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ORIGINAL RESEARCH PAPER

Effects of cadmium chloride as inhibitor on stability and kinetics of immobilized Lactoperoxidase(LPO) on silica-coated magnetite nanoparticles versus free LPO

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ABSTRACT

***Objective(s)*:** Enzyme immobilization via nanoparticles is perfectly compatible against the other chemical or biological approximate to improve enzyme functions and stability. In this study lactoperoxidase was immobilized onto silica-coated magnetite nanoparticles to improve enzyme properties in the presence of cadmium chloride as an inhibitor.

***Materials and Methods*:** The process consists of the following steps: (1) preparing magnetic iron oxide nanoparticles using the co-precipitation method, (2) coating NP with silica (SiO2) by sol–gel reaction, (3) characterizations of NPs were examined by FT-IR, XRD, AGFM and TEM. (4) Immobilization of LPO on the magnetite NPs, (5) Study kinetic and stability of both free and immobilized LPO in the presence of various concentrations of cadmium chloride.

***Results*:** The size of the Fe_3O_4 and silica-coated magnetite nanoparticles were about 9 nm and 12 nm, respectively. The results showed that the highest immobilization yield, nearly 90 %, was attained at 240 to 300 µg of LPO at 15h. It was found that the concentration of cadmium chloride directly affects the LPO activity and changes the kinetic parameters of it. Also, the results showed that immobilized LPO has better tolerance than the free LPO, so that after immobilization, Vmax of immobilized LPO was increased and Km of immobilized LPO was decreased.

***Conclusion*:** The results demonstrating that the effect of immobilized lactoperoxidase on silica-coated magnetite nanoparticles increases the stability of the LPO in the presence of cadmium chloride as inhibitor. Michaelis–Menten parameters (Km and Vmax) also revealed the considerable improvement of immobilized.

Keywords: *Cadmium chloride, Enzyme immobilization, Enzyme stability, Lactoperoxidase, Silica-coated magnetic nanoparticles*

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INTRODUCTION

Practical use of enzymes has been realized in various industrial processes and products including laundry detergents, and is being expanded in new fields: fine-chemical synthesis, pharmaceuticals, biosensors, bioremediation, biobleaching, polymerase chain reaction, protein digestion in proteomic analysis and biofuel cells [1-3]. Low thermal stability, narrow pH range, effective activity in water environment and the loss of catalytic activity after one cycle have been the greatest obstacles in the use of the enzymes in the multiple practical processes. However, the enormous catalytic potential offered by the enzymes for innumerable trans-formations, has stimulated intense studies aimed at the improvement of their properties. Fortunately, there are many techniques available that to increase the stability of enzymes: Selecting suitable source for purification of enzymes, Environment Engineering [4], Protein Engineering [5] and immobilization [6]. To be used as practical biocatalysts, especially industrial

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biocatalysts, improvement of the activity, stability and recovery of enzymes are necessary. Enzyme immobilization provides a superior approach to achieve enzyme recovery, and establishes a better way to handle the enzyme and eliminate protein contamination of the product easily [7]. Immobilized enzymes are drawing significant attention for potential commercial applications as biocatalysts by reducing operational expenses and by increasing process utilization of the enzymes. Among the possible candidates for carrier-bound enzyme immobilization, magnetic nanoparticles (MNPs) are promising due to their lack of toxicity, biocompatibility, and most importantly their magnetic properties [7-9]. In many researches MNPs such as Fe_3O_4 NPs must go through a surface modification procedure before enzyme immobilization. Magnetic core-shell Fe_3O_4@SiO_2 NPs as specially immobilizing carrier of biomolecules have stimulated great interest in current researches. iron oxide nanoparticels core with silica, stabilizes the nanoparticles in solution and also by silanol group provides good surface for subsequent functionalization for bioimaging, diagnosis, and therapeutic applications [10, 11]. Fe_3O_4@SiO_2 NPs have been used as support materials for the immobilization of enzymes such as Aspergillus niger xylanase A [8], pullulanase [9], acetyl xylan esterase [12].

