

## Purification of Human Serum Paraoxonase-1

**R. Redya Naik\* and T. Sankarshana**

Department of Pharmacy, University College of Technology,  
Osmania University, Hyderabad, India.

Email address: [naikpharmacy@gmail.com](mailto:naikpharmacy@gmail.com)

\*Corresponding author: Email address: [naikpharmacy@gmail.com](mailto:naikpharmacy@gmail.com)

Tel No. +91-9676169260

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### ABSTRACT

Membrane proteins play key roles in fundamental biological processes, such as transport of molecules, signalling, energy utilization, and maintenance of cell and tissue structures. Serum paraoxonase (PON1, EC 3.1.8.1.) is a protein of 354 amino acids with a molecular mass of 43 kDa is a high density lipid (HDL) - associated, calcium-dependent enzyme whose 3D structure, active site residues and physiological substrates are not known. It is highly conserved in mammals but is absent in fish, birds, and invertebrates, such as arthropods. Purification of PON1 has been challenging for a long time. Here, we report a novel purification technique for this enzyme, which allowed us to obtain human serum paraoxonase 1 (hPON1) using straightforward chromatographic methods. The PON1-specific inhibitor 2-hydroxyquinoline almost completely inhibited paraoxonase and lactonase activities, while only moderately inhibiting arylesterase activity. By this method the purity of the isolated PON -1 was increased many folds. Later the purified sample was assessed for its paraoxonase, lactonase and arylesterase activities.

**Keywords:** Paraoxonase; purification; human serum

### INTRODUCTION

The paraoxonase (PON) gene family contains three different members (PON1, PON2 and PON3), and exhibits antioxidative properties principally in the blood circulation. Recent interests have been directed towards a better comprehension of the functions of PON2 and PON3, but PON1 remains by far the most studied of the three enzymes [1]. Beginning in 1946, the HDL-associated human serum enzyme paraoxonase-1 (PON1) was initially discovered by Mazur et al., [2] to have the ability to hydrolyze organophosphorus compounds (OPs). Aldridge identified the enzyme as PON1 and

demonstrated its general characteristics, which included proposing that its A-esterase activity should be separated from B-esterases [3]. Mammalian PONs (PON1, PON2, and PON3) are a unique family of calcium-dependent esterases/lactonases. PON1 is a calcium-dependent glycoprotein with 354 amino acid residues, and has a molecular mass of approximately 45 kDa, depending on the degree of glycosylation. It is synthesized in the liver and bound to HDL in the bloodstream [4]. Many of the antiatherogenic properties of HDL are attributed to PON1. This enzyme reduces

macrophages' cellular oxidative stress, decreases cholesterol-biosynthesis rate, and stimulates HDL-mediated macrophage cholesterol efflux, thus protecting against foam-cell formation and atherogenesis [5, 6]. PON1 was also observed to hydrolyse homocysteine thiolactone [7], which could pose potential harm by homocysteinylation of some proteins [8]. Recent clinical investigations indicate that PON1 activity was lower in subjects with coronary heart diseases than control subjects [9–11]. Additionally, there are polymorphisms that affect the PON1 level in blood, which may alter the propensity to develop coronary vascular disease [12]. PON1's only free sulfhydryl group is present at Cys284 and is associated with its activity [13], even though crystallization of the enzyme has revealed that Cys284 is not part of its active site; rather, it is part of a highly conserved PON groove [14]. Based on the substrate specificity found in various experiments, it was proposed that PON1 could primarily be a lactonase enzyme and specifically play a role in anti-inflammatory and antioxidative response, since there were many oxidized metabolites of polyunsaturated fatty acids that are structurally similar to lactones [15].

PON1 has been purified from human serum and has also been synthesised using recombinant technology. These two types of PONs have been able to show the activity in terms of aryl esterase, phosphotriesterase and lactonase activities. Our efforts are to purify the enzyme from human serum using chromatographic procedures and check for the three activities of the enzyme after purification.

