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Expression, purification, and characterization of recombinant human paraoxonase 1 (rhPON1) in *Pichia pastoris*

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Abstract: The main purpose of the present study was to perform the expression in *Pichia pastoris* X-33 of the human paraoxonase 1 (hPON1) enzyme, which is a mammalian serum protein. Extracellular hPON1 enzyme was expressed with the pPICZαA vector using a strong AOX promoter, and enzyme secretion in the fermentation medium was achieved by means of *Saccharomyces cerevisiae* alpha factor signal sequence. The recombinant cells were grown in a shaking flask containing production medium. SDS-PAGE and Western blot analysis illustrated that the molecular mass of extracellular hPON1 enzyme produced by the recombinant *P. pastoris* strain was 59.1 kDa. Biochemical characterization of the enzyme was carried out after purification with a Probond affinity column. The purified paraoxonase 1 activity was determined as 18.9 U/mL; however, enzyme activity reached 31.27 U/mL at the end of the characterization studies. According to the results, K_M and V_{max} values were 0.025 mM and 28.4 U/mL, respectively, in 100 mM glycine-NaOH buffer (pH 10) containing 2 mM Ca^{2+} at 15 °C. This is the first report on the expression and production of hPON1 in *P. pastoris*.

Key words: *Pichia pastoris* X-33, hPON1, recombinant protein, purification, characterization

1. Introduction

Paraoxonase 1 (PON1), whose activity is related to the toxicology of organophosphorus compounds and cardiovascular health, is a mammalian serum protein (Shih et al., 1998; Lusis, 2000; Draganov and La Du, 2004). The human paraoxonase (PON) gene family is composed of *PON1*, *PON2*, and *PON3* members lying together on chromosome 7. By far, PON1 is the most studied family member (Draganov and La Du, 2004). Human serum paraoxonase 1 (hPON1) is a Ca^{2+}-dependent glycoprotein with a molecular mass of 43–45 kDa consisting of 354 amino acids and tightly associated with high-density lipoprotein (HDL) (Sorenson et al., 1999; Lu et al., 2006). This glycosylated enzyme has three Cys residues, which contain two that form a disulfide bond (Harel et al., 2004). The enzyme, synthesized principally in the liver, is named after paraoxon (an insecticide) and is one of its most studied substrates. This enzyme can hydrolyze the active metabolites of nerve agents such as sarin, soman, and several organophosphorus insecticides such as chlorpyrifos oxon and diazoxon (Costa and Furlong, 2002; Costa et al., 2003).

Bacteria and yeasts are used largely for producing recombinant proteins. In spite of expressing recombinant proteins, using *E. coli* is both inexpensive and effective (Jana and Deb, 2005; Francis and Page, 2010), as the capability of *E. coli* is inadequate for many recombinant proteins to be folded of protein and formed of disulfide bonds (Brondyk, 2009). Particularly, the expression of cysteine-rich proteins with their native conformations are difficult in bacteria (Nilsson et al., 1991; Ostermeier et al., 1996). This difficulty can be avoided by using the *Pichia pastoris* expression system. *P. pastoris*, known as a methylotrophic yeast, has become one of the most convenient yeast systems for producing recombinant proteins for industrial and research purposes (Macauley-Patrick et al., 2005). Due to being easily manipulated on a genetic level, it is possible to add posttranslational modifications, as well as use with many eukaryotic organisms and a number of strong promoters to perform heterologous expression. Thus, many scientists choose to use this exclusive yeast expression system for producing heterologous proteins (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005). Recombinant proteins can also be secreted into culture media by the *P. pastoris* secretory pathway. Consequently, the effectiveness of disulfide bond formation and protein folding can be increased and the procedure of the purification can be

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simplified by this expression system (Almeida et al., 2001; Mengwasser et al., 2011). To date, some investigators have used *E. coli* expression systems for recombinant hPON1 production (Stevens et al., 2008; Suzuki et al., 2010). However, to our best knowledge, there are no studies on the expression and production of hPON1 in *P. pastoris*. This is the first report on the production of hPON1 in a *P. pastoris* expression system and we understand that it is a definitive system for the expression of applicable hPON1 with the extracellular production in its native form.

