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The Effects of Some Antioxidant Compounds and Antilipid Drugs on the Immobilized of hPON1 Activity

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In our work, hPON1 enzyme was purified from human serum using the Three Phase Partitioning (TPP) and it was immobilized on functionalized ferric magnetic chitosan nanoparticles (MC-NPs) by the covalent binding method. The optimized amount for parameters of enzyme units (EU)/mg MC-NPs, reaction time, pH and temperature were determined to be 230(EU)/mg, 30 min, 8.0, and 37°C respectively. The amount of immobilization yield according to the enzyme activity was obtained to be 88% and also the amount of immobilized enzyme on MC-NPs was 0.23 EU/mg. Stability studies showed significant increase in immobilized enzyme stability at 4, 25 and 37°C. The stability of Immobilized enzyme showed a 6.4-fold increase in comparison to free enzyme at 37°C. The results demonstrated that the pH stability of the immobilized enzyme significantly increased in comparison with free enzyme. The immobilized enzyme was usable and recoverable for ten cycles. The results depicted that 85% of enzyme activity was retained after fifth cycle. SEM, XRD and FTIR test showed the covalent binding of enzyme to magnetic nanoparticles' surface. In the last stage of the study; the effects of some antilipid drugs (Valeric acid, phenoxy – isobutyric acid, N-desmethyl rosuvastatin) and some antioxidant compounds (Gallic acid, Quercitin, Pyrogallol, Ascorbic acid) on the activity of immobilized hPON1 enzyme were investigated. Inhibition kinetics (IC_{50} and Ki values) was determined.

Keywords: Magnetic chitosan nanoparticles. Immobilization. Human Paraoxonase 1 (hPON1). Inhibition kinetics

INTRODUCTION

Paraoxonase enzymes are Ca^{2+}-dependent plasma esterases which have both arylesterase (E.C. 3.1.1.2) and paraoxonase (E.C. 3.1.8.1) activities. Paraoxonase gene family have 3 members: PON1, PON2, and PON3 (Ceron et al. 2014). Mackness and colleagues had first demonstrated that human serum PON1 enzyme was on HDL (Mackness, et al., 1996). It has been discovered that there was a relationship between PON1 enzyme and high-density lipoproteins (HDL) in 1946 by Mazur *et al* (Mazur, 1946).

PON1 enzyme is a glycoprotein which has 354 amino acids and is synthesized in liver. It is connected to the HDL lipoproteins in blood circulation so the anti-atherogenic properties of HDL have been attributed to the PON1 enzyme. This enzyme stimulates macrophage cholesterol flow through HDL while reducing the cellular oxidative stress of macrophages and the biosynthesis ratio of cholesterol. Thus, foam cell formation and protection against atherogenesis are provided. The PON1 enzyme hydrolyzes the homocysteine thiolactone to prevent homocysteine damage of some proteins. The only free sulfhydryl group of PON1 is present in Cys284 and this amino acid also plays an active role in the activity of the enzyme (Naik and

Sankarshana 2015).

The reduction of PON1 enzyme level on blood causes a great risk to many metabolic disorders such as cardiovascular diseases, diabetes mellitus, chronic diseases, renal failure, rheumatoid arthritis, hyperthyroidism and aging. For this reason, a good characterization of PON1 enzyme inhibitors and activators is a good starting point for drug trials.

Investigations on the PON1 multigene family have gained the momentum over the past decade and researches related to two new members, PON2 and PON3 enzymes, have also increased. PON1 enzyme is used for the specific preservative against diseases related to the oxidative stress, inflammation, and liver diseases (Ceron et al. 2014).

The immobilization processes of enzymes lead to the development of economically efficient and biological continuous processes. The large surface area to volume ratio of a nanoparticle allows it to serve as an effective carrier of biomolecules.

The unique physical properties of nanoparticles allow for biomedical drug design (Atanasijevic et al. 2006; Demir et al. 2014; Demir et al. 2004, 2008; Ito et al. 2005; Lin et al. 2007) and biosensor formation (Katz and Willner 2004). It is useful for applications that require manipulation of magnetic nanoparticles (MNPs) with an external magnetic field. Such particles do not retain any residual magnetism after the magnetic field has been removed (Ito et al. 2005; Soleimani et al., 2017). The use of magnetic nanoparticles and enzyme immobilization in biological applications is considered to be one of the new immobilization methods. MN-NPs have found many applications in various processes due to their high specific surface area ratios, magnetic properties and special properties (Wang et al., 2013).

In this study, we first purified the hPON1 enzyme using the TPP method and developed a new and simple process for the preparation of magnetic chitosan nanoparticles.

