HACETTEPE JOURNAL OF BIOLOGY AND CHEMISTRY Research Article
Hacettepe J. Biol. & Chem., 2009, 37 (1) 61-66

Activity-pH-Inhibition Interaction on Purify $PON1_{Q192}$ and $PON1_{R192}$

Nahit Gençer* and Oktay Arslan

Balikesir University, Science and Art Faculty, Department of Chemistry/Biochemistry Section, Balikesir, Turkey

Article Info

Article history:

Received
December 1, 2008

Received in revised form
December 20, 2008

Accepted
December 23, 2008

Available online
March 21, 2009

Key Words

PON1,

Inhibition,

Metals,

pH

Abstract

Human serum paraoxonase (PON1, EC 3.1.8.1.) is a high-density lipid (HDL)-associated, calcium-dependent enzyme; its physiological substrates are not known. PON1 exists in 2 major polymorphic forms: Q (glutamine) or R (arginine) at codon 192. PON1Q and R were separately purified by ammonium sulfate precipitation and hydrophobic interaction chromatography. The in vitro effects of some heavy metals (Hg, Cd, Cu, Co and Ni) has been previously investigated on the purified human serum PON1Q and R isoenzyme, using paraoxon as substrate at pH= 8 tris-base buffer. In this study, in vitro effects of some heavy metals (Hg, Cd, Cu, Co and Ni) were investigated on the purified human serum PON1Q and R isoenzyme, using paraoxon as substrate at pH= 10.5 tris-base buffer. Metals were more effective inhibitors on purified human serum $PON1_{R192}$ activity than $PON1_{Q192}$ activity also at pH=10.5 tris-base buffer. The IC_{50} values of these metals exhibiting inhibition effects were found from graphs of paraoxsonase activity% by plotting the concentration of the metals.

INTRODUCTION

Paraoxonase (PON1; EC 3.1.8.1) is a calcium-dependent esterase that is hydrolyzes aromatic carboxylic acid esters, toxic organophosphate compounds, and lactones, yet the natural substrates and physiological function(s) of PON1 remain to be established [1]. Human PON1 is a member of a multigene family (PON1, PON2 and PON3 genes) encoded by a single gene on chromosome 7q21–22 [2]. PON1 is synthesized in the liver and secreted into the blood, where it is associated with high density lipoproteins (HDL). PON1 activity in serum shows a wide variation among individuals [3], and this variability is attributed to the presence of polymorphisms in PON1 gene; thus, subjects have been classified into homozygous for the low activity allele, and heterozygous, or homozygous for the high activity allele [4]. Meanwhile, PON1 coding region shows two polymorphic sites at positions 55 and 192; the latter has two isoforms (Q and R) and is described as substrate-dependent, thus the 192R alloenzyme hydrolyzes paraoxon (the parathion oxon metabolite) faster than the 192Q isoform, while diazoxon (the oxon of diazinon) is hydrolyzed faster by the 192Q isoform [5].

PON1 was proven to possess both arylesterase and organophosphatase activities [6]. Some ester substrates, such as phenyl acetate, are hydrolyzed by the PON1 Q and R isozymes at approximately equivalent rates, whereas most organophosphates

* Correspondence to: Nahit Gençer

Balikesir University, Science and Art Faculty, Department of Chemistry, 10145 Balikesir, Turkey

Tel: +90266 1278 Fax: +90266 612 1278
E-mail: ngencer@balikesir.edu.tr

are hydrolyzed at different rates by the isozymes. These differences in isozymic properties allowed our laboratory to phenotype serum samples by dividing the organophosphatase activity in the presence of 1 M NaCl (with paraoxon as the substrate) by the arylesterase activity (with phenylacetate as the substrate) [4]. Phenotyping serum samples using these simple assays aided in the purification of the two PON1 isozymes [7], which led to subsequent studies of their catalytic activities with a number of compounds.

The inhibition by metallic ions is well known for many enzymes and has been reported for A-esterases from different sources. Other compounds, as complexing agents and thiol reagents are also, typical enzyme inhibitors. Aldridge [8,9] proposed to do detailed studies with inhibitors as a tool for a better classification of esterases that hydrolyse OP compounds. In addition, inhibition studies can also be used to identify some omponents in the active centre of the enzymes.

