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RESEARCH ARTICLE

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The effect of magnetized water on the oxidation reaction of phenol derivatives and aromatic amines by horseradish peroxidase enzyme

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Abstract

The present study aimed to investigate, for the first time, the rate of the oxidation reaction of some derivatives of phenol and aromatic amines, that is, pyrogallol, catechol, resorcinol, ortho-aminophenol, meta-aminophenol, para-aminophenol, ortho-phenylenediamine, and para-phenylenediamine, in the presence of hydrogen peroxide in pure and magnetized solvents using horseradish peroxidase enzyme. The reaction was studied in the absence and presence of a magnetized solvent under completely identical conditions. The results showed that magnetized solvent could change the structure of the enzyme and reduce its activity. In addition, it affected the rate of oxidation of the selected derivatives through altering the strength of the hydrogen bonds of the system. The changes in the structure and activity of the enzyme were examined using UV–Vis and fluorescence spectroscopy as well as viscosity measurement technique. Examination of the secondary structure via the far UV-CD spectrum indicated the increase in the alpha helical structure in the magnetized solvent. When dissolved in a magnetized solvent, hydrogen peroxide as an enzyme substrate reduced the rate of enzymatic reaction and provided lower saturation conditions for the enzyme compared with when it was dissolved in the pure solvent.

KEYWORDS

HRP enzyme, magnetized solvent, oxidation reaction, pure solvent

1 | INTRODUCTION

Water, characterized by many unique properties, such as high melting and boiling temperatures and high solubility for charged and polar molecules, is a vital liquid for living organisms, such as humans, animals, and plants.[1] As a diamagnetic material, it has a mass magnetic susceptibility of about -7.2×10^{-2} JT^{-2}/kg.[2] Under a magnetic field, the intermolecular hydrogen bond in pure water (ordinary water) as a polar and associative material can be changed and transferred to lower stability state for a short time.[3] The behavior of magnetized water[4] in different processes, for example, solubility and crystallization, is different from that of pure water.[5]

Water is magnetized when it passes through a magnetic field or when located in the vicinity of a static magnetism. Strength of the magnetic field and duration of the magnetization process have a remarkable effect on the properties of magnetized water.[6,7] Mosin and Ignatov presented two magnetic devices based on permanent magnet and electromagnetism (alternating magnetic field generator).[8,9] Some of the properties of pure water and magnetized water are compared in Table 1.

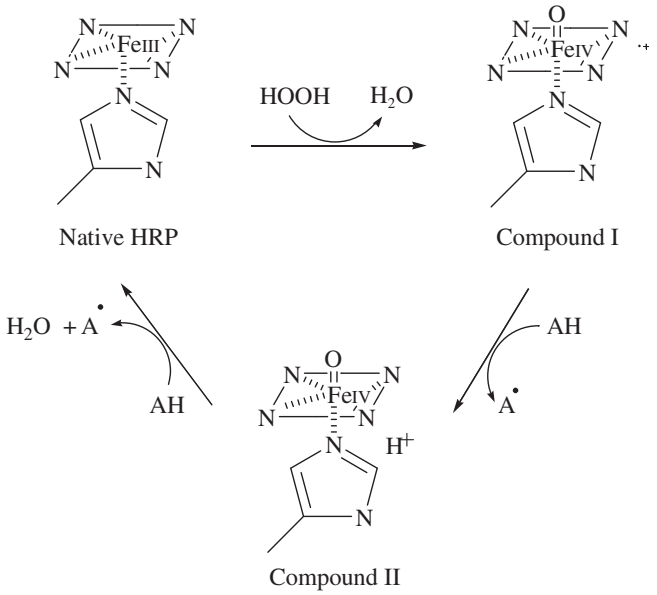
As the table shows, the structural properties of water change in the presence of magnetic field. In fact, some physical and chemical changes occur in magnetized water because of the breaking of hydrogen bonds in clusters.[15] Magnetized water retains its new properties

TABLE 1 Changes in some properties of magnetized water

Properties of water	Pure water	Magnetized water	Reference
pH	7.86@70°C	8.08@70°C	10
Self-diffusion coefficient	2.14	1.88	11
Density (g/cm^3)	0.89646	1.00267	12
Number of hydrogen bonds	3.47	3.482	11
Maximum wavelength (nm)	191	220	13
Electrical conductivity of water (μS/cm)	170@70°C	140@70°C	10
Contact angle between water and graphite (degree)	92.6	91.2	14
Diffraction intensity (counts/s)	39,417	42,872	13
Refractive index	1.3336	1.3346	14

for a while and gradually returns to its normal condition called memory effects. The time to return to normal conditions is proportional to the time of exposure to the magnetic field. There is a limit for changes in the magnetized properties of water, which is called saturation level. If the duration of water magnetization exceeds the saturation level, the magnetized property decreases until it disappears. The time to reach the saturation level and memory effect are regarded as two parameters in the process of water magnetization.[13,16-19] Magnetized water plays an important role in the synthesis of one-pot organic compounds. In the multicomponent reactions it act as a solvent and produces 1H-tetrazoles and 5-substituted compounds,[20] pyrazolopyranopyrimidines,[21] 9-substituted-9H-diuracilopyrans,[22] and the pyrano[2,3-c]pyrazole and pyrano[4′,3′:5,5]pyrazolo [2,3-*d*]pyrimidine derivatives.[23] In fact, simplicity, short reaction time, low cast, green, lack of organic solvents, easy extraction, and high efficiency are among the characteristics of these reactions.[20] The effect of water solvent in biochemical processes is one of the challenging questions that have attracted the researchers in this field. The present study investigated the effect of magnetized water as a solvent on the oxidation reaction of phenols derivatives and aromatic amines catalyzed by horseradish peroxidase (HRP) enzyme.

