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Expression and purification of biologically active recombinant human paraoxonase 1 from a *Drosophila* S2 stable cell line

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ABSTRACT

Many pesticides and chemical warfare nerve agents are highly toxic organophosphorus compounds (OPs), which inhibit acetylcholinesterase activity. Human paraoxonase 1 (PON1) has demonstrated significant potential for use as a catalytic bioscavenger capable of hydrolyzing a broad range of OPs. However, there are several limitations to the use of human PON1 as a catalytic bioscavenger, including the relatively difficult purification of PON1 from human plasma and its dependence on the presence of hydrophobic binding partners to maintain stability. Therefore, research efforts to efficiently produce recombinant human PON1 are necessary. In this study, we developed a *Drosophila* S2 stable cell line expressing recombinant human PON1. The recombinant human PON1 was fused with the human immunoglobulin Fc domain (PON1-hFc) to improve protein stability and purification efficiency. We purified the recombinant human PON1-hFc from the S2 stable cell line and characterized its enzymatic properties for OP hydrolysis. We purified the recombinant human PON1-hFc from the S2 stable cell line and characterized its enzymatic properties for OP hydrolysis compared with those of the recombinant human PON1 derived from *E. coli*. We observed that the recombinant human PON1-hFc is functionally more stable for OP hydrolyzing activities compared to the recombinant human PON1. The catalytic efficiency of the recombinant PON1-hFc towards diisopropyl fluorophosphate (DFP, 0.26×10^6 M^{-1} min^{-1}) and paraoxon hydrolysis (0.015×10^6 M^{-1} min^{-1}) was 1.63- and 1.24-fold higher, respectively, than the recombinant human PON1. Thus, we report that the recombinant PON1-hFc exerts hydrolytic activity against paraoxon and DFP.

1. Introduction

Organophosphorus compounds (OPs) are phosphorus-containing organic compounds that have been used as pesticides, including parathion, malathion and chlorpyrifos, and as nerve agents, including soman, sarin, tabun and VX [1]. Poisoning by OPs disrupts neurotransmission by inhibiting acetylcholinesterase (AChE; EC 3.1.1.7) activity, which catalyzes the rapid breakdown of acetylcholine into choline and acetic acid [2,3]. OP poisoning leads to acetylcholine accumulation in the synapse, thereby causing muscle weakness, fatigue, muscle cramps, or even respiratory muscle paralysis, resulting in death [4]. Chemical ligands such as carbamates mask the active site of AChE from OP exposure and are commonly used as a prophylactic treatment, but they elicit adverse side effects [2,3]. To reduce the serious side effects of chemical ligand therapeutics, the use of bioscavengers has recently emerged as a new approach to protect against OP poisoning.

It has been proposed that human butyrylcholinesterase (BChE; EC 3.1.1.8) acts as a stoichiometric bioscavenger for the safe and efficient prophylaxis of OP poisoning [5–8]. However, a major limitation of BChE in protecting against OP poisoning is the necessity of 1:1 stoichiometry between the enzyme and the OP [9]. Therefore, research efforts are underway to develop catalytic

Abbreviations: OPs, organophosphorus compounds; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; PON1, paraoxonase 1; DFP, diisopropyl-fluorophosphate; SMP-30, senescence marker protein 30.

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bioscavengers, which are capable of hydrolyzing multiple OPs with turnover, to overcome the limitation of stoichiometric bioscavengers. Administration of a small dose of a catalytic bioscavenger can provide rapid and efficient protection against OP exposure compared to larger doses of costly stoichiometric bioscavengers [3,10]. Because the G117H mutant of human BChE has been identified as a catalytic bioscavenger with remarkably improved activity against OPs [11–13], several potential catalytic bioscavengers, such as human paraoxonase 1 (PON1; EC 3.1.8.1), human prolidase (EC 3.4.13.9), human senescence marker protein 30 (SMP-30; EC 3.1.8.2) and bacterial phosphotriesterases, are in development [2,3,9,14]. Specifically, human PON1 is the leading enzyme among catalytic bioscavengers currently in development [2,3,15].

Human PON1 is a calcium-dependent promiscuous enzyme (phosphotriesterase, arylesterase and lactonase) with a wide range of substrates [3]. Human PON1 is primarily expressed in the liver and circulates in the blood while bound to high-density lipoprotein (HDL) [15–17]. Human PON1 is capable of hydrolyzing a broad range of OPs, including paraoxon, diisopropylfluorophosphate (DFP) and nerve agents such as sarin, soman and VX [15,18,19]. Interestingly, it has been reported that the catalytic activity of PON1 for the hydrolysis of paraoxon is 240-fold greater than the BChE G117H mutant [15,19]. Furthermore, intravenous administration of purified PON1 derived from human plasma has shown a protective effect against sarin and soman exposure in an experimental animal model; conversely, PON1-deficient mice are more sensitive to chlorpyrifos compared to wild type mice [20,21]. Despite the protective effect of human PON1 against OP exposure, it has limitations; the top two are that human PON1 is difficult to purify from human plasma and that its stability depends on the presence of hydrophobic binding partners [3,22,23]. Furthermore, it was shown that the recombinant human PON1 is not functionally expressed in *E. coli* due to aggregation and a defect in glycosylation [24,25]. Interestingly, unlike recombinant human PON1, it has been reported that the PON1 variant G3C9 clone, which is derived from random DNA shuffling of human PON1, mouse PON1, rat PON1 and rabbit PON1, shows soluble and functional expression in *E. coli* [26]. However, the recombinant PON1-G3C9 (gi: 40850544) shares only 85% of its amino acid identity with human PON1 (94% identity in the case of rabbit PON1), which may induce an adverse immune response *in vivo*; however, the OP hydrolyzing activities of the recombinant PON1-G3C9 are improved compared to the human serum PON1 [26,27]. Therefore, research efforts are required to develop an efficient system to produce recombinant human PON1.

