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RESEARCH ARTICLE

Investigation of heavy metal effects on immobilized paraoxanase by glutaraldehyde

Çiğdem Bilen, Serap Beyaztaş, Oktay Arslan, and Özen Özensoy Güler*

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

Abstract
Serum paraoxonase 1 (PON1) was purified from bovine serum using hydrophobic interaction chromatography on Sepharose 4B-coupled L-tyrosine 1-naphthylamine gel, and monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Paraoxonase enzyme was immobilized using different ratios of glutaraldehyde and the maximum activity was observed with 7% glutaraldehyde. The effects of inhibition by Mn^{+2}, Co^{+2} and Cu^{+2} heavy metals on the immobilized and free enzyme activities were studied. At the optimum pH and temperature, the K_m and V_{max} kinetic values for bovine serum paraoxonase and immobilized paraoxonase towards paraoxon substrate were determined as 0.296×10^{-3} M & 37.04 EU vs. $0.727 – 10^{-3}$ M & 36.36 EU, respectively.

Keywords: Paraoxonase, enzyme immobilization, glutaraldehyde, enzyme inhibition

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Introduction

Human plasma contains four different esterases, butyrylcholinesterase (EC 3.1.1.8), paraoxonase (EC 3.1.8.1), albumin esterase, and acetylcholinesterase (EC 3.1.1.7), although the last binds to the membrane of erythrocytes, which contain additional cholinesterases[1] and in mammalian serum, butyrylcholinesterase, paraoxonase (PON), and albumin are in high enough concentrations to contribute significantly to ester hydrolysis. In addition to these esterases, only under *in vitro* conditions, the esterase activity of carbonic anhydrase (CA) was determined[2]. CA enzyme activity was assayed both with respect to its hydratase activity (i.e. hydration of CO_2)[3,4], and to its esterase activity (i.e. hydrolysis of an ester of p-nitrophenylacetate)

PON, a calcium-dependent antioxidant glycoprotein, is synthesized in the liver and secreted into the plasma[5]. PON is a polymorphic enzyme of many tissues as well as serum, where it is associated with high-density lipoprotein (HDL)[6]. PON's Ca^{2+} dependency suggests a model of metal-catalyzed hydrolysis, such as that proposed for phospholipase A_2, in which Ca^{2+} is thought to act as an electrophilic catalyst[7]. Purified arylesterase/PON is a glycoprotein with a minimal molecular weight of about 43,000 daltons. It has up to three sugar chains per molecule, and carbohydrate represents about 15.8% of the total weight. The enzyme has an isoelectric point of 5.1[8].

PON enzyme has detoxification and antioxidant features as well as hydrophobic character and capability of hydrogen peroxide hydrolysis[9]. PON acts as an antioxidant to prevent low-density lipoprotein (LDL) oxidation by hydrolyzing lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide. PON also renders HDL resistant to oxidation, thereby maintaining the capacity of HDL to induce reverse cholesterol transport[10]. The existence of two or more enzyme forms with PON activity has been reported in sheep, rabbit, human, rat, and bovine serum[11]. The N-terminal domain of PON is associated with apolipoprotein $ApoA_1$ of HDL. HDL paraoxonase retards the oxidation of LDL and is a major antiatherosclerotic component of HDL. HDL is present in the artery wall at a much higher concentration than

*Present address: Yildirim Beyazit University Faculty of Health Sciences, Head of Department of Nursing Etlik Ihtisas Hastanesi Karsısı 06100 Etlik/Ankara

Address for Correspondence: Dr. Özen Özensoy Güler, Head of Department of Nursing, Yildirim Beyazit University, Faculty of Health Sciences, Etlik Ihtisas Hastanesi Karsısı 06100, Etlik, Ankara, Turkey. E-mail: oozensoy@hotmail.com; ooguler@ybu.edu.tr

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Abbreviations

$ApoA_1$, apoenzyme A_1
BChE, butyrylcholinesterase
CHD, coronary heart disease
CPG, controlled pore glass
EC, enzyme code
EDTA, ethylenediaminetetraacetic acid
EU, enzyme unit
GA, glutaraldehyde
HDL, high-density lipoprotein
HIC, hydrophobic interaction chromatography
IC_{50}, 50% inhibitor concentration
LDL, low-density lipoprotein
PON, paraoxonase
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED, tetramethylethylenediamine
Tris-Base, tris (hydroxymethyl)-aminomethane

LDL and should, therefore, be ideally placed to protect LDL from oxidative modification[12]. The oxidation of LDL is centrally involved in the initiation and progression of atherosclerosis[6].