2. Materials and methods

2.1. Strains, plasmids, and reagents

The yeast shuttle expression vector pPICZαA, *E. coli* strain One Shot TOP10, *P. pastoris* strain X-33, adult human liver cDNA, Zeocin, High Pure Plasmid Isolation Kit, Anti-His (C-term)-HRP antibody, and Probond Nickel Chelating Resin were purchased from Invitrogen (USA). Restriction enzymes, T4 DNA ligase, and DNA and protein ladder were obtained from Fermentas (USA). PCR primers were synthesized by Metabion Company (Germany) and the GenElute Gel Extraction Kit was purchased from Sigma (USA).

2.2. Construction of the recombinant vector

The DNA coding sequence of hPON1 from human liver cDNA (GenBank accession number NM_000446.5) was amplified. Forward and reverse primers used in amplifying the target gene by PCR were GAGAATTCATGGCGAAGCTGA (F) and GCGTCTAGAGAGCTCACAGTAAAGAG (R), respectively. The PCR products containing restriction sites of *Eco*RI and *Xba*I were digested with these enzymes and inserted into pPICZαA containing α-factor signal sequence and AOX1 promoter. The resulting construct also contained a c-Myc tag and a 6xHis-tag at the C-terminus.

PCR conditions were performed with an initial denaturation at 94 °C for 4 min and 35 cycles of amplification including denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, and extension at 72 °C for 1.5 min. The other extension was then performed at 72 °C for 5 min. The resulting PCR product purified from the gel was digested with *Eco*RI and *Xba*I and ligated into the *Eco*RI-*Xba*I digested and gel-purified vector pPICZαA to obtain a recombinant plasmid. Competent *E. coli* One Shot TOP10 cells combined with this plasmid were grown in Law salt LB agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at a pH of 7.5, as recommended. Optionally, 25–50 μg/mL of Zeocin was added. Colony PCR was performed using gene primers to confirm the positive clones and restriction enzyme digestion was performed to affirm whether the gene insertion was correct. DNA sequencing was performed by İontek Company (Turkey) after isolating recombinant plasmid from the positive transformant. The constructed expression vector was named as pPICZαA-hPON1.

2.3. *P. pastoris* transformation and selection of transformants

Competent *Pichia pastoris* X-33 cells were prepared by using LiCl as defined in the Easy Select *Pichia* Expression System manual from Invitrogen. The pPICZαA-hPON1 was linearized using restriction endonuclease *Pme*I and transformed to the competent cells. Transformants were selected on YPD plates (2% dextrose, 2% peptone, 1% yeast extract, and 2% agar) containing 400 μg/mL Zeocin after incubating 50 μL of competent cells for 1 h and 4 h at 30 °C. The positive yeast clones were confirmed by colony PCR with forward and reverse primers of the insert gene. PCR was performed with an initial denaturation at 94 °C for 4 min and 35 cycles of amplification including denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, and extension at 72 °C for 1.5 min. One negative control transformant and two positive transformants were selected for the expression of recombinant human PON1 (rhPON1). The selected transformants were precultured with 50 mL of BMGY medium (1% (v/v) glycerol, 2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6, 1.34% (w/v) yeast nitrogen base with ammonium sulfate and without amino acids and 4×10^{-5}% (w/v) biotin) in a 500-mL baffled flask at 30 °C for 18 h on a 225-rpm shaker. Harvested cells from culture media were resuspended to an OD_{600} of 1.0 in BMMY medium (0.5% (v/v) methanol, 2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6, 1.34% (w/v) yeast nitrogen base with ammonium sulfate and without amino acids and 4×10^{-5}% (w/v) biotin) for induction of expression. Methanol adjusted to 0.5% of the final concentration was added to culture media every 24 h for induction maintenance. The culture was centrifuged at 3000 rpm and 4 °C for 15 min after 48 and 72 h. The obtained supernatants were used for SDS-PAGE analysis and enzyme activity assay. For selection of the best enzyme producer clone, enzyme activities and SDS-PAGE results of clones were analyzed and compared with each other.