Peroxide enzymes (E.C 1.11.1.7) are detoxification enzymes that are important to get rid of the cells from extra hydrogen peroxide under normal and stressed conditions [13]. Even though the peroxidase enzymes in the presence of metal ions remain active, some reports show that they are inhibited by metal ions [14]. Peroxidase In the classification in oxidoreductase group and they are produced by a number of microorganisms, plants and mammals. These enzymes reduce in peroxides such as hydrogen peroxide, and catalyze the oxidation of various organic and inorganic substances [15]. Actually, peroxidas enzymes oxidation of different substrates are by using hydrogen peroxide or other peroxides. Peroxidases are widely used in clinical Biochemistry and an enzyme immunoassay. Some peroxidases applications include; Application in analysis and diagnostic kits, Enzyme immunoassays, Peroxidase biosensors, Decolorization of synthetic dyes, Deodorization of swine manure, Application in the paper pulp industry and Enzyme immunoassays(16). One of the peroxidases that can used in industrial and medicine function is Lactoperoxidase (LPO). LPO (EC 1.11.1.7) is a heme-containing glycoprotein, and it is one of the useful proteins which plays an important role in protecting the lactating mammary gland and the intestinal tract of newborn infants against pathogenic microorganisms. LPO is secreted into milk that is natural host-defense systems against bacterial infections by killing or inhibiting their growth via blocking their metabolism [15, 17, 18]. LPO is an efficient enzyme in many applications, for instance; bleaching of fluid whey [19], antimicrobial activity in fish and meat Industry, in milk and dairy Industry, preservation of pharmaceuticals and iodine detection(20).

One of the substances that reduces the activity of the enzymes especially peroxide enzymes by their presence are heavy metals as; Lead, aluminum, mercury, copper, cadmium, nickel and arsenic [21]. They are dangerous toxins in breathing air, drinking water, building materials, kitchen appliances and even clothing. Cadmium is a toxins dangerous in environments, Cadmium is a strong carcinogens in human and animal that destroy skeletons, reduced bone strength and break the DNA strand (*In vitro* and *In vivo*). Cadmium exists in dental alloys, batteries, motor oil, seafood, ceramics, smoke cigarettes, tea and coffee, fertilizers, solders, galvanized pipes, water or wells, welding, smoke from burning rubber and plastics, cereals and no bran cereal [22].

According to previous studies on other peroxidases as HRP we founded cadmium is a one of peroxidase enzymes groups inhibitors,if it is peresnt in environments of LPO it would effect on on LPO activity. According to the ame of this study, to investigate the stability of immobilize LPO in the presence of cadmium chloride as its inhibitor, we wanted to find out to whether immobilized enzyme can be stable against inhibitors or not? In addition, in this study given the importance of the enzyme kinetics, the enzyme kinetics before and after immobilized was studied. There is not enough facts for using nanoparticles in order to immobilize this enzyme (LPO) yet.

MATERIALS AND METHODS

Materials

The chemical reagents used for Synthesis of nanoparticles are: Ferric chloride ($FeCl_3$), Ferrous chloride ($FeCl_2$), Sodium hydroxide (NaOH), Tetraethyl

orthosilicate ($C_8H_{20}O_4Si$), Sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7.2H_2O$), Citric acid ($C_6H_8O_7$) which are provided with high purities from Sigma-Aldrich company, Ethanol (C_2H_6O) and Acetone (C_3H_6O) are from Merck company.The chemical reagents used for enzyme immobilization and assaying enzyme activity are: Lactoperoxidase, 2,2-Azinobis [3-Ethyl Benzothiazo- line-6-suiphonic acid] diammonium salts (ABTS), Hydrogen peroxide(H_2O_2), Phosphate Buffer (PBS) and Cadmium chloride ($CdCl_2$) which are also provided with high purities from Sigma-Aldrich company.

Preparation of silica-coated magnetite nanoparticles (Fe_3O_4@SiO_2)

Fe_3O_4 NPs, were synthesized by a coprecipitation method by Ferric chloride ($FeCl_3$), Ferrous chloride ($FeCl_2$), and Sodium hydroxide (NaOH) respectively with concentrations (2:1:8) at 70 C f. Fe_3O_4 nanoparticles washed by distilled deionized water many times to remove the byproducts of the reaction. Then citric acid ($C_6H_8O_7$) (0.1 M) was added to neutralize the anionic charge on the particle surface, and stirred for one hour in room temperature. After washing the cationic colloidal particles Sodium citrate tribasic dihydrate (TSC= 0.01 M) was added under magnetic stirring. After finishing the TSC solution reaction solution heated at $90C^0$ for 30 min under magnetic stirring. An appropriate amount of acetone was added to remove the excessive citrate groups adsorbed in the nanoparticles and collected with a magnet. Immediately after the synthesis of Fe_3O_4 nanoparticles, Following the Stober method, with some modifications, the coating of citrate-modified Fe_3O_4 with silica were carried out in a basic in ethanol/ water mixture (3:1) and TEOS (0.5 ml) was added every three seconds a drop under magnetic stirring, after finishing the TEOS the mixture solution was continuously stirred for 12 h in room temperature. After 12 hours they were washed with ethanol and deionized water several times and by using XRD, TEM, AGFM and FTIR techniques were characterized [23].