## MATERIALS AND METHODS

Acrylamide, Calcium chloride dehydrated, Cholesterol, EDTA, Ethyl acetate, Glycerol,  $\gamma$ -nonanoic lactone, Human Plasma, M-cresol purple, Molecular weight marker Sigma marker TM (Wide range), Paraoxon-ethyl pestanal, Phenyl acetate, Protease Inhibitor cocktail, Tergitol NP-10 Non ionic, Trizma hydrochloride reagent grade, Sodium deoxycholate, Sodium

dodecyl sulfate (SDS) all the chemicals used were purchased from Sigma Aldrich and were of analytical grade. Instruments used were pseudo affinity chromatography (Cibacron blue column) and ion exchange chromatography (DEAE Sepharose column) Fast flow (Sigma Aldrich).

### Blood samples collection

O<sup>+</sup> human blood (250 ml) was taken from local blood bank (Usha blood bank, Osmania University) and added to EDTA containing tubes and to tubes without anticoagulant, in order to obtain plasma, buffy-coat and serum, and processed within 2 h of collection. Centrifuged at 10000 g, for 15 min for separate plasma, the frozen human plasma (100 ml) was taken and allowed to thaw. The plasma was allowed to clot by adding stock CaCl<sub>2</sub> (final concentration of 10 mM). The fibrin clot was then removed by centrifugation at 15000g; 20 min at 4 °C and the clear serum was collected and used as a source of human PON1.

## PURIFICATION METHOD

### Purification of PON1

PON1 was purified from the human plasma by following the procedure of Gan *et al* with little modifications [16]. PON1 was purified from human plasma by (pseudo) affinity chromatography using Cibacron Blue 3GA (Sigma Aldrich). The column was equilibrated in buffer A (50 mM Tris/HCl, pH 8.0, 1.0 mM CaCl<sub>2</sub>, 5  $\mu$ M EDTA) and 3 M NaCl. The serum was loaded for 4 hrs and recirculated two times for the maximum binding of protein. The column was washed with buffer A with 3 M NaCl, which mostly reduces or prevents albumin and other soluble serum proteins from binding to the matrix. Then column was washed with buffer A without NaCl to reduce the ionic strength. The bound protein was finally eluted with buffer A containing 0.1% deoxycholate. During elution 1 ml fractions were collected and assayed for optical density. Eluted fractions with highest optical density were pooled for the next chromatographic procedure.

The pooled sample was diluted with equal volume of buffer containing 0.2% tergitol, 40% glycerol, 25 mM Tris/HCl, pH 8.0, 1 mM CaCl<sub>2</sub> and 5 μM EDTA. This sample mixture was loaded on to DEAE-Sepharose (Sigma Aldrich) according to a slight modification of the published procedures [16]. Ion exchange column was equilibrated with buffer containing 25 mM Tris/HCl, pH 8.0, 0.1% Tergitol, 20% glycerol, 1mM CaCl<sub>2</sub> and 5μM EDTA. The column was washed with same buffer and eluted with buffer containing 0.35 M NaCl. During elution 1 ml fractions were collected and assayed for aryl esterase activity. The fractions with highest aryl esterase activity were pooled, concentrated and subjected to SDS PAGE analysis to check the purity of the preparation. The identification of PON1 from human plasma has been reported previously [16]. The purified enzyme is homogeneous, with a molecular mass of ≈45 kDa in SDS/PAGE analysis.

Protein concentration of samples was determined by Bradford reagent using bovine serum albumin as standard. SDS-PAGE (12.0 %) was done using standard procedure [16]. After electrophoresis the bands were detected with Coomassie brilliant blue staining.

### Activity measurements

Paraoxonase activity (phosphotriesterase activity):

Paraoxonase activity was determined by measuring the change of absorbance at 405 nm in 50 mM Tris buffer, pH 8.0, containing 1 mM CaCl<sub>2</sub> and 1 mM paraoxone after adding enzyme solution [17].

Phenyl acetate activity (arylesterase activity)

Arylesterase activity of PON1 was measured by adding enzyme solution to 0.5 ml of 50 mM Tris buffer, pH 8.0, containing 1 mM CaCl<sub>2</sub> and 1 mM phenyl acetate, and the rate of generation of phenol was monitored at 270 nm [17].