We previously reported effect of inhibition by metals on purify Q and R isoform at pH= 8 tris base buffer. In this paper we have investigated the inhibitory effect of some metals on purified Q and R isoform, as well as the kinetics of the inhibition data at pH= 10.5 tris base buffer. The possible implication of differential inhibition of pH= 8 and pH= 10.5 tris base buffer by metals is also discussed.

MATERIALS AND METHODS

The materials used include sepharose 4B, L-tyrosine, 9-aminophenantrene, paraoxon, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. (St. Louis, USA) All other chemicals used were analytical grade. Metals were of commercial origin and at the highest available purity (99%).

Paraoxonase Enzyme Assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al [10]. The reaction was followed for 2 min at 37°C by monitoring the appearance of p-nitrophenol at 412 nm in Biotek automated recording spectrophotometer (Winooski, VT, United States) at pH= 10.5 trise base buffer. At pH= 10.5, 1 mM of final substrate concentration was used during enzyme assay and all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis. PON1 activity (1 U/liter) was defined as 1 μmol of p-nitrophenol formed per minute.

Phenotyping and Purification of Human PON1 Types Q and R.

In order to classify individual phenotypes, two parameters were used. According to Eckerson et al [4], phenotypic distribution of the paraoxonase activity was determined by the basal and stimulation of paraoxonase activity by 1 M NaCl.

Individuals were classified for paraoxonase phenotype using the antimode at 60% stimulation as the dividing point between the non-salt-stimulated, Q type, and the salt stimulated, QR (60%-200%) and R (200%- up) types [4].

Purification of PON1 Q and R Isoforms from Human Serum by Hydrophobic Interaction Chromatography

Human serum was isolated from 35 ml fresh human blood and put into a dry tube. The blood samples were centrifuged at 1500 rpm for 15 min and the serum was removed. Firstly, serum paraoxonase was isolated by ammonium sulfate precipitation (60—80%) [11]. The precipitate was collected by centrifugation at 15000 rpm for 20 min, and redissolved in 100 mM Tris–HCl buffer (pH= 8.0). Then, we synthesized the hydrophobic gel, including Sepharose 4B, L-tyrosine and 9-aminophenantrene,

for the purification of human serum paraoxonase [7]. The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH= 8.00) including 1 M ammonium sulfate. The paraoxonase was eluted with an ammonium sulfate gradient using 0.1 M Na_2HPO_4 buffer with and without 1 M ammonium sulfate (pH= 8.00). The purified PON1Q and R isoenzymes were stored in the presence of 2 mM calcium chloride in order to maintain activity.

Total Protein Determination

The absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulfate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [12], with bovine serum albumin standard.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrilamide gel electrophoresis was performed after purification of the enzyme. It was carried out in 12% and 3% acrylamide concentration for the running and stacking gel, respectively, containing 0.1% SDS according to Laemmli [13]. A 20 mg sample was applied to the electrophoresis medium. Gel was stained overnight in 0.1% Coomassie Brilliant Blue R- 250 in 50% methanol and 10% acetic acid, then destined by frequently changing the same solvent, without dye. The electrophoretic pattern was photographed with the system of produce as an image of the gel.

In Vitro Inhibition Kinetic Studies and Determination of Ki Values

For the inhibition studies of these metals, different concentrations of metals were added to the enzyme activity. PON1Q and R enzymes activity with metals were assayed by following the hydration of paraoxon. Activity % values of paraoxonase for five different concentrations of each metal were determined by regression analysis using Microsoft Office 2000 Excel. Paraoxonase activity without a metal was accepted as 100% activity. For the metals having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC_{50} values) was determined from the graphs.