The main innovation of our Fe_3O_4/CS nanocomposites, compared to previous reports, mainly includes the following points:

(1) Our high magnetic susceptibility Fe_3O_4/CS nanocomposites facilitate the separation of chitosan.

(2) Fe_3O_4/CS carriers have pH-dependent hydrophobic / hydrophilic properties which can alter the size of the chitosan by changing the pH value. The chitosan crustal structure yields the attractive biomedical properties of chitosan and has a pH-dependent property and stability of magnetic chitosan carriers.

(3)The production of the supports is appropriate and green synthesis and there is no need for any sorting procedure during support preparation.

(4)Carriers may be regenerated after the immobilized enzyme has lost its activity completely after repeated use.

(5)Fixing enzymes on external surfaces of non-porous supports can overcome the diffusion limitation (Yu et al. 2006; Liu et al. 2009).

The advantages of immobilized enzymes are ease of use of immobilized enzymes, ease of separation of enzymes from the reaction mixture, reusability, low cost, temperature and pH stability. Because of these advantages, immobilized enzymes are regarded as commercial products. In recent times, nanoparticles have been used extensively for enzyme immobilization due to their small size and high surface area (Ansari and Husain 2012).

MATERIALS AND METHODS

Chemicals

Human serum, paraoxone ($C_{10}H_{14}NO_6P$), *n*-butanol, ammonium sulfate ($(NH_4)_2SO_4$), calcium chloride ($CaCl_2$), phenyl acetate, glutaraldehyde, sodium acetate, sodium carbonate (Na_2CO_3), sodium phosphate (Na_3PO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl), Coomassie Brilliant blue R250, glycine, iron (III) oxide NPs (Fe_3O_4), chitosan were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Purification of PON1 Enzyme

Human blood was collected from Blood Centre, Ataturk University. Blood samples were stored at +4 ^oC until use and then, were centrifuged at 4000 x*g* for 10 min to separate serum. The obtained serum was used to purify PON1 enzyme *via* the three-phase separation method. (Demir et al., 2004; Demir et al., 2008; Gagaoua and Hafid 2016)

For this purpose, after determining the *n*-butanol ratio in the first stage, the highest activity value was determined by optimizing ammonium sulfate in the second stage. 10 ml of serum was added to 4 beakers to determine the *n*-butanol ratio, and *n*-butanol was added at ratios of 1:0.5, 1:1, 1:1.5 and 1:2, 4 g of ammonium sulfate $(NH_4)_2SO_4$ was then added to each reaction mixture medium. In the second step, ammonium

sulfate optimization was made by using determined concentration of *n*-butanol. According to this, 10 ml of serum and 5 ml of *n*-butanol were added to different beakers. Ammonium sulfate content was added to the ratios by 20%, 40%, 60%, 80% (w/v) according to the volumes of serum. Then, the second ammonium sulfate precipitation was repeated at 25%, 30%, 35%, 40%, 45%, and 50% ratios (Onem et al., 2016; Onem and Nadaroglu 2018).

Protein determination

Concentration of protein amount of PON1 enzyme was determined *via* Bradford method. In this method, 100 µL of enzyme was added to 5 ml of Coomassie brilliant solution and incubation at room temperature for 10 minutes. After the mixture was vortexed, the change in the absorbance of the medium was read in the spectrophotometry at 595 nm (Bradford MM 1976).

Enzyme Activity Determination

PON1 Activity

Paraoxone was used for PON1 enzyme activity and phenyl acetate as a substrate was used for the hydrolysis of aryl esters. The activity of PON1 enzyme was determined spectrophotometrically. 0.1 ml of enzyme, previously prepared 0.4 ml of buffer (50 mM tris/HCl pH:8.0 containing 1 mM $CaCl_2$) and 0.5 ml paraoxone were added to the medium and incubated at 37°C for 10 minutes. The absorbance change of the medium was read spectrophotometrically at 412 nm (Renault et al. 2006).

Determination of the Molecular Weight of the PON1 Enzyme by SDS-PAGE Method

10% SDS-PAGE polyacrylamide gel electrophoresis was used to the molecular weight and size of the purified PON1 enzyme (Laemmli 1970).