The expanded number of PON genes has important implications for future experiments designed to discover the individual functions, catalytic properties, and physiological roles of the PONs[13]. For example, the PON1 gene contains a number of functional polymorphisms in the coding and the promoter regions, which affect either the level or the substrate specificity of PON1[14], This difference is related to the position-192 polymorphism of the PON gene. The PON A-phenotype is associated with low PON activity and the PON B-phenotype with high PON activity[15]. The enzyme was originally characterized as an organophosphate hydrolase, thus its name derived from the commonly used substrate, paraoxon[16]. By hydrolyzing paraoxon, PON1 provides protection against exogenous organophosphate poisoning[17]. Moreover, PON1 status has relevance beyond the toxicology community, since low PON1 status has been associated with cardiovascular and other diseases. PON1 is also involved in the activation and inactivation of specific drugs and the inactivation of bacterial quorum-sensing factors[18].

The PON1 gene, as well as the other members of the gene family PON2 and PON3 (both of which also have antioxidant properties[19], and are 65 and 70% similar at the nucleotide level[20], are located on chromosome 7q21.3–q22.1[21] and linked with diabetic retinopathy. There are conflicting reports of an association between PON1 and Parkinson's disease[22], and also PON1 status (i.e. activity or concentration) is more closely related to coronary heart disease (CHD), and indeed, PON1 has been shown to be an independent risk factor for CHD in a prospective study, compared to the genetic polymorphisms[14]. For any given LDL concentration, the HDL-cholesterol concentration is inversely correlated with the risk of CHD and stroke[23].

PON has been shown to play an important role in lipid metabolism as an antioxidant–antiatherosclerotic molecule through (i) hydrolysis of active oxidized phospholipids (phospholipase A_2-like activity), (ii) destruction of platelet-activating factor, lipid hydroperoxides and H_2O_2 (peroxidase-like activity), (iii) reduction of monocyte chemotaxis and adhesion to endothelial cells, (iv) preservation of HDL integrity and function[13], and (v) protection of macrophages from oxidative stress and inhibition of macrophage cholesterol biosynthesis[24]. In diabetic patients, PON1 activity was shown to be decreased under oxidative stress conditions[25], so oxidative stress might be an important predictor of the development of complications in type 2 diabetic individuals[26]. Moreover, PON contributes to detoxification of several organophosphorus compounds, such as pesticides, neurotoxins and arylesters[27]. In terms of these properties of PON, purification and immobilization of PON is very important.

Materials and methods

Materials

Sepharose 4B, L-tyrosine, 1-naphthylamine, paraoxon, and all other chemicals were obtained from Merck (Milan, Italy).

Purification of PON1 enzyme from bovine serum

Ammonium sulfate precipitation

In order to purify PON from bovine serum, ammonium sulphate precipitation and hydrophobic interaction chromatography (HIC) methods were used. Ammonium sulfate has been the most widely used protein precipitant, because it has high solubility and is relatively inexpensive[28]. It is used early in a protein purification procedure to precipitate out contaminants. Bovine serum was centrifuged at 2208*g* for 15 min before precipitation with 60% ammonium sulfate to remove the bulk of contaminants. PON1 was then precipitated from the supernatant with 80% ammonium sulfate, and collected by centrifugation at 19,872*g* for 30 min.

