Turkish Journal of Chemistry

Volume 39 | Number 4 Article 5

1-1-2015

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DEMİR, YELİZ and BEYDEMİR, ŞÜKRÜ (2015) "Purification, refolding, and characterization of recombinant human paraoxonase-1," *Turkish Journal of Chemistry*: Vol. 39: No. 4, Article 5. https://doi.org/10.3906/kim-1501-51
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Turkish Journal of Chemistry
http://journals.tubitak.gov.tr/chem/
Research Article

Turk J Chem
(2015) 39: 764 – 776
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doi:10.3906/kim-1501-51

Purification, refolding, and characterization of recombinant human paraoxonase-1

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Received: 19.01.2015 • **Accepted/Published Online:** 15.05.2015 • **Printed:** 28.08.2015

Abstract: A high density lipoprotein (HDL)-linked enzyme with antioxidant and antiatherogenic properties, paraoxonase-1(PON1), prevents the formation of atherosclerotic lesions in humans. In the present study, a recombinant hPON1 gene was produced using a small ubiquitin-related modifier (SUMO) fusion protein expression system. To that end, the hPON1 gene was amplified from human liver-ready cDNA, cloned into the expression vector pET SUMO, and expressed in *Escherichia coli* BL21 (DE3). The predominance of the expressed fusion SUMO-hPON1 protein was inclusion bodies and purified using 6xHis affinity chromatography under natural and denaturing conditions. Subsequently, the enzyme was purified and refolded directly on the affinity matrix under redox conditions to obtain a bioactive protein in a single step. The inclusion bodies were solubilized with urea, guanidine hydrochloride, and Triton X-100 and refolded in vitro. After purification, 0.045 mg/mL protein in soluble fraction and 0.108 mg/mL protein from inclusion bodies were obtained. Optimum temperature, pH, and ionic strength for rhPON1 activity were determined as 40 °C, 10.0, and 100 mM, respectively. The kinetic parameters K_m and V_{max} for rhPON1 were determined as 0.94 mM and 110.01 EU/mL, respectively, by using Lineweaver–Burk plots.

Key words: Recombinant DNA, cloning, HDL, SUMO expression system, 6xHis affinity chromatography

1. Introduction

The paraoxonase (PON) gene family consists of three members: paraoxonase 1 (PON1), paraoxonase 2 (PON2), and paraoxonase 3 (PON3). They are very similar to each other in terms of base sequence, sharing 65% amino acid and 70% nucleotide identity [1] and located on the long arm of human chromosome 7 (q21.22). [2] Particularly, human PON1 is one of the most extensively studied paraoxonases due to its esterase/lactonase activity, as well as its antiatherogenic activity. It is a glycoprotein with 354-amino acid and a molecular mass of 43–47 kDa. [1,3] The crystal structure of human serum PON1 has not been determined yet, but it is available for recombinant PON1 variant (G1A5, G1C4, G3C6, G2E6 expressed in *E. coli*) [4] and described as a six-bladed β-propeller each blade of which contains four strands. Two calcium ions are located in the central part of the tunnel in the propeller. One of these ions is known to play a structural role, while the other serves catalytic functions. The enzyme contains three cysteine residues at positions 42, 284, and 353, two of which (Cys42 and Cys353) form disulfide bridges (Figure 1). PON1 may contain a presumptive HDL binding site including polar residues.

hPON1 is synthesized as HDL-linked in the liver. The mature protein contains the hydrophobic N-terminal secretory signal sequence that provides the structural basis for its interaction with phospholipids and HDL. [5,6] Moreover, it is well known that hPON1 plays a role in protection against atherosclerosis by inhibiting

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low density lipoprotein (LDL, bad cholesterol) oxidation. Due to its physiological effects, paraoxonase is also a lactonase. Physiological substrate of this enzyme has not been illuminated yet. However, it can also catalyze the cleavage of ester bonds.[7] As a matter of fact, PON1 had been initially characterized as a hydrolyzer for organophosphates including paraoxon. The enzyme got its name from this reaction.