Preparation of Iron-Magnetic Chitosan Nanoparticles

For preparation of magnetic chitosan NPs, 2 g of chitosan (2 mg/mL) was completely dissolved in acetic acid (1%, v/v) solution and it was stirred at 200 x*g* for 24 hours with solved Fe_3O_4 NPs (1.54 g) in 100 mL of pure water. And then, 5 mL of 1 M NaOH solution was added to the mixture. 5 mL of L-glutaraldehyde (1%) was added to the medium. After the reaction, the magnetic chitosan NPs were repeatedly washed with pure water and then dried at 35°C (Colak et al., 2012; Nadaroglu and Onem, 2014; Nadaroglu and Sonmez 2016; Soleymeni et al., 2017).

hPON1 Enzyme Immobilization on Fe_3O_4-chitosan NPs

Purified PON1 enzyme, Glycine buffer, pH:10.0 and solved Fe_3O_4-chitosan NP were added to tube. This mixture was kept in the ultrasonic bath at 30°C for 1 h. All reaction steps were illustrated in the Figure 1. After covalent attachment of the enzyme onto the iron-chitosan, optimum pH, temperature and time values were determined (Onem et al., 2016; Nadaroglu and Onem 2014; Onem and Nadaroglu 2017; Nadaroglu and Sonmez 2016; Soleymani et al., 2017).

Characterization of Immobilized PON1 Structures

For the characterization of magnetic PON1 immobilized structures, SEM and FT-IR analyzes were performed.

For the SEM analysis of immobilized PON1 enzyme structures, Zeiss brand Sigma 300 model scanning electron microscope. Bruker VERTEX 70v was used for FT-IR analysis. All analyzes were performed to determine the surface and structural characterization of the nanoparticles (Zhao et al., 2013).

***In Vitro* Investigation of the Effects of Some Antioxidant Compounds on MC-PON1 Enzyme Activity**

The effects of some antioxidant compounds (Gallic acid, Quercetin, Pyrogallol and Ascorbic Acid) and antilipid drugs (Valerian acid, Phenoxy-zobutyric acid, N-desmethyl rosuvastatin) on free and immobilized PON1 enzyme activities were investigated *in vitro*. For this purpose; concentrated stock solutions of the determined antioxidants were prepared. For this purpose, reactions were made at 5 different drug concentrations. Activities according to the control reaction without inhibitor were calculated. Blind experiment; prepared by adding pure water instead of enzyme.

After incubation at 37 °C for 15 min, the absorbance changes were recorded at 412 nm and PON1 enzyme activities were determined. According to the data obtained, Activity-Inhibitor % versus concentration graphs were calculated for all three drugs and the IC_{50} values from the

curve in the graphs were calculated (Demir et al., 2011).

RESULTS

In order to determine the best Three Phase Partitioning (TPP) system for the purification of the PON1 enzyme, the various process parameters including the amount of $(NH_4)_2SO_4$ for precipitation, the t-butanol ratio of the crude extract and the pH are also optimized to obtain the highest purity and yield (I: Ammonium sulphate (%60-%80); II. n-Butanol (1:0,5); III: 1.Ammonium sulphate (20%) (Table 1).

After selecting $(NH_4)_2SO_4$ saturation, the ratio of the volume of the crude extract to the t-butanone, which is also very important in TPP, has been optimized. Tetr-Butanol was chosen as the organic co-solvent to separate the studied PON1 enzyme in TPP because it was generally reported to give the best results (Gagaoua and Hafid 2016; Kiss 1998; Mondal et al., 2006).

PON1 enzyme was purified from human serum using ammonium sulfate precipitation (60-80%) and the Three-phase partitioning (TPP) method. The obtained results, activity, percent yield, purification fold number values were calculated and the findings were shown at Table 1.

The highest activity (372.32 EU/ml) was found in rate 1:0.5 serum: butanol as seen Figure 2(A). The lowest activity value was seen at 1:2 serum: butanol rate (290.86 EU/ml). The highest PON1 activity was obtained with 20% (313.82 EU/ml) ammonium sulphate saturation compared Figures 2(B) and 2(C). The lowest activity value was obtained in 75% (170 EU/ml) ammonium sulphate saturation in Figure 2.

TPP was used to purify the enzyme and proteins with high recovery and purity levels were obtained. For example, invertase (Akardere 2010), mannanase (Nadaroglu and Sonmez 2016), α-galactosidase (Dhananjay and Mulimani 2009), phytase (Onem and Nadaroglu 2018; Onem and Nadaroglu 2014), lipase (Soleymeni et al., 2017) and many other widely used to purify the substance. All these articles have shown that TPP is an attractive process for primary purification of enzymes compared to conventional chromatographic methods (Nadaroğlu and Dikbas 2018).