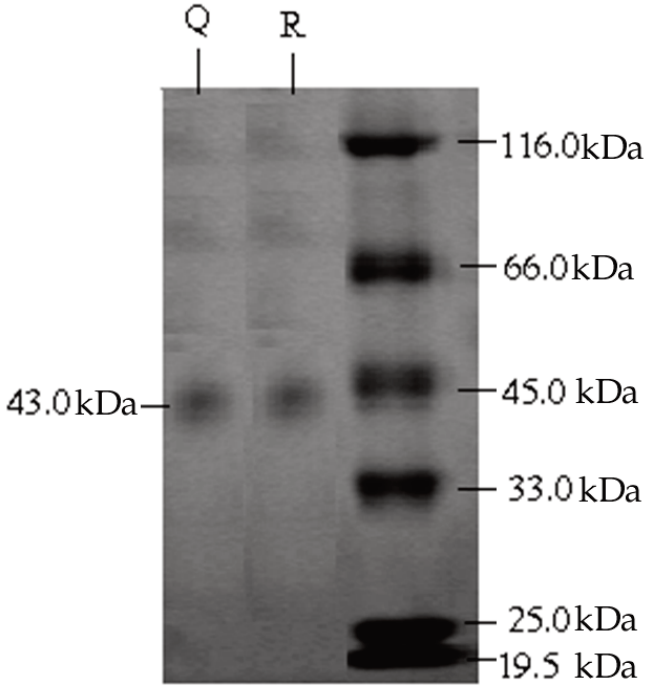


Figure 1. SDS-PAGE of human serum paraoxonase. The pooled fractions from ammonium sulfate precipitation and hydrophobic interaction chromatography (sepharose-4B, L-tyrosine, 9-aminophenantrene) were analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 3 contained 3 µg of various molecularmass standards: ß-galactosidase, (116,0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase, (33,0 kDa), ∞-lactoglobulin (25.0 kDa), lysozyme (19.5 kDa). Thirty microgram of purified human serum paraoxonase Q type (lane 1) and paraoxonase R type (lane 2) migrated with a mobility corresponding to an apparent Mr 43.0 kDa

RESULT AND DISCUSSION

In order to classify individual phenotypes, two parameters were used. According to Eckerson et al., phenotypic distribution of the paraoxonase activity was determined by the basal and stimulation of

paraoxonase activity by 1 M NaCl [4]. PON1 was purified from the healthy human volunteers previously identified as homozygous for PON1Q or for PON1R

In a previous paper [7] we reported that PON1Q and R isoform activity was inhibited by different metal ions (Co, Hg, Cu, Cd, and Ni) at pH=8 trise base buffer. Here we determined PON1Q and PON1R's effect of inhibition by metals at pH=10.5 tris base buffer. And inhibition-activity-pH effects was investigated.

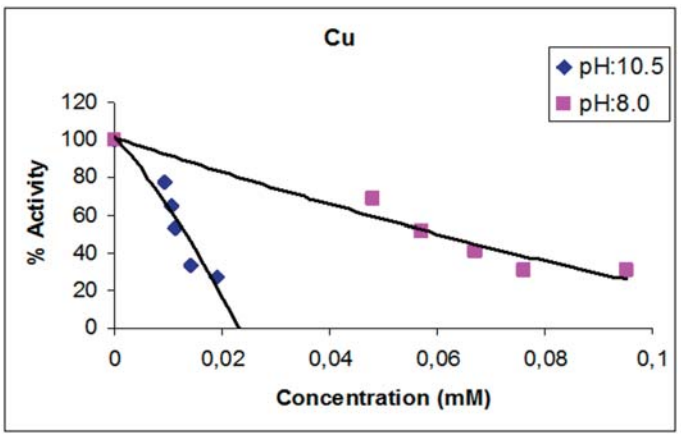
In order to investigate the effect of these pesticides on PON1Q and R allozymes activity in vitro, human serum paraoxonase Q and R were purified by ammonium sulfate precipitation at 60—80% intervals [11], and subjected to hydrophobic interaction chromatography. We previously reported a purification strategy designed for the human PON1 enzyme consisting of two-step procedures resulting in a shorter and more straightforward approach in contrast to other purification procedures [7].

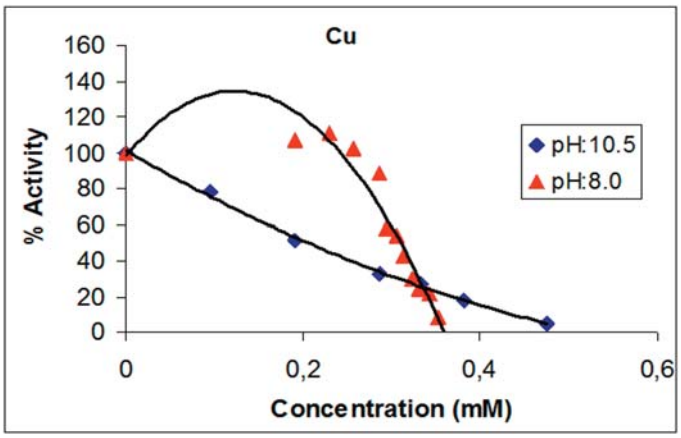
The gel for hydrophobic interaction chromatography was synthesized using Sepharose 4B, L-tyrosine and 9-aminophenantrene. Overall purification rate of our method was found 901-fold for R isoenzyme and 453-fold for Q isoenzyme. The V_{max} and K_M of the purified enzyme were determined for Q isoenzyme 55 EU and 0.599 mM and for R isoenzyme 50 EU 0.492 mM, respectively [7].

