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Expression and purification of biologically active recombinant human paraoxonase 1 from inclusion bodies of *Escherichia coli*

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ABSTRACT

Human PON1 (h-PON1) is a Ca^{2+}-dependent serum enzyme and can hydrolyze (and inactivate) a wide range of substrates. It is a multifaceted enzyme and exhibit anti-inflammatory, anti-oxidative, anti-atherogenic, anti-diabetic, anti-microbial, and organophosphate (OP)-detoxifying properties. Thus, h-PON1 is a strong candidate for the development of therapeutic intervention against these conditions in humans. Insufficient hydrolyzing activity of native h-PON1 against desirable substrate affirms the urgent need to develop improved variant(s) of h-PON1 having enhanced activity. Production of recombinant h-PON1 (rh-PON1) using an *Escherichia coli* expression system is a key to develop such variant(s). However, generation of rh-PON1 using *E. coli* expression system has been elusive until now because of the aggregation of over-expressed rh-PON1 protein in inactive form as inclusion bodies (IBs) in the bacterial cells. In this study, we have over-expressed rh-PON1$_{(wt)}$ and rh-PON1$_{(H115W;R192K)}$ proteins as IBs in *E. coli*, and refolded the inactive enzymes present in the IBs to their active form using *in vitro* refolding. The active enzymes were isolated from the refolding mixture by ion-exchange chromatography. The catalytic properties of the refolded enzymes were similar to their soluble counterparts. Our results show that the pure and the active variant of rh-PON1 enzyme having enhanced hydrolyzing activity can be produced in large quantities using *E. coli* expression system. This method can be used for the industrial scale production of rh-PON1 enzymes and will aid in developing h-PON1 as a therapeutic candidate.

Introduction

Human paraoxonase 1 (h-PON1)[1] (EC 3.1.8.1) is a ~45 kDa, Ca^{2+}-dependent serum enzyme that can hydrolyze a variety of substrates [1,2]. It is primarily synthesized in the liver and is secreted into the bloodstream where it is associated with a category of high density lipoprotein particles [3,4]. The h-PON1 is a multitasking enzyme and various hydrolytic activities of h-PON1 can be grouped into three categories: arylesterase, phosphotriesterase and lactonase [5]. The precise physiological function(s) of h-PON1 is not known yet; however, the enzyme has shown to exhibit anti-inflammatory, anti-oxidative, anti-atherogenic, anti-diabetic, anti-inflammatory and organophosphate (OP)-hydrolyzing properties [6–13]. Recent reports suggest that h-PON1 also plays an important role in the metabolism of certain drugs [14,15].

The level and the activity of serum PON1 in individuals suffering from cardiovascular diseases, liver diseases, diabetes, renal diseases, cancer, and obesity are considerably lower than in the normal subjects [6,16,17]. Animals deficient in PON1 have been found to be more susceptible to these disease conditions and the over expression of h-PON1 or administration of purified PON1 in these animals has been shown to prevent/retard the development of these disease conditions [7–10]. Thus, h-PON1 is a strong candidate for the development of therapeutic intervention against these disease conditions in humans.

The beneficial role of h-PON1 in OP-poisoning is also well demonstrated. OP-compounds are toxic chemicals that exert their harmful effect by inhibiting the function of neurotransmitter-metabolizing enzymes [18]. OP-compounds are easy to manufacture and are widely used as pesticides, fungicides, insecticides, herbicides and petroleum additives in agriculture and other industries. Certain OP-compounds developed by the armies as chemical warfare nerve agents (CWNAs) are much more dangerous and have become important terrorist chemical weapon in today's world [18,19]. Animals deficient in PON1 have been found to be more susceptible to OP-poisoning and the over-expression of h-PON1 or administration of purified h-PON1 into transgenic animals has been shown to

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[1] *Abbreviations used:* AHL, acyl homoserine lactone; CPO, chlorpyrifos oxon; CWNA, chemical warfare nerve agent; h-PON1, human paraoxonase 1; rh-PON1, recombinant human paraoxonase 1; OP, organophosphate; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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prevent/retard their susceptibility to OP-poisoning [20–24]. In humans, the amount and the level of activity of serum PON1 have been demonstrated to have an impact on determining their susceptibility towards OP-poisoning [25–27]. Thus, h-PON1 is a strong candidate for the development of antidote against OP-poisoning in humans.