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed to determine the subunit number of PON1 enzyme. Figure 3 showed SDS PAGE analysis on 10% polyacrylamide gel for PON1 enzyme purified by TPP from human serum according to Laemmli (Laemmli 1970). The single band in the TPP treated fractions showed that purification took place. At the end of the SDS PAGE analysis, the purified enzyme showed a single band with a molecular weight of 45 kDa. In addition, the molecular weight of PON1 enzyme purified by gel filtration chromatography was found 45 kDa.

Results of Optimum pH and Temperature for Free and immobilized PON1 Enzymes

The effect of temperature on pure PON1 activity (Figure 4A) was determined spectrophotometrically using paraoxone as the substrate. The optimum activity of the purified enzyme was observed at 37 °C (Figure 4A).

The effect of pH (Figure 4B) on pure PON1 activity was also determined spectrophotometrically. As shown in Figure 4B, the optimum pH of the enzyme was found to be 8.0 with paraoxone as the substrate.

Results of V_{max} and K_M of PON1 Enzyme

The kinetic parameters Km and Vmax for hPON1 were found as 0.94 mM and 110.01 μmol/L.min, respectively, by using Lineweaver–Burk plots. The Lineweaver-Burk graphs of free and nano flower hybrid PON1 enzymes belonged to paraoxone and phenyl acetate substrates were drawn. For this purpose, activity measurements were made at 5 different substrate concentrations and Lineweaver-Burk graphs were drawn by plotting the obtained values 1/S versus 1/V. K_M and V_{max} values were calculated *via* these graphs. The V_{max} and K_M values of immobilized (IMB) PON1 enzyme were 8.65 and 0.215 by using phenyl acetate substrate, respectively. The V_{max} and K_M values of immobilized PON1 enzyme were 2.88 and 0218 by using paraoxon substrate, respectively. It was found that the immobilized PON1 enzyme exhibited more activity against the paraoxone and phenyl acetate substrates compared to the free PON1 enzymes. Data are presented in Table 2.

As shown in Table 2, the Vmax and K_M values of immobilized magnetic chitosan NPs of the PON1 enzyme did not change compared to the free PON1 enzyme. However, for both substrates, the Vmax values of the immobilized PON1 enzyme were found to be increased and the K_M was decreased. It is clear that the immobilized PON1 enzyme has increased affinity for both paraoxone and phenyl acetate substrates. It is

possible to say that the increase in Vmax is due to the fact that the IMB-PON1 enzyme is more stable than the free structure.

Table 1: Purified PON1 enzyme profile

	Volume (mL)	Activity (EU/mL)	Total Activity	%	Protein (mg protein /mL)	Specific Activity (EU/mL)	Purification coefficient
Serum	50	379.9±0.05	18999±6.4	100	1318±5.05	0.288	---
Ammonium Sulphate (%60-%80)	15	329.6±0.11	4944±1.23	86.7	568±2.14	0.58	2.01
n-Butanol (1:0,5)	15	320.3±0.45	4804.5±2.41	84.3	236±2.74	1.35	4.68
2. Ammonium sulphate (20%)	15	307.8±1.21	4617±6.12	81	41.8±1.78	7.36	25.56

Table 2: Vmax and KM values of free and immobilized PON1 enzymes for phenyl acetate and Paraokson substrates

	Phenyl acetate		Paraoxon	
	Vmax (μmol/Lmin)	K_M (mM)	Vmax (μmol/Lmin)	K_M (mM)
Free PON1	8.36	0.233	1.77	0.232
IMB hPON1	8.65	0.215	2.88	0.218

