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Immobilization of Lactoperoxidase on Graphene Oxide Nanosheets and Copper Oxide Nanoparticles and Evaluation of Their Stability

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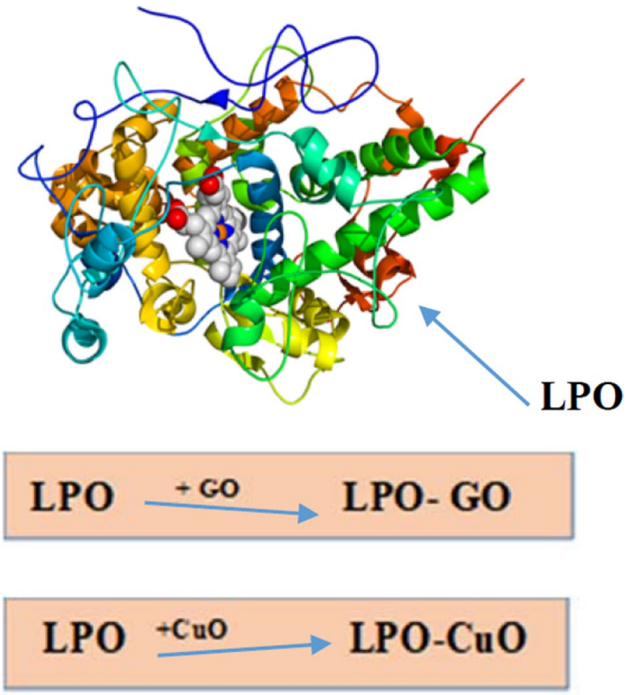
Abstract

Lactoperoxidase (LPO) is a peroxidase enzyme that functions as a natural antibacterial, antiviral, antioxidant and antitumor agent. Stabilization of LPO is a key factor in its industrial applications. In this respect, this work focused on immobilizing LPO on graphene oxide (GO) nanosheets and copper oxide (CuO) nanoparticles using glutaraldehyde, as a cross-linking reagent, and investigating its stability. The Km values of free LPO and LPO immobilized on GO (LPO–GO) and CuO (LPO–CuO) were found to be 53.19, 83.33 and 98.7 mM and their Vmax values equaled to 0.629, 0.504 and 0.41 U/mL min, respectively. The LPO–GO and LPO–CuO samples retained 35 and 12% of their primary activity within 30 days at 25 °C whereas the free enzyme lost its activity after 7 days at the same temperature. Moreover, evaluation of the thermal stability of LPO at 75 °C determined conservation of 24 and 8% of the primary activity of LPO in the LPO–GO and LPO–CuO samples, respectively, after 60 min whereas the free enzyme lost its activity after 5 min. As the findings demonstrated, GO nanosheets are more appropriate for immobilization of LPO, compared with CuO.

Graphical Abstract



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Keywords Lactoperoxidase · Immobilization · Enzyme activity · Stability · Graphene oxide nanosheets · Copper oxide nanoparticles

1 Introduction

Lactoperoxidase (LPO) (EC.1.11.1.7), a peroxidase enzyme found in mammalian milk, is a hemoprotein and glycoprotein with an extensive antimicrobial performance. Bovine LPO contains a single peptide chain of 595 amino acid residues with a molecular weight of about 78 kDa [1]. LPO catalyzes the conversion of the thiocyanate (SCN^-) to hypothiocyanate ($OSCN^-$) in the presence of hydrogen peroxide (H_2O_2). Hypothiocyanate is a potent antibacterial agent because of its tendency to oxidize sulfhydryl groups of microbial enzymes. LPO with SCN^- and H_2O_2 is called LPO system (LPOS), which is a naturally antibacterial system in milk [1, 2] that can be utilized as a preservative agent in Food stuff, cosmetics, tumor therapy and associated products, and in developing drugs, antiviral agents, and dental pastes [3].

LPO, like all enzymes, requires optimum experimental conditions including temperature and pH for efficient catalytic reaction. The importance of this issue can be well understood by knowing that the activity of LPO is considerably reduced after its storage at temperatures above 70 °C for a few minutes [4]. Similarly, LPO is highly sensitive to pH levels below 5.3, and loses its activity under acidic conditions due to the release of calcium ions from the enzyme, and free LPO deactivates when optimal conditions are not met. Therefore, some new approaches should be proposed to promote its stability. For this purpose, enzyme immobilization can be employed as a promising method [5, 6].

Immobilization improves many attributes of enzymes including pH tolerance, selectivity, heat stability or the functional stability [7]. Immobilized enzymes have numerous benefits including retention and repetition of their catalytic performance compared to soluble enzymes [8]. Today, the commercial use of immobilized enzymes has increased because of their high efficiency [8]. Nanoparticles are appropriate to support enzyme immobilization owing to their minimized diffusional limits, the highest surface area per unit mass and high enzyme loading ability [9]. Graphene oxide (GO) is an important solid substrate for enzyme immobilization [10]. It is an extremely oxidized form of chemically modified graphene and is the water-soluble derivative of graphene [10]. The edges of GO sheets are hydrophilic because of functional groups, while the basal plane has a hydrophobic nature, and thus, it shows an amphiphilic behavior giant sheet-like molecule [11]. Immobilization of enzymes has been performed on GO by covalent bonding for functional groups on the GO surface or by using a cross-linker and/or non-covalent binding via weak interactions [12]. GO with a large surface area, two-dimensional structures, and accessibility of surface functionalization can facilitate the mass transfer of substrates and their products through the reaction processes. It is an appropriate carrier in enzyme immobilization due to its good suspending ability and mechanical performance [13]. Furthermore, in vitro and in vivo biological studies showed nontoxic effects of GO-based materials under low dose administration due to their surface functional groups [14]. GO is used for biomedical researches specifically for drug and gene delivery. It has been reported that some enzymes immobilized on GO including Cicer α-galactosidase, β- Galactosidase and Fenugreek β-Amylase are used for food industries [15–17].

