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Synthesis of Cholesterol-Conjugated Magnetic Nanoparticles for Purification of Human Paraoxonase 1

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Abstract Human serum paraoxonase 1 (PON1) is known as an antioxidant and is also involved in the detoxification of many compounds. In this study, a novel purification strategy was employed to purify the PON1 by using cholesterol-conjugated magnetic nanoparticles. Magnetic nanoparticles were synthesized and conjugated with cholesterol through diazotized *p*-aminohippuric acid. In Fourier transform infrared spectrum of cholesterol-*p*-aminohippuric acid-Fe_3O_4 nanoparticles, the appearance of peaks at 3,358.3, 1,645 cm^{-1}, and at 2,334.9 cm^{-1} confirmed the conjugation. The molecular weight of purified PON1 was nearly 45 kDa on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and isoelectric point was 5.3. The specific activity was 438 U mg^{-1} protein, and the purification fold was 515 with 73% yield. The K_m values were 1.3 and 0.74 mM with paraoxon and phenyl acetate, respectively. Western blot of 2D-PAGE confirmed the homogeneity and stability of the enzyme. Mg^{+2}, Mn^{+2}, glycerol, $(NH_4)_2SO_4$, PEG 6000, Triton X-100, and phenylmethylsulfonyl fluoride did not show any effect on activity. Pb^{+2}, Co^{+2}, Zn^{2+}, ethanol, β-mercaptoethanol, and acetone reduced the activity while Ni^{2+}, Cd^{2+}, Cu^{2+}, iodoacetic acid, SDS, dimethylformamide, DMSO inhibited the activity. In vitro enzyme activity was slightly reduced by acetyl salicylic and acetaminophen and reduced 50% with amino glycosides and ampicillin antibiotics at concentrations of 0.6 and 30 mg ml^{-1}, respectively. This is the first report for the synthesis of cholesterol-conjugated magnetic nanoparticles for simple purification of PON1 enzyme.

Keywords Paraoxonase 1 · Nanoparticles · FTIR · Antibiotics · 2D-PAGE · Western blot

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Introduction

Paraoxonase 1 (PON1; aryldialkylphosphatase, EC 3.1.8.1) is the glycosylated esterase enzyme synthesized in liver and released into serum after association with high-density lipoprotein (HDL) [1, 2]. PON1 name is derived from its property to hydrolyze paraoxon and to detoxify the broad range of substrates such as aryl esters, carbamates, cyclic carbonates, lactones, commonly used insecticides, and chemical warfare organophosphate compounds [3–8]. Paraoxon inhibits acetyl cholinesterase and is metabolically generated in vivo from parathion (insecticide) by mitochondrial oxidation [9]. PON1 is conserved in mammals, but its activity is not detected in fish, birds, and arthropods. In mammals, PON1 is a member of a multigene family and located on human chromosome 7 [10, 11].

PON1 is known as a bioscavenger that protects against cardiovascular diseases by inhibiting the formation of oxidized high-density lipoproteins and low-density lipoproteins [12]. The pharmacological modulation of PON1 expression may be a useful approach for protection of cardiovascular diseases and detoxification of organophosphate compounds. Due to the active role of PON1 in lipid metabolism, cardiovascular disease, and atherosclerosis, it has also gained attention to understand its role in the metabolism of pharmaceutical drugs [13]. Serum PON1 level depends upon a number of physiological and pathological conditions such as renal disease, diabetes mellitus, HDL deficiencies, liver cirrhosis, high-fat diets, and nutritional and environmental factors [5, 14].

With the advent of nanotechnology, the affinity purification of proteins, enzymes, and immunoglobulins through ligands or biomolecules immobilized onto magnetic nanoparticles has become an important tool since last decade [15, 16]. The affinity purification onto ligand-conjugated magnetic nanoparticles has the following advantages (a) enhanced stability, (b) easy separation from mixture, (c) possible modulation of the catalytic properties, and (d) prevention of microbial growth [17]. Due to the specific size and shape of magnetic nanoparticles, these have applications as drug delivery [18, 19], tissue repair [20], cell engineering [21], and for diagnosis [22, 23]. The surface of the magnetic nanoparticles can be modified chemically, and the binding can be done by carbodiimide activation method due to the simplicity and efficiency of the reagent [24, 25]. Cumbersome chromatographic procedures about the purification of paraoxonase 1 are available [13, 26–29]. By utilizing these procedures, a moderate level of enzyme activity is lost. There is a need to develop the strategy for easy purification of paraoxonase 1 from any source.

Due to affinity of human paraoxonase 1 with cholesterol (CHO), we describe a simple and novel method for purification of human paraoxonase 1 by using cholesterol-conjugated magnetic nanoparticles. The effect of some analgesics and antibiotics is also studied on the activity of purified PON1 due to physiological importance of these medicines.

Materials and Methods

Analytical-grade reagents such as iron(II) chloride tetrahydrate, iron(III) chloride hexahydrate, paraoxon, phenyl acetate, cholesterol, *p*-aminohippuric acid, acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), Triton X-100, ethanol, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), β-mercaptoethanol, dithiothreitol, iodoacetate, ethylenediaminetetraacetic acid (EDTA), ammonia solution, acetyl salicylic acid, acetaminophen, sodium ampicillin, gentamicin sulfate, kanamycin sulfate, streptomycin sulfate, alkaline-phosphatase-conjugated rabbit antimouse immunoglobulin G (IgG), and other chemicals required for routine analysis were

purchased from Sigma-Aldrich. Bradford reagent and nitrocellulose membrane were purchased from Bio-Rad. Protein markers were purchased from Fermentas Inc. Sephadex G-75 was purchased from Pharmacia. Healthy human plasma (after screening for hepatitis B and C virus) was used in this study. Deionized water produced by Milli-Q was used throughout the experiments.

Synthesis of Magnetic Nanoparticles and Determination of Amino Group

Magnetic nanoparticles (Fe_3O_4) were synthesized by mixing iron(II) and iron(III) ions in 1:2 ratios under alkaline hydrothermal conditions [30]. The synthesized magnetic nanoparticles were analyzed by Fourier transform infrared (FTIR) spectroscopy. The number of amino groups ($–NH_2$) attached to the Fe_3O_4 was determined spectrophotometrically using molar absorption coefficient 6,623 $M^{-1}cm^{-1}$ [31].