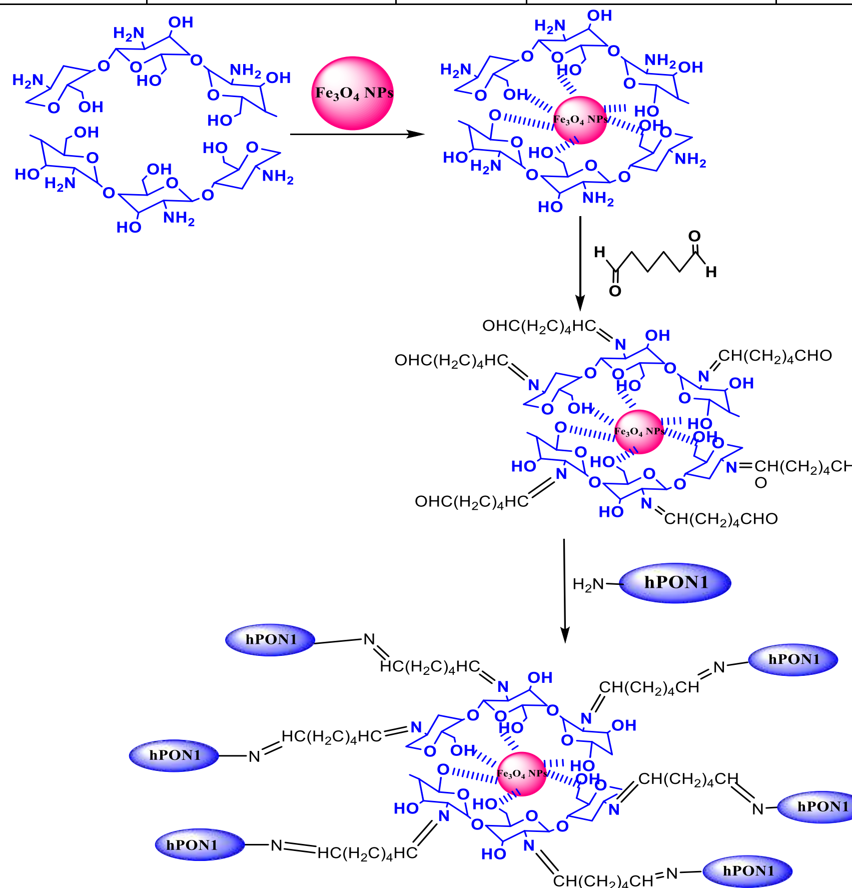

Figure 1: A schematic showing the formation mechanism of immobilized of hPON1 enzyme to the CS-coated Fe_3O_4 NPs by the covalently.

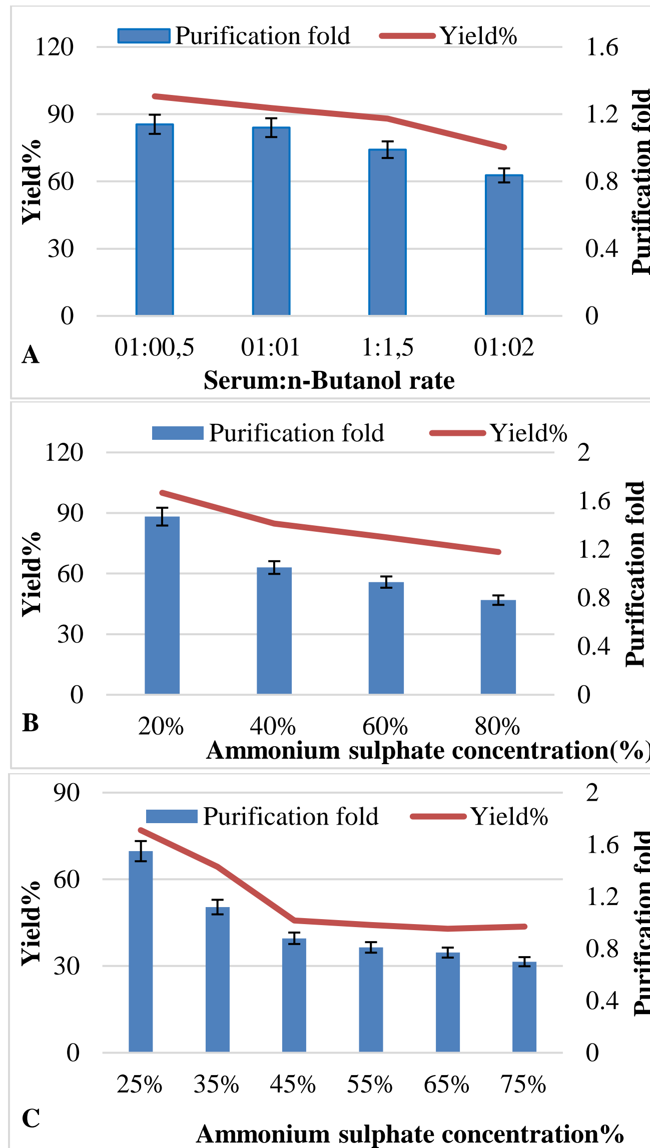

Figure 2: N- butanol optimization (A), First Ammonium sulfate optimization (B), Second Ammonium sulfate optimization (C)

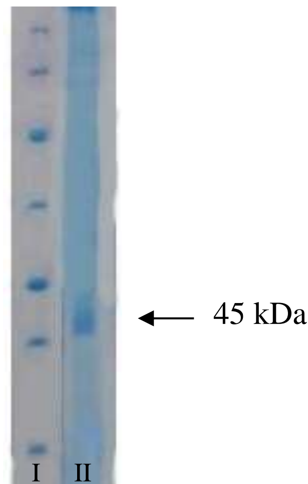

Figure 3: Paraoxonase enzymes SDS-page gel electrophoresis photography
(I: Standard protein: 30kDa, 40kDa, 50kDa, 100kDa, 130kDa, 150kDa, 250kDa : Carbonic anhydrase; 40 kDa: II: Purified PON1 from human serum