Metal oxide nanoparticles are extensively used as industrial catalysts, chemical sensing instruments, in medical usages, disinfection, as antimicrobials, and for enzyme immobilization because of physical constancy, resistance to microbial attacks, biocompatibility, and being very active electrochemically [18, 19]. CuO nanoparticles have high electrocatalytic activity properties, uniform quantum size, and good crystallinity, and several applications for immobilization of enzymes [20]. In organisms, Cu is one of the indispensable elements for maintaining homeostasis. High levels of Cu ions that exceed the physiologically tolerable level have been proved to cause toxicity in vivo [21]. To ensure that NPs are safe to organisms and the environment, toxicity must be decreased to the significant level [22]. This objective requires further work that focuses on toxicity factors of metal oxide NPs.

In the present study, LPO was purified from bovine whey through a modified Fong Yoshida approach [23]. Then, it was immobilized on GO and CuO by covalent attachment by glutaraldehyde to promote the stability under harsh conditions as well as its reusability [24]. Finally, the stability and kinetics of the free and immobilized LPO samples were studied under various conditions and were compared with each other.

2 Experimental Section

2.1 Materials

Fresh unpasteurized bovine milk (raw milk) was purchased from the local dairy. GO nanosheets and CuO nanoparticles were prepared from US Research Nanomaterials, Inc. and were utilized as received. Glutaraldehyde solution (50% (v=v) in water), Guaiacol and the CM-cellulose resin were obtained from Sigma–Aldrich (USA). chemicals and buffers

for electrophoresis were obtained from Merck (Germany), and Bradford reagent was purchased from Bio-Rad (USA).

2.2 Purification of the Lactoperoxidase

2.2.1 Skim Milk Preparation

Fresh bovine milk was centrifuged at 1048 × *g* for 20 min at 4 °C. Then the upper layer (lipid layer) was separated by pipetting, and it was discarded [1].

2.2.2 Rennet Whey Preparation and Precipitation of Whey Globulins

The skim milk was warmed to 37 °C and then rennet (Sigma Co, St.Louis, Mo, USA) was added in the proportion of 0.1 g/L and kept at 37 °C for 1 h so that the caseins could be coagulated. The coagulated caseins were subsequently removed by filtration using Whatman No.1 filter paper. In the next step, to eliminate globulin portions, the solution of ammonium sulfate (242 g/L) was added to the whey in three steps, and the mixture was shaken for 3 h at 4 °C and, finally, it was centrifuged (Laborzentrifugen, Sigma, Heraus Sepatech Suprafuge 22, Hanau, Germany) at 25,155 × *g* rate for 20 min at 4 °C to remove its globulin portions. Then, the solution of ammonium sulfate (202 g/L) was added to the obtained supernatant and centrifuged at 25,155 × *g* for 20 min at 4 °C to isolate the proteins portion. The obtained pellet was dialyzed overnight against the 50 mM Tris–HCl buffer (pH 8.6) with a ratio of 1/100. The dialyzed sample was applied to a CM-Cellulose column.

2.2.3 Cation Exchange Chromatography

Carboxy methyl cellulose (CM-cellulose), was used as a cation exchanger resin. 2 g of dry resin was slowly suspended to double distilled water to swell for 1 h. The resin was packed in a 1.2 cm × 30 cm column and equilibrated with 50 mM Tris–HCl buffer (pH8.6). The dialyzed sample was applied to a CM-Cellulose column following the equilibration with Tris–HCl buffer (50 mM, pH 8.6). After that, the matrix was washed with an amount of the Tris–HCl buffer (50 mM, pH 8.6) equaling two times the volume of the column, and the same buffer containing a linear gradient of 0.1 to 0.5 M NaCl with 0.7 mL/min flow rate was applied to fractionate the adsorbed proteins. For each NaCl gradient step, 50 mL of the related buffer was used and the collected fraction volume was about 5 mL. Then, the value of Reinheits Zahl (Rz; A412 nm/A280 nm) or the purity number of the collected fractions was calculated and the fractions with Rz values equaling or higher than 0.7 were pooled and salted out using a solution of ammonium sulfate with 90% saturation. The enzyme pellet was dialyzed overnight against 30 mM sodium phosphate buffer (pH 6.8) [1]. To monitor all steps of the purification procedure, SDS-PAGE electrophoresis was performed according to the Laemmli method using a 5% stacking gel and a 10% separating gel [25]. In order to calculate the purification fold and specific activity of LPO, the protein concentration was determined based on the Bradford method using bovine serum albumin as standard [26].

2.3 Enzyme Assay

Lactoperoxidase peroxidase activity was determined in 30 mM sodium phosphate buffer (PH 6) and guaiacol was used as enzyme substrate. This method is based on the oxidation of guaiacol by H_2O_2 in the presence of lactoperoxidase. The absorbance was measured at 470 nm every 15 s for 3 min (Molar extinction coefficient of tetra-guaiacol is 26.6/mM/cm). One unit of enzyme activity was known as the concentration of enzyme catalyzing the alteration of 1 µmol guaiacol to tetraguaiacol per min [27].

2.4 Protein Estimation and Immobilization Efficiency

To determine the amount of immobilized LPO, centrifugation was carried out at 25,155 × *g* for 10 min, 0.8 mL of the obtained supernatant, which contained free LPO, was removed and mixed with 0.2 mL of the Bradford's reagent [5]. After 5 min, spectrophotometry was performed to measure the concentration of free LPO at the wavelength of 595 nm. Then, the amount of immobilized LPO was determined by subtracting the total amount of LPO from the amount of free LPO.

$$\text{Immobilization efficiency \%} = \frac{\text{Specific activity of immobilized enzyme}}{\text{Initial specific activity of soluble enzyme}} \times 100$$

2.5 Immobilization Procedure

The GO nanosheets (1000 µg) were modified using glutaraldehyde as cross-linking reagent at a concentration of 1% (v/v) and the mixture was slowly stirred (4 h) at room temperature (25 °C). After this reaction, the glutaraldehyde modified GO material was separated by centrifugation at 25,155 × *g* for 10 min [24]. The material was washed with sodium phosphate buffer (pH 6.8) three times. Then, 1000 µL of LPO (0.345 mg/mL) was added and the mixture was shaken at room temperature for 1 h as the cross-linking procedure. The immobilized LPO was separated by centrifugation at 25,155 × *g* for 10 min. The material was washed three times with sodium phosphate buffer (pH 6.8) and the

obtained material was suspended in the buffer and stored at 4 °C.