Attachment of *p*-Aminohippuric Acid with Fe_3O_4

The *p*-aminohippuric acid (PAHA) was conjugated with Fe_3O_4 by the modification of method [32]. PAHA (200 mg) was dissolved in 120 ml distilled water and mixed with 200 mg of Fe_3O_4. The mixture was sonicated for 10 min at 4 °C on ice, and pH 8.0 was adjusted with 1.0 M NaOH. The mixture was warmed for 15 min at 37 °C. After cooling, 200 mg of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCL (ECD) was added. The pH 6.4 was adjusted with 2.0 M HCl and stirred for 6 h in dark at 25 °C. ECD (100 mg) was again added to the mixture and kept at 25 °C for another 15 h. The PAHA-Fe_3O_4-conjugated particles were separated by applying external magnetic force and washed with 1.0 l deionized water and finally with 2.0 ml acetone. The particles were air-dried and processed for FTIR analysis.

Coupling of Cholesterol with PAHA-Conjugated Fe_3O_4

The CHO was coupled with PAHA-conjugated Fe_3O_4 by the modifications in method [33]. PAHA-conjugated Fe_3O_4 (200 mg) was suspended in 30 ml of deionized water, and pH was adjusted to 1.5 with 1.0 M HCl. The solution was cooled to 0 °C for 30 min, and 0.5 ml of $NaNO_2$ solution (100 mg/ml distilled water) was slowly added with constant shaking. After 5 min, the excess nitrite was destroyed by adding 0.5 ml of ammonium sulfamate solution (60 mg ml^{-1} distilled water). The cholesterol solution (8 mg per 10 ml of 50% (*v*/*v*) acetone in 0.1 M borate buffer, pH 9.0) was added dropwise with constant stirring at 0 °C. After 1 h, conjugated CHO-PAHA-Fe_3O_4 was removed and washed with water to remove the unconjugated cholesterol. The particles were dried at 37 °C and processed for FTIR analysis.

Fourier Transform Infrared Spectroscopy

Fe_3O_4, PAHA, PAHA-Fe_3O_4, CHO, and CHO-PAHA-Fe_3O_4 were homogenized separately in Nujol, and the FTIR spectrum was taken on FTIR spectrophotometer (Perkin-Elmer) at 25 °C.

Protein Determination and Enzyme Assays

During purification of PON1 enzyme, the protein concentration was determined by Bradford reagent assay using bovine serum albumin as standard [34]. Paraoxonase and aryl

esterase activities were determined using diethyl-*p*-nitrophenyl phosphate (paraoxon) and phenyl acetate as substrate, respectively [23]. The final substrate concentration of paraoxon and phenyl acetate was 2 mM at pH 8.0. The activity of PON1 enzyme was determined by using the molar extinction coefficients of paraoxon and phenyl acetate as 18,290 and 1,310 $M^{-1} cm^{-1}$ at 412 and 270 nm, respectively. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1.0 μM of substrate per minute at 37 °C. All assays were conducted in duplicate.

Purification of PON1 Enzyme

All purification steps were carried out at 4 °C unless otherwise stated. Before purification, total protein concentration and PON1 activity of human plasma were measured.

Triton X-100 Treatment and Ammonium Sulfate Precipitation

Human plasma (10 ml) was mixed with Triton X-100 (1% *v*/*v*) and $CaCl_2$ solution (final concentration 10 mM) and gently stirred for 16 h. The mixture was centrifuged for 20 min at 6,000 rpm. The supernatant was separated and mixed with ammonium sulfate to 60% saturation and then to 70% saturation. The solution was stirred slowly for 5 h. The precipitates were collected at 12,000 rpm for 25 min. The precipitates were dissolved in 5.0 ml of PON buffer (20 mM Tris–HCl pH 8.0, 1.0 mM $CaCl_2$, and 5.0 μM EDTA) and dialyzed against 2.0 l of the same buffer.

Affinity Cholesterol-Conjugated Magnetic Nanoparticles

The dialyzed solution was mixed with 0.2 g of cholesterol-conjugated Fe_3O_4 nanoparticles and gently shaken for 30 min. The particles were separated and washed twice with 20 ml of PON buffer. The washed particles were mixed again with 5.0 ml of PON buffer containing 0.2% deoxycholate for 30 min. The magnetic nanoparticles were separated again. The supernatant was dialyzed against 1 l of PON buffer and concentrated to 1.5 ml on microcentrifuge filter unit (10,000-Da cut).

Gel Permeation Chromatography

Concentrated fractions from affinity cholesterol-conjugated magnetic nanoparticles were applied on Sephadex G-75 column (1.5×30 cm) pre-equilibrated with PON buffer. A 2.0-ml fraction was collected at a flow rate of 0.5 ml min^{-1}, and fractions containing the PON1 activity were pooled, concentrated, and stored at −20 °C until further use. The protein concentration was determined by Bradford reagent.

Production of Anti-PON1Antibodies

Purified human paraoxonase 1 (1.0 mg ml^{-1}l) was mixed with denaturing buffer (50 mM Tris–HCl, pH 7.4, 0.01% SDS, 0.005 M β-mercaptoethanol) in 1:1 ratio and kept at 60 °C for 30 min to denature the enzyme to avoid the self-tolerance of mammalian antigen. Male Balb/C mice, 6–7 weeks old, were subcutaneously injected with denatured PON1 (80–100 μg per injection) after mixing with Freund's complete adjuvants in 1:1 ratio at 2-week intervals with a total of five injections. After checking the anti-PON1 antibody titre in mice blood by indirect enzyme-linked immunosorbent assay, whole blood was isolated by

cardiac puncture, and serum was isolated. Preimmune serum was obtained from mice tail before immunization and used as control.