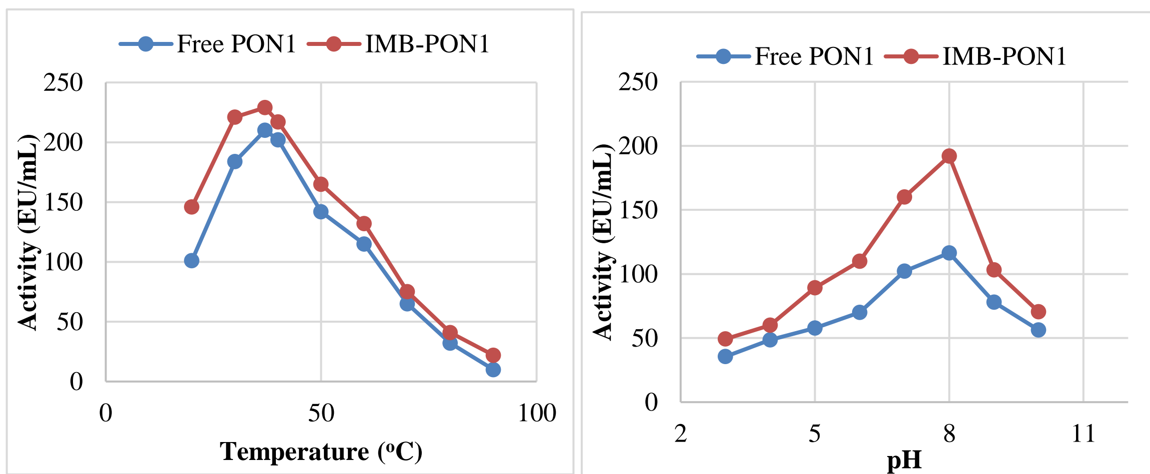

Figure 4: The effect of pH (A) and temperature (B) on free and immobilized PON1 enzyme