The CuO nanoparticles (1000 µg) were modified using glutaraldehyde as cross-linking reagent at concentration 1% (v/v) and the mixture was slowly stirred (4 h) at room temperature (25 °C). After this reaction, the glutaraldehyde modified CuO material was separated by centrifugation at 25,155 × *g* for 10 min. The material was washed with sodium phosphate buffer (pH 6.8) three times. Then, 1000 µL of LPO (0.345 mg/mL) was added and the mixture was shaken at room temperature for 1 h as the cross-linking procedure. The immobilized LPO was separated by centrifugation at 25,155 × *g* for 10 min. The material was washed three times with sodium phosphate buffer (pH 6.8) and the obtained material was suspended in the buffer and stored at 4 °C.

2.6 Structure Characterization

The FT-IR spectra of samples were recorded by a Fourier Transform Infrared Spectroscopy (JASCO FT/IR-4200, Easton, USA), and for FT-IR studies, samples were prepared by mixing the material with KBr powder. The range of 350–4000 cm^{-1} was selected to record the spectra. The morphology of the specimens was characterized by using Field Emission Scanning Electron Microscopy (FESEM, TESCAN MIRA3 XMU VP, Brno, Czech Republic).

2.7 Determination of the Michaellis–Menten Constants

Km and Vmax values of the Free and immobilized enzyme were determined by measuring the enzyme activity using four different concentrations of guaiacol (100, 200, 400, and 800 mM) as substrates. The Km and Vmax values were calculated by the Lineweaver–Burk graph [28].

2.8 Optimum pH

The optimal pH for the activity of free and immobilized LPO was measured by incubation of the enzyme in pH ranging from 3 to 8. Buffers were 30 mM sodium citrate (pH 3–5.5) and 30 mM sodium phosphate (pH 6–8) [29].

2.9 Optimum Temperature and Thermal Stability

The optimum temperature for activity of the free and immobilized LPO was determined by incubating the enzyme in temperatures ranging from 20 to 80 °C in a water bath, and their activities were assayed. The highest activity was taken as 100%. LPOs were incubated at 75 °C in a water bath for 60 min to determine the thermal stability for immobilized and free LPO. At certain time intervals, LPOs were immediately transferred to an ice bath and their activities were assayed [29].

2.10 Evaluation of Enzyme Storage Stability

Immobilized and free enzymes were kept at room temperature (25 °C) to evaluate the storage stability. Then, for 30 days, on a regular basis, the activity of the immobilized and free enzyme was measured.

2.11 Reusability of Immobilized LPO

To evaluate the reusability of the immobilized LPO proteins, they were studied under optimal conditions. After each run, the immobilized LPOs were isolated by centrifugation at 25,155 × *g* for 10 min and they were washed carefully with respective buffer solution to remove any residual substrate (guaiacol) and product before the next assay. The initial activity of the immobilized LPO enzymes was considered to be 100% and their residual activity after each assay was determined, accordingly [5].

2.12 Statistical Analysis

The data were analyzed using SPSS software (version 20, SPSS Inc., Chicago, IL, USA) and reported as a mean ± standard deviation. Independent t-test was utilized to compare the mean activity of the immobilized and free enzyme. If $p < 0.05$, the differences were considered statistically significant.

3 Results and Discussion

3.1 Purification of the Lactoperoxidase

In this study, LPO was purified from bovine whey using two steps of precipitation by ammonium sulfate and one step of CM-cellulose cation-exchange chromatography. After loading the sample solution to the CM-cellulose column and using a linear gradient of 0.1–0.5 M of NaCl in the elution buffer, for each fraction, absorbance values were measured at 412 nm and 280 nm, and the results are shown in Fig. 1. The enzyme was eluted with a 0.4 M NaCl gradient with Rz value of 0.8, and it was salted out at 90% saturation of ammonium sulfate. Table 1 shows the specific activities and protein concentrations related to all steps of LPO purification. According to this table, LPO was purified 54.3 fold with yield of 11.05% and 5.579 IU/mg specific activity. The present method provides a higher LPO purity index relative to the previously reported techniques [30, 31]. This may be attributed to the application of the 50% ammonium sulfate solution to remove globulins from bovine whey. Overall,

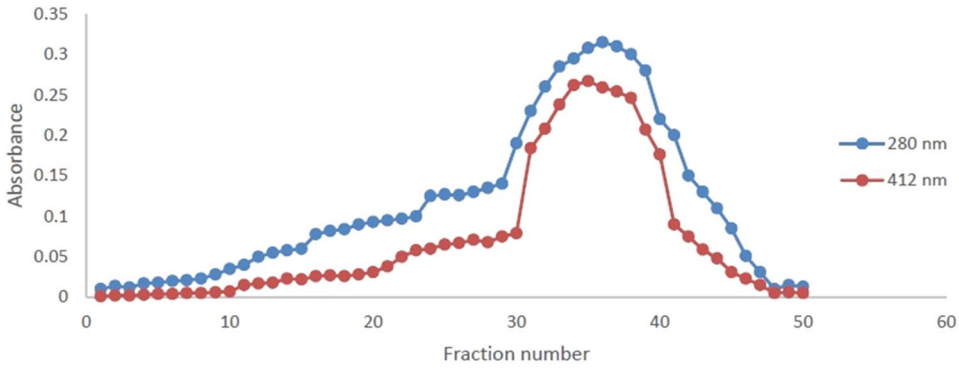

Fig. 1 Ion-exchange chromatography of LPO with CM-Cellulose column

Table 1 Purification steps of lactoperoxidase (LPO) from bovine whey

Purification steps	Total volume (mL)	Enzyme activity (IU/mL)	Total enzyme activity (IU)	Protein (mg/mL)	Total protein (mg)	Specific activity (IU/mg)	Recovery (%)	Purification fold
Whey	510	0.2936	149.77	3.1	1581	0.1027	100	1
Dialyzed whey proteins precipitated with 70% ammonium sulfate	50	0.7358	36.79	4.87	243.5	0.151	24.56	1.47
Collection of chromatography fraction with Rz value > 0.7	50	0.369	18.45	0.072	3.6	5.125	12.32	49.9
Dialyzed LPO precipitated with 90% ammonium sulfate (purified)	8.6	1.925	16.55	0.345	2.967	5.579	11.05	54.3

it can be stated that using 50% ammonium sulfate solution for precipitation of globulins is so effective that it can lead to very pure LPO with high yields comparable with those reported by Nandini et al. [30] and Mecitoglu et al. [32] who employed various successive methods for the extraction of LPO from bovine milk while the procedure used in the present study involved simple removal of globulins and using just CM-cellulose ion exchange chromatography as a fast and cost effective technique. In a similar research, the purified LPO by Borzouee et al. [1] showed 59.13-fold with a yield of 10.26%.