Lactonase activity:

Lactonase activity was determines by a pH - indicator colorimetric assay using γ-nonanoic lactone as a substrate [17]. Proton released from carboxylic acid formation was followed using pH indicator m-cresol purple. Briefly, the

reaction was initiated by adding substrate solution to the reaction mixture (bicine buffer pH 8) containing 0.1 mM cresol purple and PON1 and the decrease in absorbance at 577 nm was monitored as a function of time.

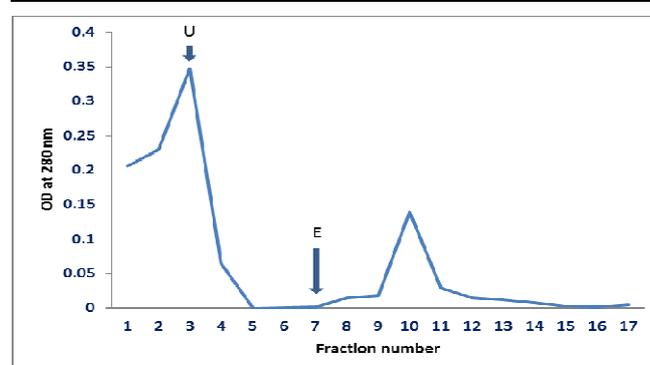
## RESULTS AND DISCUSSION

Purification of serum PON1 started with the separation of serum from the pooled blood. Pseudo affinity chromatography [Fig. 1] and ion exchange chromatography [Fig. 2] resulted in purification of the enzyme from human serum. First few fractions in the blue sepharose column have been listed in Table 1. These separation techniques were previously reported, however, we have used sodium deoxycholate in the last step for the elution of the enzyme from the ion exchange chromatography column. Finally obtained protein is very pure enzyme which is clear from the SDS-PAGE analysis [Fig. 3]. Purified enzyme showed only one protein band in the analysis which suggests that the enzyme is very pure.

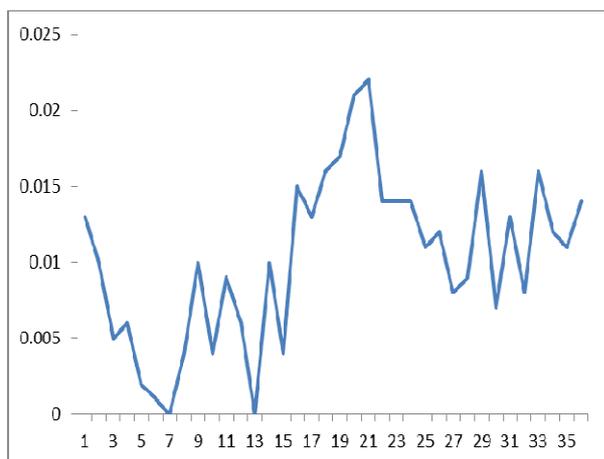
The activities of the enzyme after purification showed that the enzyme retained its activity which suggests that the purification process retained the enzyme activity.

**Table 1.** Binding profile of PON1 from Blue agarose-Cibacron blue column (Pseudo-affinity chromatography).

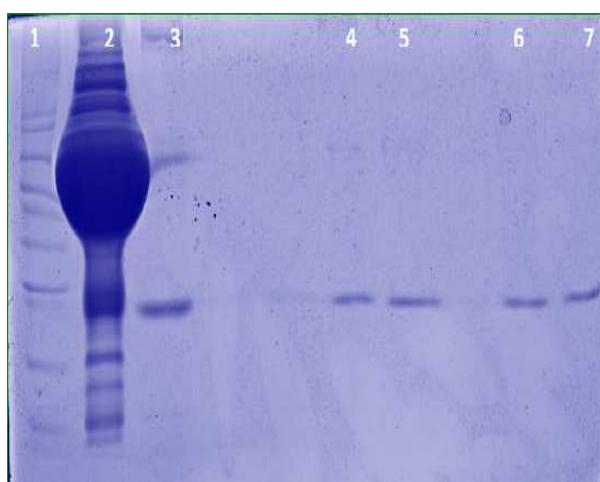
Human plasma treated with CaCl <sub>2</sub>	fraction 1
Unbound I	fraction 2
Unbound II	fraction 3
Washes with 3M NaCl +buffer A	fraction 4
Washes with buffer A	fraction 5 & 6
Elution	fraction 7 onwards



**Fig. 1.** Purification of PON1 from Blue agarose-Cibacron blue column (Pseudo-affinity chromatography). U- Unbound protein, E- elution of bound protein.