Characterization of PON1 Enzyme

SDS–Polyacrylamide Gel Electrophoresis and Western Blot

Protein samples obtained during purification steps were analyzed on slab 10% SDS–polyacrylamide gel electrophoresis (PAGE) as described [35]. The gel was stained with Coomassie Brilliant Blue R-250 and destained in solution containing methanol, acetic acid, and water in 40:10:50 ratio, respectively. The proteins samples were electrophoretically transferred onto nitrocellulose membrane [36]. The nitrocellulose membrane was treated with blocking buffer (3% BSA in Tris-buffered saline–Tween 20) and incubated with primary antibodies (mouse anti-PON1 antibodies) followed by incubation with secondary antibody (alkaline-phosphatase-conjugated rabbit antimouse IgG). After washing in Tris-buffered saline–Tween 20, color reaction was developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Preimmune serum and secondary antibody were used as control.

Effects of pH and Temperature

The effect of pH on purified human serum paraoxonase 1 was measured at 37 °C over the pH range 5–11 in 50-mM buffer (sodium acetate buffer, pH 5–6; Tris–HCL buffer pH 7–9; glycine–NaOH buffer pH 9–11) using 2.0-mM paraoxon concentration. All assays were conducted in duplicate.

The effect of temperature on paraoxonase 1 was determined by incubating the enzyme at different temperatures (10, 20, 30, 40, 50, and 60 °C) for 30 min. The enzyme activity was measured under standard assay conditions which were considered as 100%. All assays were conducted in duplicate.

Effects of Metal Ions, Detergents, Solvents, and Reducing Agents

The effects of metal ions ($MgCl_2$, $MnCl_2$, $PbCl_2$, $CoCl_2$, $CuCl_2$, $CdCl_2$, $ZnCl_2$, and $NiCl_2$,) were studied by adding the metal ion separately in a final concentration of 0.5 to 3.0 mM. The effects of other reagents such as, PMSF, iodoacetate, SDS, β-mercaptoethanol, DMF, DMSO, ethanol, acetone, glycerol, ammonium sulfate, and polyethylene glycol (PEG) 6000 were also determined separately by adding compounds in final concentration of 2% to 7%. The purified PON1 enzyme was preincubated separately with above reagents dissolved in PON buffer for 15 min at 37 °C, and the activity was measured under standard assay conditions. The activity of the enzyme without the addition of above reagents was considered as 100%. All assays were conducted in duplicate.

Effect of Stabilizers on PON1 Activity

The effect of stabilizers was checked as described [24] with little modification. Purified paraoxonase 1 (200 U ml^{-1}) was taken in 5 ml of PON buffer pH 8.0 containing 20% concentration of $(NH_4)_2SO_4$, PEG 6000, and glycerol separately and incubated for 30 days at 4 °C. Enzyme solution (0.05 ml) was taken on each day, and the enzyme activity was

checked under standard assay conditions for 30 days. Control enzyme assays were conducted without stabilizers. All assays were carried out in duplicates.

Effect of Medicines on PON1 Activity

The inhibitory or noninhibitory effects of different final concentration of some medicines such as acetyl salicylic acid (2, 4, 6, 8, 10 mg ml^{-1}), acetaminophen (2, 4, 6, 8, 10 mg ml^{-1}), sodium ampicillin (10, 20, 30, 40, 50 mg ml^{-1}), gentamicin sulfate (2, 4, 6, 8,10 mg ml^{-1}), kanamycin sulfate (2, 4, 6, 8,10 mg ml^{-1}), and streptomycin sulfate (2, 4, 6, 8,10 mg ml^{-1}) was determined (in vitro). Purified paraoxonase 1 (50 U ml^{-1}) was mixed with the above medicines separately for 15 min, and the enzyme activity was checked under standard assay conditions. Paraoxonase 1 enzyme activity without medicines was considered as 100%.

2D-PAGE, Western Blot, and Kinetic Study

The purity of the enzyme was checked by 2D-PAGE. The isoelectric point of PON1 enzyme was determined in isoelectric focusing (IEF) gel in tube (1.0×50 mm) according to the O'Farrell method, and the isoelectric focused gel was also placed on SDS–polyacrylamide gel (10%) for second dimension [37, 38]. The protein separated in 2D-PAGE was transferred onto nitrocellulose membrane and processed further for immunochemical reaction as described above. The kinetic parameters of PON1 enzyme were studied under standard assay conditions using different concentrations of substrate (0.5 to 3.0 mM of paraoxon and phenyl acetate). All assays were carried out in duplicates. The Michaelis–Menton constant (K_m) was determined by Lineweaver–Burk plot method.