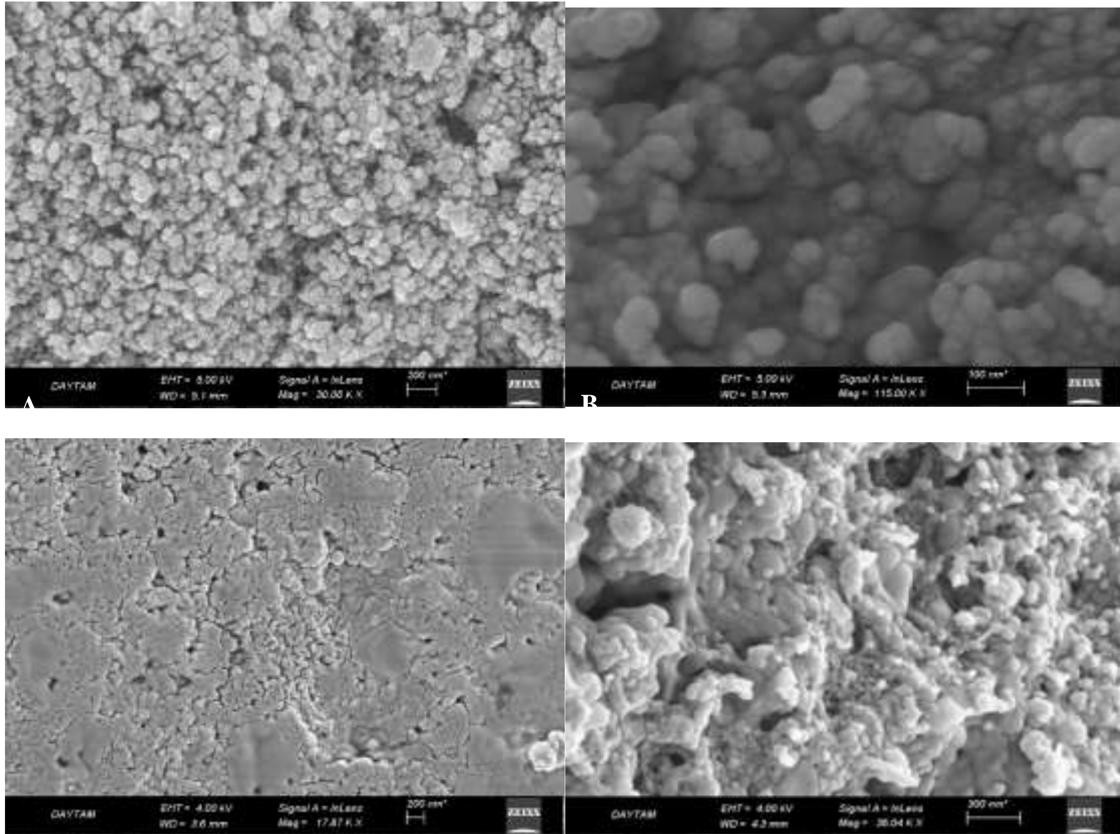

Figure 5: SEM images of Fe_3O_4- chitosan NP structure (A and B) and the Fe_3O_4-chitosan NPs immobilized by PON1enzyme

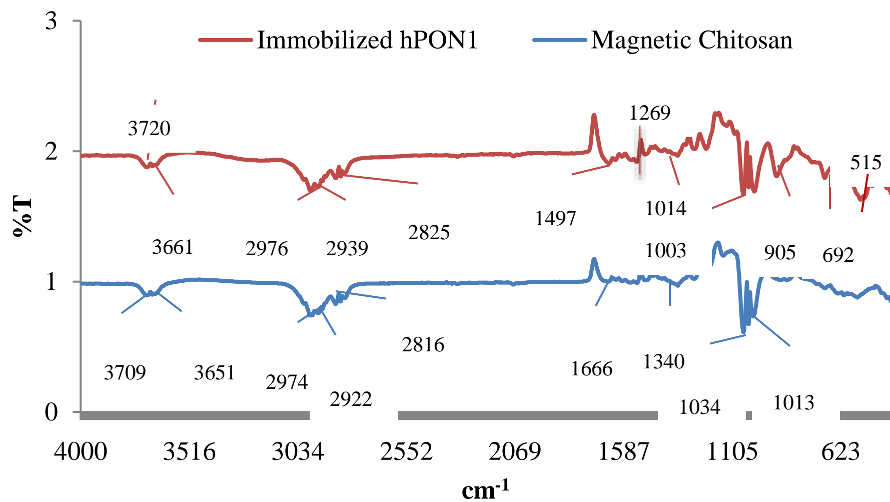

Figure 6: FTIR patterns of magnetic chitosan and immobilized-PON1 enzyme

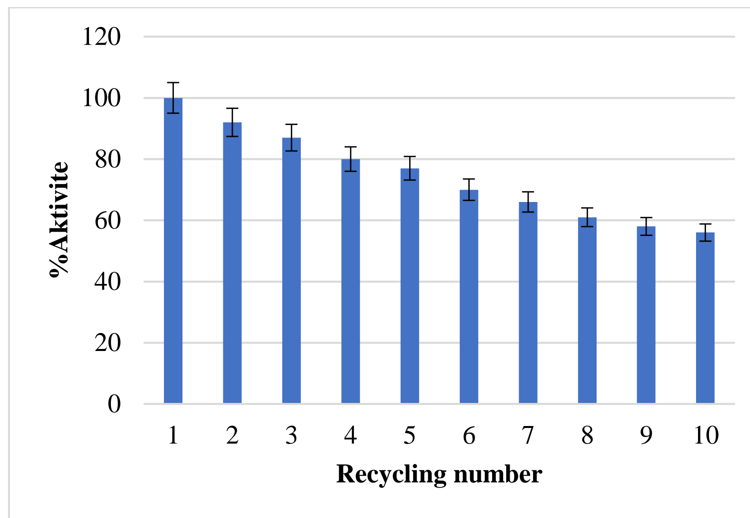

Fıgure 7: Re-usability stability graph of immobilized paraoxonase on Fe_3O_4-Chitosan NPs

Table 3. The results of *IC_{50}* and Ki values of free and IMB-PON1 enzymes and types of inhibition