As seen in Figure 2, all of the selected metals in vitro inhibited the human serum PON1Q and R activity. The kinetic parameters for the various metals are presented in Table 1. The IC_{50} values obtained with purified Q and R isoenzyme are in different value. These metals were more effective inhibitors on purified human serum PON1Q192 activity than PON1R192 activity. Effect of inhibition Ni, Co, Cd and Hg at pH:10.5 is more than at pH=8.0 for R phenotype. Effect of inhibition Co, Hg and Cu more than at pH=10.5 is more than at pH=8.0 for Q phenotype.

Table 1. IC_{50} values and type of inhibition (mM) of pesticides on paraoxonase enzyme Q and R type.

Phenotype	Metals	I_{50} (mM) at pH=10.5	I_{50} (mM) at pH=8.0 [7]
R	Cu	0.013	0.061
	Hg	0.314	0.106
	Ni	0.142	1.026
	Cd	0.219	0.152
	Co	0.314	0.781
Q	Cu	0.21	0.310
	Hg	0.327	0.891
	Ni	1.844	1.144
	Cd	0.484	0.218
	Co	0.346	3.910





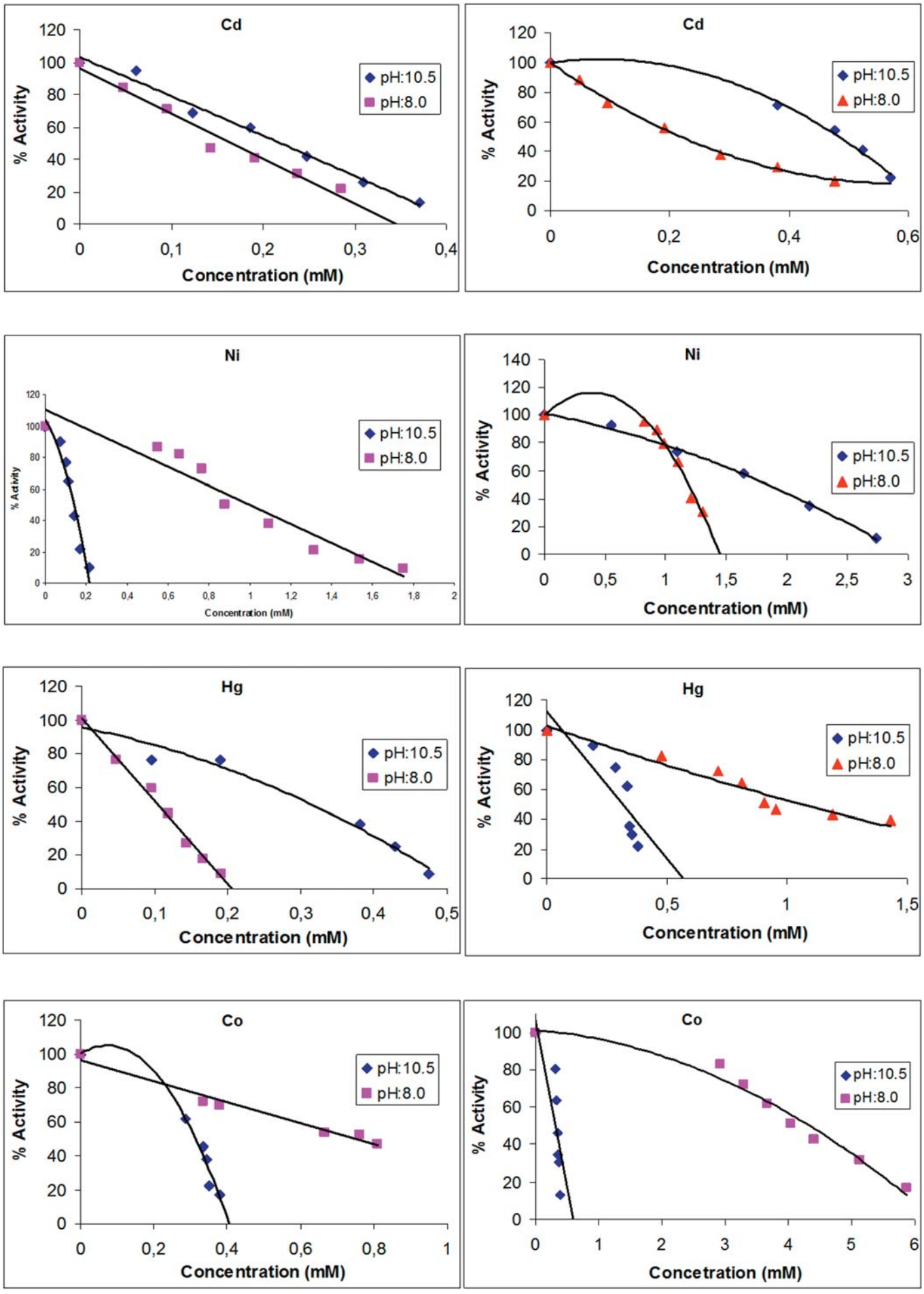


Figure 2. Inhibition of metals on paraoxonase enzyme R and Q phenotype, respectively

In conclusion, due to the lack of inhibition studies on paraoxonases from different sources there is not enough data with which to compare our results and consequently it is not possible to establish definite differences or similarities among paraoxonases. In spite of the contribution of our study to increase in the knowledge of the biochemical properties of paraoxonase, more extensive inhibition studies are necessary before the identity of paraoxonases could be stated.

ACKNOWLEDGEMENT

This work has been supported by Balikesir University Research Project (2007/11) and carried out at the Balikesir University Research Center of Applied Sciences (BURCAS). Special thanks to M. Lütfi BORA for his help and advice during the study.

REFERENCES

1 Draganov, D. I., and B. N. La Du. Pharmacogenetics of paraoxonases: a brief review. Naunyn Schmiedebergs Arch. Pharmacol., 369, 78–88, 2004.