Hydrophobic interaction chromatography

Sepharose-4B gel (10 mL) was washed with distilled water and then CNBr (4 g) was added to equal volumes of Sepharose 4B and water. The mixture was titrated to pH 11 with 4 M NaOH in an ice bath. The reaction was stopped by filtering the gel on a Buchner funnel and washing with cold 0.1 M $NaHCO_3$ pH 10 giving CNBr-activated Sepharose-4B. Tyrosine (15 mg) was added to the activated matrix in 0.1 M $NaHCO_3$ buffer, pH 10 and stirred with a magnet for 90 min. After this, the suspension was kept at 4°C for 16 h, and then washed with distilled water

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followed by 100 mL of 0.2 M $NaHCO_3$ (pH 8.8). The hydrophobic gel was then completed by diazotization of 40 mL of the Sepharose-4B-L-tyrosine with 1-naphthylamine. The pH was adjusted to 9.5 with 1 M NaOH and, after gentle stirring for 3 h at room temperature, the coupled red Sepharose derivative was washed with 1 L of water and then 200 mL of 0.01 M Na_2HPO_4 (pH 6.0)[29,30].

Hydrophobic interaction chromatography (HIC) is ideal as the next step, as the protein of interest is present in the supernatant at a high ammonium sulphate concentration, and the sample can be directly applied to the HIC column. Purification occurs concomitant with a reduction in volume[31]. HIC is a technique used to separate peptides, proteins, and other biological molecules based on their degree of hydrophobicity[32]. HIC requires a minimum of sample pre-treatment and can thus be used effectively in combination with traditional protein precipitation techniques. Protein binding to HIC adsorbents is promoted by moderately high concentrations of anti-chaotropic salts (e.g. ammonium sulphate), which also have a stabilizing influence on protein structure. Elution is achieved by a linear or stepwise decrease in the concentration of salt in the adsorption buffer. Recoveries are often very satisfactory[28].

The hydrophobic interaction column was equilibrated with 0.1 M Tris-HCl pH 8.0 including 1 M $(NH_4)_2SO_4$. A linear, decreasing, salt gradient was applied from 1 M to zero $(NH_4)_2SO_4$ in 0.1 M Tris-HCl pH 8.0. Fractions of 1.5 mL were collected in eppendorf tubes and was followed for 2 min at 37°C at 412 nm in CARY 1E, UV-Visible Spectrophotometer[29].

A large number of techniques and supports are now available for the immobilization of enzymes or cells onto a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme and the substrate, and its ultimate application. In this study, a cross-linking immobilization method is applied for paraoxanase enzyme using glutaraldehyde as the cross-linker.

Cross-linked enzyme aggregates were constituted between free enzyme molecules and glutaraldehyde (GA), which is a homo cross-link reagent (Figure 1). Because GA is bivalent, it can cross-link soluble proteins into insoluble aggregates comprised of many protein molecules. The advantages of this method include simplicity, ease of use with no carrier required, and cheapness. Glutaraldehyde at concentrations varying from 1–10% (w/w), relative to protein, was added to different portions of free enzyme molecules. The mixtures of GA and PON were incubated with shaking at 1 g, 25°C to form cross-linked aggregates. At first, the free enzyme was colourless, but with time the immobilized enzyme turned brown. After 24 h, the immobilized PON was centrifuged at 2208*g* (4°C, 5 min.) and recovered in the solid phase, which was then reconstituted in 100 mM Tris-HCl buffer, included 2 mM $CaCl_2$. Enzyme activities of free and immobilized enzymes were compared by spectrophotometric assay.

Paraoxonase enzyme activity method

PON activity towards paraoxon, either as a free enzyme or cross-linked by GA, was determined spectrophotometrically at 412 nm[33] using 850 μL 100 mM Tris-HCl, pH 8 + 100 μL 2 mM paraoxon, 2 mM $CaCl_2$ + 100 μL enzyme solution. The assay components were mixed at 37°C and the change at absorbance during a minute was read by spectrophotomoter, as the paraoxon was converted to p-nitrophenol. Enzyme units were calculated from the absorbance change according to the formula below[29,30]:

$$EU = \frac{\text{Reaction volume (mL)} \times dA/dT \times 1000}{\in_{412} \times \text{Enzyme volume (mL)} \times d \text{ (cm)}}$$

Comparison of IC_{50} values for different heavy metal inhibitors of PON activity

Many functional assays seek the total inhibitor concentration that reduces these activities by 50% (i.e. IC_{50})[34,35]. The heavy metal ions Mn^{+2}, Co^{+2} and Cu^{+2} were tested over a range of concentrations using the spectrophotometric assay at 412 nm to determine their respective IC_{50} values upon PON activity.