Phenols as the main part of the biological molecules are involved in various activities, such as growth, development, and defense of plant,[24-26] and can be found in hormones, antioxidants, vitamins, and amino acids.[27] Phenols play an important role in biological properties, such as anti-inflammatory, antibacterial, antitumor, and antioxidant properties. Furthermore, they are hydrogen donor compounds that act as antioxidant and can transport hydrogen or electrons to convert them to the corresponding radicals. The resulting radical, that is, phenoxyl, is able to attack another phenol molecule and convert it to phenoxyl radical, which, due to having a nonlocalized electron, is relatively stable and has a low reactivity.[28] Since almost all of the aromatic amines are hazardous, carcinogenic, and harmful for human health, they are categorized as toxic compounds. Today, they are found in industrial waste generated in paint, resin, textiles, and factories.[29]



SCHEME 1 Mechanism of horseradish peroxidase enzymatic oxidation

Peroxidases are a group of oxidoreductase that catalyzes oxidation reactions in the presence of hydrogen peroxide. They have a prostatic group, which oxidizes the phenols to quinones and includes trivalent iron and protoporphyrin (ferriprotoporphyrin) enzymes known as heme proteins.[30] Peroxidases catalyze the oxidation of electron donor substrates, such as phenols, aromatic amines, indoles, sulfonates, thioanizoles, and iodides, through the transfer of two electrons by hydrogen peroxide. The catalytic process of HRP enzyme is a cyclic process comprising of three steps: (a) the enzyme loses two electrons through interaction with hydrogen peroxide, forming radical cation compound I; (b) due to the transfer of one electron from the electron substrate to the ferric enzyme group, the intermediate 1 is reduced and the intermediate 2 is formed; and (c) compound II also reacts with another substrate and causes the formation of radical substrate, water molecule, and initial enzyme. This catalytic cycle continues in the same way (Scheme 1).[31]

2 | MATERIALS AND METHODS

2.1 | Material and reagent

HRP P8250 was a sigma product (type VIA, RZ = 3.0); pyrogallol (PGL), catechol (CTL), resorcinol (RSL), ortho-aminophenol (OAP), meta-aminophenol (MAP), para-aminophenol (PAP), ortho-phenylenediamine (OPD), and para-phenylenediamine (PAP); and hydrogen peroxide (30% vol/vol) were purchased from Aldrich and used without further purification. All the other reagent chemicals were of the highest available grade. Deionized water was also employed throughout the experiment.

2.2 | Characterizations

UV spectra were recorded on a UV-2550 (SHIMADZU), from 200 to 600 nm and visible measurements were performed on Vis 2100 single beam spectrophotometer. Fluorescence spectra were determined by F-2700 (HITACHI) fluorescence spectrophotometer, equipped with xenon pulse lamp and a 1.0 cm quartz cell. The excitation wavelength was 280 nm, and the emission spectra were read in the range of 300–500 nm. Far-UV CD experiments were performed on a Jasco-815 spectropolarimeter equipped with a Jasco 2-syringe titration mechanism. Spectra were recorded in the UV region (190–240) with the same protein concentration in a 1-mm path length quartz cuvette. A bandwidth of 1 nm was used together with a response time of 2 sec with a scanning rate at 50 nm/min to obtain the final spectrum as the average of three scans. The instruments were calibrated with ammonium d-10-camphorsulfonic acid. NMR spectra were recorded on Avance Bruker-300MHz spectrometers. All chemical shifts in NMR experiments were referenced to residual solvent and reported in parts per million. The signals were denoted as s (singlet), br (broad), d (doublet), and m (multiplet). FT-IR spectra were recorded on an AVATAR-370-FTIR Thermo Nicolet instrument. Ostwald viscometer was employed to measure viscosity. Doubly-distilled water which was deionized by a Millipore Q-Plus 185 system was used as deionized water in the preparation of reagent and in experiment at 25°C. The solvents magnetizing apparatus (Figures 1 and 2) is made of stainless steel 316 with two solvent containers (1 and 2) and sampling valves (3 and 4) placed right after the solvent storage. There is a linking valve (5) between the two containers (1) and (2) and connections are linked via a ¾ in steel pipe. A flange (8) is between the container (2) and the first sampling valve (3) and also between the second sampling valve (4) and the steel pump (9). Furthermore, a flange (7) exists between the stainless steel pump (9) and the AQUA CORRECT device (10) (The static magnetic field in a compact form, a unit called "AQUA CORRECT," was used). Having a coaxial static magnetic system of 6000G field strength, the equipment was imported from Germany HPS Co. [DN = 20,3/4 in flow rate $2m^3/h$].[20]

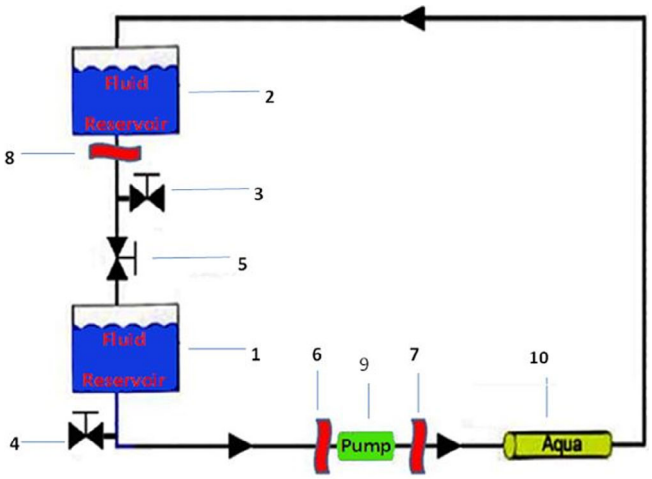


FIGURE 1 Solvent magnetizing apparatus contains solvent containers (1 and 2), sampling valves (3 and 4) placed right after the solvent storage, linking valve (5) between the two containers (1) and (2). A flange (8) is between the container (2) and the first sampling valve (3) and also between the second sampling valve (4) and the steel pump (9). A flange (7) is between the stainless steel pump (9) and the AQUA CORRECT device (10)

