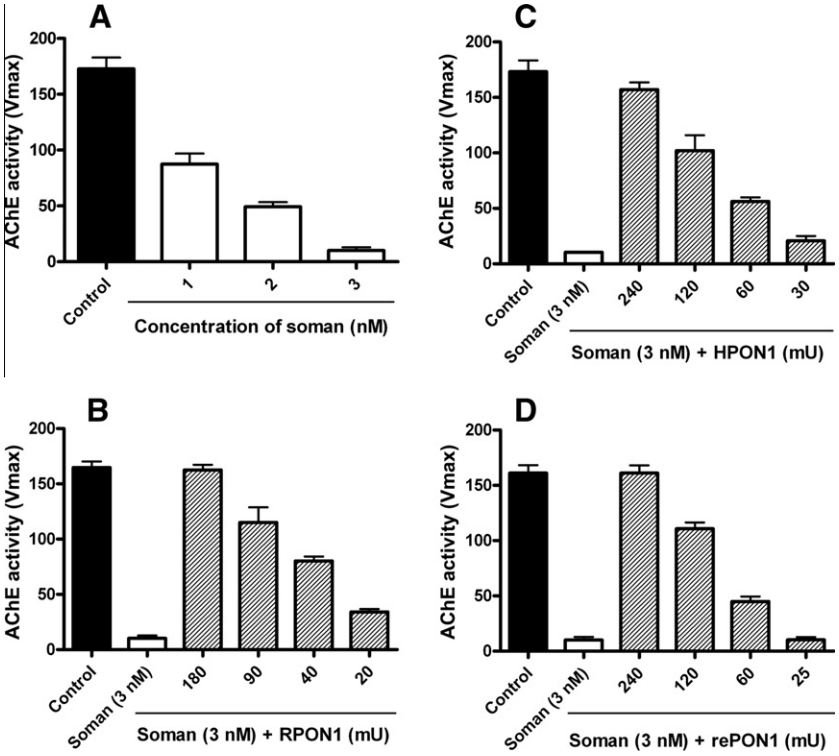

Fig. 3. Hydrolysis of soman with PON1. (A) Indicated concentrations of soman were pre-incubated with 5 ng of AChE for 15 min, followed by Ellman assay. (B–D) Indicated concentrations of RPON1 (B), HPON1 (C) and rePON1 (D) were incubated with 3 nM of sarin for 30 min followed by Ellman assay with 1 mM of ATCh and DTNB.

the PON1 in the purified preparation is indeed responsible for the hydrolysis of nerve agents, PON1 activity was first inhibited with 2-HQ and then the nerve agent hydrolysis was carried out and monitored by AChE back-titration assay. As show in Fig. 4C, incubation of PON1 with 2-HQ resulted in the complete loss of the hydrolyzing ability of the enzyme for tabun, sarin, or soman. These