LPO activity assay

LPO activity was measured spectrophotometrically (using Shimadzu spectrophotometer uv260- model TCC-240A, Tokyo, Japan; equipped with a constant temperature cell holder working at 22 °C). Before activity determination, the lyophilized LPO was diluted 5 fold with 0.1 M potassium phosphate buffer at pH 6.4. The reaction mixture consisted of 1860 ìlit of 1 mM ABTS, prepared in 0.1 M potassium phosphate buffer at pH=6.4, 1030 ìlit of 0.3 mM H_2O_2 solution, and 0.1 mL of enzyme solution. The absorbance was monitored continuously at 412 nm for 2 min. The enzyme activity was calculated with the following equation (Barrett et al., 1999). The average of the three measurements was used to calculate enzyme activity. The activities of immobilized enzymes were determined in the same way.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{412nm}/\Delta T)\ (3.1)\ (df)}{(3.24)(0.01)}$$

3.1 = Total volume (in milliliters) of assay
df = Dilution factor
3.24 = Millimolar extinction coefficient of oxidized ABTS at 412nm
0.01 = Volume (in milliliter) of enzyme used

Protein assay

The Lowry assay is an often-cited general use protein assay. For some time it was the method of choice for accurate protein determination for cell fractions, chromatography fractions, enzyme preparations, and so on. In this study, to measure the amount of protein in supernatant and wash fractions, Lowry method was used [24].

Immobilization procedure

Silica-coated magnetite nanoparticles (1.0 mg) were dispersed in 970-650 µl of phosphate buffer (0.1 M, pH 6.4).

Then, various volumes of the LPO solution (30-350µl (The concentration of the enzyme solution was 1mg/ml)) were added into the suspension and the mixture was shaken at $4C^f$ for 24 h. To investigate the effect of time on immobilization of enzymes, silica-coated magnetite nanoparticles (1.0 mg) were dispersed in 650 µl of phosphate buffer (0.1 M, pH 6.4).

Then 300µl LPO solution were added into the suspension and the mixture was shaken at $4C^f$ for 5-24 h. The immobilized LPO was removed by magnetic decantation and washed three times with phosphate buffer (0.1 M, pH 6.4). The amount of LPO immobilized on MNPs was determined by measuring the initial and final concentration of LPO in the immobilization medium using the Lowry method.

Stability in present of $CdCl_2$ as inhibitor

After successful immobilized enzyme on silica-coated magnetite nanoparticles, immobilized enzyme and free enzymes as control in various concentrations of $CdCl_2$ (0.1, 0.2, 0.3 M) at different times (5 and 10 min) in room temperature were incubated. After that activities of immobilized enzyme and free enzyme were measured and compared. The amount of $CdCl_2$ and incubation time was selected on previous studies with little modification [25].

Determination of kinetic parameters

Km and Vmax values of the native and immobilized enzyme were determined by measurement of the enzyme activity with various concentrations of ABTS. Then, Km and Vmax values were obtained from a Lineweaver- Burk plote.

Characterization

Size and morphology of magnetic nanoparticles were determined by using transmission electron microscopy (Philips CM 10 HT-100 KV); the FT-IR spectra were recorded by a Fourier transform Infrared Spectroscopy (JASCO FT/IR-6300, Japan), X-ray diffraction measurement was recorded on a X-Ray Diffractometer Bruker, D8ADVANCE [(Germany) using Cu K_ radiation (Ž= 0.1540 nm)] and the AGFM (AGFM, Meghnatis Daghigh Kavir Co, Iran).

Statistical analysis

The data were analyzed using SPSS software version 20 (SPSS Inc, Chicago, IL, USA). Kolmogrov-Smirnov test was utilized to evaluate the normality and values were examined by the One Way ANOVA to illustrate whether there are any significant differences between the different times and concentration of LPO or $CdCl_2$. Tow Way ANOVA was used, In statistics, the two-way analysis of variance (ANOVA) is an extension of the one-way ANOVA that examines the influence of two different categorical independent variables on one continuous dependent variable and it used for examined the time and concentration of LPO or $CdCl_2$ simultaneously.

independent T-test to detect the differences between the groups such as Kinetic Parameters before and after immobilizion. Moreover, the data was demonstrated as Mean±SEM and a P-value of less than 0.05 that was considered significant.