Results

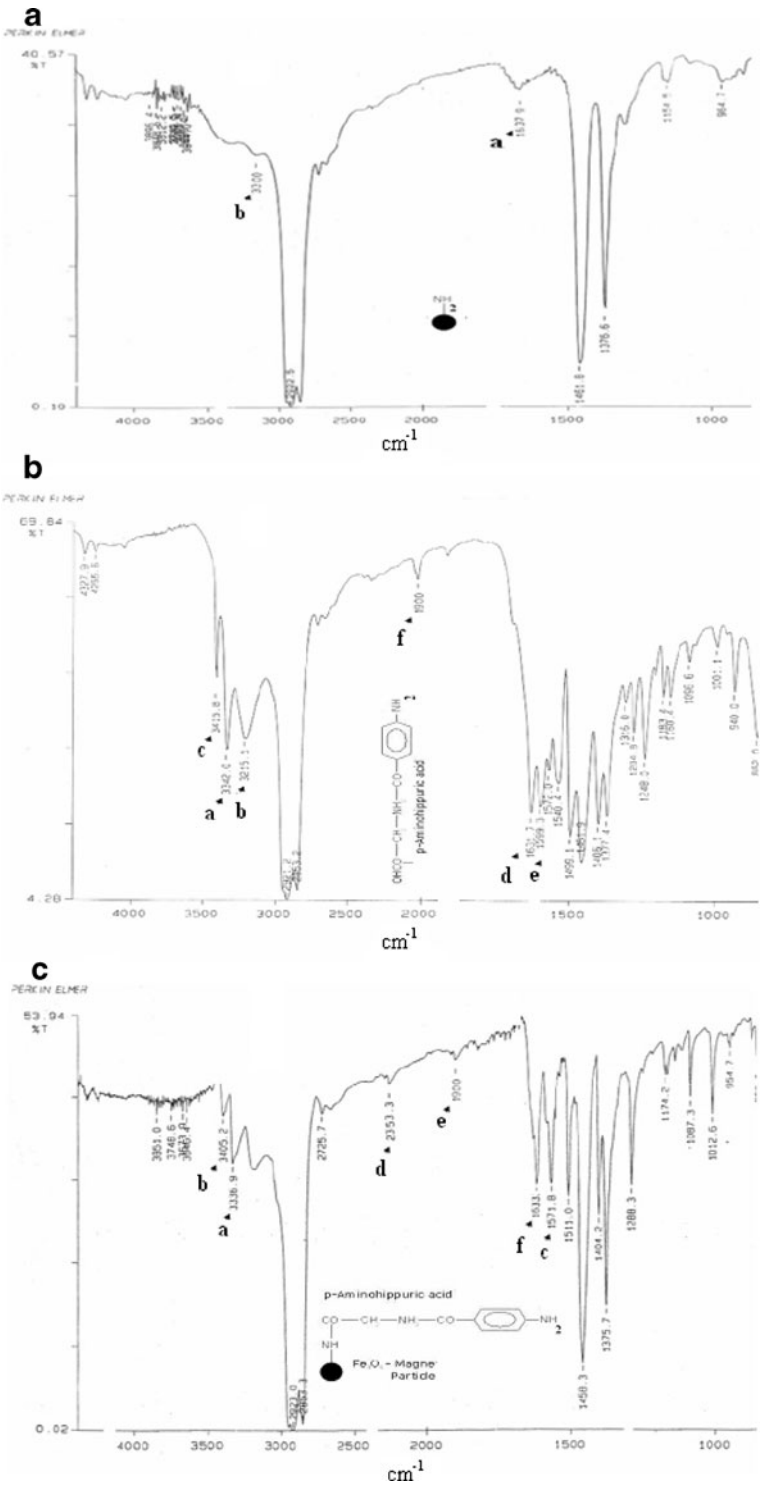
FTIR Analysis

The binding of cholesterol with magnetic nanoparticles was achieved through diazotization reaction of *p*-aminohippuric acid. The conjugation was confirmed by FTIR spectroscopy. The FTIR spectra of Fe_3O_4, PAHA, PAHA-Fe_3O_4, CHO, and CHO-PAHA-Fe_3O_4 are shown in Fig. 1a–e, respectively.

In FTIR spectrum of Fe_3O_4, the bend and stretch peaks of $–NH_2$ group present on Fe_3O_4 were observed at 1,637 and at 3,300 cm^{-1}, respectively (Fig. 1a (a and b, respectively)). In FTIR spectrum of PAHA, the bend peaks of $–NH_2$, –NH, and –COOH groups were observed at 3,342, 3,415, and 3,215 cm^{-1}, respectively (Fig. 1b (a, b, and c)). The stretch peaks of –amino group, aromatic ring, and –C=O group were observed at 1,631.4, 1,599.3, and 1,900 cm^{-1}, respectively (Fig. 1b (d, e, and f)).

In FTIR spectrum of PAHA-Fe_3O_4, the stretch peaks of $–NH_2$, –NH, and –C=O groups of PAHA were observed at 3,336.9, 3,405, and 1,571 cm^{-1}, respectively (Fig. 1c (a, b, and c)).

Fig. 1 FTIR spectra for conjugation of cholesterol with magnetic nanoparticles through diazotized *p*-aminohippuric acid. *The details about the peaks are explained in text section*. **a** FTIR spectrum of magnetic nanoparticles, **b** FTIR spectrum of *p*-aminohippuric acid, **c** FTIR spectrum of conjugation of magnetic nanoparticles with *p*-aminohippuric acid, **d** FTIR spectrum of cholesterol, **e** FTIR spectrum of conjugation of cholesterol through diazotized *p*-aminohippuric-acid-conjugated magnetic nanoparticles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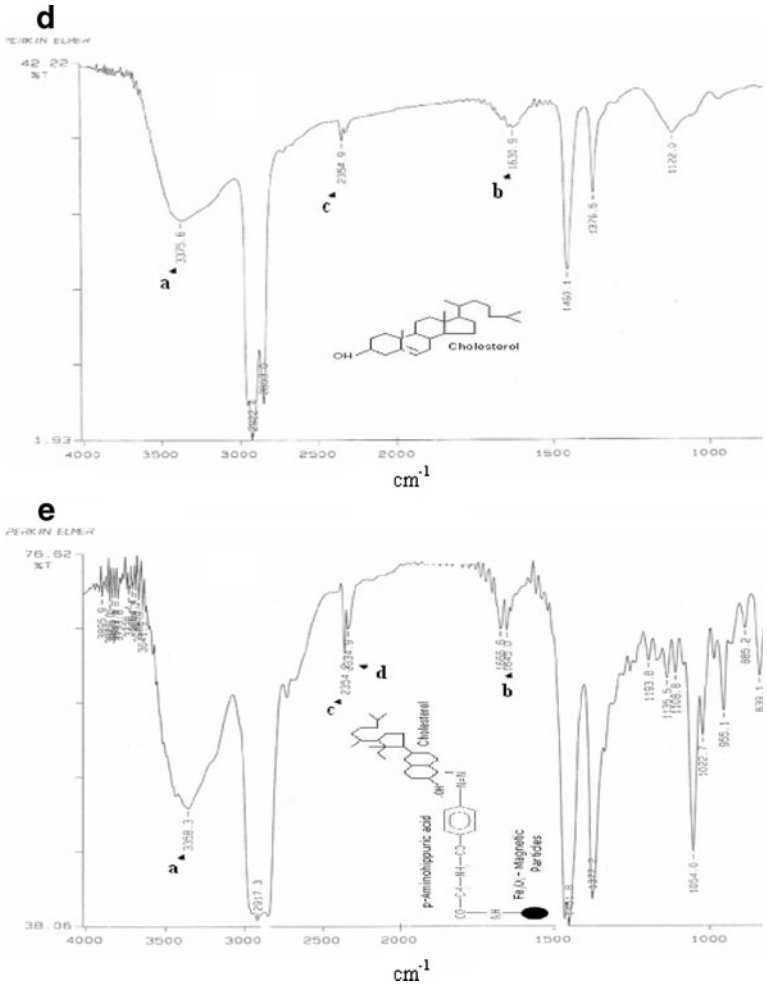