Results and discussion

For this study, PON1 was purified from bovine serum on Sepharose 4B-coupled L-tyrosine 1-naphthylamine gel (Figure 1), and monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 2). Paraoxonase enzyme was immobilized using different ratios of glutaraldehyde and the maximum activity was observed with 7% glutaraldehyde (Figure 3).

PON was subsequently immobilized by cross-linking into protein aggregates using GA, which reacts with

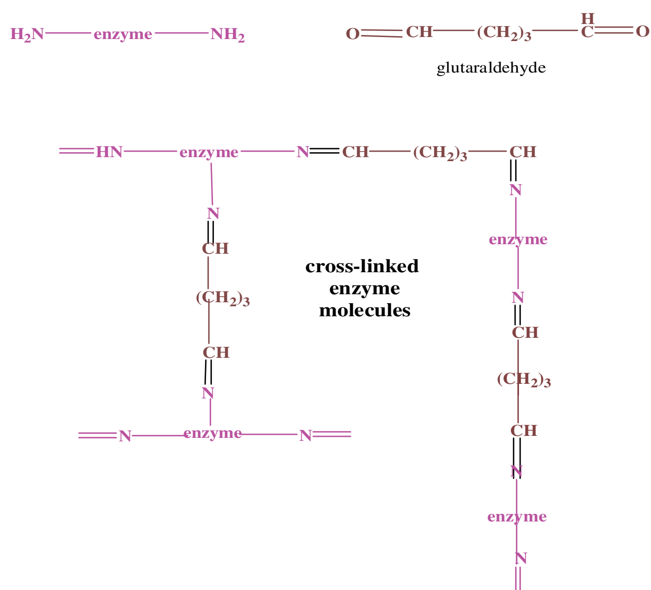

Figure 1. Immobilization of paraoxonase with glutaraldehyde.

several functional groups of proteins, such as amine, thiol, phenol, and imidazole groups[36] and maximum activity was observed at the ratio of cross-linker concentration 7% GA. Consequently, this ratio was used in all subsequent assays. Glutaraldehyde has been previously and successfully used for various other enzymes. For instance, trypsin immobilization with GA, either by covalent attachment to aminopropyl controlled pore glass (CPG) particles, or by cross-linking of the enzyme in solution, gave a product that showed an increase in the apparent Michaelis constant, K_m, app (i.e. a decrease in enzyme-substrate affinity) relative to free trypsin. This effect was more pronounced for the GA-crosslinked trypsin than for the CPG-coupled trypsin[37]. Similarly, in another study, the *Bacillus licheniformis* ATCC 21415 alkaline protease was immobilized by covalent binding through a spacer group of GA giving a good loading efficiency and immobilization yield. Summary of the activity of bovine serum PON1 immobilized using different concentrations of GA is seen in Table 1.

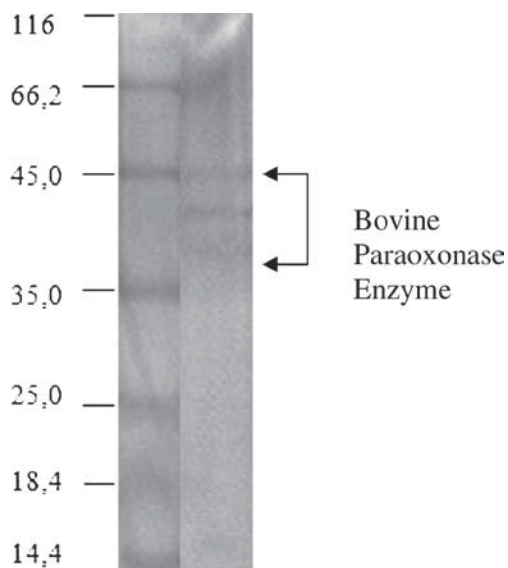

Figure 2. Purification of bovine PON monitored by SDS-PAGE. The pooled fractions from ammonium sulfate precipitation and hydrophobic interaction chromatography were analyzed by SDS-PAGE (10% separating gel and 3% stacking gel) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 1 contained 3 µg of various molecular-mass standards: Bovine serum albumin (66.7 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa). Thirty microgram of purified bovine serum PON (lane 2) migrated as a series of three bands with mobilities corresponding to apparent masses of between 38–45 kDa.