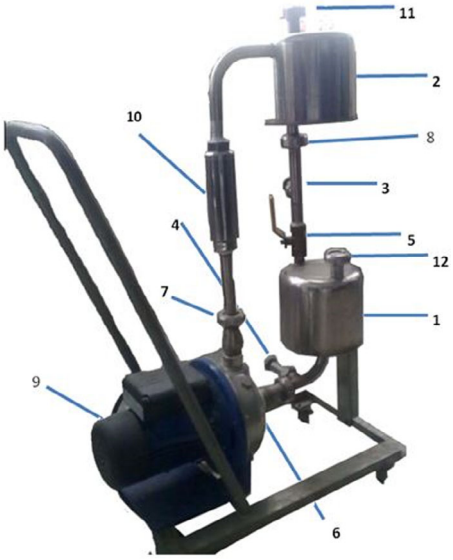


FIGURE 2 Solvent magnetizing apparatus contains solvent containers (1 and 2), sampling valves (3 and 4) placed right after the solvent storage, linking valve (5) between the two containers (1) and (2). A flange (8) is between the container (2) and the first sampling valve (3) and also between the second sampling valve (4) and the steel pump (9). A flange (7) is between the stainless steel pump (9) and the AQUA CORRECT device (10)

2.3 | Solution preparation

Evaluation of HRP enzyme activity at 25°C, and the preparation of potassium phosphate buffer (pH 6.0) and hydrogen peroxide solution were carried out according to the procedure.[32] The potassium phosphate buffer (pH 6.0) was employed in all enzymatic reactions in pure and magnetized solvents. To prepare a solution from phenolic derivative, 2.0 mg of each substrate was dissolved in 1.0 ml of deionized water. Then, 0.32 ml of each substrate solution PGL, CTL, and RSL was added to 0.39 ml of phosphate buffer and 0.16 ml of hydrogen peroxide solution to obtain 3.0 ml with deionized water. The oxidation reaction was performed after the addition of 0.01 ml of the enzyme and the absorption changes over the time were recorded for PGL, CTL, and RSL at wavelengths of 420, 395, and 330 (corresponding to the absorption peak of each reaction). The final concentration of each substrate was 2.0×10^{-3} M in solution. To prepare a solution from aromatic amines, 2.0 mg of each substrate was dissolved in 1.0 ml of deionized water. The appropriate volume of substrate solution OAP, MAP, OPD, PAP, and PPD (50, 50, 50, 100, and 0.20 ml, respectively) was added to 0.39 ml of phosphate buffer and 0.16 ml of hydrogen peroxide solution to reach to the volume of 3.0 ml with deionized water. Then, the reaction was performed after the addition of 0.01 ml of the enzyme

and the absorption changes over the time were recorded at their maximum wavelengths (λ_{max}). Then 0.05 ml of solution containing OPD, OAP, and MAP; 0.68 ml of phosphate buffer; 0.16 ml of hydrogen peroxide solution; and 0.01 ml of enzyme dissolved in phosphate buffer (pH 6.0), were added to 2.10 ml of deionized water, causing the final concentration of each of the substrates to reach 3.0×10^{-4} M in solution. At the next step, 0.20 ml of solution containing PAP, 0.53 ml of buffer phosphate, 2.10 ml of deionized water, and 0.01 ml of enzyme dissolved in buffer were added to 0.16 ml of hydrogen peroxide solution, due to which the final concentration of substrate reached to 6.0×10^{-4} M in solution. Next, 0.10 ml of solution containing PPD, 0.63 ml of phosphate buffer, 2.10 ml of deionized water, and 0.01 ml of enzyme dissolved in buffer were added to 0.16 ml of hydrogen peroxide solution. Consequently, the final concentration of substrate was 1.0×10^{-3} M in solution. The absorption changes over the time were recorded for OAP, MAP, PAP, OPD, and PPD at wavelengths of 436, 432, 492, 423, and 500 (corresponding to the absorption peak of each reaction). To study the magnetic effect, magnetized water was used in the preparing of all the solutions.

2.4 | Determination of molecular absorption coefficient (epsilon) of products

First, after the oxidation reactions of PGL, CTL, RSL, OAP, MAP, PAP, OPD, and PPD, preparation of several solutions with different concentrations from the products and termination of the reaction, absorbance of solutions was recorded at their maximum wavelength and the product epsilon of each reaction was determined.

2.5 | Initial rate experiment

In order to identify the initial rate, the absorbance of the solution was read at specified intervals over time after enzyme injection. Epsilon, which converts the absorbance to concentrations, was estimated based on the results of the absorbance. Then the initial rate was determined using the concentration data.

2.6 | Enzyme incubation

The HRP enzyme was incubated with potassium phosphate buffer in the presence of hydrogen peroxide for 10 min before the addition of PGL. The conditions of enzyme incubation were quite similar to those applied in enzyme activity assay (based on the literature).[32]

2.7 | Product identification and characterization

In order to purify the products after the completion of the reaction, 50 ml of ethyl acetate was added to the solution and then, organic phase was extracted. Next, vacuum distillation was used to evaporate the organic solvent and pure product was obtained.

2.7.1 | Purpurogalline

Dark brown powder, IR (KBr) νmax/cm^{-1}:3456, 3,382 (OH), 1,626 (C═O), 1,590 (C═C), 1,235 (C—O), 1,425–1,600 (C═C aromatic), 1,068 (C═O bending), 1,010, 841, 803, 648 cm^{-1}.

^{1}H NMR (C_3H_6O-d_6 300MHz): δ 6.74 (dd, 1H, J_{HH} = 11.4, J_{HH} = 9.6 Hz), 6.92 (s, 1H), 7.08 (d, 1H, J = 9.3), 7.35 (d, 1H, J = 11.4), 9.74 (s, 1H, arom-H), 15.33 (s, 1H). ^{13}C NMR (C_3H_6O-d_6, 75 MHz): δ 110.74 (1C), 115.32 (1C), 116.98 (1C), 124.04 (1C), 133.49 (1C), 134.79 (1C), 135.20 (1C), 125.04 (1C), 152.24 (1C), 155.52 (1C), 182.70 (1C).

2.7.2 | Cyclohexa-3,5-diene-1,2-dione

Dark brown powder, IR (KBr) νmax/cm^{-1}:1711 (C═O), 1,620.44 (C═C), 650–1,000 (C—H bending).

^{1}H NMR (C_3H_6O-d_6 300MHz): δ 5.24 (s, 2H), 7.16 (dd, 1H, J = 5.1, J = 3.6), 7.27 (dd, 1H, J = 6.3, J = 3.6). ^{13}C NMR (C_3H_6O-d_6, 75 MHz): δ 113.97 (2C), 118.78 (2C), 141.59 (2C).