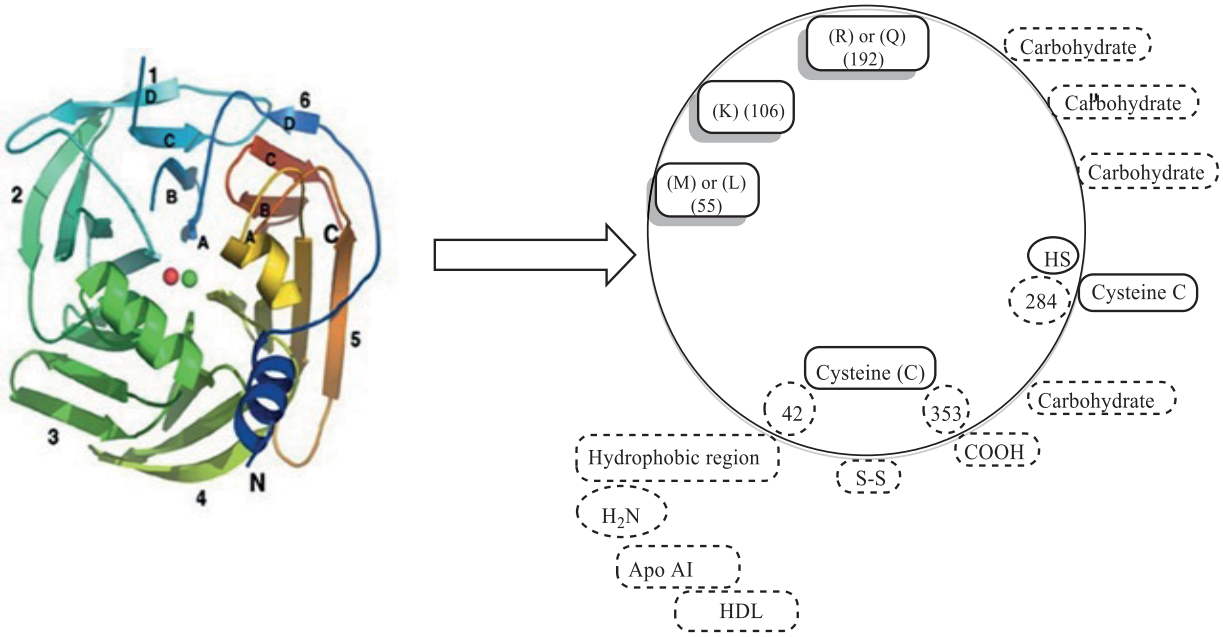


Figure 1. Structure of PON1 is described as a six-bladed β-propeller with each blade containing four strands. Two calcium ions are located in the central part of the tunnel in the propeller. The enzyme contains three cysteine residues at position 42, 284, and 353. Two of them (Cys42 and Cys353) form a disulfide bridge. (Reprinted from reference 4 with permission of the published journal editor.) Cys-284 residue is considered to provide the antioxidant effects at the three-dimensional structure of PON1 enzyme.

The large number of recombinant proteins has been overexpressed in molecular biotechnology studies. *Escherichia coli* has usually been used as a good expression system for the production of recombinant proteins. *E. coli* has several advantages over the other expression systems, including fast growth, low cost, and easy handling.[8,9] If expressed in *E. coli*, human recombinant proteins may not fold correctly in some cases. If the protein does not take its correctly folded tertiary structure, it often becomes misfolded and deposits insoluble inclusion bodies within the bacterium.[10–12] This can lead to protein degradation and improper native structure. Sometimes overexpression of proteins may cause the formation of inclusion bodies. In this case, the protein must be refolded to take the native form. Particularly, endogenous proteins also accumulate as inclusion bodies when overexpressed in *E. coli*. To obtain an active product, inclusion bodies should be solubilized, refolded, and then purified.[13,14] The solubilization of the inclusion bodies is performed by using high concentrations of denaturants such as urea or guanidine hydrochloride, together with a reducing agent such as mercaptoethanol.[13,15,16] Solubilization of inclusion body proteins under mild solubilization conditions protects the existing native like the secondary structure of proteins. Thus, it diminishes protein aggregation during the refolding step and promotes the recovery of bioactive proteins from inclusion bodies.[17,18] Extreme pHs are used successfully for solubilization. Solubilized proteins are refolded to their native state in the presence of an oxidizing agent.[11,19]

It has recently been reported that L-arginine[20] and lauryl glutamate are used for solubilization of inclusion bodies.[21] Many times, the overall yield of bioactive protein from inclusion bodies is around 15%–25% of the total protein.[18] Moreover, some additives such as acetone, acetoamide, dimethyl sulfoxide, and polyethylene glycol are used to enhance the yield of folded bioactive protein.[22]

Due to its antioxidant and antiatherosclerotic effects, PON1 enzyme has been studied extensively all over the world. For instance, in our previous studies, PON1 enzyme was purified from human serum using a simple three-step purification method: ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration chromatography.[7,23–25]

In the present work, hPON1 was cloned from Human Liver Marathon-Ready cDNA and expressed in *E. coli* as inclusion body by using a small ubiquitin-related modifier (SUMO) fusion protein expression system. Furthermore, some kinetic properties of the recombinant hPON1 were also determined.

2. Results and discussion

2.1. Results

2.1.1. Expression of rhPON1

After amplification of the hPON1 gene from the human fetal liver cDNA library by PCR, obtained fragments were transferred into the pET-SUMO vector with T4 DNA-ligase as described in the Experimental section. pET-SUMO-rhPON1 containing (His)6tag at the N-terminus was extracted (Figure 2). Plasmid was transformed into competent *E. coli* BL21 (DE3). The soluble fraction of cell lysate was loaded on SDS-PAGE. Expression time is shown in Figure 3.