Antioxidand compounds	*IC_{50}* (mM)		K_i		K_i Average		Type of Inhibition
	Free PON1	IMB PON1	Free PON1	IMB PON1	Free PON1	IMB PON1	
Gallic acid	0.682	0.771	0.278	0.558	0.407	0.516	Competitive
	0.582	0.671	0.334	0.671			
	0.482	0.571	0.61	0.319			
Quercetin	0.722	0.831	0.16	0.278	0.143	0.22	Competitive
	0.622	0.731	0.214	0.393			
	0.522	0.631	0.408	0.568			
Pyrogallol	0.577	0.706	0.297	0.950	0.444	1	Semi-Competitive
	0.477	0.606	0.925	1.16			
	0.377	0.506	0.111	0.9			
Ascorbic acid	0.623	0.714	0.769	1.89	0.454	2.71	Non-competitive
	0.523	0.614	0.371	2.08			
	0.423	0.514	0.222	4.17			
Antilipid Drug	**IC_{50} (mM)**		**Ki**		**Ki Average**		**Type of Inhibition**
	Free PON1	IMB PON1	Free PON1	IMB PON1	Free PON1	IMB PON1	
Gemfibrozil	0.569	0.785	0.256	0.441	0.318	0.913	Competitive
	0.469	0.685	0.341	0.628			
	0.369	0.585	0.359	1.67			
Fenofibrate	0.558	0.756	0.686	1.41	0.583	1.61	Semi-Competitive
	0.458	0.656	0.598	1.43			
	0.358	0.556	0.467	1.99			
Ropixon	0.61	0.872	1.65	1,61	0.983	1.79	Non-competitive
	0.51	0.772	0.76	1,79			
	0.41	0.672	0.54	1,97			

Synthesis and Characterization of Hybrid PON1 Structures

Surface topographic and structural characterizations of the synthesized immobilized PON1 structure were performed using SEM and FT-IR analyzes. SEM images of samples for immobilization on magnetic chitosan nanoparticles following purification of PON1 enzyme from human serum using TPP method were presented in Figure 5. Fe_3O_4 NPs linked with chitosan were found to have a well-formed spherical form with a smooth surface and average particle size was about 16 ± 3 nm with a narrow size distribution. The SEM images clearly showed that the initial surface roughness of the iron chitosan NPs had reduced and the PON1 enzyme had immobilized on the surface.

FT-IR Analysis Results

The FT-IR spectra of the magnetic-chitosan and immobilized PON1 enzyme structures were presented in Figure 7. The vibrations at 2879 cm^{-1} and 2116 cm^{-1} belonged to the $–CH_3$ and $–CH_2$ groups in the chitosan structure and the stretching vibrations at 1638-1029 cm^{-1} belonged to the –C=O and –N-H in the amide and secondary amide structures in the FT-IR spectrum of magnetic-chitosan structure. The vibrations belonged the –C-H bond in amide structure were seen at 891 cm^{-1} and 608 cm^{-1}. The peaks at 1340 cm^{-1} belonged to the -NH stretching in O-H, hydroxyl and free amino groups in chitosan structure (Figure 6). The sharpness of the peak at 1340 cm^{-1} belonged to the magnetic chitosan was reduced compared with the magnetic chitosan immobilized PON1 enzyme. A new sharp peak was observed at 1627 cm^{-1} in the FT-IR spectrum of immobilized PON1 enzyme and it belonged to the N-H group. The peaks at 906-758-700 cm^{-1} fields belonged to POP and 3651-1649 cm^{-1} fields belonged to (O-H)(HOH) (Wang et al., 2013).

Analysis of Reusability of Immobilized PON1 and PON1 MC-hPON1 Hybrid Structure

The reusability of PON1 enzymes immobilized on magnetic chitosan NPs was investigated and the results are shown in Figure 7. It was shown that PON1 enzyme immobilized on Fe_3O_4-chitosan NP still retained 56% of its activity after re-use of ten times. The obtained findings showed that PON1 enzyme in immobilized structure was more stable (Nadaroglu et al., 2017).

CONCLUSION

In conclusion, inhibition of drug and drug active compounds on enzyme activities is always known as a major problem for biochemical applications and drug design. PON1 is known to play an important role in detoxification of organophosphate toxicity in living metabolism and is an important drug target enzyme (Cao et al 2014; Wang et al., 2013). It is known that decreased activity of hPON1 causes some very important metabolic diseases such as atherosclerosis, arteriosclerosis and elevated levels of bad cholesterol in the blood, and therefore all factors affecting PON1 need to be well identified. In this study, hPON1 enzyme was purified from human serum for the first time using TPP method and covalently immobilized to non-toxic magnetic chitosan NPs which are biodegradable (Zowalaty et al., 2015; Assa et al., 2017). Subsequently, some antilipid drugs (Valeric acid, phenoxy–isobutyric acid, N-desmethyl rosuvastatin) and some antioxidant compounds (Gallic acid, Quercitin, Pyrogallol, Ascorbic acid) were found to inhibit IMB-hPON1 enzyme activity. According to the findings, it is concluded that taking the dosage into consideration both antioxidant and antilipid drugs will have important effects.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

This article does not contain any studies with human participants or animals performed by any of the authors.

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