2.7.3 | 2-Amino-3-H-phenoxazin-3-one

Brown powder, IR (KBr) νmax/cm^{-1}:3411.31, 3,338.02 (primary N—H), 3,303.49 (aromatic C—H), 1,729 (C═O unsaturated), 1,655 (C═N), 1,586.60, 1,462.19 (C═C unsaturated), 1,379 (C—N═), 1,222 (C—N), 1,272, 1,040 (C—O), 582, 463 (aromatic plane).

^{1}H NMR (C_3H_6O-d_6 300MHz): δ 2.52 (s, 2H, NH_2), 3.36 (s, 2H), 6.4 (d, 1H, J = 15), 6–84-7.73 (m, 3H, arom-H). ^{13}C NMR (C_3H_6O-d_6, 75 MHz): δ 98.83 (1C), 103.91 (1C), 116.42 (1C), 125.76 (1C), 128.46 (1C), 129.29 (1C), 134.21 (1C), 142.40 (1C), 147.86 (1C), 148.72 (1C), 149.36 (1C), 180.70 (1C).

2.7.4 | 2,3-Diaminophenazine

Yellow powder, IR (KBr) νmax/cm^{-1}:3309, 3,432 (—NH_2), 1,642 (C═N), 1,564, 1,466 (C═C), 1,222 (C—NH_2), 1,336 (C—N═), 585, 489 (aromatic plane).

^{1}H NMR (C_3H_6O-d_6 300MHz): δ 6.27 (s, 4H, -NH_2), 6.93 (s, 2H), 7.55 (dd, 2H, J = 9, J = 6, arom-H), 7.91 (dd, 2H, J = 9, J = 3, arom-H). ^{13}C NMR (C_3H_6O-d_6, 75 MHz): δ 102.66 (2C), 126.89 (2C), 128.36 (2C), 140.79 (2C), 142.53 (2C), 144.52 (2C).

3 | RESULTS AND DISCUSSION

3.1 | Effect of magnetic field on water

In the presence of a magnetic field, water has higher viscosity because of stronger hydrogen bonds.[33] As can be seen from Table 2, the observed behavior was the same for water and magnetized buffer. The viscosity of the magnetized buffer, like the magnetized water,

TABLE 2 The viscosity of water and phosphate buffer, pH 6, in pure and magnetized state at 27°C

	Pure water	Magnetized water	Pure phosphate buffer	Magnetized phosphate buffer
Viscosity	0.997	1.004	0.997	1.078

Note: Concentration of HRP enzyme 3.50×10^{-1} mM.

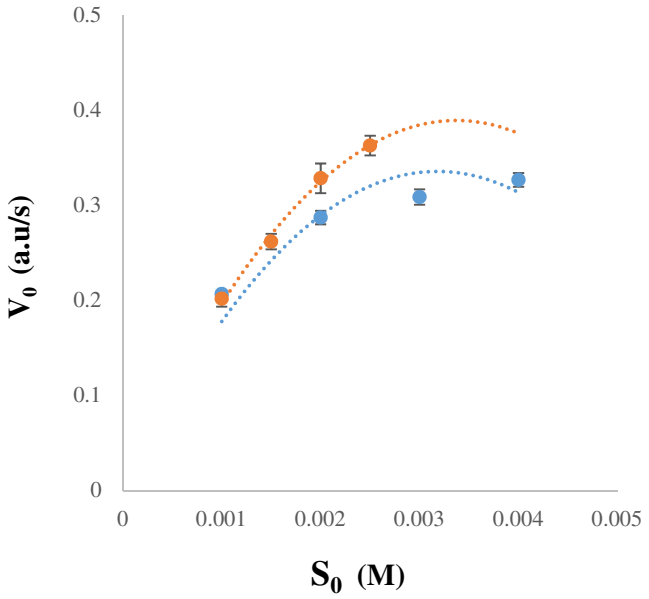


FIGURE 3 Saturation diagram of the horseradish peroxidase enzyme in the presence of various concentrations of hydrogen peroxide. Pure solvent (orange graph) and magnetized solvent (blue graph)

increased when compared with the pure state. Agata Wasak et al. found that by placing the buffer in the magnetic field, the pH was not altered, but if the water was exposed to the magnetic field, the hydrogen bond network, and distribution and polarization of its molecules may change. Hence, change of buffer pH was eliminated as a factor disturbing the enzyme activity.[34] Therefore, due to the same effect of the magnetic field on water and buffer and the partial volume of the buffer relative to that of water in our reaction solution, it is logical to investigate the effect of magnetized water on structure, enzyme function, and ultimately the speed of enzymatic oxidation reaction.

3.2 | Evaluation of PGL oxidation reaction rate in pure and magnetized solvent

The activity of HRP enzyme was measured in the presence of PGL in a pure solvent and the initial rate, as a measure of enzyme activity, was determined to be 20×10^{-6} M/s. In order to reveal the effect of magnetized solvent on the oxidation reaction of PGL in the presence of HRP enzyme, the reaction was performed in the same condition and the initial rate was estimated to be 21.40×10^{-6} M/s (Table 5). It

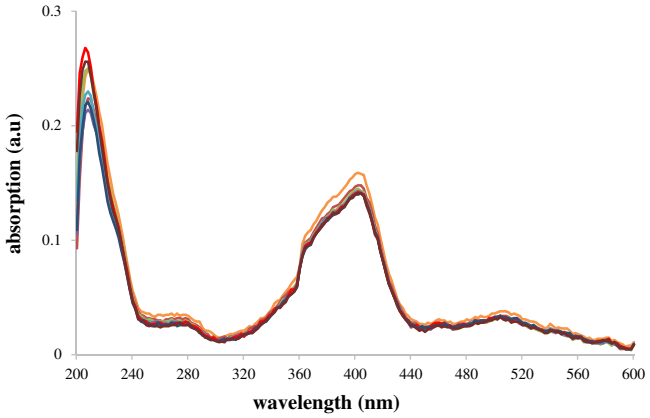


FIGURE 4 UV–Vis absorption spectrum of horseradish peroxidase (HRP) enzyme. HRP enzyme in a magnetized solvent at different times: t = 0 (orange graph); 30 sec (brown graph); 1 min (green graph); 2 min (violet graph); 4 min (light blue graph); 6 min (red graph); 8 min (dark blue); and 10 min (pink graph), concentration of HRP enzyme, 13.6×10^{-1} mM. Phosphate buffer (100 mM, pH 6)

is worth noting that the magnetized solvent slightly increased the oxidation reaction. Since PGL is a usual method in determining the activity of HRP, the initial rate of PGL was considered as the basis for comparing the rate of oxidation of other substrates, here.