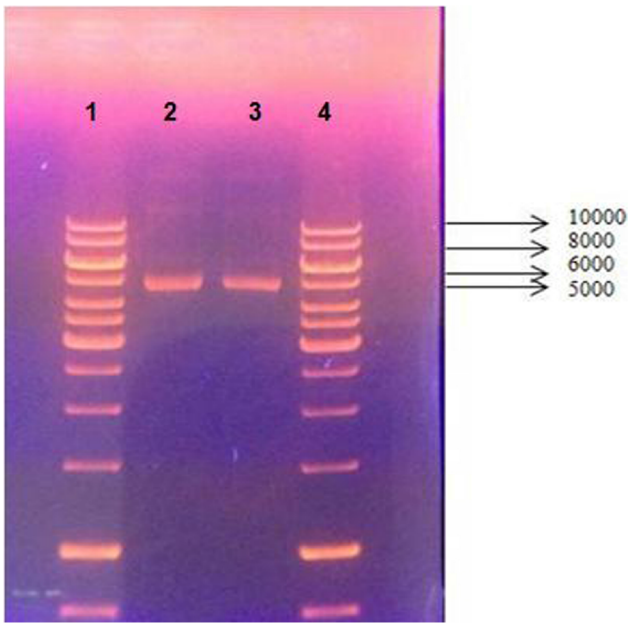


Figure 2. Plasmid isolation, Lane 1, 4: 1 kb DNA ladder standard, Lane 2, 3: pET-SUMO-rhPON1 plasmid.

2.1.2. Purification of soluble rhPON1

The rhPON1 was purified in two steps by using PEI precipitation and 6xHis affinity chromatography according to the purification procedure from the soluble fraction in *E. coli.* It is summarized in Table 1. Because PEI

precipitation did not affect enzyme activity, it is not shown in the purification table. The target protein was eluted with 250 mM imidazole. Protein was quantified by the Bradford method[26] using bovine serum albumin as a standard. The soluble fractions were also loaded onto two separate SDS-PAGE gels. One of the gels was stained with Coomassie brilliant blue G-250 and a single band of the enzyme was obtained. The molecular weight of soluble rhPON1 was determined as 51.52 kDa with this application (Figure 4). The other gel was stained with silver staining because of its sensitivity. The molecular weight of the enzyme was found to be identical in both methods.

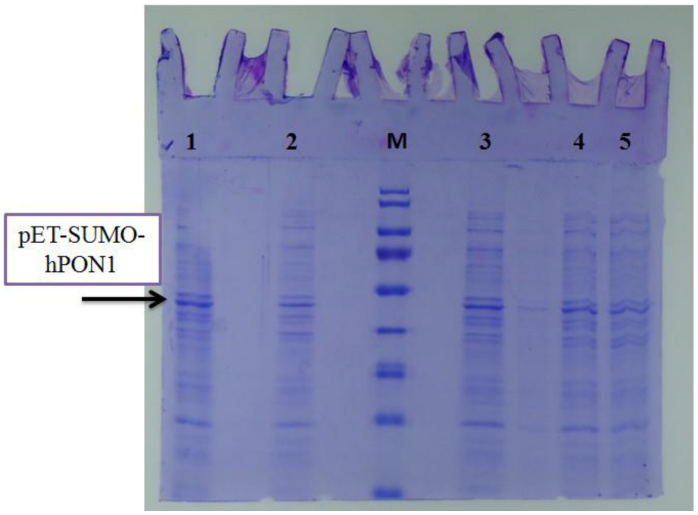


Figure 3. SDS-PAGE analysis of rhPON1 produced in *E. coli* BL21(DE3). All samples were prepared by boiling for 5 min in sample loading buffer containing 10% of 2-mercaptoethanol. Lane 1: soluble fraction of cell lysate of IPTG induced 2 h BL21(DE3) pET-SUMO-hPON1. Lane 2: Soluble fraction of cell lysate of IPTG induced 3 h BL21(DE3) pET-SUMO-hPON1. Lane 3: Soluble fraction of cell lysate of IPTG induced 4 h BL21(DE3) pET-SUMO-hPON1. Lane 4: Soluble fraction of cell lysate of IPTG induced 6 h BL21(DE3) pET-SUMO-hPON1. Lane 5: Soluble fraction of cell lysate of IPTG induced 8 h BL21(DE3) pET-SUMO-hPON1. *M Standard proteins (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa).

Table 1. Summary of rhPON1 purification from soluble form in nature conditions.

Step	Activity (EU/mL)	Protein (mg/mL)	Volume (mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Recovery (%)	Purification fold
Homogenate	5.46	0.412	8	43.68	3.296	13.252	100	1
Ni-NTA SUMO-hPON1	10.21	0.045	4	40.84	0.18	226.88	93.5	17.12

2.1.3. Isolation, solubilization, and purification of rhPON1 from inclusion bodies and refolding

Isolation, solubilization, and purification of rhPON1 from inclusion bodies are described in the Experimental. The purification procedure from the inclusion bodies is summarized in Table 2. Eluted fractions were loaded onto SDS-PAGE (8%). The gel was stained with Coomassie brilliant blue. Molecular weight of purified rhPON1 from the inclusion bodies was found to be 53.48 kDa (Figure 5a). Two methods were applied for the refolding

process: on-column refolding and dialysis. Refolding is greatly preferred because it does not require filtration or concentration on-column and is less time consuming.