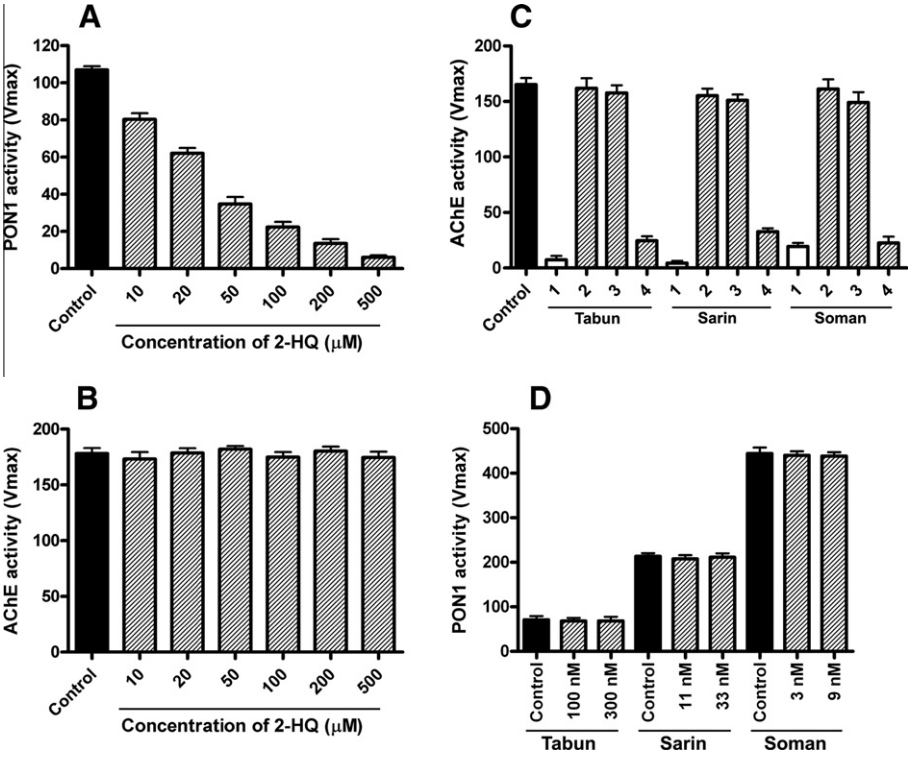

Fig. 4. Efficient hydrolysis of higher concentration of nerve agents by PON1 and inhibition of PON1 activity by 2-HQ. (A) Five milliunits of RPON1 were pre-incubated with indicated concentrations (10–500 μM) of 2-HQ for 15 min, followed by PON1 activity assay with *p*-NPA substrate. (B) Five nanograms of AChE was pre-incubated with indicated concentrations of 2-HQ (10–500 μM), followed by Ellman assay. (C) 25–180 mU of RPON1 was pre-incubated with or without 2-HQ (1–5 mM) for 15 min, followed by incubation with tabun, sarin or soman for 30 min or 1 h and the AChE back-titration assay was performed as described in "Section 2". 1 – tabun (100 nM), sarin (11 nM), or soman (3 nM); 2 – RPON1 (25 mU) + tabun (100 nM), RPON1 (80 mU) + sarin (11 nM), or RPON1 (180 mU) + soman (3 nM); 3 – RPON1 (25 mU) + tabun (300 nM), RPON1 (80 mU) + sarin (33 nM), or RPON1 (180 mU) + soman (9 nM); 4 – RPON1 (25 mU) + 2-HQ (1 mM) + tabun (100 nM), RPON1 (80 mU) + 2-HQ (3 mM) + sarin (11 nM), or RPON1 (180 mU) + 2-HQ (5 mM) + soman (3 nM). (D) PON1 enzyme activity in the presence of nerve agents. 25–180 mU of RPON1 was pre-incubated with tabun (100 or 300 nM), sarin (11 or 33 nM), or soman (3 or 9 nM) for 1 h, followed by the PON1 activity assay using *p*-NPA substrate.

results confirm that the hydrolysis of nerve agents by PON1 is exclusively due to PON1 enzyme activity. Moreover, PON1 was also able to hydrolyze 3-fold concentrated tabun, sarin, and soman as shown in Fig. 4C. The intrinsic activity of PON1 was found to be unaltered in the presence of nerve agents even after 1 h (Fig. 4D). These data demonstrate that PON1 functions as an efficient catalytic bioscavenger against nerve agents.

4. Discussion

The catalytic efficiency of PON1 to hydrolyze OPs including chloropyrifos oxon, and diazoxon and nerve agents such as soman and sarin has been reported previously [10,16,28,29]. None of these studies investigated the ability of recombinant or human and rabbit serum PON1 to hydrolyze tabun. The major finding in the present study is that purified PON1 from human and rabbit serum and human recombinant hydrolyzes tabun more effectively than sarin and soman. Moreover, lower concentration of PON1 is sufficient to hydrolyze maximum amount of tabun as compared to sarin and soman (compare Figs. 1–3). It is also observed that rabbit PON1 is more effective in hydrolyzing tabun than sarin or soman as compared to the other two sources. The reason for the efficient hydrolysis of tabun by PON1 is still unclear. However, it could be related to the stereo-specificity of tabun and its alignment in the active site to create a differential charge in the molecule to induce bond breaking or in brief hydrolysis. We also found that PON1 hydrolyzed sarin more efficiently than soman. In general, rabbit serum PON1 showed higher hydrolyzing activity with all three nerve agents compared to recombinant and human serum PON1.

It has been reported that the enzymes present in the extracts of various tissues (mainly liver, kidney, small intestine, and plasma) hydrolyze DFP and have DFPase activity indicating that other enzymes can also hydrolyze nerve agents [21,22]. The loss of hydrolytic potential of PON1 by specific inhibitor suggests that the PON1 enzyme is indeed responsible for the hydrolysis of the nerve agents. Moreover, PON1 has several advantages to be considered as therapeutic bioscavenger as it is unaltered after the reaction with nerve agents with promising turn over. To summarize, the development of a universal catalytic bioscavenger against known OPs and CWNAs is highly warranted and our in vitro data strongly support the idea of developing PON1 as a broad spectrum catalytic bioscavenger against CWNA exposure.

5. Conclusion

In conclusion, recombinant and human and rabbit serum purified PON1 hydrolyze chemical warfare nerve agent tabun more efficiently compared to that of sarin and soman. This is the first report in the literature comparing the efficiency of different types of PON1s against tabun, sarin, and soman. Hydrolysis of the nerve agents by purified PON1 was specific to PON1 enzyme activity. We also demonstrate that purified PON1 acts like a catalytic bioscavenger against nerve agents by hydrolyzing higher amounts of nerve agents and retaining PON1 activity after nerve agent hydrolysis. The efficient hydrolysis of tabun and other nerve agents by PON1 suggests that it can be potentially used as a catalytic bioscavenger for prophylactic treatment against organophosphate insecticide and chemical warfare nerve agent exposure.

Disclosure

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army, the Navy, or the Department of Defense, USA.

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