2.4. Production of recombinant enzyme

The selected best clone was inoculated on BMGY medium for precultivation. After 18 h of incubation, the culture was centrifuged and the obtained cells were transferred to 50 mL of BMMY medium. The cells in BMMY medium were incubated by methanol induction at 30 °C at 24-h intervals for 120 h on a 280-rpm shaker. The culture supernatants were used for SDS-PAGE analysis and enzyme activity assay, and concentrated supernatants using the TCA protein precipitation method (Bensadoun and Weinstein, 1976) were also analyzed by western blot using an anti-His (C-term)-HRP antibody. Casamino acid (1%) and sorbitol (3%) were added to the medium to increase production of rhPON1 by determining optimum incubation time.

2.5. Enzyme activity assay

Recombinant paraoxonase activity was detected at 25 °C using paraoxon (diethyl p-nitrophenyl phosphate) (2 mM) as a substrate in 50 mM glycine-NaOH (pH 10.5) containing 1 mM $CaCl_2$. The enzyme assay was performed according to estimation of p-nitrophenol at 412 nm. Calculation of the enzyme activity was done with the molar extinction coefficient of p-nitrophenol (ε = 18,290 M^{-1} cm^{-1} at pH 10.5) (Renault et al., 2006). The enzyme amount that hydrolyses 1 µmol of the substrate at 25 °C was described as one enzyme unit. A spectrophotometer (Beckman Coulter, USA) was used to perform the assays.

2.6. Purification and quantification of rhPON1 protein

Protein purification was carried out for the analysis of the biological activity of rhPON1. The highest enzyme producer colony was grown in 50 mL of BMMY medium to induce expression. The supernatant of the culture was collected after 96 h of induction and protein was concentrated to 10-fold with an Amicon filter. The filtrate was bound to the Probond affinity column, which was equalized with native binding buffer (0.5 M NaCl and 50 mM NaH_2PO_4, pH 8). After elimination of the proteins without His tag using wash buffer (0.5 M NaCl, 20 mM imidazole, and 50 mM NaH_2PO_4, pH 8), the recombinant protein elution from the nickel-chelating resin was performed using elution buffer (0.5 M NaCl, 250 mM imidazole, and 50 mM NaH_2PO_4, pH 8). Dialysis of the elution was performed twice for protein desalting using sodium phosphate (pH 8). The obtained sample was analyzed by SDS-PAGE and the Bradford method was used for determination of total protein concentration using bovine serum albumin as the standard (Bradford, 1976). All samples were analyzed in triplicate.

2.7. Purified rhPON1 characterization

The eluted fractions of rhPON1 protein were collected and the obtained products were analyzed by SDS-PAGE. The effect of pH on enzyme activity was determined at 25 °C in 50 mM potassium phosphate buffer for pH values of 5.5–8.0, Tris-HCl buffer for pH values of 7.5–9.0, and glycine-NaOH buffer for pH values of 9.0–10.5. For the determination of optimum buffer concentration on enzyme activity, different concentrations of glycine-NaOH buffer (20–250 mM) were studied and the effect of temperature (ranging from 4 to 90 °C) on enzyme activity was determined at optimum pH and optimum glycine-NaOH buffer concentration. Finally, enzyme activity assay was performed using at least five different paraoxon concentrations in optimum conditions for the determination of K_M and V_{max} values.

3. Results

3.1. Construction and transformation of pPICZαA-hPON1

The *hPON1* gene was amplified (S1) and integrated into the vector (Figure 1). Colony PCR, DNA sequencing, and restriction digestion of the pPICZαA-hPON1 proved that the *hPON1* gene was inserted in the correct position on pPICZαA (S2, S3, and S4). *Pme*I-linearized pPICZαA-hPON1 was transformed into *P. pastoris* X-33 strains and the cells were spread on YPD plates to screen transformants. After the incubation, colony PCR was done with selected colonies (S5).