Fig. 1 (continued)

The bend peak of –COOH group appeared at 3,215 cm^{-1} in PAHA spectrum (Fig. 1b (b)) which disappeared in spectrum of PAHA-Fe_3O_4 and new peak of *N*=C– has appeared at 2,353.3 cm^{-1} (Fig. 1c, (d)). The appearance of new peak indicated the successful conjugation of –COOH group of PAHA with $–NH_2$ group of Fe_3O_4 through carbodiimide reagent. The bend peak of aromatic ring and –C=C– of PAHA was observed at 1,900 and 1,630.9 cm^{-1}, respectively (Fig. 1c (b and f)).

In FTIR spectrum of CHO, the broad stretch peak of phenol group was observed at 3,375.6 cm^{-1}, and stretch peak of –C=C– was observed at 1,630.9 cm^{-1} (Fig. 1d (a and b)). In CHO-PAHA-Fe_3O_4 spectrum, the appearance of bend peaks of phenol group at 3,358.3 cm^{-1} (Fig. 1e (a)) and slightly shifted peak of aromatic ring at 1,645 cm^{-1} (Fig. 1e (b)) confirmed the binding of CHO with PAHA-Fe_3O_4. The additional stretch peaks at 2,354.9 cm^{-1} in pure CHO spectrum (Fig. 1d (c)) and CHO-PAHA-Fe_3O_4 spectrum (Fig. 1e (c)) advocated the binding confirmation. The stretch peak at 2,334.9 cm^{-1} (Fig. 1e

(d)) confirmed the diazotization (–N=N–) bonding. The characteristic peaks of Nujol were observed in all spectra at 2,928, 1,461.8, and at 1,376 cm^{-1}. The proposed structure of CHO-PAHA-Fe_3O_4 is shown (Fig. 2).

Purification

Human serum paraoxonase 1 was purified to homogeneity from human plasma by using affinity cholesterol-conjugated magnetic nanoparticles and Sephadex-G75 chromatographic techniques. Different concentrations of sodium deoxycholate in PON buffer (0.1%, 0.2%, 0.3%, 0.4%, 0.5%) were used to elute the PON1 enzyme from CHO-PAHA-Fe_3O_4 particles. The complete elution of adsorbed enzyme on CHO-PAHA-Fe_3O_4 was observed with 0.2% deoxycholate in PON buffer. The dialyzed and concentrated PON1 fractions were loaded on to Sephadex-G75 column and eluted in PON1 buffer. The fractions indicating the enzyme activity were pooled and concentrated (Fig. 3). The purified enzyme was analyzed on SDS–PAGE and only single band of nearly 45 kDa was observed which clearly demonstrated the homogeneity of the purified enzyme (Fig. 4a). The purification of enzyme was 516-fold with 73% yield. The specific activity was 438 U mg^{-1}. The purification steps and activity of human serum paraoxonase 1 are shown (Table 1). In Western blot analysis, a single protein band was observed which advocated the purity and integrity of the enzyme stored at −20 °C for 1 month (Fig. 4b).

Biochemical Properties of Purified PON1 Enzyme

The enzyme is functionally active at pH 8.0 with paraoxon and phenyl acetate. The enzyme activity was reduced below pH 7.0 and at alkaline pH (Fig. 5a). The optimal activity of paraoxonase 1 was observed at 37 °C and completely lost at 50 °C (Fig. 5b). The enzyme was stable for 8 to 10 h at 37 °C in PON buffer.

Various metal ions and organic compounds were tested to investigate the inhibitory or stimulatory effect on PON1 enzyme activity; Mg^{+2} and Mn^{+2} did not show any inhibitory or stimulatory effect whereas Pb^{+2}, Co^{+2}, and Zn^{2+} reduced the enzyme activity while Ni^{2+}, Cd^{2+}, and Cu^{2+} inhibited the enzyme activity. Glycerol, PEG 6000, ammonium sulfate, and PMSF did not inhibit the enzyme activity while β-mercaptoethanol, ethanol, and acetone

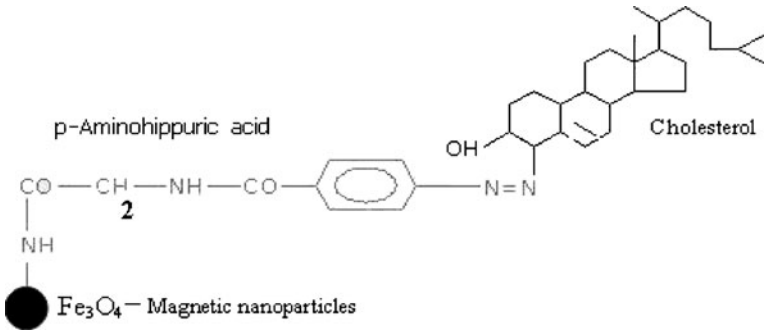

Fig. 2 Proposed structure of conjugation of cholesterol with diazotized *p*-aminohippuric acid magnetic nanoparticles

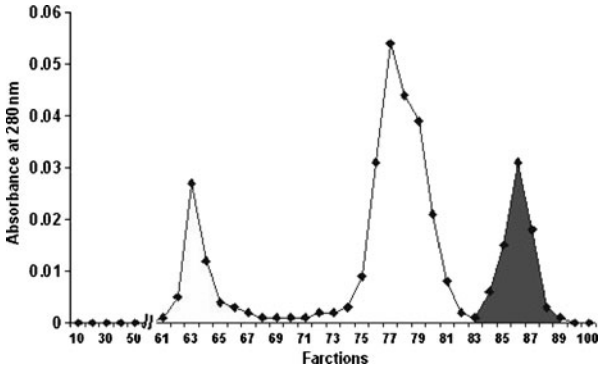

Fig. 3 A chromatographic profile of purification of PON1 enzyme on Sephadex G-75 resin. The fractions collected from affinity cholesterol-conjugated magnetic nanoparticles were applied on Sephadex G-75 column (1.5×30 cm) pre-equilibrated with PON buffer. A 2-ml peak fraction was collected and assayed for PON1 enzyme activity. The fractions showed that the enzyme activity (*shadowed area*) was pooled and characterized

moderately reduced the enzyme activity. Iodoacetic acid, SDS, DMF, and DMSO completely destroyed the enzyme activity (Table 2).