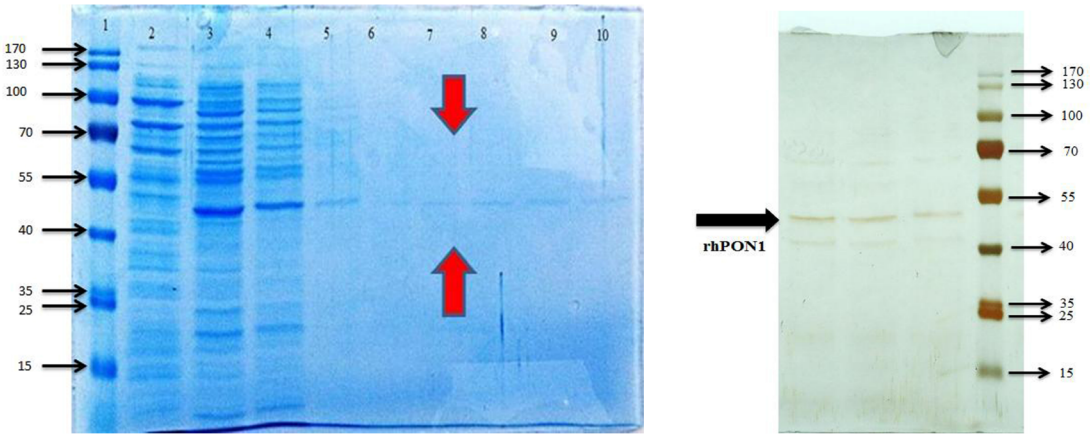


Figure 4. SDS-PAGE analysis of pET-SUMO-rhPON1 purified from soluble fraction by Ni-NTA affinity chromatography. The gel was stained with Coomassie brilliant blue and silver after electrophoresis. Lane 1: Standard proteins (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa). Lane 2: Nonbinding to column, flow through. Lane 3: Wash II, Lane 4: Wash III, Lanes 5–10: Purified pET-SUMO-hPON1.

Table 2. Summary of rhPON1 purification from inclusion body in denaturating conditions and refolding.

Step	Activity (EU/mL)	Protein (mg/mL)	Volume (mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Recovery (%)	Purification fold
Homogenate	8.38	0.478	6	50.3	2.868	17.53	100	1
Ni-NTA SUMO-hPON1	23.32	0.108	2	46.64	0.216	215.93	92.72	12.31
hPON1	12.57	0.027	1	12.57	0.027	465.56	25	26.56

2.1.4. Cleavage of fusion protein in vitro using SUMO protease

Since the amount of protein was not sufficient in soluble fractions, in accordance with manufacturer's protocol we did not cleave SUMO fusion protein. After purification of rhPON1 from inclusion bodies, SUMO fusion protein was cleaved by the highly specific SUMO (ULP-1) protease. The other gel was stained with silver staining. In addition to hPON1, two other bands were also detected. These proteins were not visible on the gel that stained with Coomassie brilliant blue G-250, because they had a minimal amount of protein. Molecular weight of hPON1 was determined as 43.45 kDa (Figure 5b). After cleaving, hPON1 lost its activity in insoluble fractions. Therefore, we used rhPON1 to determinate kinetic parameters.

2.1.5. Effect of pH, ionic strength, and temperature on rhPON1 and determination of kinetic parameters

Effect of pH, ionic strength, and temperature on rhPON1 and the kinetic parameters were determined and are summarized in Table 3.

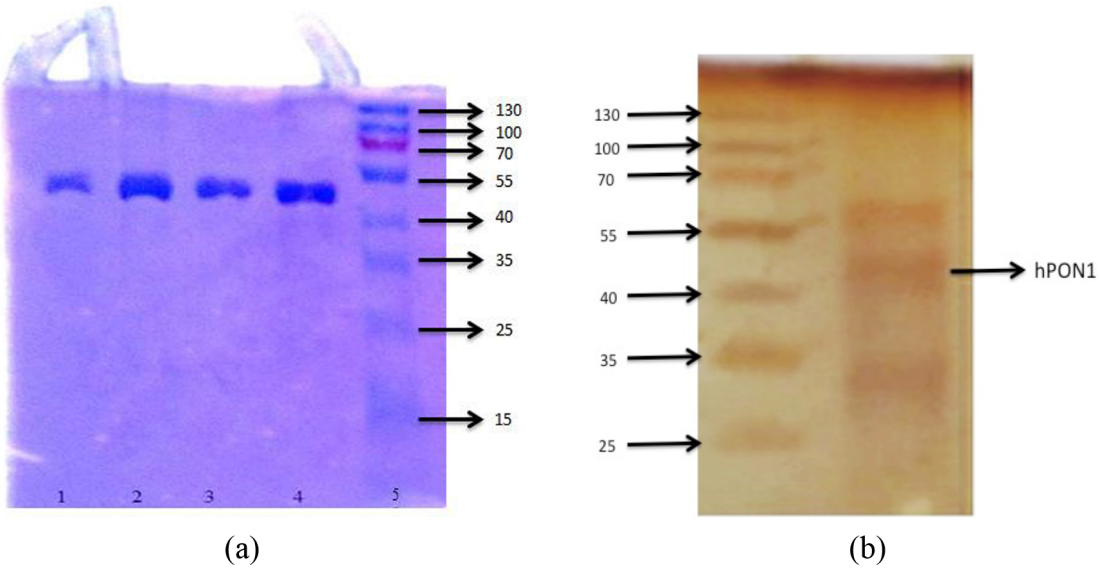


Figure 5. (a) SDS-PAGE analysis of rhPON1 purified from inclusion bodies by Ni-NTA affinity chromatography. Lanes 1–4: Purified pET-SUMO-hPON1 Lane 5: Standard proteins. (b) SDS-PAGE analysis of hPON1, cleaved from SUMO fusion protein. Lane 1: Standard proteins, Lane 2: hPON1.