3.2 SDS-PAGE Results

SDS-PAGE electrophoresis was used to analyze all purification steps of LPO from bovine whey. Figure 2 shows the SDS-PAGE electrophoresis of the isolated LPO from bovine whey. As it can be seen, the analysis has resulted in the appearance a single band, as described previously with 78 kDa molecular weight [1, 5]. It shows that the present method has been efficient in purifying LPO.

3.3 Immobilization Efficiency

The results showed that the protein concentration of the free and immobilized LPO on GO and CuO were 0.345, 0.293 and 0.269 mg/mL, respectively. In addition, the results indicated that the activity of free and immobilized LPO on GO and CuO was1.925, 1.315 and 0.945 IU/mL, respectively and the Specific activity of free and immobilized LPO on GO and CuO was 5.57, 4.48 and 3.51 IU/mg, respectively. Accordingly, the immobilization efficiency of LPO on GO was 80%, and, that of LPO on CuO was 63%.

3.4 Sample Characterization

The FT-IR spectra of GO, GO- glutaraldehyde, and GO-glutaraldehyde-LPO are shown in Fig. 3. Moreover, The FTIR spectra of CuO, CuO-glutaraldehyde, and LPO–CuO-glutaraldehyde are presented in Fig. 4. The main functional groups were identified in GO including peaks at 1720–1740 cm^{-1} are subjected to C=O stretching vibration, and 3300–3400 cm^{-1} are related to O–H stretching vibrations [33]. Moreover, characteristics band for C=C from unoxidized sp^2 C–C bonds appeared in 1590–1628 cm^{-1}, and also C–O vibrations and epoxide groups appeared at 1228 cm^{-1}, 849, 1048 cm^{-1}, respectively (Fig. 3a) [33].

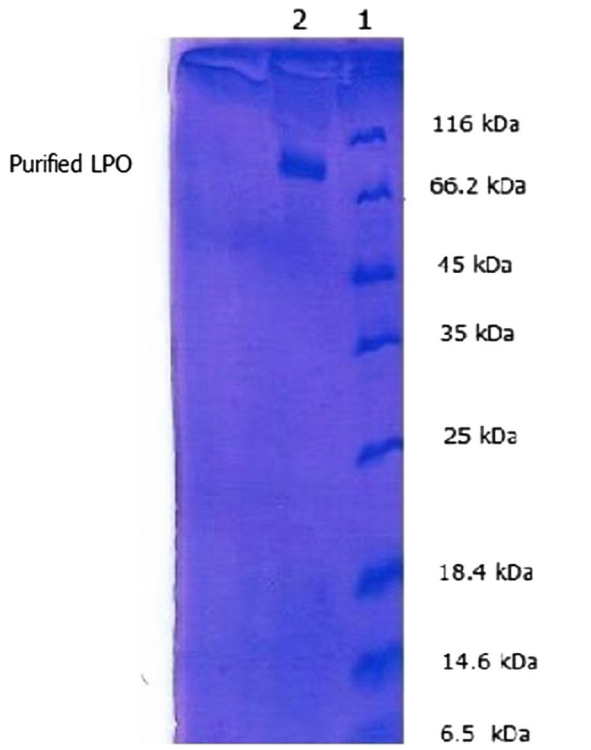

Fig. 2 SDS-PAGE electrophoresis of purified LPO from bovine whey; lane 1 shows standard proteins and lane 2 illustrates the purified LPO

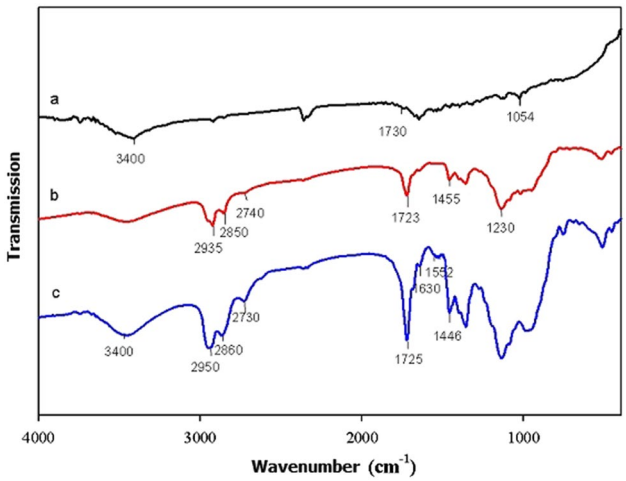

Fig. 3 FT-IR spectrum: *a* GO, *b* GO–Gul, and *c* GO–Gul–LPO

Figure 4a presents the FT-IR spectra of CuO, wherein the functional groups identified at 400–800 cm^{-1} are indexed to Cu–O stretching vibration [19]. The Characteristic bands at 1720–1740 cm^{-1} (C=O), 2720–2850 (C–H) cm^{-1} which correspond to the stretching vibration of the aldehyde group in glutaraldehyde has confirmed the successful modification GO and CuO NPs with it (Figs. 3b, 4b) [33, 34]. After