Native h-PON1 does not have sufficient catalytic activity against all of its substrates; therefore, there is a need to develop improved variant(s) of h-PON1 having enhanced activity against desired substrate(s). Production of recombinant h-PON1 (rh-PON1) using *Escherichia coli* expression system, a most preferred system for the manufacture of recombinant proteins, is a key to develop better variants of h-PON1. However, various attempts to generate active rh-PON1 with high purity and high yield using this expression system were unsuccessful [28–31]. Being a eukaryotic protein, over-expression of rh-PON1 in *E. coli* leads to aggregation of recombinant protein in inactive form as inclusion bodies (IBs), making it difficult to produce rh-PON1 enzymes in active form with high yield using *E. coli* expression system [28–31].

Production of active recombinant proteins by *in vitro* refolding of inactive proteins present in IBs have emerged as an attractive alternative method over soluble production of these recombinant proteins [32–34]. However, the process of refolding of inactive proteins present in IBs to their active form is recognized to cause a major bottleneck in the protein production scheme and method(s); hence, refolding of recombinant proteins should be developed on a case-by-case basis [33,34].

In this study, we have over-expressed rh-PON1$_{(wt)}$ and rh-PON1$_{(H115W;R192K)}$ proteins as IBs in *E. coli* and refolded the inactive enzymes present in the IBs to their active form using *in vitro* refolding. The active enzymes were isolated from the refolding mixture by ion-exchange chromatography. The catalytic properties of the refolded enzymes were similar to their soluble counterpart.

Materials and methods

Materials

5-bromo-4-chloro-3′-indolyphosphate (BCIP), nitro-blue-tetrazolium (NBT) reagent, alkaline phosphatase-labeled anti-mouse secondary antibody, arginine, CHES buffer, dextrose, guanidine HCl, Hepes buffer, isopropyl-1-β-D-galactopyranoside (IPTG), lactose, lysine, lysozyme, maltose, mannitol, mannose, *m*-cresol purple, methyl β-cyclodextrin, MOPS buffer, NDSB201, NDSB256, oxidized glutathione, paraoxon ethyl, PIPES buffer, polyethylene glycol (PEG)-400, -1000 and -3350, proline, protease inhibitor cocktail, reduced glutathione, sorbitol, TAPS buffer, Tris (2-carboxyethyl) phosphine (TCEP), trehalose, Tris–HCl, Triton X-100, Tween-20, Tween-80 and urea were purchased from Sigma–Aldrich, Bangalore, India. *E. coli* BL21 (DE3), DH5α cells and pET23a(+) plasmid were purchased from Merck (Novagen) Bangalore, India. Gene JET™ gel extraction kit, T4 DNA ligase, dNTPs, restriction enzymes, nuclease free water and DNA ladder were obtained Fermentas, CA, USA. Protein molecular weight markers and Bradford reagent were purchased from Bio-Rad, Gurgaon, India. Q-Sepharose columns were from GE Healthcare (GE Healthcare Bio-Sciences Ltd., Uppsala, Sweden). All other reagents used were of highest analytical grade.