The isoelectric point of purified PON1 was observed at 5.3, and the PON1 enzyme showed a single spot in Western blot of 2D-PAGE which corresponds to purified PON1 enzyme. It indicated that the enzyme was purified to homogeneity (Fig. 6). The K_m values of PON1 enzyme were found as 1.3 and 0.74 mM for paraoxon and phenyl acetate, respectively, by Lineweaver–Burk plot (Fig. 7).

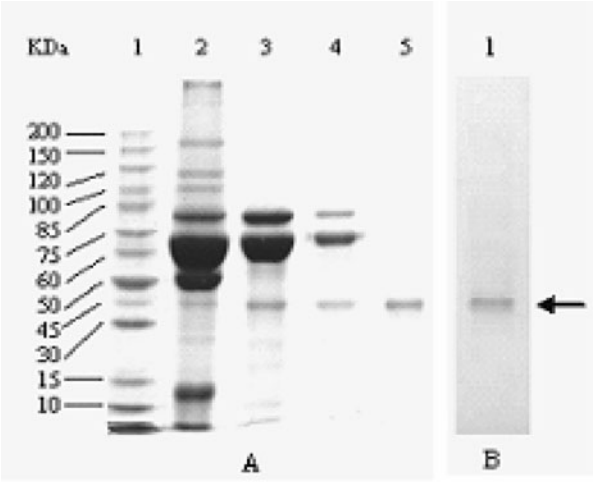

Fig. 4 SDS–PAGE (10%) and Western blot analysis of purified PON1 enzyme. **a** (*lane 1*) standard molecular weight protein marker, (*lane2*) Triton-X-100-treated human serum, (*lane 3*) 60–80% ammonium-sulfate-precipitated protein fraction, (*lane 4*), partially purified PON1 enzyme from affinity cholesterol-conjugated magnetic nanoparticles, (*lane 5*), purified PON1 enzyme from Sephadex G-75 resin. After electrophoresis, gel was stained with Coomassie brilliant blue R-250. A single protein band of nearly 45 kDa of PON1 enzyme is detected. **b** (*Lane 1*) a single immunoreactive protein band of nearly 45 kDa indicated the purity and stability of the enzyme (*arrow*)

Table 1 Purification scheme of human plasma PON1enzyme.

Fractions	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Human plasma	10	605	535	0.85	1.0	100
Triton-X-100-treated plasma	9.3	460	507	1.1	1.29	94.76
60–80% $(NH_4)_2SO_4$ Precipitation	5.0	165	490	2.97	3.49	91.58
Affinity cholesterol-conjugated magnetic nanoparticles	2.0	14.3	428	29.93	35.21	80.00
Sephadex G-75	9.0	0.89	390	438.20	515.52	72.89

The PON1 activity was measured at 37 °C using paraoxon as substrate

Effects of Stabilizers and Medicines

The activity of paraoxonase 1 was estimated every day in the presence of stabilizers. The optimal concentration of stabilizers that did not have an effect on enzyme activity was found to be 20% (*w*/*v*). The purified PON1 enzyme was stable in 20% glycerol at 4 °C. Paraoxonase 1 activity (8% to 10%) was lost on storage in glycerol for 1 month at 4 °C while 20% to 24% activity was lost on storage in $(NH_4)_2SO_4$ and PEG 6000.

The effects of some medicines such as acetyl salicylic acid, acetaminophen, sodium ampicillin, gentamicin sulfate, streptomycin sulfate, and kanamycin sulfate on PON1 activity were also studied. The data indicated that acetyl salicylic acid and acetaminophen slightly reduce the PON1 activity by 10% and 18% concentration, respectively (Fig. 8a). Sodium ampicillin reduced the 50% of PON1 enzyme activity at 30mg/ml (Fig. 8b) while gentamicin sulfate, streptomycin sulfate, and kanamycin sulfate reduce the 50% enzyme activity at 0.6 mg/ml (Fig. 8c).

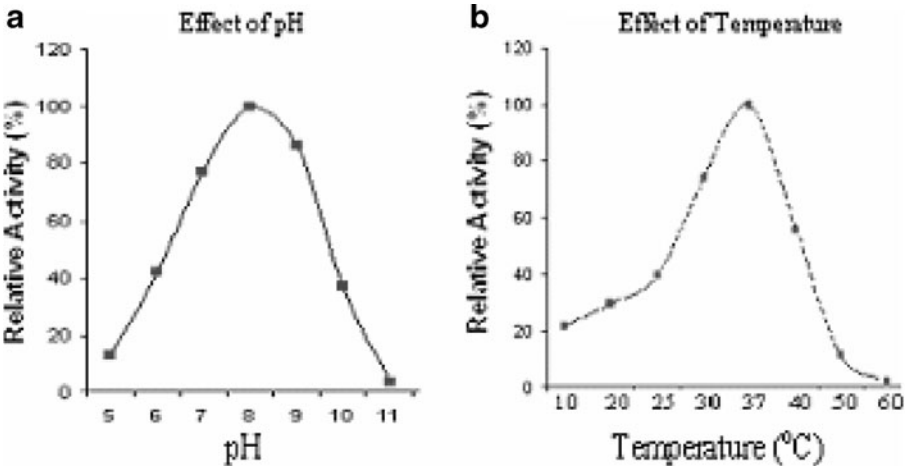

Fig. 5 Effects of temperature and pH on the activity of purified human PON1 enzyme using paraoxon as substrate. **a** Effect of pH on PON1 enzyme activity was studied at 37 °C at different pH ranges (5–11) in 0.05 M buffers (sodium acetate buffer pH 5–6; Tris–HCL buffer pH 7–9; glycine–NaOH, 9–11) by using the same substrate concentration. The optimal pH of the enzyme activity is 8.0. **b** Effect of temperature on PON1 enzyme activity at pH 8.0 was studied. The enzyme was incubated at required temperature, and activity was measured using standard assay conditions. The optimal activity of the enzyme is at 37 °C

Table 2 Effect of metal ions and organic compounds on human PON1 enzyme activity.