3.2. Selection of high-expression yeast clone

The results of enzyme activity assay obtained from two correct yeast transformants by using culture supernatants showed that the maximum activity obtained by colony 6 was 10.12 U/mL, whereas less activity (7.23 U/mL) was observed in the supernatant of colony 3 at 72 h. This result was consistent with the SDS-PAGE analysis, suggesting that there was no band related to the control, and no almost visible amount of hPON1 was seen in the cultivation medium of colony 3 (Figure 2a). On the other hand, 9.8 g/L and 9.0 g/L cell dry weights were obtained by colony 3 and colony 6 at 72 h, respectively. Colony 6 was selected for recombinant enzyme production in the following experiments.

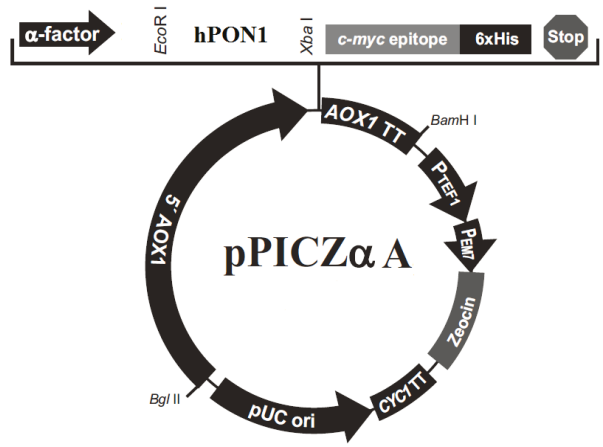

Figure 1. Construction of recombinant plasmid pPICZαA-hPON1. The insert gene (*hPON1*) was cloned into the *Eco*RI and *Xba*I restriction enzyme sites of the pPICZαA vector. AOX1, alcohol oxidase 1 promoter; AOX1 TT, transcriptional termination region from *AOX1* gene; PTEF1, transcription elongation factor 1 gene promoter from *Saccharomyces cerevisiae*; PEM7, synthetic prokaryotic promoter; Zeocin, resistance gene; CYC1 TT, transcription termination region from *Saccharomyces cerevisiae CYC1* gene; pUC ori, pUC origin of replication.

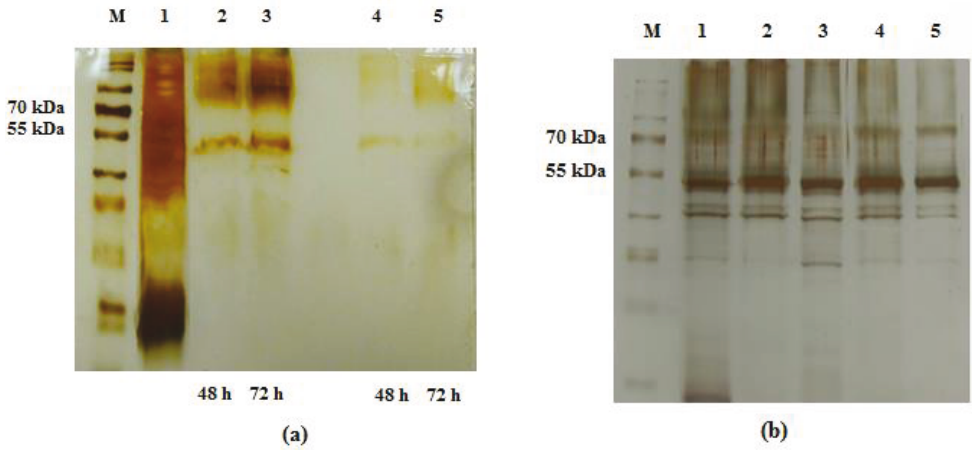

Figure 2. a) SDS-PAGE analysis for selecting the best producer recombinant yeast colony. M: Marker; 1: control (*P. pastoris* containing empty vector); 2, 3: colony 6; 4, 5: colony 3. **b)** SDS-PAGE analysis of recombinant protein in culture media. M: Marker, 1: 24 h, 2: 48 h, 3: 72 h, 4: 96 h, 5: 120 h.