RESULTS AND DISCUSSION

Characterization of Fe_3O_4@SiO_2 nanoparticles

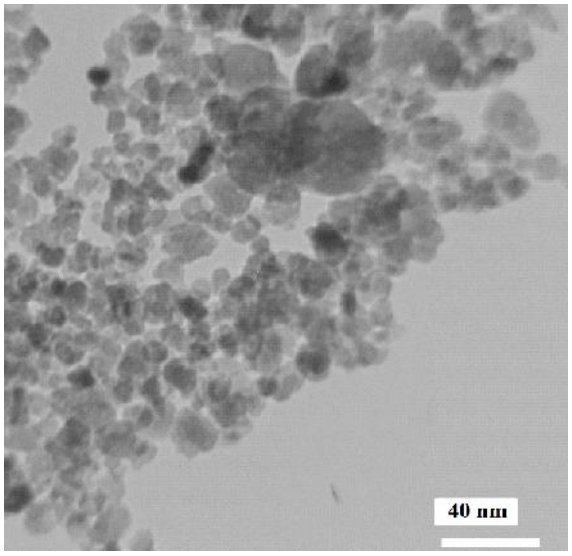
The mean particle size was examined by TEM imaging, as in Figure 1, a and b shows the representative TEM image of the Fe_3O_4 and Fe_3O_4@SiO_2. The magnetic Fe_3O_4 and Fe_3O_4@SiO_2 nanoparticles were found to be spherical in shape in TEM imaging. The mean size of magnetic Fe_3O_4 and Fe_3O_4@SiO_2 nanoparticles was 9 and 12 nm. The figure 1 (a and b) demonstrate that after coating of Fe_3O_4 by SiO_2 the shape of Fe_3O_4 NPs did not change but size was increased. Figure 2, shows the XRD patterns of pure Fe_3O_4 and Fe_3O_4@SiO_2 nanoparticles. The diffraction peaks were perfectly indexed to the inverse cubic spinel structure of Fe_3O_4, implying that the MN were single-phase spinel. These peaks correspond to the 220, 311, 400, 422, 511, 440 and 533 lattice planes. All diffraction peaks of Fe_3O_4@SiO_2 were almost identical to those of the pure Fe_3O_4, indicating that the structure of Fe_3O_4 was not changed. The average particle size of Fe_3O_4 and Fe_3O_4@SiO_2 were determined as 9 and 11 nm calculated by Debye–Scherrer formula from the half-maximum width of the (311) X-ray diffraction line.

Magnetic properties of the Fe_3O_4@SiO_2 nanoparticles were assessed by AGFM. Fig.3 illustrates the AGFM curve of the Fe_3O_4 and Fe_3O_4@SiO_2. As it can be seen, both of them have the super paramagnetic property. The saturation magnetization was determined by extrapolation of magnetization curve on the basis of H/1 when 0/1'!H. It was measured 55 emu/g for Fe_3O_4 where as it was determined 47 emu/g for Fe_3O_4@SiO_2.The chemical interaction between Fe_3O_4 and SiO_2 were investigated by FTIR.

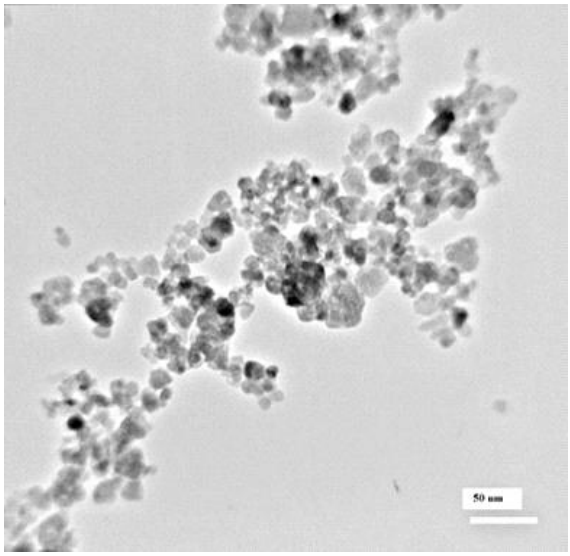
Fig. 4 shows the FTIR curve of the Fe_3O_4, SiO_2 and Fe_3O_4@SiO_2. As it can be observed, in the Fe_3O_4 curve, 1628 and 3400 peaks are related to OH junctions and it means that there exists water in the material structure. The 576 peak shows that the spinel structure was formed and we find that is well-agreed with XRD results. Compared to the untreated Fe_3O_4 NPs, absorptions at 1622 and 1397 cm^{-1} are due to the COO–Fe bond.