3.3 | Effect of magnetized water on PGL oxidation reaction rate in the presence of hydrogen peroxide with different concentrations

The oxidation reaction of PGL was carried out at different concentrations of hydrogen peroxide in the presence of pure and magnetized solvent (Figure 3). The results showed that the use of magnetized water as a solvent in the oxidation process reduced the initial rate for all concentrations of hydrogen peroxide. Moreover, the enzyme saturation occurred at lower concentrations of hydrogen peroxide.

3.4 | Effect of magnetized water on the structure of the peroxidase enzyme

The structural change of enzymes is a common reason for the changes in their activity. To find out why the rate of enzyme reaction decreased in the presence of magnetized solvent, the effect of this solvent on the structure of the enzyme was investigated by UV–Vis spectroscopy and fluorescence emission spectroscopy. The UV–Vis absorption spectrum of HRP in magnetized solvent at different times is shown in Figure 4. In this figure, the effect of different times of the presence of the enzyme in magnetized water is demonstrated. Considering the soret peak, the magnetic effect increased in short times and decreased over the time so that the changes were too low after about 10 min; hence, in subsequent experiments, the absorbance of the solution in the enzymatic reaction was investigated just for

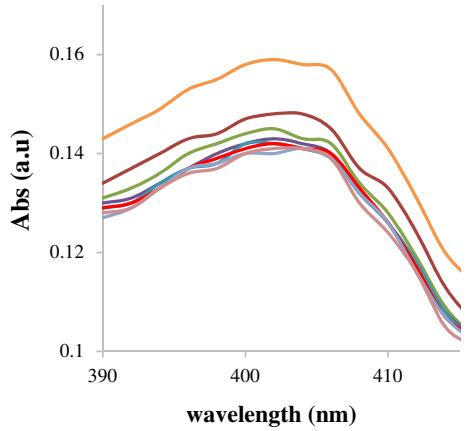


FIGURE 5 Soret peak magnification in the UV–Vis absorption spectrum of horseradish peroxidase (HRP) enzyme. HRP enzyme in a magnetized solvent, high intensity to low intensity spectrum, t = 0 (orange graph); 30 sec (brown graph); 1 min (green graph); 2 min (violet graph); 4 min (light blue graph); 6 min (red graph); 8 min (dark blue graph); and 10 min (pink graph), respectively. Phosphate buffer (100 mM, pH 6)

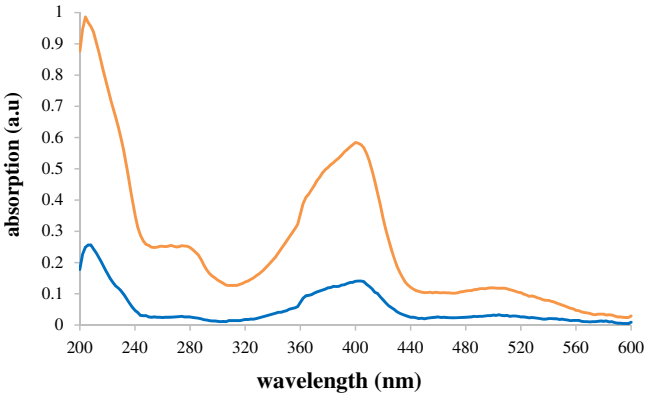


FIGURE 6 UV–Vis spectrum of horseradish peroxidase (HRP) enzyme solution. 0.05% of HRP enzyme solution in pure solvent (orange spectrum) and in magnetized solvent (blue spectrum). Phosphate buffer (100 mM, pH 6), concentration of HRP, 13.6×10^{-1} mM

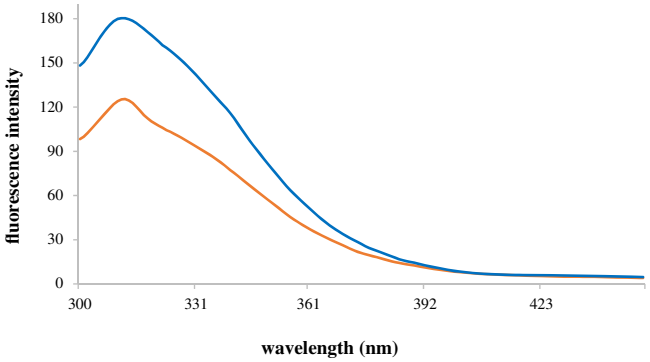


FIGURE 7 Fluorescence spectrometry of horseradish peroxidase (HRP) enzyme solution. 0.05% HRP enzyme solution excited at 280 nm. Pure (orange graph) and magnetized solvent (blue diagram), concentration of HRP, 13.6×10^{-1} mM

10 min. The magnification of soret peak in the UV–Vis absorption spectrum at different times is presented in Figure 5, indicating the decrease in the intensity of the UV–Vis spectrum in the soret region. Figure 6 shows the UV–Vis absorption spectra of HRP in pure and magnetized solvent. Although absorption intensity decreased in all wavelength range, the adsorption pattern was similar in the two solvents.

Fluorescence spectroscopy is a useful method to study the structure and dynamics of protein.[35] Environmental changes in the third structure of HRP protein was investigated by intrinsic fluorescence spectroscopy at its excitation wavelength (280 nm). Figure 7 displays the fluorescence emission spectrum of the enzyme in pure and magnetized solvents after 10 min of enzyme incubation. Spectrometry studies revealed the increase in the fluorescence emission intensity in a magnetized solvent, which can be associated with alteration of enzyme structure and causes a change in the enzyme activity (Figure 7). The effect of the magnetized solvent on the structure of the enzyme was also observed here.