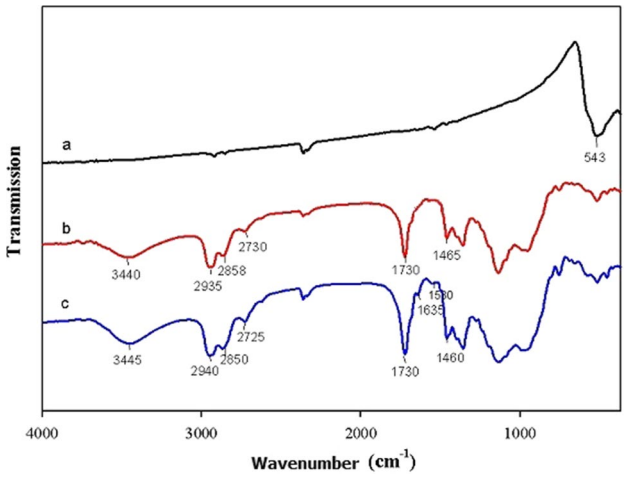

Fig. 4 FT-IR spectrum: *a* CuO, *b* CuO–Gul, and *c* CuO–Gul–LPO

immobilizing enzyme on modified GO and CuO NPs, the characteristics of bands related to amide groups, especially the appearance of 1640–1690 cm^{-1} (C=O), 3100–3500 cm^{-1} (N–H stretching), 1550–1640 cm^{-1} (N–H bending), 1455 cm^{-1} (C–N stretching) [35] has indicate covalent attachment LPO (Figs. 3c, 4c). However, it should be considered that due to the presence of common amid bonds in both enzyme and glutaraldehyde the increase of the intensity of bonds mode can confirm that the after cross-linking reaction, enzyme immobilized on support successfully. In fact, these changes might be associated to the formation of chemical bonds with nitrogen and oxygen, signifying that –OH and –NH were involved in the reaction with glutaraldehyde and conjugation was successfully established between enzyme and modified supports.

Morphology study of the GO revealed layers with about 70 nm thickness in FESEM image (Fig. 5), and the appearance of spherical particles in the case of GO–LPO samples was compared with those of GO in FESEM image (Fig. 5b). Morphology study of CuO revealed nanoparticles with various shapes in the FESEM image (Fig. 6), and the appearance of spherical particles in the case of CuO–LPO samples was compared with those of CuO in FESEM image (Fig. 6b). FESEM images clearly show the enzyme attachment. The loaded enzyme had a spherical morphology.

3.5 Determination of Kinetic Parameters for Free and Immobilized LPO

Kinetic parameters, Km and Vmax were determined for native and immobilized enzyme on GO nanosheets and CuO nanoparticles (Figs. 7, 8). Km values were calculated as 53.19 mM for free enzyme and 83.33 and 98.07 mM for immobilized enzyme on GO nanosheets and CuO nanoparticles, respectively. Vmax values of the free LPO and