It reveals that trisodium citrate has been successfully grafted on to the surface of Fe_3O_4 NPs through the reaction of hydroxide radical groups on the surface of Fe_3O_4 with carboxylate anion of

trisodium citrate. On the other hand, in the Fe_3O_4@SiO_2 curve, the broad high-intensity band at 1092 and 824 are characteristic peak of the symmetrical and asymmetrical vibrations of Si–O–Si in SiO_4 tetrahedron associated with the motion of oxygen in Si–O–Si antisymmetrical stretch, the band at 463 cm^{-1} is an indication of the presence of Si–O–Fe.



a



b

Fig. 1. a and b show the representative TEM image of the Fe_3O_4 and Fe_3O_4@SiO_2

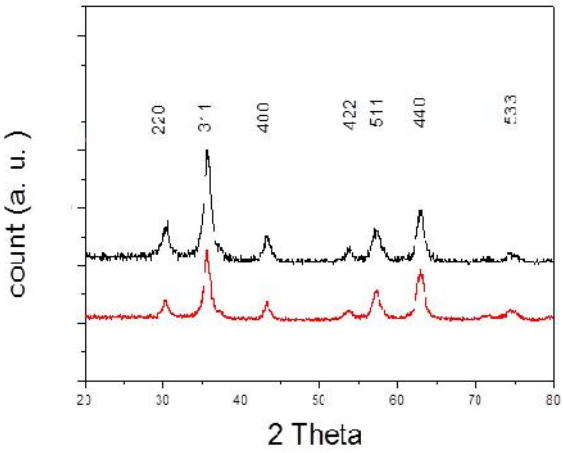


Fig. 2. XRD patterns of Fe_3O_4 and Fe_3O_4@SiO_2

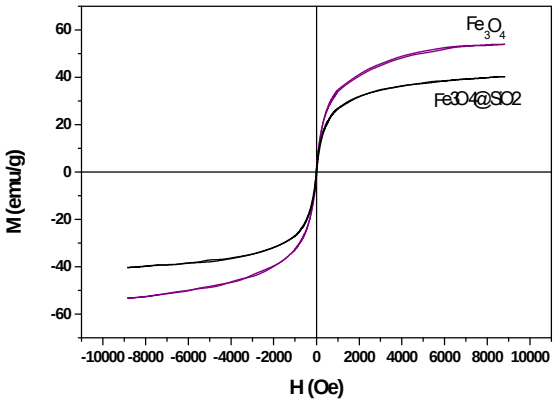


Fig. 3. AGFM plots of Fe_3O_4 and Fe_3O_4@SiO_2

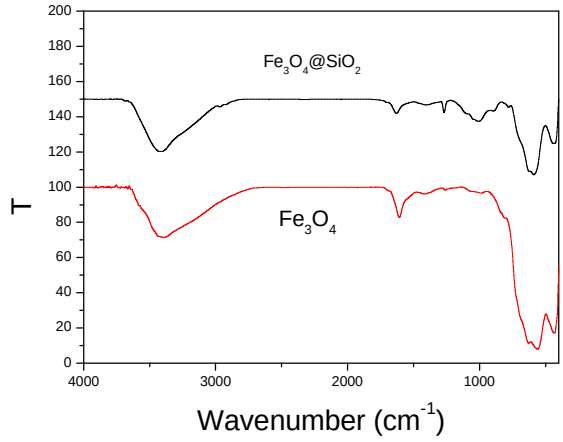


Fig. 4. FTIR curve of the Fe_3O_4 and Fe_3O_4@SiO_2

Table 1. Summary of characteristics of nanoparticles

sample	Size by TEM	Size by XRD	Hc(oe)	Mr(emu/gr)
Fe_3O_4	9nm	9nm	5	53.5 emu/gr
Fe_3O_4@SiO_2	12nm	11nm	5	47.33 emu/gr