Construction of expression plasmid containing gene for rh-PON1 enzyme

Construction of expression plasmids containing codon optimized gene encoding soluble rh-PON1 enzymes is described elsewhere [30,31]. These recombinant enzymes contained 355 amino acids of native h-PON1 along with one extra amino acid (glutamic acid) at 356th position and a $(His)_6$-tag. The soluble recombinant proteins were 'humanized' by removing the extra amino acid, as well as a C-terminal $(His)_6$-tag *via* a PCR amplification reaction. For this, plasmids containing gene encoding rh-PON1 enzymes were purified and amplified in a PCR reaction using following primers; F-(5′-GGAATTCCATATGGCGAAACTGATTGCCCTG-3′) and R-(5′-CCGCTCGAGTCAGAGTTCGCAATACAGCGCTTT-3′). The forward primer contain *Nde*1 restriction site and the reverse primer contain a stop codon followed by a *Xho*1 restriction site. The amplified genes and empty pET23a(+) plasmid (1 μg each) were separately subjected to restriction digestion by mixing with 1 U of *Nde*1/*Xho*1 restriction enzymes and 1 × of appropriate digestion buffers in a final volume of 20 μl. All the digestion reactions were carried out for 12 h at 37 °C and the digested DNA products were resolved on (1%) agarose gel electrophoresis. The linearized plasmid backbone and open reading frame (ORF) dropouts were purified from the agarose gel using Gene JET™ gel extraction kit and ligated using T4 DNA ligase (plasmid backbone and ORF ratio of 1:3) to generate pET23a(+) plasmids containing gene for 'humanized' rh-PON1 enzymes. The ligation reaction was incubated at 16 °C for 16 h and transformed into *E. coli* DH5α cells. The presence of desirable ORF in the transformed plasmids was confirmed by restriction digestion of the purified plasmids with *Nde*1/*Xho*1 restriction enzymes, as well as by direct DNA sequencing (Eurofinn, Bangalore, India). The plasmid containing rh-PON1 enzymes were then transformed into *E. coli* BL21(DE3) cells and glycerol stocks of the transformed cells were prepared and stored at −80 °C.

Expression of rh-PON1 and purification of IBs

Glycerol stock of *E. coli* BL21(DE3) cells containing pET23a(+)-rh-PON1 was streaked on a LB-agar plate containing 50 μg/ml carbenicillin and 1 mM $CaCl_2$ and incubated overnight at 37 °C. A single colony from the plate was picked and inoculated into LB-broth supplemented with 50 μg/ml carbenicillin and 1 mM $CaCl_2$ and was grown at 37 °C overnight (seed culture). Of the seed culture, 1% was withdrawn and then inoculated into fresh LB-broth supplemented with 50 μg/ml carbenicillin and 1 mM $CaCl_2$ and the main culture was grown at 37 °C until OD_{600} reached 0.6–0.8. The culture was then induced with 1 mM IPTG and the cells were further allowed to grow at 37 °C for 8 h. The cells were harvested by centrifugation (∼3–4g wet cell mass per liter of culture) and were used to purify IBs containing rh-PON1 enzyme, by following a procedure described in ref [32,35], with slight modification. Briefly, the cell pellet was re-suspended in ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0 containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM β-ME, 0.1 mM of protease inhibitor cocktail and 10 μg/ml lysozyme) at a ratio of 1:10 (w/v). The cell suspensions were subjected to sonication. The sonicated cell suspensions were immediately cooled on ice and treated with DNase (10 μg/ml) and 0.3 mM $MgCl_2$ for 1 h. Then 2 V of buffer (100 mM Tris–HCl, pH 7.0 and containing 2% Triton X-100, 1.5 M NaCl and 20 mM EDTA) were added to the sample to make the total volume 3 V. The samples were centrifuged (10,000g, 30 min, 4 °C) to separate clear cell lysates from insoluble fraction containing rh-PON1-enriched IBs. The IBs were then washed twice with 400 ml of IB-washing buffer (100 mM Tris–HCl, pH 7.0 containing 20 mM EDTA) and centrifuged (10,000g, 30 min, 4 °C) to remove the contaminants present in the IBs. Purified IBs were then collected and stored at −80 °C until further use. Fractions containing proteins were analyzed by SDS–PAGE and western blot analysis. Monoclonal mouse anti-human PON1 antibody was used as a primary antibody in the western blot analysis.

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