Table 3. Summary of characterization of purified rhPON1 from inclusion bodies.

Enzyme	Opt. pH	Opt. °C	Opt. ionic strength (mM)	K_M (mM)	V_{max} (EU/mL)
rhPON1	Glycine–NaOH pH 10.0	40 °C	100	0.94	110.01

2.2. Discussion

Paraoxonase (arylesterase, EC 3.1.8.1, PON1), the calcium-dependent enzyme, is mainly synthesized in liver as HDL-associated serum esterase.[27] PON1 enzyme protects both HDL (the good cholesterol) and LDL (the bad cholesterol) against oxidation in the metabolism. Radicals like hydrogen peroxide are neutralized by the antioxidant property of the enzyme.[28] Thus, PON1 activity is critical for the prevention of atherosclerotic lesions. PON1 has three activities: paraoxonase, arylesterase, and diazoxon.[7] The enzyme can hydrolyze O–P ester linkage in paraoxon by its esterase activitiy.[29,30] It is known that paraoxon is used as an insecticide, due to its strong inhibitory action against acetylcholine esterase.[31]

PON1 includes two calcium ions: one is structural and the other is catalytic. Asn 224, 270, 168, Asp 269, and Glu 53 interact with the catalytic Ca^{2+} in the active site of PON1. Because of this, purification buffer contained Ca^{2+} to maintain the activity and stability of PON1. It is known that the active site of PON1 contains tryptophan, histidine, lysine, phenylalanine, and aspartate/glutamine.[32] Additionally, the sulfhydryl group of the cystein 284 in the PON1 structure is also known to provide antioxidant properties[4] (Figure 1). In addition to its antiatherogenic effects, PON1 enzyme is also the hydrolyzer of other synthetic esters like phenyl-acetate with high efficiency.[33] Many studies have been performed on PON isoforms, especially on PON1. The enzyme has been purified from different sources via various techniques. For instance, Furlong et al.[34] obtained the enzyme from human serum in four steps including agarose blue, Sephadex G 200, DEAE-trisacryl M, and Sephadex G-75 chromatography. In another study, hPON1 was purified from human serum with 9.95 U

mg^{-1} specific activity and yield of 9.96% using ammonium sulfate precipitation and Sepharose-4B-l-tyrosine-1-naphthylamine hydrophobic interaction chromatography.[35] In our previous study, hPON1 was purified in three steps consisting of ammonium sulfate precipitation (60-80%), DEAE-Sephadex anion exchange, and Sephadex G-200 gel filtration chromatography. The enzyme was purified with 4612.4 U mg^{-1} protein specific activity, yield of 34.2%, and approximately 231-fold.[25] Additionally, Lu et al.[36] purified refolded rhPON3 from *E. coli* using DEAE-Sepharose fast flow anion exchange chromatography.

In the present study, rhPON1 was expressed as a SUMO fusion protein containing 6xHis Tag. The cell lysate included soluble and insoluble proteins. The purification was performed by chromatography with a Ni-NTA affinity column for both soluble and insoluble proteins. During the purification of the soluble protein, PEI precipitation was also performed. PEI is a positively charged molecule at neutral pH. Thus, it binds to the negative charge of the nucleic acids and prevents the precipitation of the proteins.[37] However, PEI precipitation did not cause any change in the activity of rhPON1. Insoluble proteins contained inclusion bodies with rhPON1. To obtain natural rhPON1 protein, denatured rhPON1 was refolded by dialysis after Ni-NTA affinity chromatography. There are similar studies involving purification of PON3 enzyme in the literature.[36] Furthermore, we managed refolding using a Ni-NTA affinity column by making changes in some solutions. We did not encounter any study regarding this technique for PON1 purification in the literature. Thus, we purified the rhPON1 from the inclusion bodies by a single step procedure that is simple and efficient. After purification 0.045 mg/mL protein in soluble fraction and 0.108 mg/mL protein from inclusion bodies were obtained. Optimum temperature, pH, and ionic strength for rhPON1 activity were determined as 40 °C, 10.0, and 100 mM, respectively. The kinetic parameters K_m and V_{max} for rhPON1 were found as 0.94 mM and 110.01 EU/mL, respectively, by using Lineweaver–Burk plots.

Fusion tags are generally used to enhance the protein solubility and the protein expression level. In the present study SUMO fusion protein was used to improve the expression and folding of recombinant human PON1. SUMO tag is small in size and it is known to be highly specific to SUMO protease. The protease can also be combined with a number of other conventional tags in a variety of configurations.[38] Moreover, we saw that the SUMO fusion tag did not increase the solubility of rhPON1. Thus another fusion tag may be considered for increasing the solubility of rhPON1 in *E. coli*. This might also be caused by *E. coli*. It is well known that some eukaryotic proteins tend to aggregate when *E. coli* is used as the protein expression system.[10–12] In addition, hPON1 is reported to be produced in soluble, folded form in *E. coli* with great difficulty. Stevens et al.[39] used the large-scale fermentation to produce soluble hPON1, along with other variants of PON1 in *E. coli*. However, they were not able to obtain good yields for PON1 protein. They obtained 5.5 mg of 90% pure protein from 12 L of fermentation. This confirms the difficulty of expression and purification of soluble human protein within a bacterial system.