Metal ions (1.0 mM)	Residual activity (%)	Organic compounds (5%)	Residual activity (%)
Control	100	Glycerol	100
Mn^{2+}	100	Ammonium sulfate	100
Mg^{2+}	100	PEG 6000	100
Co^{2+}	70	Triton X-100	100
Pb^{2+}	65	PMSF	100
Zn^{2+}	30	Ethanol	57
Cd^{2+}	0	β-mercaptoethanol	48
Cu^{2+}	0	Acetone	18
Ni^{2+}	0	SDS	0
		DMF	0
		DMSO	0
		Iodoacetate	0

The purified enzyme was incubated with the reagents separately and then enzyme activity was measured at 37 °C paraoxon as substrate. (All assays were conducted in duplicate)

PMSF phenylmethylsulfonyl fluoride, *SDS* sodium dodecyl sulfate, *DMF* dimethyl formamide, *DMSO* dimethyl sulfoxide

Discussion

A new methodology was developed for the affinity purification of human serum paraoxonase 1. Paraoxonase 1 is an important enzyme to hydrolyze the toxic organophosphorus, nerve gasses, insecticides, and pharmaceutical drugs [9, 39]. Human serum paraoxonase 1 was purified in three sequential steps: ammonium sulfate precipitation, affinity separation by

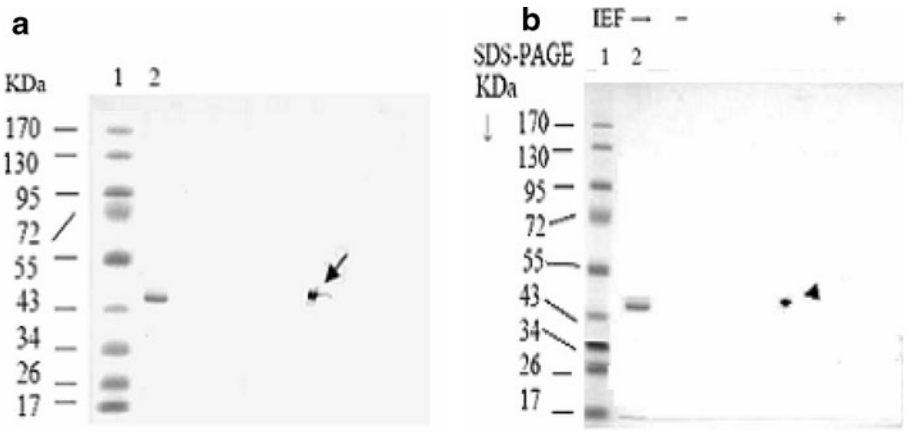

Fig. 6 2D-PAGE of purified human PON1 enzyme and Western blot analysis of 2D-PAGE. **a** First dimension IEF was placed onto 10% SDS–PAGE for second dimension. A 45-kDa protein spot (*arrowhead*) corresponds to purified PON1 enzyme and indicates the purity of the protein. (*lane1*) Standard molecular weight protein marker, (*lane 2*) purified PON1 enzyme. **b** The purified PON1 enzyme separated on 2D-PAGE was transferred onto nitrocellulose membrane and proceeded for immunochemical reaction. An immunoreactive protein spot of 45 kDa (*arrow*) was highlighted that corresponds to purified PON1 enzyme. (*lane1*) Standard molecular weight protein marker, (*lane 2*) an immunoreactive PON1 protein

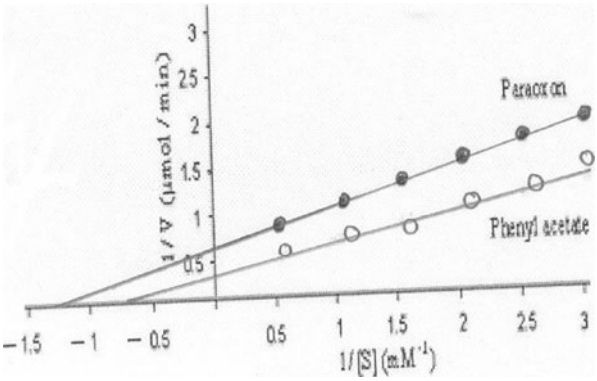

Fig. 7 Lineweaver–Burk plot for human serum paraoxonase 1. Paraoxon (*filled circles*) and phenyl acetate (*empty circles*) are used as substrates under standard assay conditions

cholesterol-conjugated magnetic nanoparticles, and gel permeation chromatography. Paraoxonase 1 was detached from HDL by treating the serum with nonionic detergent Triton X-100. The maximum enzyme activity was observed at 60–70% ammonium sulfate saturation. A 0.2% deoxycholate solution was found suitable for the complete elution of