LPO immobilization parameters

Concentration of LPO

In this part, different amounts of the LPO (30-350 µg) were used for immobilization on 1 mg of Fe_3O_4@SiO_2 nanoparticles. As shown in Fig. 5, the amount of immobilized LPO increased with increasing the initial amount of LPO and amount of LPO decreasing in supernatant and washing solution. As shown in Fig. 5, at different enzyme concentrations complete binding of LPO onto magnetic nanoparticles was achieved at the enzyme concentrations ranging from 250 to 300 ìg. The amount of the immobilized LPO increased at the same time as LPO concentration increased. A complete immobilization of enzyme onto magnetic nanoparticles surface was accomplished in the enzyme concentration range from 250 to 300 ìg. In case when lower concentration of enzyme was used, only small amounts of LPO were successfully bound onto magnetic supports. It seems that the over loading of enzyme molecules on magnetite NPs causes some unfavorable protein–protein interactions that, subsequently, reduces the enzyme activity.

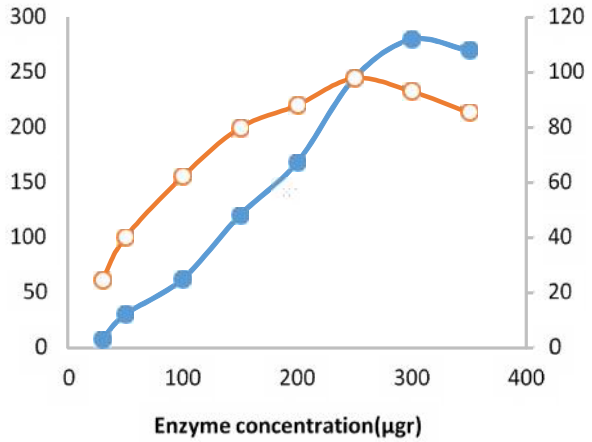


Fig. 5. Effect of the initial amount of LPO on the amount of immobilized LPO (●) and relative activity (o). Immobilization condition: the initial amount of LPO (30-350 µgr), 1 mgr of NPs, phosphate buffer (0.1M, pH 6.4), t =4C [f]

Time of immobilization

The amount of immobilized LPO of versus reaction time are shown in Fig. 6. It was found that, by increasing the reaction time from 5 to 15 h, the amount of immobilized LPO is increased.

It seems that most surface of magnetite nanoparticles were blocked by LPO after this time. Finally The immobilization yield was more than 90% at optimum conditions.

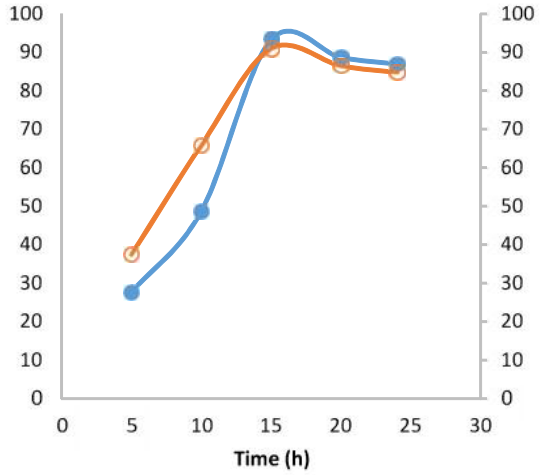


Fig. 6. Effect of reaction time on amount of immobilized LPO (●) and relative Activity% (o). Immobilization condition: the initial amount of LPO(300µgr), 1 mgr of NPs, phosphate buffer (0.1M, pH 6.4), t =4C [f]

Stability of free and immobilized LPO in present of $CdCl_2$

In order to investigate the $CdCl_2$ tolerance we tested the activity of free and immobilized LPO at different $CdCl_2$ concentrations (0.1, 0.2 and 0.3 M) and different time (5 and 10 min). The results on $CaCl_2$ tolerance in different $CdCl_2$ concentrations and time are illustrated in Figs. 7 and 8. The immobilized enzyme showed a higher $CdCl_2$ tolerance than the free form of the same $CdCl_2$ concentration and time.

The results show that, Immobilize LPO by using Fe_3O_4@SiO_2 nanoparticles are able to reduce percentage of inhibition of enzyme activity by maintaining the structure of the enzyme. Enzymes bind with nanoparticles causing a conformational change in the enzyme that can reduce the cadmium

ion binding site on the enzyme, and thus reduce the percent inhibition.