N-linked protein glycosylation is a posttranslational modification in eukaryotes and it is extremely uncommon in bacteria.[40] PON1 is a glycoprotein; thus it may not undergo glycosylation in *E. coli* correctly. PON1 has four potential N-glycosylation sites (Asn 227 and Asn 270; Asn 253 and Asn 324). On the other hand, it is suggested that glycosylation is not important for the hydrolytic activities of PON enzymes, but it may be essential for increasing their solubility and stability.[41,42] We consider that deficiency of glycosylation and some aggregations may result in insoluble and unstable protein in *E. coli*. In the present study, we found high expression levels with minimal solubility and minimal protein. Thus, we measured low activity of PON1 in the soluble fraction. However, in order to improve the folding stability, and solubility of the protein, site-directed rational mutations can be performed. Similar results were obtained in several studies on this subject.

For example, Sarkar et al.[43] expressed hPON1 in *E. coli*, and examined the structure of hPON1 and G2E6 as chimeric recombinant PON1s. G2E6 differs by multiple amino acids from hPON1. These amino acids are outside the putative active site of the enzyme. hPON1 was detected mostly in the insoluble fraction. Authors examined how mutations affect the solubility and soluble expression of hPON1 in *E. coli*. They suggest that three different types of mutations might increase the solubility of hPON1. These mutations may include the removal of the hydrophobic N-terminal leader sequence and mutations of hydrophobic amino acids in the presumptive HDL binding site to polar residues. Moreover, the surface residues that were mutated to be more polar amino acids during the directed evolution of G2E6. PON1 were mostly responsible for the increased solubility. Additionally, Suzuki et al.[44] reported that human PON1 was expressed with GST-tagged fusion protein in the *E. coli* expression system and demonstrated that active rhPON1 fusion protein was expressed and purified from *E. coli*.

It is apparent from the above-mentioned statements that rhPON1 has been mostly expressed in insoluble form by *E. coli*. Hence, in vitro refolding is necessary to recover bioactive proteins but successful refolding of proteins is not guaranteed. In the present study, we performed refolding for the insoluble form of hPON1. Thus, protein quantity and activity of hPON1 were increased approximately three-fold. We encountered a study on refolding of rhPON1. Bajaj et al.[45] investigated hPON1 as a pharmacologic agent. They refolded the rh-PON1 enzyme in vitro and detected a dramatic increase in the yield of the active enzyme. These results showed that refolding is effective for protein quantity and activity, which verify our data.

Consequently, the Ca^{2+} dependent enzyme, PON1, was produced using a SUMO fusion protein expression system. The recombinant PON1 was expressed in *E. coli* BL21(DE3). After expression, inclusion bodies were purified using 6xHis affinity chromatography under nature and denaturing conditions and refolded directly on the affinity matrix under redox conditions to obtain a bioactive protein. Furthermore, refolding studies were performed using dialysis, but the effects of this method were at minimum level. Following this, the kinetic properties of rhPON1 were determined. It is well known that obtaining PON1 enzyme by cloning in *E. coli* is very difficult. Therefore, there is a need for discovery of novel host cells. In addition, some mutations in amino acids of the PON1 structure may help us to understand several aspects of the enzyme's activity and folding mechanism, along with improving its stability properties.

3. Experimental

3.1. Materials

Human Fetal Liver Marathon-Ready cDNA was provided from Clontec (USA); pET-SUMO cloning and expression vector were obtained from Invitrogen; GeneJET Plasmid Miniprep, paraoxon, urea, guanidine hydrochloride, DTT, NaH_2PO_4, NaCl, imidazole, glycerol, $CaCl_2$, PEI, and Triton X-100 were obtained from Sigma (Sweden). Protein molecular weight marker was provided by Thermo and IPTG was obtained from BBI Fermentas (USA). Primers were synthesized by Metabion (Germany).

3.2. Cloning of hPON1 cDNA in pET-SUMO

Using forward primer (Primer 1: 5-ATGGCGAAGCTGATTGCG-3) and the reverse primer (Primer 2: 5-TTAGAGCTCACAGTAAAGAGCTTTG-3) hPON1 was constructed by PCR from Human Fetal Liver Marathon-Ready cDNA. The PCR was applied in a final volume of 15 μL under the following conditions: 94 °C for 4 min, 35 cycles [94 °C for 1 min, 60 °C for 30 s, and 72 °C for 1 min], and a final extension at 72 °C for 5 min. The purity of the PCR product was checked by agarose gel electrophoresis. The hPON1 gene was

ligated into pET-SUMO vector with His6-sites in N-terminus. After overnight ligation at 16 °C, the plasmid containing our gene of interest was transformed into competent *E. coli* One Shot Mach1-T1^R cells. These cells were recovered, plated on LB kanamycin plates and incubated overnight at 37 °C. Colonies were collected and then were grown in LB media. pET-SUMO-rhPON1 with a (His)6tag located at the N-terminus was extracted (GeneJET Plasmid Miniprep), and sequenced by Iontek (Turkey).