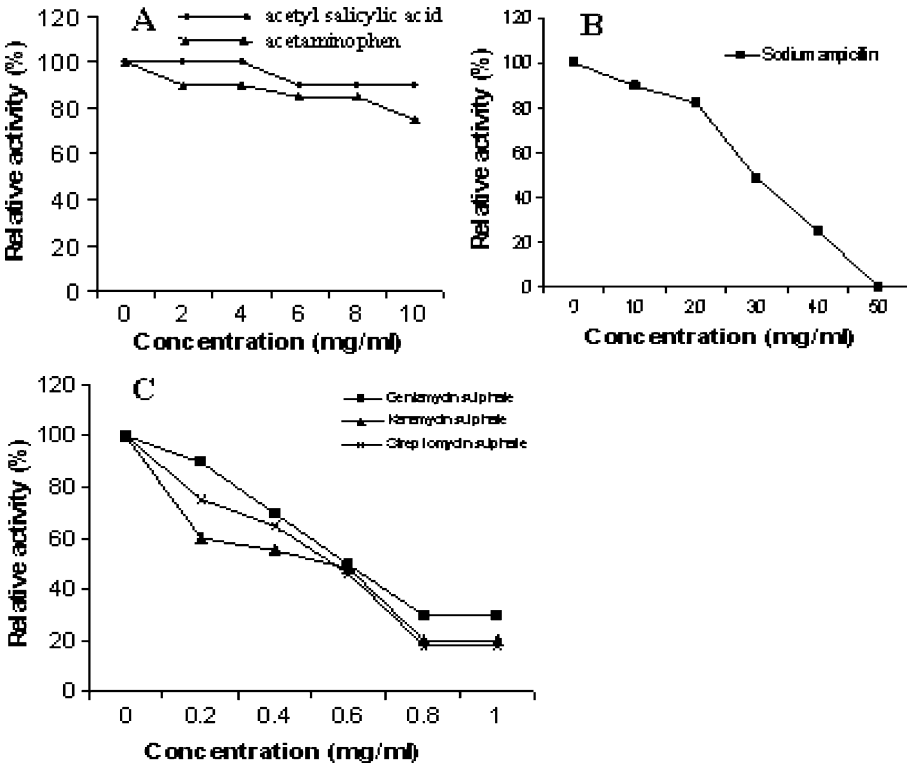

Fig. 8 Effects of medicines on purified PON1 enzyme activity (in vitro). **a** Acetylsalicylic acid (*circles*) and acetaminophen (*triangles*) slightly reduced the enzyme activity. **b** Sodium ampicillin (*squares*) reduced the 50% enzyme activity at 30 mg/ml. **c** Gentamicin sulfate (*squares*), kanamycin sulfate (*x marks*), and streptomycin sulfate (*triangles*) reduced the 50% enzyme activity at 0.6 mg/ml

PON1 from cholesterol-conjugated magnetic nanoparticles. The single spot of PON1 was obtained by Western blot of 2D-PAGE that confirmed the homogeneity of purified enzyme. The optimum pH of the paraoxonase 1 activity was found to be 8.0 which is similar to the previous reports [27]. A Lineweaver–Burk plot was made by using different concentrations of paraoxon and phenyl acetate (0.5 to 5 mM) at 37 °C, pH 8.0. The K_m values were 1.3 and 0.67 mM with paraoxon and phenyl acetate substrates, respectively, which is in close agreement with the reported values [13, 27].

Paraoxonase 1 activity was also studied in the presence of some metal ions and organic compounds due to their biological importance. Co^{2+} is used in diagnosis while Zn^{2+} is used in therapy as well as in the agricultural field. Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+} are used in metal alloy and are environmentally hazardous metals. The Triton X-100 and SDS are nonionic detergents, and PMSF is used as protease inhibitor. β-Mercaptoethanol and iodoacetate are used as reducing and oxidizing agents. Ammonium sulfate, ethanol, and acetone are used as protein precipitant. The optimal concentration of Mn^{2+} and Mg^{2+} ions that did not have any effect on paraoxonase 1 activity was 1.00 mM while 5% concentration of organic reagents was optimal to study the enzyme activity. It was observed that the enzyme activity was not inhibited by metal ions (Mn^{2+} and Mg^{2+}), glycerol, ammonium sulfate, PEG 6000, Triton X-100, and PMSF while Co^{2+}, Pb^{2+}, Zn^{2+}, ethanol, β-mercaptoethanol, and acetone moderately inhibit (70% to 30%) the enzyme activity. Cd^{2+}, Cu^{2+}, Ni^{2+}, SDS, DMF, DMSO, and iodoacetate totally inhibited the enzyme activity (Table 2).

Paraoxonase 1 not only protects from the oxidation of lipids conjugated with HDL and LDL but can also hydrolyze many pharmaceutical compounds. It is also reported that some diuretic and hypocholesterolemic drugs, such as spironolactone, mevastatin, lovastatin, and simvastatin, pravastatin, and prulifloxacin inhibit the PON1 activity [40–42]. So there is need to study the effect of some pharmaceutical medicines on paraoxonase 1 activity to understand its role during therapy. The analgesic (acetylsalicylic acid and acetaminophen) and amino glycoside antibiotics (gentamicin sulfate, kanamycin sulfate, and streptomycin sulfate) are commonly used in fever and during infections. In order to study the effect of these medicines, purified human serum paraoxonase 1 was incubated (in vitro) with medicines, and the enzyme activity was noted. It was observed that acetylsalicylic acid and acetaminophen slightly reduced the PON1 enzyme activity while ampicillin and amino glycoside antibiotics effectively reduced the PON1 enzyme activity at low concentration.

PON1 is polymorphic in nature as R and Q allozyme and is substrate dependent. The paraoxon and fenitroxon are hydrolyzed by the R allozyme while phenyl acetate is hydrolyzed by both allozymes. The other compounds such as diazoxon and the nerve gasses (soman and sarin) are hydrolyzed by Q allozyme [3]. The effect of macronutrients on PON1 enzyme level in human diet has not been studied, but in rodents it is reported that monounsaturated fatty acid increased the PON1 enzyme activity than saturated or highly polyunsaturated fatty acid [43]. An atherogenic diet and reused or degraded cooking oil decrease PON1 enzyme level activity in rabbit, mice, and human while polyphenols of tea and fruit juice increase the PON1 enzyme activity in both mice and humans [44, 45].

The purification strategy described here is simple, easy, less expensive, and less time consuming. A high yield of purified PON1 with optimal activity was obtained from small volume as compared to reported cumbersome methodology for purification of PON1. The study of effect of pharmaceutical medicines on PON1 enzyme activity may be helpful in antibiotic therapy. This is the first report about the new purification strategy which may be equally important like other purification procedures for PON1 enzyme.

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