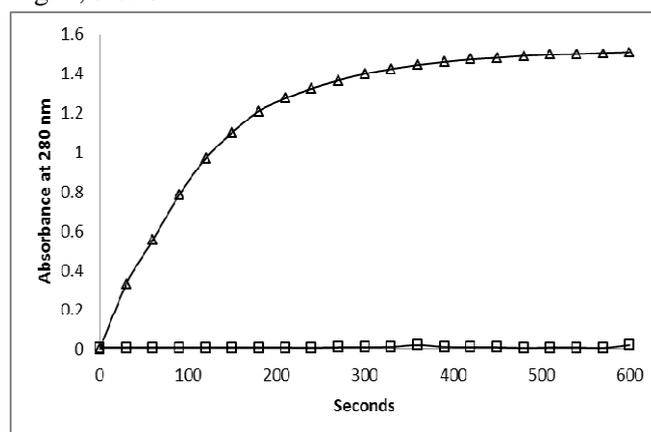
**Fig. 2.** Purification of PON1 from DEAE column.



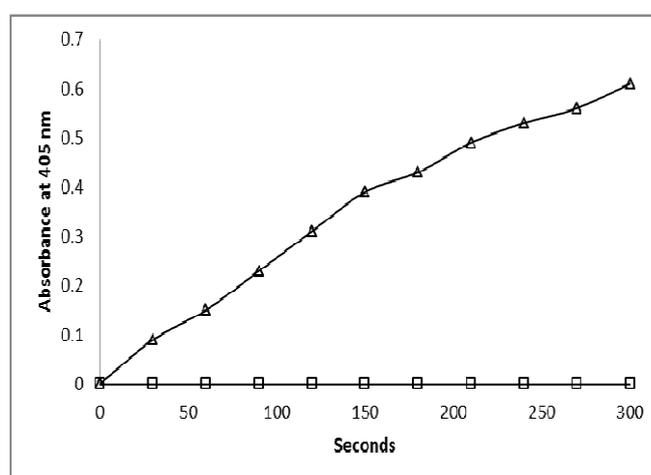
**Fig. 3.** SDS PAGE of purified PON1 showing band at 43 kD. Lane 1- Molecular weight marker; Lane 2 – Human serum; Lane 3 - Cibacron Blue agarose elution fraction; Lane 4,5,6 & 7- DEAE-Sepharose elution fractions. PON1 band is clearly visible at 43 kD in all the lanes.

Since PON1 is known to possess various activities, we checked all the activities (ie. lactonase, arylesterase and phosphotriesterase) of PON1. The lactonase activity of PON1 is responsible for the antioxidant and anti-atherogenic properties of the enzyme, while its phosphotriesterase (paraoxonase) activity imparts an important role to the enzyme in natural defense against (by hydrolyzing and inactivating) various organophosphates (OPs) intoxication, including nerve gases. The enzyme is also able to hydrolyze aromatic esters, such as phenyl acetate (this activity is referred to as arylesterase activity). Different activities of PON1 were measured, as described in materials

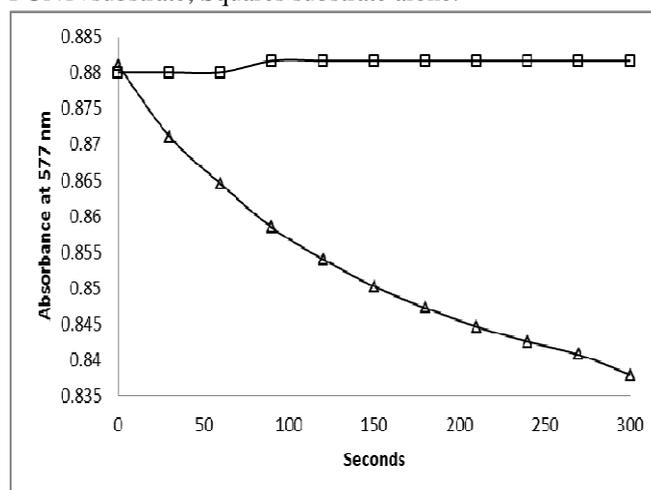
and methods, and the results are presented in Fig. 4, 5 & 6.