3.3. Expression of hPON1 gene in *E. coli*

Once the pET-SUMO-rhPON1 gene was confirmed by sequencing, it was transformed into competent *E. coli* BL21(DE3) cells for protein expression. Bacteria were grown in 10 mL of LB medium containing kanamycin (1 mg/mL) and 1 mM $CaCl_2$ in a shaking incubator at 37 °C until the OD at 600 nm reached 0.8, and then transferred to 1 L of LB medium containing kanamycin 1 mg/mL with 1 mM $CaCl_2$ and grown at 37 °C, at 225 rpm. Subsequently the gene was induced with 0.5 mM (IPTG) after log phase of OD 600 0.6–0.8 was reached, and then placed at 28 °C for 6 h. The cells were centrifuged and cell lysis was performed by resuspending the pellet using 8 mL of lysis buffer. Then 8 mg of lysozyme was added for cell lysis and the cells were incubated on ice for 30 min. The cell lysate was sonicated on ice (15 × 15 s pulses with 15 s intervals). The lysate was centrifuged at 5000 rpm for 15 min. The rhPON1 inclusion bodies containing pellets were kept at –80 °C and soluble rhPON1 containing supernatant was transferred into a fresh tube. The soluble rhPON1 was approximately 10% of the expressed protein. The clear lysate was loaded onto SDS-PAGE gel (8%) for analysis.

3.4. Purification of hPON1 gene in *E. coli*

Primarily, polyethyleneimine (PEI) precipitation was performed to purify the soluble rhPON1. The linear form of PEI has the structure $H_2N(C_2H_4NH)_xC_2H_4NH_2$, and the pKa value of the imino groups is 10–11.[37] PEI is a positively charged molecule in solutions of neutral pH. Binding of greatly negatively charged nucleic acids to PEI can prevent protein precipitation.[37] First 10% PEI solution was prepared in water at pH 7.9 and next it was added dropwise to the crude supernatant (1% PEI final) with constant stirring for 35–40 min and then centrifuged at 5000 rpm for 15 min. To get rid of the PEI binding, the supernatant was subjected to ammonium sulfate precipitation. The precipitate was obtained after centrifugation at 15,000 $\times g$ for 15 min and redissolved in lysate buffer (pH 8.0) and dialyzed in 5 mM Tris-HCl buffer (pH 8.0).

After dialysis, lysozyme (1 mg/mL), DTT (1 mM), and 10% glycerol were added to the sample and incubated on ice for 45 min. Sonication helped the final lysis of the pellet. Triton X-100 (0.1%) was added followed by shaking for 2–3 h. The clear cell lysate was collected after centrifugation and added to 2 mL of Ni-NTA resin. After allowing overnight binding of the protein, the lysate was run through the column. It was then washed with 25 mL of buffer I (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, 1 mM $CaCl_2$, 10% glycerol, 0.1% Triton X-100, pH 8.0) and subsequently washed with 25 mL of buffer II (50 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole, 1 mM $CaCl_2$, 10% glycerol, 0.1% Triton X-100, pH 8.0). Finally, the column was washed with buffer III (50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, 1 mM $CaCl_2$, 10% glycerol, 0.1% Triton X-100, pH 8.0). The protein was eluted with 10 mL of elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, 1 mM $CaCl_2$, 10% glycerol, 0.1% Triton X-100, pH 8.0). The samples of the clear lysate, flow through, wash, and elution were loaded onto SDS-PAGE gel for analysis.

3.5. Measurement of PON1 activity

PON1 activity was measured at 25 °C with paraoxon (diethyl p-nitrophenyl phosphate; 1 mM) in 50 mM glycine/NaOH (pH 10.5) containing 1 mM $CaCl_2$. The paraoxonase enzyme assay was based on the estimation of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol (ε = 18.290 M^{-1} cm^{-1} at pH 10.5) was used to calculate PON1 activity.[46] One enzyme unit was described as the amount of enzyme catalyzing the hydrolysis of 1 mmol of substrate at 25 °C. The assays were carried out using a spectrophotometer (Chebios UV-VIS).[25]

3.6. Isolation and solubilization of inclusion bodies

The cell pellet including inclusion bodies was resuspended from 1 L of culture in 40 mL of resuspension buffer (20 mM Tris-HCl, pH 8.0). The cells were disrupted by sonication on ice (6 × 10 s) and centrifuged at 11,000 $\times g$ for 15 min at 4 °C. Supernatant was removed and the pellet was resuspended three times in 30 mL of cold isolation buffer (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, 4% Triton-X 100, pH 8.0) and sonicated as described above. It was centrifuged at 11,000 $\times g$ for 15 min at 4 °C.

The pellet was resuspended in binding buffer including 6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 1 mM DTT, pH 8.0, and stirred for 60 min at room temperature for solubilization and sample preparation. The samples were centrifuged for 15 min at 11,000 $\times g$ and 4 °C.