2 Primo-Parmo, S.L., Sorenson, R.E., Teiber, J., La Du, B.N., The human serum paraoxonase/arylesterase gene (PON1) is a member of a multigene family. Genomics. 33, 498–509, 1996.

3 La Du, B.N., Piko, J.I., Eckerson, H.W., Vincent-Viry, M., Siest, G., An improved method for phenotyping individuals for the human serum paraoxonase arylesterase polymorphism. Ann. Boil. Clin. 44, 369–372, 1986.

4 Eckerson, H.W., White, C.M., La Du, B.N., The human serum paraoxonase/arylesterase polymorphism. Am. J. Hum. Genet. 35, 1126–1138, 1983.

5 Davies, H.G., Richter, R.J., Keifer, M., Broomfield, C.A., Sowalla, J., Furlong, C.E., The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin, Nat. Genet., 14, 334–336, 1996.

6 Sorenson RC, Primo-Parmo SL, Kuo CL, Adkins S, Lockridge O and La Du BN, Reconsideration of the catalytic center and mechanism of mammalian paraoxonase/arylesterase. Proc. Natl. Acad. Sci. USA, 92, 7187–7191, 1995.

7 Gençer, N., and Arslan, O., Purification human $PON1_{Q192}$ and $PON1_{R192}$ isoenzymes by hydrophobic interaction chromatography and investigation of the inhibition by metals, J. Chromatogr. B, 87(3) 134-140, 2009.

8 W.N. Aldridge, A-esterases and B-esterases in perspective, in: E. Reiner, W.N. Aldridge, F.C.G. Hoskin (Eds.), Enzymes Hydrolising Organophosphorus Compounds, Ellis Horwood, Chichester, New York, l-14, 1989.

9 W.N. Aldridge, The esterases: perspectives and problems, Chem.-Biol. Interact., 87, 5513, 1993.

10 Adkins, S., Gan, K.N., Mody. M. & La Du, B.N, Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B alloenzymes, Am. J. Hum. Genet., 52, 598-608, 1993.

11 Sinan, S., Kockar, F., Arslan, O., " Novel purification strategy for human PON1 and inhibition of the activity by cephalosporin and aminoglikozide derived antibiotics. Biochimie, 88(5) 565-574, 2006.

12 Bradford M.M., A rapid and sensitive method for the quotation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248–251, 1976.

13 Laemmli, D. K., "Cleavage of Structural Proteins During in Assembly of the Head of Bacteriohhoge T_4. Nature, London, 227, 680, 1970.