**Fig. 4** Phenyl acetate hydrolyzing (arylesterase) activity of purified PON1. Triangles PON1+substrate; Squares substrate alone.



**Fig. 5** Paraoxonase hydrolyzing (phosphotriesterase) activity of purified PON1. Triangles PON1+substrate; Squares substrate alone.



**Fig. 6.** Lactonase activity of PON1. Hydrolysis of nonalactones was assayed in 2.5mM Bicine buffer pH 8, 0.2 M NaCl, and 1 mM CaCl<sub>2</sub> in the presence of 0.1mM of cresol purple as indicator. Proton

release by the carboxylic acid product was detected at 577 nm. PON1 enzyme triangles + Substrate alone square.

## CONCLUSION

Present research work demonstrates a simple procedure for the purification of PON1 from human serum. The purified PON1 showed a single band in the SDS-PAGE indicating the separation of this enzyme from all other proteins in the serum. The procedure has little effect on the activity of the enzyme PON1 as it showed all the three tested activities of the enzyme.

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## REFERENCES

1. Louis-Philippe Précourta, et al., (2011) The three-gene paraoxonase family: Physiologic roles, actions and regulation *Atherosclerosis* 214, 20–36.
2. Mazur A. (1946) An enzyme in animal tissue capable of hydrolyzing the phosphorus–fluorine bond of alkyl fluorophosphates. *J Biol Chem*; 164:271–89.
3. Aldridge WN. (1953) Serum esterases I. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate and a method for their determination. *Biochem J*; 53:110–7.
4. Lenz, DE, et al., (2010) “Human paraoxonase I: A potential bioscavenger of organophosphorous nerve agents.” *The Paraoxonases: Their Role in Disease Development* (February 19): 1–20.
5. M. Rosenblat, et al., (2010) “Pomegranate juice (PJ) consumption antioxidative properties on mouse macrophages, but not PJ beneficial effects on macrophage cholesterol and triglyceride metabolism, are mediated via PJ induced stimulation of macrophage PON2,” *Atherosclerosis*, vol. 212, no. 1, pp. 86–92.
6. M. Aviram and M. Rosenblat, (2004) “Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development,” *Free Radical Biology and Medicine*, vol. 37, no. 9, pp. 1304–1316.
7. Jakubowski, H. (2000) Calcium-dependent human serum homocysteine thiolactone hydrolase: a protective mechanism against protein N-homocysteinylation. *J. Biol. Chem.* 275, 3957–3962.
8. Ferguson, E., et al., (1998) Generation and initial characterization of a novel polyclonal antibody directed against homocysteine thiolactone modified low density lipoprotein. *J. Lipid Res.* 39, 925–933.
9. Ruiz, J., et al., (1995) Gln-Arg192 polymorphism of paraoxonase and coronary heart disease in type 2 diabetes. *Lancet* 30, 869–872.
10. Roberts, D. H., et al., (2001) Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? *Arterioscler. Thromb. Vasc. Biol.* 21, 1451–1457.
11. Mackness, B., et al., (2002) The paraoxonase gene family and coronary heart disease. *Curr. Opin. Lipidol.* 13, 357–362.
12. Furlong, C. E., et al., (2002) Pharmacogenomic considerations of the paraoxonase polymorphisms. *Pharmacogenomics* 3, 341–348.
13. Aviram, M.; et al., (2000) Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* 101: 2510–2517.
14. Harel, M. et al., (2004). S. Structure and evolution of the serum paraoxonase family of detoxifying and antiatherosclerotic enzymes. *Nat. Struct. Mol. Biol.* 11:412–419.

15. Draganov DI, et al., (2005) Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res*; 46:1239–47.
16. Gan KN, et al., (1990). Purification of human serum paraoxonase/arylesterase. *Drug Met and Disposition*; 19:100-106.
17. Khersonsky O, Tawfik DS. (2005) Structure-reactivity studies of serum paraoxonase RPN1 suggest that its native activity is lactonase. *Biochemistry*. 44:6371-6382.