3.7. Refolding and purification of rhPON1

Refolding of polypeptides is significant because it can supply soluble native protein for structural, regulational, and functional studies. There are some procedures for refolding insoluble recombinant proteins such as gentle dialysis, dilution in large volume of refolding buffer, or using packed columns.

3.7.1. In vitro refolding of rhPON1 on-column

Ni-NTA resin (Invitrogen) was loaded into a column equilibrated in buffer containing 6 M guanidine hydrochloride (GuHCl), 20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazol, 1 mM DTT, 4% Triton X-100, pH 8.0. First, the column was washed using the denaturing buffer containing 10 mM imidazole to eliminate nonspecifically bound contaminants. Subsequently, solubilized inclusion bodies were bound on Ni-NTA resin by batch-absorption overnight at room temperature. Renaturation and purification were carried out the next day with slight modifications in buffers on-column.

All renaturation steps were performed in buffer A (20 mM Tris–HCl, 0.5 M NaCl pH 8.0). The pH of buffer A can be at least 1.0 pH units away from the pI of the protein to prevent protein precipitation. In the next step, the column was washed with 30 mL (20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 1 mM DTT, and 4% Triton X-100 at pH 8.0). Refolded protein was eluted with buffer (20 mM Tris-HCl, 0.5 M NaCl, 250 mM imidazole, 1 mM DTT, 4% Triton X-100, pH 8.0). The eluted fractions containing soluble refolded protein were run on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).

3.7.2. In vitro refolding of rhPON1 by dialysis

The inclusion body pellet was washed with buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.1% NaN_3, 0.5% Triton X-100) and sonication was applied. After sonication 10 mM $MgSO_4$ was added to chelate the EDTA, and about 0.1 mg/mL lysozyme to the lysate, followed by incubation at 4 °C for 30 min. Inclusion

bodies were centrifuged at 20,000 $\times g$ for 15 min at 4 °C The supernant was loaded on the column and inclusion bodies were purified according to the manufacturer's protocol of denaturing conditions. Protein concentration, which was purified from inclusion bodies, was measured by Bradford methods and regulated to 1 mg/mL using inclusion body refolding buffer (20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.0) with 6 M urea. Then 5 mM glutathione and 0.5 mM oxidized glutathione were added to the solution and slowly stirred overnight to reduce the protein. Next, 10 mL of the reduced protein solution was dialyzed with 500 mL of inclusion body refolding buffer containing 4, 2, 1 M urea to slowly eliminate urea at 4 °C over the next 36 h; 5 mM DTT and 0.4 M L-arginine were also added at this stage. After removal of urea denaturant, the protein was further dialyzed with inclusion body refolding buffer by step-wise decrease of DTT concentration from 5, 3, to 1 mM to enable reshuffling of disulfide bond and oxidation in the next 48 h. The folded protein was then dialyzed with buffer (20 mM Tris-HCl, 0.1 M NaCl, pH 8.0) for 48 h, and during this process the buffer was replaced six times. Finally, the protein was centrifuged at 24,000 $\times g$ for 30 min at 4 °C to eliminate unfolded or aggregated proteins. Concentration of the refolded protein was determined by the Bradford protein assay method.

3.8. Protein quantity assay

The quantitative protein amount was determined by the Bradford method, which is based on complexation of Coomassie brilliant blue G 250 with protein. The measurement of the absorbance was performed at 595 nm.[26]

3.9. SDS-polyacrylamide gel electrophoresis

In accordance with Laemmli's procedure[47] SDS polyacrylamide gel electrophoresis was used to confirm the purity of the enzyme. The single band obtained was photographed (Figures 3–5).

3.10. Cleavage of fusion protein in vitro using SUMO protease

After purification, 11 kDa SUMO fusion protein was cleaved by the highly specific and active SUMO (ULP-1) protease at the carboxyl terminal, producing a native protein according to the manufacture's protocol.

3.11. Effect of pH on rhPON1 activity

The optimum pH of the enzyme was determined using different buffers with pH ranges between 5.0 and 10.5. Prepared buffers are 50 mM potassium phosphate buffer pH 5.0–8.0, 50 mM Tris-HCl buffer pH 7.5–9.0, and 50 mM glycine–NaOH pH 9.0–10.5 to determine the optimum pH of rhPON1. The enzyme activity was assayed for each pH range.

3.12. Effect of ionic strength on rhPON1 activity

Glycine–NaOH buffer at pH 10.0 with different ionic strengths (10, 25, 50, 100, 200, 400, 600, 800, and 1000 mM) was used to determine its effect on enzyme activity.

3.13. Effect of temperature on rhPON1 activity

The optimum temperature was determined for the enzyme assay selecting the temperatures 0.5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 °C. The reactions were performed in 100 mM glycine–NaOH at pH 10.0.

3.14. Determination of kinetic parameters of rhPON1

The kinetics of rhPON1 was characterized in terms of Michaelis–Menten kinetic parameters (K_m and V_{max}) using the Lineweaver–Burk double reciprocal plot.[48] The rhPON1 activities were determined using a range of paraoxon concentrations varying from 1.1 to 6.66 mM obtained by preliminary tests in glycine–NaOH (pH 10.0).

Acknowledgments

The authors are thankful to Dr Harun Budak and Dr Deniz Ekinci for their helpful suggestions during the preparation of the manuscript.

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