3.3. Production, purification, and quantification of recombinant enzyme

The selected colony (colony 6) was grown in BMMY medium containing 1% casamino acid and 3% sorbitol and induced by 0.5% methanol at 24-h intervals for 120 h. According to the SDS-PAGE analyses of culture supernatants (Figure 2b) as well as purified enzyme (Figure 3a), the induced protein had a molecular mass of 59.1 kDa. In the case of the SDS-PAGE analyses, western blot analysis could also show that the molecular mass of the produced protein was 59.1 kDa (Figure 3b). As for enzyme activity analyses, it was determined that although the highest biomass amount (24.8 g/L) was obtained after 120 h, the enzyme activity reached its highest value (16.49 U/mL) after 96 h (Figure 4a). The purified protein was 10.1 mg/mL and enzyme activity was 18.9 U/mL. Thus, the specific enzyme activity was calculated as 1.87 EU/mg.

3.4. Enzyme characterization of purified rhPON1

For determining the properties of purified rhPON1, paraoxon was used as a substrate and the reactions were performed as described above at 25 °C. The maximum activity of rhPON1 was at pH 10.0 with glycine-NaOH buffer (21.5 U/mL). On the other hand, activities were observed in Tris-HCl buffer at pH 8.5 and 9.0, while no activity was determined in potassium phosphate buffer at any of the pH ranges tested (Figure 4b). The optimum buffer concentration for enzyme activity was determined as 100 mM (30.29 U/mL) (Figure 4c) when the different concentrations of glycine-NaOH buffer from 20 to 250 mM were studied. The optimum temperature for the enzyme was determined as 15 °C (31.27 U/mL) (Figure 4d). Finally, K_M and V_{max} values were determined to be 0.025 mM and 28.4 U/mL under the optimum conditions, respectively.

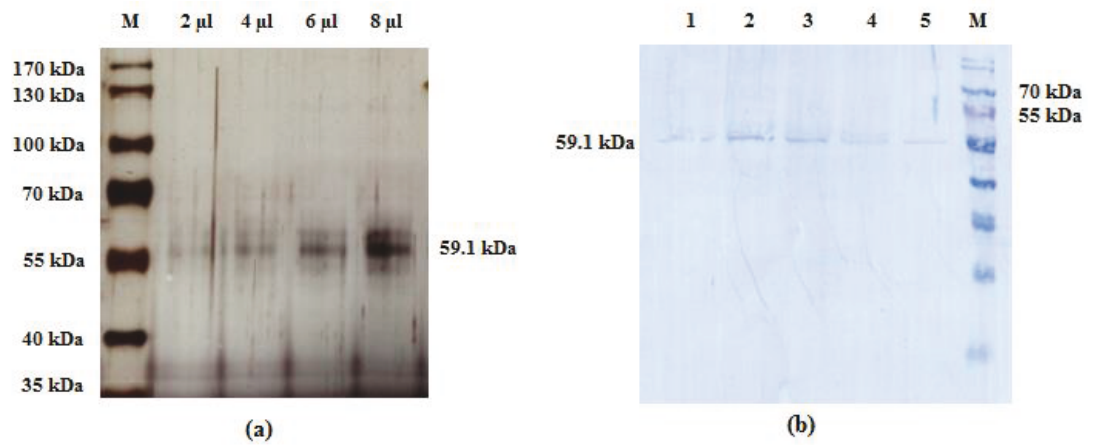

Figure 3. a) SDS-PAGE analysis of purified recombinant protein (silver staining). **b)** Western blot analysis of recombinant protein from culture media. M: Marker, 1: 120 h, 2: 96 h, 3: 72 h, 4: 48 h, 5: 24 h.