In the present study, we developed a *Drosophila* S2 stable cell line that expresses recombinant human PON1. The recombinant human PON1 was fused with the human immunoglobulin Fc domain (PON1-hFc) to improve protein stability and purification efficiency. We purified the recombinant human PON1-hFc from this S2 stable cell line and compared the enzymatic characteristics of PON1-hFc for OP hydrolysis with the recombinant PON1 derived from *E. coli*. Here, we report that recombinant PON1-hFc has hydrolytic activity against paraoxon and DFP.

2. Materials and methods

2.1. Cloning of recombinant human PON1

The open reading frame of human PON1 was amplified by RT-PCR from the human liver cell line HepG2, and the amplified sequences were analyzed by DNA sequencing. For the construction of a human PON1 expression plasmid (pMTa-hygro-PON1-hFc), the active region of human PON1 (amino acids 16–355) produced by PCR amplification was subcloned into a pMTa-hygro vector [28]. The following primers were used for PCR amplification: 5′-AAG CTT ACC ATG CTC TTC AGG AAC CAC CAG-3′ and 5′-GTC GAC GAG CTC ACA GTA AAG AGC-3′. The active region of human PON1 (amino acids 16–355) was fused with a preprotrypsin signal sequence and FLAG epitope at the N-terminus and the Fc region of human immunoglobulin G (hFc) at the C-terminus (Fig. 1A and C). For the bacterial expression plasmid of human PON1 (pET21b-PON1), the active region of human PON1 (amino acids 16–355) was inserted into the pET21b vector (Novagen, Madison, WI, USA) using the *Hin*d III and *Sal* I restriction sites (Fig. 1B).

2.2. Drosophila S2 stable cell line

A *Drosophila* S2 stable cell line secreting PON1-hFc was generated as previously described [28]. Briefly, S2 cells were cultured in Ex-Cell 420 media (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) at 25 °C. Cultured S2 cells (3.0×10^6 cells/ml) were transfected with pMTa-hygro-PON1-hFc (5 μg) using a *Drosophila* expression system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were selected by hygromycin treatment (300 μg/ml) for 14 days. The hygromycin-resistant cells were further cultured for at least 20 passages to establish a stable cell line, and the expression of recombinant PON1-hFc in the stable cells was identified using western blotting analysis with anti-FLAG antibody (Sigma-Aldrich) and anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described [29].

2.3. Purification of recombinant PON1-hFc

To induce recombinant PON1-hFc expression, the stable S2 cells were seeded at 3.5×10^7 cells in 300 ml of Ex-Cell 420 media supplemented with 10% FBS and spinner-cultured for 5 days. The cultured cells were transferred into 2 L of Ex-Cell 420 media without FBS and spinner-cultured for an additional 2 days. After this, the expression of PON1-hFc in the spinner-cultured S2 stable cells was induced by treatment with 500 μM $CuSO_4$ for 14 days. To purify the secreted recombinant PON1-hFc, the culture supernatant was harvested by centrifugation at 10,000 *g* for 10 min. The recombinant PON1-hFc was purified using 1 ml of protein A agarose in a column (50% slurry, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Non-specific proteins bound to the column were washed with 50 ml of phosphate-buffered saline (PBS). Recombinant PON1-hFc was collected by adding 10 ml of elution buffer (0.1 M glycine (pH 3.0)). Finally, the purified recombinant PON1-hFc was dialyzed in PBS at 4 °C for 16 h. The purified recombinant PON1 was analyzed by SDS-PAGE and western blot analysis using an anti-FLAG antibody as previously described [28].

2.4. Expression and purification of the recombinant human PON1

The bacterial expression plasmid pET21b-PON1 was transformed into the *E. coli* BL21 (DE3) strain. To induce the soluble expression of the recombinant human PON1 in *E. coli*, pET21b-PON1 were further transformed into BL21 cells harboring the pG-Tf2 plasmid expressing GroEL-GroES and TF chaperones [30,31]. The production of the recombinant human PON1 in the transformed BL21 cells (0.3 OD_{600}) was induced by treating the cells with 0.1 mM IPTG for 3 h at 30 °C. After induction, the BL21 cells were lysed in lysis buffer (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 1 mg/ml lysozyme) by sonication. The cell lysates and cell debris were then separated by centrifuging at 12,000 × g for 10 min at 4 °C. The recombinant proteins were analyzed using 10% SDS-PAGE gels, which were then stained with Coomassie Blue.