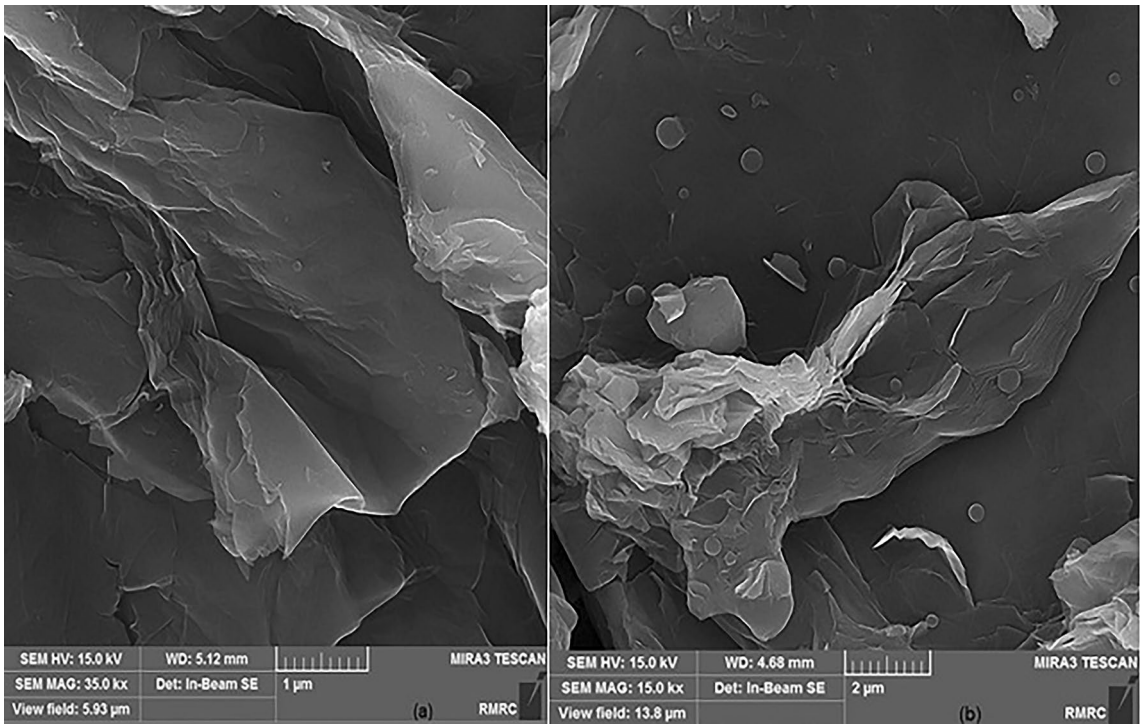

Fig. 5 FESEM imaging of: **a** the GO nanosheets and **b** GO–LPO

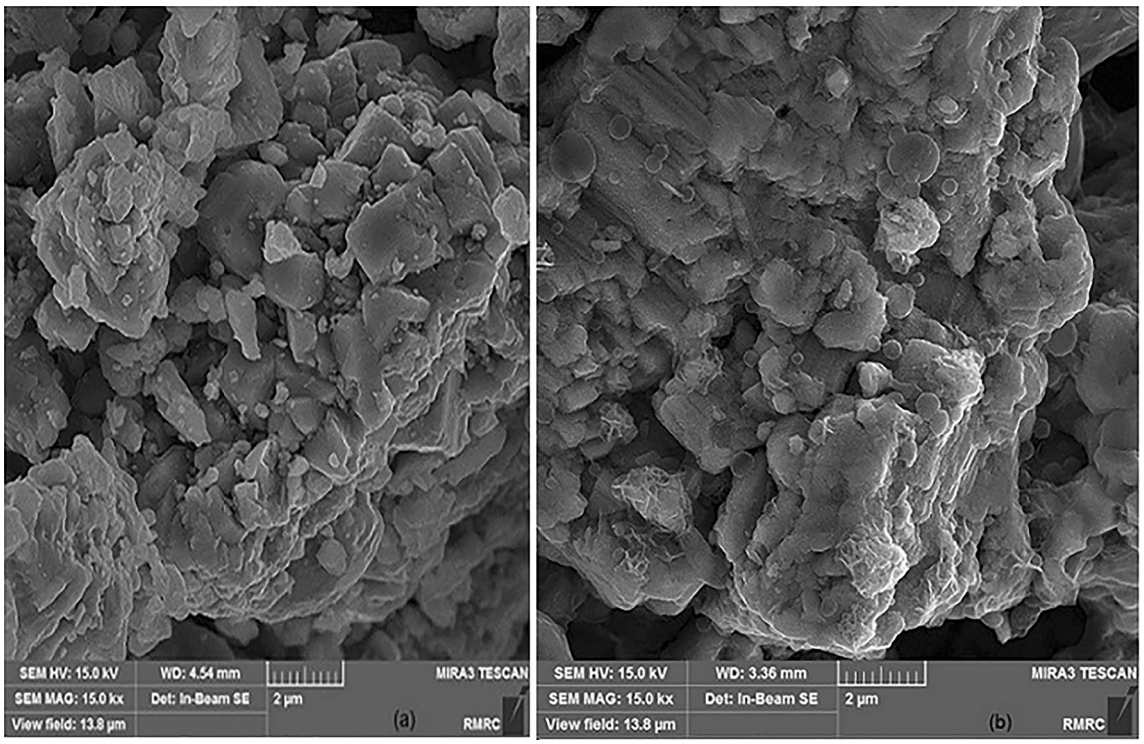

Fig. 6 FESEM imaging of: **a** the CuO nanoparticles and **b** CuO–LPO

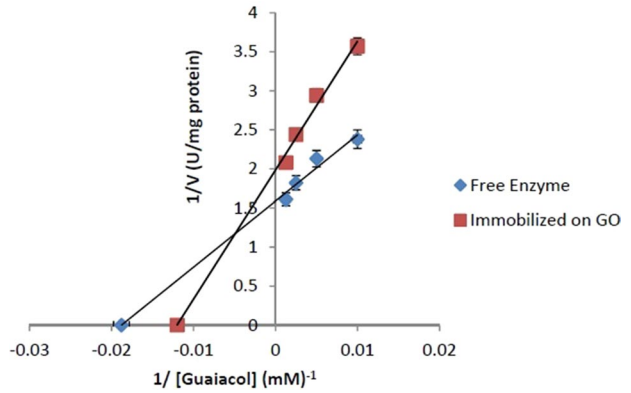

Fig. 7 Lineweaver–Burk plot relating Free LPO and LPO immobilized on GO; each data point represents the average value of three independent measurements ± standard deviation

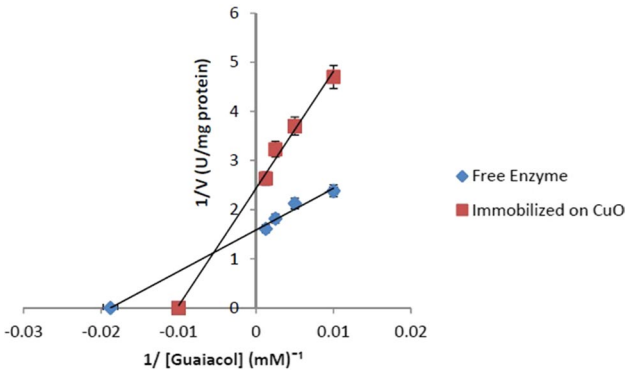

Fig. 8 Lineweaver–Burk plot relating Free LPO and LPO immobilized on CuO; each data point represents the average value of three independent measurements ± standard deviation

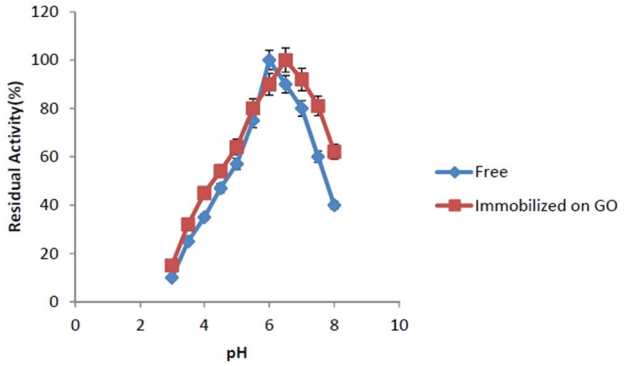

Fig. 9 Optimum pH for the activity of free and immobilized lactoperoxidase on GO; each data point represents the average value of three independent measurements ± standard deviation

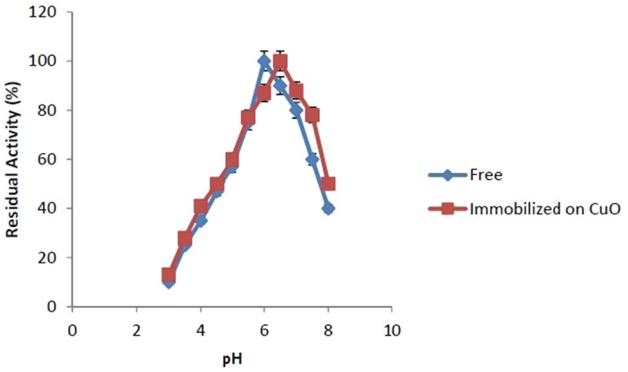

Fig. 10 Optimum pH for the activity of free and immobilized lactoperoxidase on CuO; each data point represents the average value of three independent measurements ± standard deviation