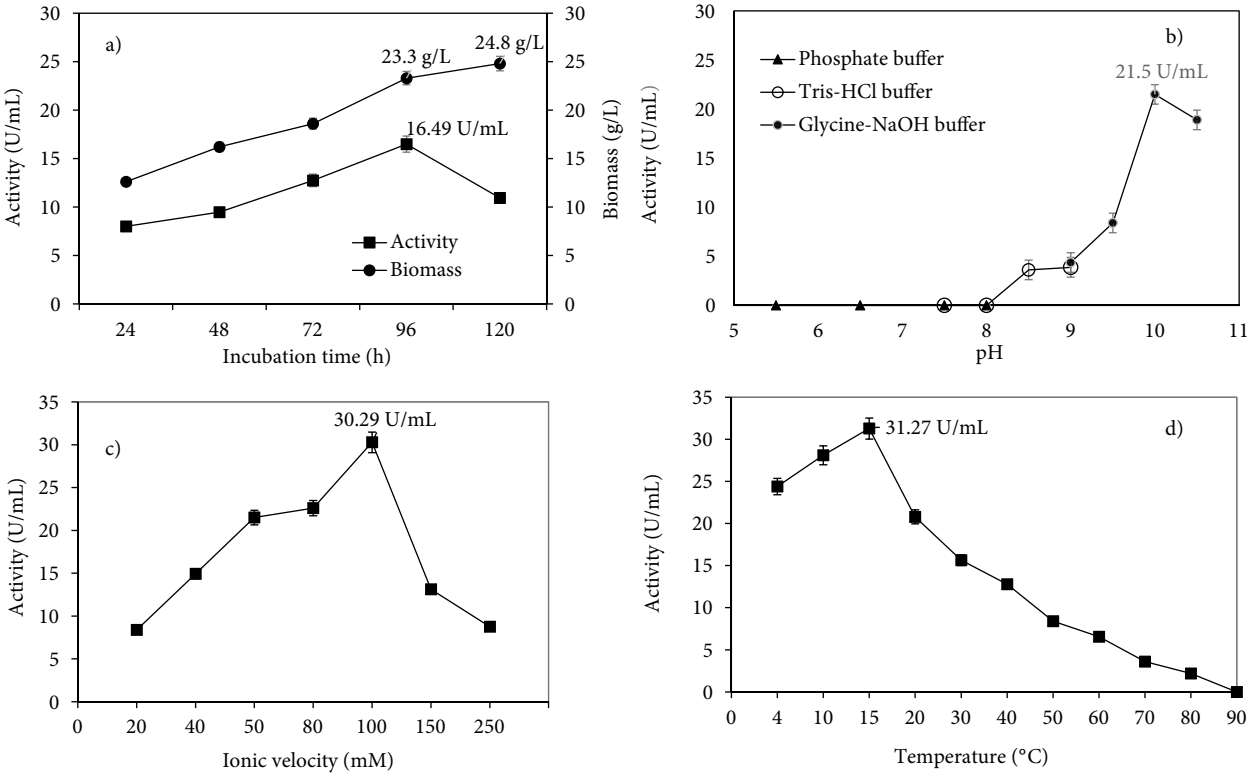

Figure 4. Activity assays of recombinant hPON1. **a)** Effect of incubation time on activity and biomass. **b)** Effect of pH on activity of rhPON1. **c)** Effect of ionic velocity on activity of rhPON1. **d)** Effect of temperature on activity of rhPON1.

4. Discussion

Recombinant proteins obtained for in vitro and in vivo activity studies need to correct disulfide bond formation. Although in bacteria, the expression of proteins containing disulfide bond generally collapses, the *P. pastoris* yeast system can effectively express functional proteins containing a wide range of disulfide bonds by means of its secretory pathway (Almeida et al., 2001; Li et al., 2009). As endogenous proteins belonging to *P. pastoris* are secreted at low levels, the expressed recombinant proteins can be easily obtained from the culture medium (Gurkan and Ellar, 2005). Similarly, Li et al. (2012) used *E. coli* for producing recombinant fish IGF-1 and reported that the protein mainly expressed within the cytoplasm needed refolding and additional cell disruption steps to produce a biologically active peptide. However, the protein was successfully secreted and purified from the medium when they studied with same protein in *P. pastoris*.

In this study, the expression vector containing the hPON1 sequence was constructed and combined with the *P. pastoris* X-33 genome. The 6xHis-tagged rhPON1 protein expressed by the methanol-inducing AOX1 promoter was secreted into the culture medium by means of the α-factor signal peptide. The recombinant strain could effectively secrete the hPON1 for 96 h with 0.5% methanol induction and the protein was indicated by SDS-PAGE and western blot. The rhPON1 activity analysis was detected as quantitative. The secreted rhPON1 protein was purified through a Probond affinity column and some biochemical characteristics of the purified recombinant enzyme were also examined.