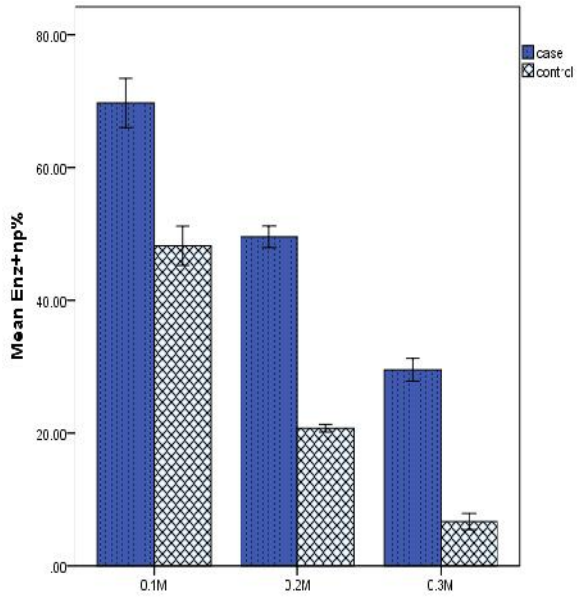


Fig.7. Effect of different concentrations of $CdCl_2$ (0.1, 0.2, 0.3 M) on remaining activity% of immobilized LPO and free LPO

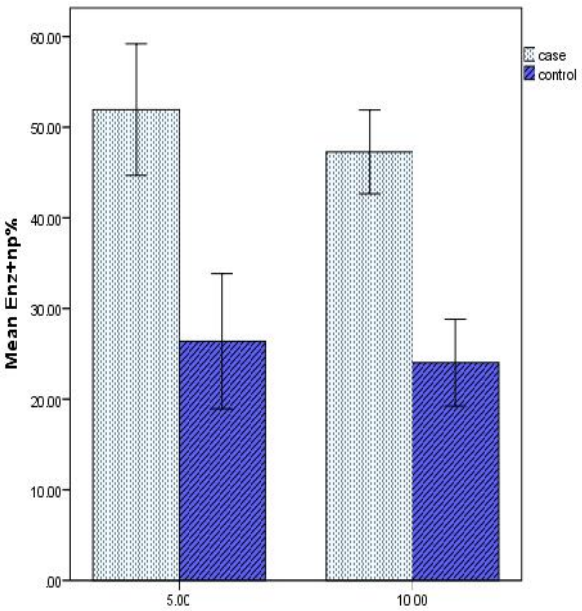


Fig.8. Effect of different incubation times (5 and 10 min) by different of $CdCl_2$ (0.1, 0.2, 0.3 M) on remaining activity% of immobilized LPO and free LPO

Determination of kinetic parameters for free and immobilized LPO

Kinetic parameters of free and immobilized enzymes were determined from the Lineweaver–Burk plots and using ABTS (1×10^{-4} to 3×10^{-2} mol l^{-1}) as substrate.

The results (Km, Vmax) showed that the Km value of immobilized LPO (0.34mM) was lower than that of free LPO (0.55mM), which represents the higher affinity of immobilized LPO to substrate. Since magnetic nanoparticles were so small and non-porous, it could be imagined that the LPO molecule might be expanded over the particle surface with a better orientation leading to a higher affinity to substrate and more available active sites.

The results also represent that the increase of Vmax is due to immobilization (1.09 mmol/min mg for immobilized LPO and 0.59 mmol/min mg for free LPO). The improvement of Vmax may also be as a result of more efficient conformation of immobilized LPO respect to free LPO. It looks that immobilization induces conformational changes in three dimensional structure of enzyme in order to improve its catalytic efficiency.

An enzyme with a pocket complementary to the reaction transition state helps to destabilize the substrate, contributing to catalysis of the reaction. In fact, this complementary interaction has been improved due to immobilization.

The improvement of kinetic parameters of immobilized LPO can be a good probable industrial application. According to results, addition of cadmium chloride makes both of Km and Vmax change, as Km was increased and Vmax was decreases that shows inhibition was the uncompetitive type.

In this type of inhibition enzyme inhibitor binds only to the complex formed between the enzyme and the substrate (the E-S complex). reduction in the effective concentration of the E-S complex increases the enzyme's apparent affinity for the substrate through Le Chatelier's principle (Km is lowered) and decreases the maximum enzyme activity (Vmax), as it takes longer for the substrate or product to leave the active site.

Free and immobilized LPO kinetics in presence of cadmium chloride showed that both have been affected by the inhibitor($CdCl_2$) but as is clear from Fig 6 and 7, immobilized enzyme was less affected by inhibitor($CdCl_2$).