Circular dichroism is an important technique for studying the structure of enzymes. Studies conducted in the far UV-CD spectrum provide practical information on the secondary structure of the enzyme because the peptide bond is the major attractor in this region.[36] Figure 8 shows the far UV-CD spectrum of HRP enzyme in conventional and magnetized solvent. This spectrum has two minima at 210 and 222, and a maximum at 193 nm for peroxidase. Examination of the spectrum intensity reveals the role of the magnetic solvent in increasing the alpha-helix and reducing the beta-structure and random coils.

Table 3 represents the estimation of the contents of the secondary structure of HRP enzyme in conventional and magnetized solvent. It is observed that, in the magnetized solution, alpha-helix increases and the coil random and folded plates decrease.

3.5 | Effect of enzyme incubation in the magnetized water on PGL oxidation reaction rate

As the changes observed in spectroscopy indicated, 10 min of incubation has the greatest effect on the structure. Therefore, to assess this effect for magnetized water, the PGL oxidation reaction in the presence of incubated enzyme was examined for 10 minutes (Table 4). Based on the observations, the reaction was delayed about 5 sec, color changed and the absorption increased due to the formation of the product. All of these implied that the enzyme structure was degraded and further changed in the magnetized medium and caused more structural changes in this condition. Therefore, the enzyme needed about 5 sec to reconstruct its structure and carry out biocatalytic activity to be effective in the reaction progress, but this reconstruction was not enough to increase the reaction rate to an amount above that of pure condition. As shown in Table 4, the enzyme activity was influenced by magnetized solvent as an inhibitor and as a

result, the oxidation rate reduced. The effect of the magnetized solvent had on the structure and activity of the enzyme led to designing of a reaction in which the magnetized solvent had the minimum effect on the enzyme and other parts of the reaction, such as substrate. Subsequently, the substrate was incubated in the reactions and the spectra were recorded immediately after the addition of the enzyme.

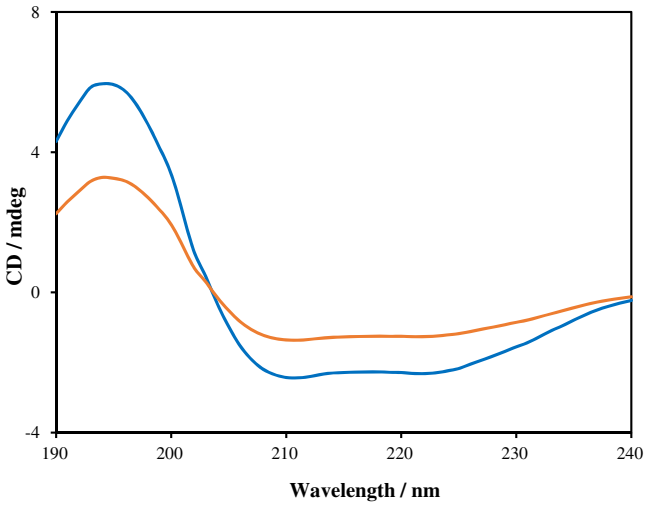


FIGURE 8 Far UV-CD spectrum of horseradish peroxidase (HRP) enzyme. HRP enzyme in pure solvent (orange diagram) and magnetized solvent (blue diagram). CD spectra obtained by dissolving the HRP enzyme in phosphate buffer (100 mm, pH 6.0). Protein concentration in both solutions 7.0×10^{-3} mM

TABLE 3 The percentage of secondary structure elements of HRP in pure and magnetized solvent, estimated from CD spectra using the SELCON III software

Enzyme	Alpha-helix (%)	Beta-structure (%)	Turn (%)	Unordered coil (%)
HRP	43.19	12.29	21.38	23.14
HRP in magnetized buffer phosphate	44.11	12.02	21.51	22.36

Abbreviation: HRP, horseradish peroxidase.

3.6 | Oxidation reaction

The oxidation of phenols and aromatic amine derivatives in the presence of hydrogen peroxide using the HRP enzyme has already been reported. After the oxidation of PGL to its orthoquinone using peroxidase, orthoquinone, which was dehydrogenated and lost its carbon, was coupled with one molecule of PGL and formed purpurogallin as the end product.[37] PGL reaction mechanism is presented in Scheme 2.

As Scheme 3 shows, the enzymatic oxidation of CTL in aqueous solution was carried out with the loss of two hydrogen in the process of transforming CTL to o-benzoquinone.[38]

OPD was oxidized via a free-radical mechanism by HRP enzyme (Scheme 4) which coupled free radicals to yield the 2,3-diaminophenazine through a ring-forming reaction, not by diazo formation.[39]

2-amino-3-Hphenoxazin-3-one was produced by the reaction of OPD with HRP enzyme in the presence of hydrogen peroxide. Since OAP is easily oxidized in the presence of oxidant, it is considered as a reactive compound (Scheme 5).[40] The mechanism of product formation is similar to the mechanism by which the product of OPD oxidation is formed.

3.7 | Determination of the molar absorption coefficient of the product

Molecular absorption coefficient is required for the specification of the initial rate for each substrate. In this regard, a reaction was designed as follows: The solutions of PGL with some specific concentrations were prepared (Table 5) after the enzyme was added. Then, enough time was given to the oxidation reaction to be completed.