This good loading efficiency for the immobilization by covalent binding might have been due to the formation of stable cross-linking between the carrier and the enzyme through a spacer group;[38] but, in the present study bovine serum paraoxonase enzyme was immobilized with glutaraldehyde without any carrier or a spacer group.

In a previously published study, EDTA and also barium, lanthanum and copper compounds gave rise to competitive inhibition of PON, whereas zinc ions showed noncompetetive inhibition[39]. Later studies also demonstrated that some metal ions (cobalt, nickel, cadmium, etc.) displayed an inhibitory effect upon human serum PON1[40,41]. Here, we specifically investigated and compared the activities of Co^{+2}, Mn^{+2}, and Cu^{+2} upon both free and immobilized PON activity. By comparison, Cu^{+2} was a significantly less effective inhibitor of the immobilized enzyme: IC_{50} value of 0.171×10^{-3} M (Figure 4). With free enzyme, the observed IC_{50} values were 0.286×10^{-3} M for Co^{+2}, 0.129×10^{-3} M for Mn^{+2} and 0.031×10^{-3} M for Cu^{+2} (Figure 5), respectively, making Cu^{+2} the most effective inhibitor. In contrast, Co^{+2} and Mn^{+2} proved to be activators of the immobilized enzyme. The comparison of $CoCl_2$, $MnCl_2$ and CuCl heavy metals effect upon the immobilized paraoxonase and free paraoxonase enzyme activities are seen in Figures 4 and 5. In literature, Ekinci and Beydemir also investigated the *in vitro* effects of some heavy metals (Pb, Cr, Fe, and Zn) on the only purified human serum

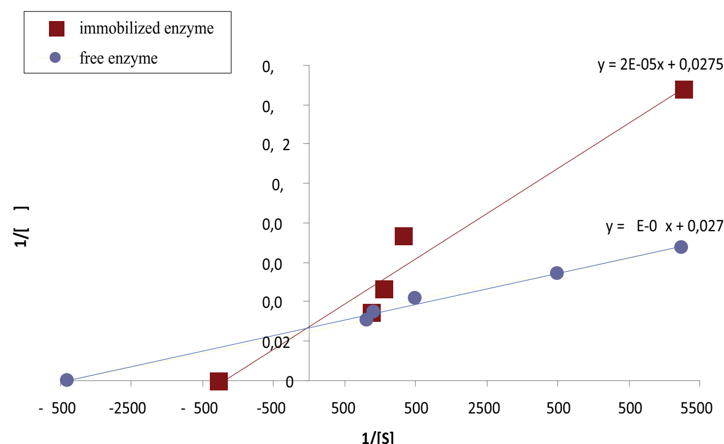

Figure 3. Graph comparing the enzyme activities of free PON and 7% GA-immobilized PON.

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PON1. In that study, Pb^{+2} was found to be more effective inhibitor on purified human serum PON1. IC_{50} value was 0.838 mM[42].

Catalytic efficiencies (V_{max}/K_m) and K_{cat}/K_m ranges were compared for immobilized and free enzymes as shown in Table 2. As the V_{max} value of immobilized PON was slightly lower than that of free PON, the affinity of the immobilized PON for the substrate was lower than that for free PON.

Conclusions

Bovine paraoxonase enzyme activity was studied as free and immobilized enzyme. The latter was obtained by cross-linking with glutaraldehyde through enzyme amino groups (as seen in Figure 1) to form cross-linked enzyme aggregates. Among the advantages of this approach are: (i) an easy immobilization procedure, (ii) high immobilization capacity, and (iii) the substrate

Table 1. Summary of the activity of bovine serum PON1 immobilized using different concentrations of glutaraldehyde.