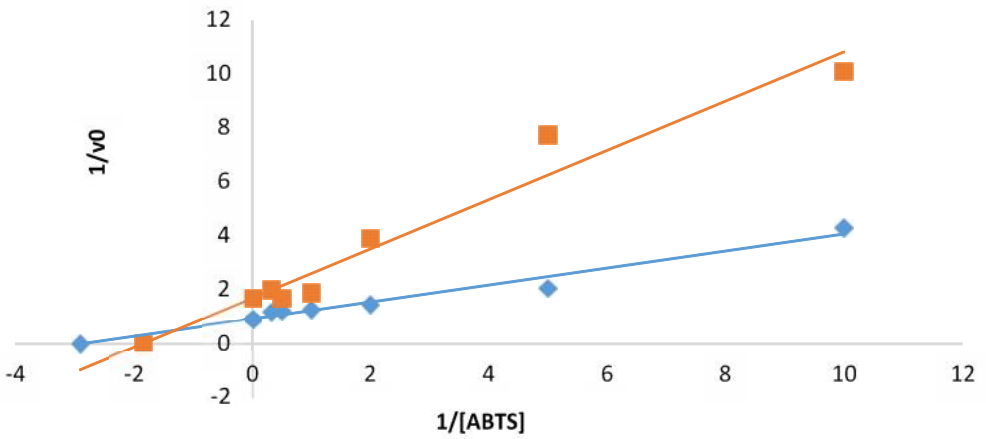


Fig.9. Lineweaver–Burk plots of free LPO (■) and immobilized LPO(♦). ABTS concentration varying from (1 × 10^{-4} to 3 ×10^{-2} mol l^{-1})

Table 2. Kinetic parameters of Free LPO and immobilized LPO on silica-coated magnetite nanoparticles in presence or absence of cadmium chloride

Free LPO		Immobilized LPO		$CdCl_2$ (M)
Km and Km appearance mmol	Vmax and Vmax appearance mmol min^{-1}	Km and Km appearance mmol	Vmax and Vmax appearance mmol min^{-1}	
0.55	0.59	0.34	1.09	0
0.5	0.497	0.318	0.950	0.1
0.431	0.374	0.329	0.781	0.2
0.361	0.32	0.321	0.595	0.3

Values are the mean ± standard deviation (n = 9)

CONCLUSION

This work described a simple method for the absorbent immobilization of Lactoperoxidase (LPO) on the surface of silica-coated magnetite nanoparticles. The TEM image showed that the diameter of silica-coated magnetite NPs was about 12 nm and XRD pattern proved that the immobilization process did not result in the phase change of Fe_3O_4. The FT-IR spectrum was used for confirming the coating of SiO_2 onto Fe_3O_4NPs. The optimal reaction conditions used for LPO immobilization onto magnetic nanoparticles (1 mg) were found at medium pH 6.4, 300 ìg of concentration of LPO and at the emperature of 4 ºC. The results demonstrated the substantial improvement of the kinetic parameters of LPO due to immobilization. The immobilized LPO demonstrate more stability in the presence of various concentrations of $CdCl_2$ to free LPO. when LPO immobilized, the enzymes may have their catalytic properties altered in a manner permitting them to preserve their activities in conditions in present of inhibitors such as $CdCl_2$. It seems that immobilizing the enzymes by nanoparticles is an effective and economic way for productivity of enzymes. Nanoparticles are good supports for enzyme immobilization, since the high surface area: volume ratios of nanoparticles can effectively improve the enzyme loading and the catalytic efficiency of the immobilized enzyme. Among many methods proposed for the enzyme immobilization by nanoparticles, the most important and useful is the immobilization by adsorption. Adsorption makes use of the physical interactions generated between the carrier and

enzyme that include van der Waals forces, ionic interactions and hydrogen bonding. The binding are rather weak and, what is important, typically are does not change the native structure of the enzyme. This prevents the active sites of the enzyme from disturbing and allows the enzyme to retain its activity. It seems Maintain the normal structure of LPO after the immobilization is one of the reasons for the increase the kinetic and activity of immobilization LPO versus free LPO.

It is necessary to mention that the results depend on the type of enzyme and nanoparticles. To get a better result in similar studies, It suggests that after the synthesis of nanoparticles immobilization process done and use nanoparticles with its small size (Below 20 nm), and also surface functionalized nanoparticles with active groups like amine and carboxyl can improve the reliability of the results.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest

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