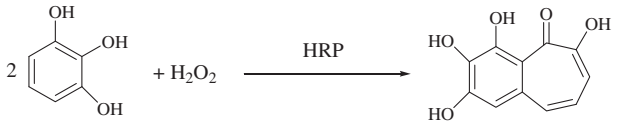
TABLE 5 Determination of pyrogallol molecular absorption coefficient

Concentration (mol/lit)	0.1531 $\times 10^{-3}$	0.28 $\times 10^{-3}$	0.387 $\times 10^{-3}$	0.48 $\times 10^{-3}$	0.56 $\times 10^{-3}$
Absorption 1 (a.u)	0.218	0.394	0.552	0.694	0.803
Absorption 2 (a.u)	0.255	0.425	0.613	0.758	0.89

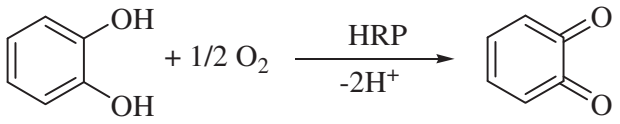
TABLE 4 The enzymatic oxidation rate of derivatives of phenols and aromatic amines in pure and magnetized solvents

Initial rate/substrate	PGL	CTL	RSL	OPD	PPD	OAP	MAP	PAP
V_0 pure solvent $\times 10^{-6}$	20.0	9.38	13.7	3.1	10.9	4.01	2.98	14.0
V_0 magnetized solvent $\times 10^{-6}$	21.4	6.4	17.0	2.17	8.02	4.45	5.2	25.5
V_0 pure solvent with enzyme incubation $\times 10^{-6}$	18.0	–	–	–	–	–	–	–
V_0 magnetized solvent with enzyme incubation $\times 10^{-6}$	15.4	–	–	–	–	–	–	–

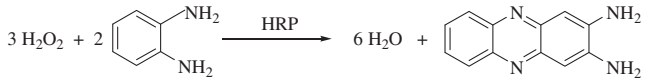
Abbreviations: CTL, catechol; MAP, meta-aminophenol; OAP, ortho-aminophenol; OPD, ortho-phenylenediamine; PAP, para-aminophenol; PGL, pyrogallol; PPD, para-phenylenediamine; RSL, resorcinol.



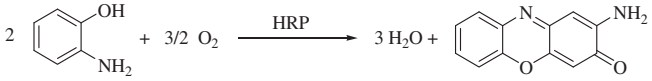
SCHEME 2 Oxidation of pyrogallol by horseradish peroxidase (HRP)



SCHEME 3 Oxidation of catechol by horseradish peroxidase (HRP)



SCHEME 4 Oxidation of ortho-phenylenediamine by horseradish peroxidase (HRP)



SCHEME 5 Oxidation of ortho-aminophenol by horseradish peroxidase (HRP)

After the completion of the reaction, the concentration of purpurogalline in the solution was equal to the initial concentration of PGL. Next, the absorbance of the solution containing the product was read at 420 nm and the concentration was plotted versus the absorption with the slope representing the molar absorption coefficient. The same process was repeated twice for the other substrates (Figure 9).

3.8 | Determination of the initial rate of the reaction

After the preparation of the solution and injection of the enzyme, the absorbance of the solution was recorded for 30 sec at a time interval of 4 sec in order to evaluate the initial rate of the reaction. The results were converted to concentration using epsilon, the concentration graph was plotted against time and then the rate equation was obtained via the derivative of the resulting equation. The constant value of the obtained equation is the initial rate (Figure 10).

Docking method was used in order to get more information at the molecular level (Figure 11). The structure of HPR with PDB code of P8250 was obtained from protein data bank. Then, the docking of substrates with HRP enzyme was investigated using the AutoDock tools software. The results of docking were uploaded in the LIGPLOT software to find more about the interaction between the substrate

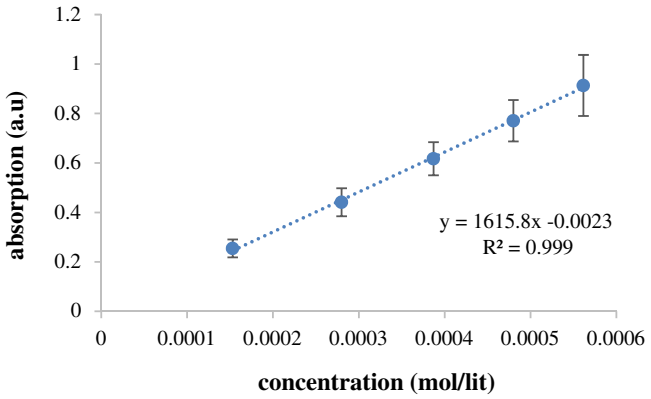


FIGURE 9 Characterization of pyrogallol (PGL) molecular absorption coefficient. After performing the oxidation reactions of PGL, several solutions with different concentrations from the product were prepared: 1.5×10^{-4}, 2.8×10^{-4}, 3.8×10^{-4}, 4.8×10^{-4}, and 5.6×10^{-4}. Absorbance of solutions was recorded at their maximum wavelength (λ = 420 nm) and the product epsilon of oxidation reaction was determined

and the active site of enzyme (Figure 12).[31] PGL was found to be linked to the two amino acids, that is, aspargine 247 and histitidins170, in the active site of enzyme by hydrogen bond. Since the structure of compound I of the enzyme was not available, docking of the substrate in the active site of the enzyme was investigated. The docked conformation of PGL is represented as an example in Figure 11. The docked and ligplot conformation for other substrates are reported in Figure S2–Figure S14.

Considering the structure of compound I, it is predicted that the interaction of the substrate with compound I is of hydrogen bond type and each factor that influences on hydrogen bond may affect the reaction rate. This is resulted from the presence and role of water molecules and the hydrogen bonding between water molecules and hydrogen peroxide with the heme group in the active site of the enzyme in the formation of compound I.[41,42] Since the interaction of aromatic ring with heme (iron) is similar in all the compounds, the structures can have hydrogen bond with appropriate residues in the active site, and therefore, the factors affecting the hydrogen bond may influence the reaction rate. In addition to playing the role of a solvent, some molecules of water placed in the active site during the interaction may have a magnetic effect on the hydrogen bond of water molecules and consequently, on the enzyme reaction.