immobilized LPO on GO nanosheets and also immobilized LPO on CuO nanoparticles were 0.629, 0.504 and 0.41 (U/mL/min), respectively. Our results showed that LPO immobilization on GO nanosheets and CuO nanoparticles results in an increase in Km value of the enzyme. It means that affinity of the enzyme for bounding to the enzyme substrate has decreased. However, Km value of the enzyme immobilized on GO nanosheets is less than that of the enzyme immobilized on CuO nanoparticles. Thus, the rate of the enzyme reaction for enzyme—GO nanosheets complex is more than that of the enzyme—CuO nanoparticles complex. Similarly, some other researches have reported the change of kinetics properties upon immobilization of enzymes. Tenovuo et al. [36] who immobilized the LPO on Con A-agarose reported an increase in Km of immobilized enzyme. The Km values of the immobilized enzyme and free enzyme were 1.9 mM and 1 mM, respectively. Su et al. [24] indicate that Km values of free and immobilized protease on graphene Oxide were 4.85 and 8.57 mM, respectively. Saleh et al. [37] who immobilized Horseradish peroxidase on the magnetic nanoparticles reported that the Km values of the free enzyme and immobilized enzyme were 31 and 45 mM for guaiacol; moreover, the Vmax values of free and immobilized enzyme were found to be 2.5 and 1.2 U/mL/min, respectively, indicating the reduced affinity of immobilized enzyme to substrate. These alterations may be due to structural alterations in the enzyme presented through the current immobilization process or the lower accessibility of the substrate to the active sites of the immobilized enzyme. However, immobilization poses some limitations due to the restriction of mass transfer and active site accessibility. This can be significantly suppressed through intensive optimization of the immobilization process and continuous stirring of the mixture containing immobilized enzyme, substrate and released product at optimum velocity [5].

3.6 Optimum pH

Optimum pH of free and immobilized LPO was evaluated by incubation of these preparations in buffers with pH values in the range of 3–8 (Figs. 9, 10). The highest activity was obtained at pH 6 and 6.5 for the free and immobilized LPO on GO, respectively, also the optimal pH for the LPO immobilized on CuO was 6.5. Both forms of immobilized LPO retained a greater amount of primary activity in acidic and alkaline pH compared to the free enzyme. It is obvious that the LPO immobilized on GO retained higher enzyme activity in acidic and alkaline pH compared to the LPO immobilized on CuO. These findings are in accordance with the results previously reported about the application of GO and CuO as a supporting material for enzyme immobilization [24, 38, 39]. This change in the optimum pH is explained by an alteration in the micro-environment of the enzyme due to immobilization. Depending on the residual charges on the surface of the support and the chemical nature of the bonds between the support material and enzyme, the pH value in the micro-environment of the enzyme molecule might be different, which might alter the optimal pH of the enzyme [6, 32].

3.7 Optimum Temperature

The optimum temperatures for activity shifted from 45 °C for free LPO to 55 °C for immobilized LPO on GO (Fig. 11). The optimum temperature for activity of LPO immobilized on CuO was unchanged at 45 °C (Fig. 12). This optimum temperature for LPO activity was in good agreement with that observed by other researchers. Miroliaei et al. [40] showed that the optimum temperature for free LPO and LPO immobilized on Con A-Sepharose 4B was 45 °C. Tenovuo et al. [36] indicated that the optimum

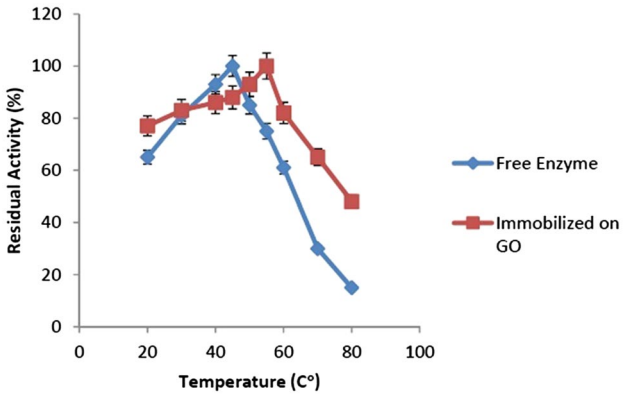

Fig. 11 Optimum temperature for the activity of free and immobilized lactoperoxidase on GO; each data point represents the average value of three independent measurements ± standard deviation

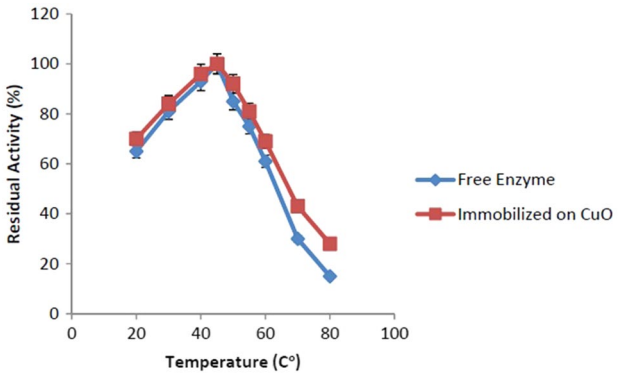

Fig. 12 Optimum temperature for the activity of free and immobilized lactoperoxidase on CuO; each data point represents the average value of three independent measurements ± standard deviation

temperature for free LPO was between 45 and 50 °C, whereas the immobilized LPO onto Con A- agarose had its maximum activity at a temperature from 50 to 60 °C. Su et al. [24] reported an increase in optimum temperature after enzyme immobilization on GO, which might be due to the fact that immobilization on GO restricts free movement of LPO [1].

3.8 Thermal Stability

The thermal stability corresponding to free and immobilized LPO at 75 °C was studied and the activities of the immobilized and free LPO were assayed (Fig. 13). The immobilized enzyme on GO maintained 24% of its primary activity after 60 min at 75 °C. In comparison, the immobilized enzyme on CuO maintained 8% of its primary activity after 60 min at 75 °C, and free enzyme inactivated entirely after 5 min at the same temperature. The results showed that the immobilized enzymes had a higher thermal stability compared to the free enzyme. In