As seen from Figure 2a, there was no band in the negative control as well as in colony 3, which could not produce a visible amount of hPON1. One possible reason for this result might be the hydrolysis of hPON1 by various yeast proteases synthesized in the cell. Therefore, we can conclude that high amounts of proteases might have caused rapid degradation of hPON1 in colony 3 because of high cell concentration (9.8 g/L cell dry weight). Moreover, the extent of induction time influences proteolysis; increasing the residence time for the hydrolysis of hPON1 enzyme is known to degrade the enzyme (Daly and Hearn, 2005). Therefore, colony 6 was selected for recombinant enzyme production. Casamino acid (1%) and sorbitol (3%) were added to the media for protease inhibition and increasing of the cell growth, respectively. According to western blot analysis (Figure 3b), protein bands that were specific to anti-6xHis-tag antibodies were observed in every well. Western blot assay showing 6xHis-fused hPON1 was successfully performed and it confirmed that the protein secreted into the culture medium by *P. pastoris* was rhPON1. On the other hand, protease inhibition was

Table. Summary of different kinetic results for paraoxon hydrolysis of hPON1 from different sources.

Source	Buffer	Temperature (°C)	K_M (mM)	Reference
Serum	50 mM Tris-HCl, pH 7.45	25	0.5	Gan et al. (1991)
Serum	100 mM Tris-HCl, pH 8	25	1.38	Li et al. (2000)
Serum	100 mM Tris-HCl, pH 8.5	37	0.81	Rodrigo et al. (2001)
Recombinant human PON1 in *E. coli*-G2E6	50 mM Tris-HCl, pH 8	Not reported	0.8	Khersonsky and Tawfik (2005)
Recombinant human PON1Q from silkworm	50 mM Tris-HCl, pH 8	37	0.6	Lu et al. (2006)
Recombinant human PON1 in *P. pastoris*	100 mM glycine-NaOH, pH 10	15	0.025	This study

observed with increased enzyme activity every 24 h, up to hour 96. However, as seen from Figure 4a, decreased enzyme activity after 96 h of incubation may be attributed to excessive protease secretion caused by increased biomass. As mentioned above, the probable reason for the low activity value of hPON1 might be the existence of proteases in the culture medium.

It is well known that the hPON1 sequence has four potential N-glycosylation sites. Two of them (Asn 227 and Asn 270) are in the center of a beta-pleated sheet, while the others (Asn 253 and Asn 324) are in the zone of the interior (Draganov and La Du, 2004). The present results of SDS-PAGE and western blot suggested that the molecular mass of the rhPON1 was slightly larger than the molecular mass of hPON1, which is 43–45 kDa glycoprotein. Similarly, Li et al. (2011) reported that human interleukin-3 from *P. pastoris* showed molecular mass of 19–22 kDa in comparison with the calculated molecular mass (15–17 kDa) and explained this result as different degrees of N-linked glycosylation. Based on these results, we thought that this enhancement was derived from the glycosylation of the target protein, which is an extensive posttranslational modification in *P. pastoris*.

After determination of activity and molecular mass of the recombinant enzyme, enzyme purification was performed and the eluted enzyme was used for characterization studies. According to the results, K_M value was determined as 0.025 mM in 100 mM glycine-NaOH buffer (pH 10) containing 2 mM Ca^{2+} at 15 °C. In the literature, different K_M values were reported for paraoxon substrate, as shown in the Table. These differences in K_M (affinity) values can derive from purity grade of enzyme extract and substrate, buffer pH, environment temperature, and substrate concentration.

The results of the present study show that *P. pastoris* is a suitable system for the effective expression of hPON1. The purified paraoxonase activity was 18.9 U/mL and the activity could reach 31.27 U/mL by means of the characterization studies. In conclusion, production of hPON1 at an industrial scale may be improved by using cell immobilization technology and performing optimization of the culture conditions. Besides, the purified enzyme may be tested in cell culture in order to elucidate its antioxidant potential and used for another functional and structural study as well as possible clinical therapy. However, further studies are required to prove these assumptions.

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