The results presented in Table 4 show that magnetized water as a solvent increases the oxidation rate of PGL, RSL, OAP, MAP, and PAP; and decreases that of CTL, OPD, and PPD. The comparison of the initial rate of oxidation reactions of CTL and PGL in the pure and magnetized solvent reveals their decrease and increase, respectively. The intramolecular hydrogen bond in CTL is amplified in the presence of a magnetized solvent. Moreover, because of the steric hindrance caused by the increase in the molecular level and the average number of hydrogen bonds in each water molecule,[43] less hydrogen was available in the active site of the enzyme resulting in an decrease in the rate. The rate of PGL reaction showed an increase in both pure and

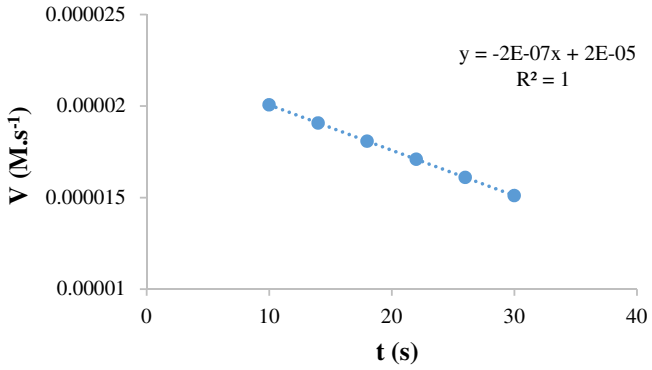


FIGURE 10 The rate graph of pyrogallol oxidation reaction versus time. The absorbance of the oxidation reaction solution was read at specified intervals over time after enzyme injection. With the use of epsilon, the absorption was converted to concentration. Finally, the initial rate was determined using the concentration data

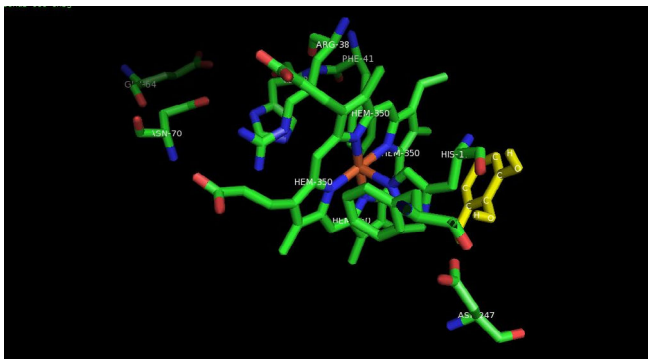


FIGURE 11 Dockings of pyrogallol (PGL) in the horseradish peroxidase (HRP) active site. Docking shows the location of PGL molecule relative to active site of HRP enzyme

magnetized states in comparison to that of the CTL, which can be associated with the higher number of hydroxyl groups of PGL in comparison to the other two substrates for oxidation. In addition, this substrate indicated a slight increase in the magnetized state which was caused by the competition between the two factors of intramolecular hydrogen bonds and a greater number of hydroxyl groups. In RSL, there is no possibility of intramolecular hydrogen bonding, and it can make intermolecular hydrogen bonds only with solvent molecules and other RSL molecules. When solvent is magnetized, the intermolecular hydrogen bonds are weakened, while intramolecular hydrogen bonds become stronger.[15] In addition, RSL molecules become isolated and find more ability to penetrate to the active site of the enzyme and accordingly, increase the reaction rate as shown in the Table 4. In fact, the magnetized solvent does not change the rate of oxidation reaction of these three substrates relative to each other, but only changes the rate of reaction of each substrate relative to its amount in pure solvent. Therefore, depending on the structure of the substrate, solvent can decrease or increase the oxidation rate in the presence of the enzyme.

As previously reported, in the enzymatic oxidation of aminophenol derivatives for radical formation, hydrogen bound to the

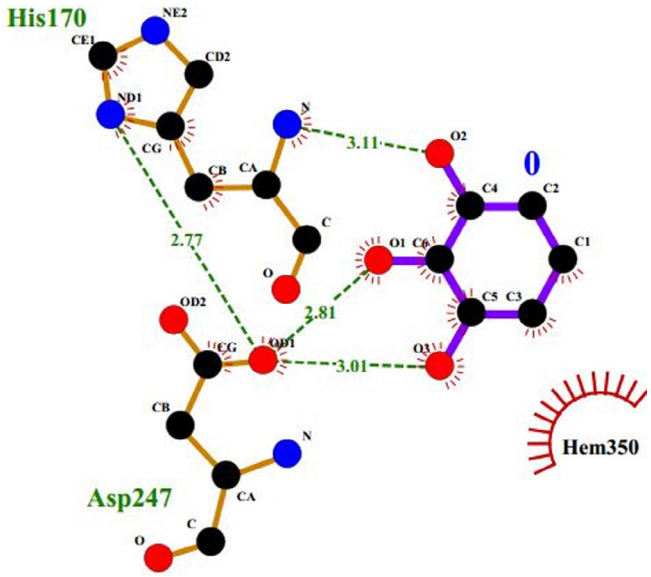


FIGURE 12 Active site of horseradish peroxidase (HRP) enzyme. The interaction of pyrogallol hydroxyl groups with aminoacids in active site of HRP enzyme can be observed. Dotted lines represent hydrogen bond

amine leaves the substrate molecule,[44] leading to an increase in the initial rate of OAP oxidation in the magnetized solvent. The factors that increase the rate include the involvement of hydrogen of the hydroxyl group in hydrogen bonding (due to the greater electronegativity of oxygen relative to nitrogen), the hydrogen bond between the amine hydrogens and the solvent molecules (it can also be said that at least one hydrogen of the amine group is engaged in the hydrogen bonding with solvent molecules), and the stability of amine radical caused by near hydroxyl group. The rate of oxidation of the two MAP and PAP derivatives also raises because they are placed in a magnetized solvent. The electron pair of amine group in PAP is involved in resonance with the aromatic ring and rarely, in hydrogen bond. Therefore, the molecule remains single and more accessible in the active site of the enzyme, which results in an increase in the rate. PPD and OPD in a magnetized solvent have lower reaction rate due to increased number of hydrogen bonds.[43] As a result, steric hindrance happens and makes the substrates less available in the active site of the enzyme.

4 | CONCLUSIONS

Based on the present study, magnetized solvent can affect enzymatic oxidation reaction through changing its activity and consequently, altering the structure of the HRP enzyme. Due to the conversion of the solvent from pure to magnetized type, the oxidation rate of the derivatives of phenol and aromatic amines changes depending on the structure of the substrate and its ability to form an intermolecular or intramolecular hydrogen bond. In fact, the magnetized solvent can affect the oxidation rate of different species in different ways.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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