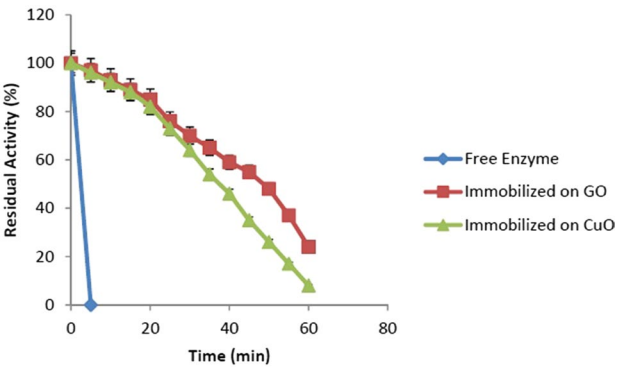

Fig. 13 Thermal stability of free and immobilized lactoperoxidase; each data point represents the average value of three independent measurements ± standard deviation ($p < 0.05$)

contrast, LPO immobilized on GO was considerably more thermostable than LPO immobilized on CuO, and these differences are statistically significant ($p < 0.05$). Boscolo et al. [41] indicated that 70 °C is an apparent midpoint transition temperature for irreversible thermal denaturation of LPO. Immobilized enzyme on GO showed a higher resistance against thermal denaturation, suggesting that the immobilized enzyme makes a strong binding with GO nanosheets through the immobilization and less conformational change by heat. A similar result was achieved by Samsam Shariat et al. [5] who reported that Lactoperoxidase immobilized on GO had an improved thermal stability. In another study, Zhang et al. [42] showed that immobilization of Horseradish Peroxidase on GO would significantly increase the thermal stability of the enzyme. Improved thermal stability after immobilization also was reported in other works [24, 43, 44]. The findings of this study suggest that immobilizing LPO on GO would make the enzyme more stable at high temperatures.

3.9 Storage Stability

The storage stabilities of free and immobilized LPO during storage at 25 °C were determined at regular time intervals. The LPO immobilized on GO retained 35% of its primary activity after 30 days. LPO immobilized on CuO retained 12% of its initial activity after 30 days, and free LPO was inactive after 7 days (Fig. 14). The results clearly indicate that the immobilized enzymes had a higher storage stability compared to the free enzyme. In contrast, the storage stability of LPO immobilized on GO was considerably higher than LPO immobilized on CuO, and these differences are statistically significant ($p < 0.05$). The findings of the current study are in agreement with those obtained by Su et al. [24] who showed immobilization of alkaline protease on GO enhanced storage stability of the enzyme. Improved storage stability may be caused by the decrease in the denaturation speed and less conformational change of the immobilized enzyme [5, 42]. GO nanosheets has high loading capacity for the enzyme due to its large surface area, covalent bonding for functional groups on the GO surface, two-dimensional structures.

3.10 Reusability of Immobilized LPO

The lack of ability of free enzymes for reusing is one of major limitations for their applications in continues reactions. Figure 15 shows the residual activity of the immobilized LPO on GO and CuO following several times of applying residual activity (54%) and (24%) observed after 10 cycles of using the immobilized LPO on GO and CuO, respectively. In comparison, the reusability of LPO immobilized on GO was considerably higher than LPO immobilized on CuO, and these differences are statistically significant ($p < 0.05$), indicating that the immobilized LPO on GO has appropriate stability and can be reused more. Su et al. [24] have also reported the residual activity of up to 40% for immobilized alkaline protease on GO after using it three times. The main reason that the immobilized LPO on GO has more appropriate reusability might be attributed to that GO nanosheets has high loading capacity for the enzyme due to its large surface area, covalent bonding for functional groups on the GO surface. High reusability of enzymes is important for the development of biosensors [38].

4 Conclusion

In this study, a facile and efficient method was presented for the purification of LPO from bovine whey and its immobilization onto the GO nanosheets and CuO nanoparticles by the covalent method. In both immobilizations, the Km value shifted towards a higher value, suggesting that the

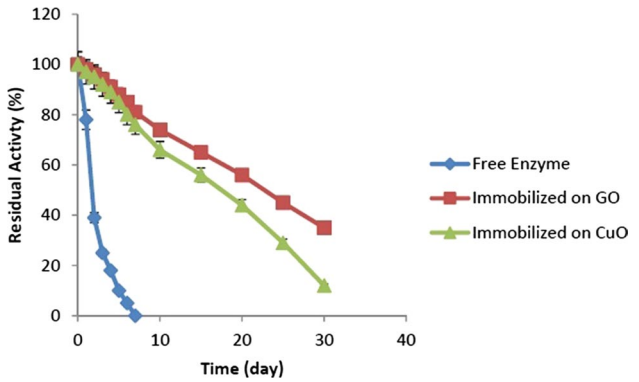

Fig. 14 Storage stability of free and immobilized lactoperoxidase; each data point represents the average value of three independent measurements ± standard deviation ($p < 0.05$)

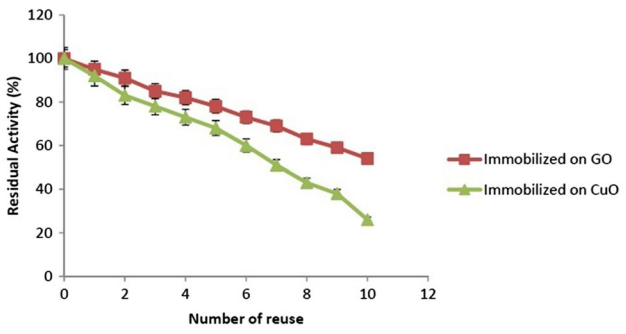

Fig. 15 Reusability of immobilized lactoperoxidase; each data point represents the average value of three independent measurements ± standard deviation ($p < 0.05$)

affinity between immobilized LPO and substrate has not been improved. The optimum pH for the activity of the immobilized enzyme on GO and CuO also increased in comparison to the free enzyme, and the optimum temperature for the activity of the immobilized enzyme on GO increased in comparison to that for immobilized enzyme on CuO and free enzyme. Immobilized LPO displayed increased thermal stability and storage stability and reusability of the immobilized LPOs in contrast to the free enzyme. In comparison, the thermal stability and storage stability and reusability of LPO immobilized on GO was significantly higher than those of LPO immobilized on CuO, which are important factors in commercial and industrial scales. Our results showed the suitable GO nanosheets compared to CuO nanoparticles because GO nanosheets have high loading capacity for the enzyme due to their large surface areas, covalent bonding for functional groups on the GO surface, and two-dimensional structures; moreover, the accessibility of surface functionalization can facilitate the mass transfer of substrates and their products through the reaction processes. Therefore, the immobilization of LPO on GO nanosheets by mediated glutaraldehyde could be suggested as an efficient, simple and useful method for extending the more stable enzyme in large scale applications.

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