Glutaraldehyde/free enzyme (weight %)	1	2	3	4	5	6	7	8	9	10
Solid phase $\lvert A_2 - A_1 \rvert$	0.071	0.003	0.044	0.021	0.004	0.018	0.078	0.002	0.012	0.030
(GA + free enzyme) V (mL)	1.0094	1.0187	1.0283	1.0377	1.0479	1.0582	1.0685	1.0781	1.0884	1.0979
Immobilized enzyme activity EU (U/mL)	43.59	1.84	27.02	12.89	2.45	11.05	47.89	1.23	7.37	18.42
Total activity (U)	43.99	1.87	27.78	13.38	2.57	11.69	51.17	1.33	8.02	20.22
Specific activity (U/mg)	43.21	1.837	26.71	12.73	2.411	10.73	46.09	1.192	7.078	17.74

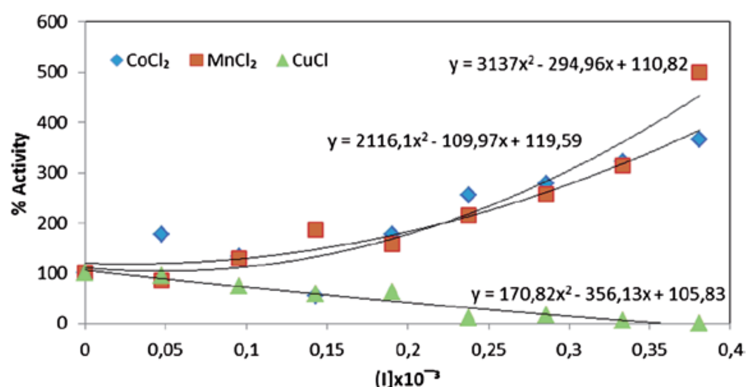

Figure 4. Comparison of $CoCl_2$, $MnCl_2$ and CuCl heavy metals effect upon the immobilized paraoxonase enzyme activity.

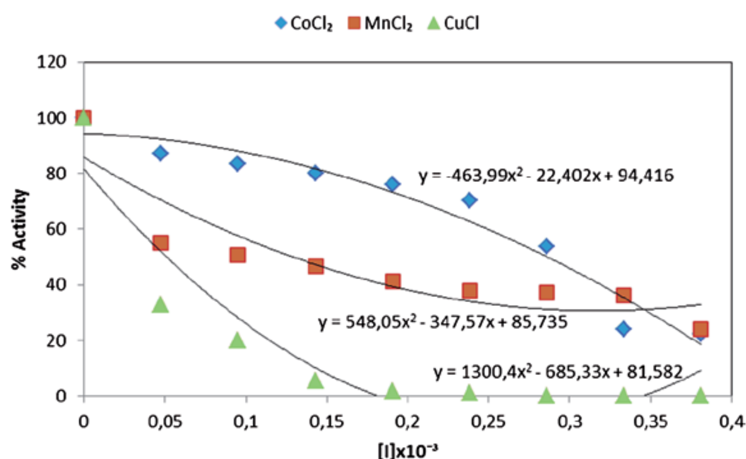

Figure 5. Comparison of $CoCl_2$, $MnCl_2$ and CuCl heavy metals effects upon the free paraoxonase enzyme activity.

Table 2. Influence of immobilization process on kinetic constants.

Derivative	V_{max} (EU)	K_m (mM)	Catalytic efficiency V_{max}/K_m (10^3 EU/M)	K_{cat} (V_{max}/E_t) (EU/mg)	K_{cat}/K_m (EU/M × mg)
Free enzyme	37.04	0.296	125	33.369	1.127×10^5
Immobilized enzyme	36.36	0.727	50	30.743	0.423×10^5

affinity of the immobilized enzyme was lower than for the free enzyme.

In addition, our experiments have shown that the bovine serum paraoxonase, when immobilized using glutaraldehyde, demonstrates different activation/inhibition properties in the presence of some heavy metals. From this point of view, the benefit of paraoxonase immobilization with glutaraldehyde is that it provides simplicity in stabilization of enzyme, being quick and low cost. The increased activity of the immobilized paraoxonase, together with its greater tolerance for heavy metals, compared to the free enzyme, is also advantageous, and could prove beneficial in potential biotechnological and pharmaceutical applications, such as a new approach for the treatment of organophosphorus poisoning, or as a candidate catalytic bioscavenger.

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Declaration of interest

The authors report no conflicts of interest.

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