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Purification and enzymatic properties of a textile dye-decolourizing peroxidase from *Moringa oleifera* roots

Purificação e propriedades enzimáticas de uma peroxidase descorante de corantes têxteis a partir de raízes de Moringa oleifera

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ABSTRACT

Peroxidases are ubiquitous enzymes involved in the oxidation of a variety of aromatic substrates including textile dyes, which are harmful for aquatic life and human health. Hence, the present study describes the purification of a peroxidase, named *Mo*POX, from *Moringa oleifera* roots using DEAE-Sephacel and gel filtration chromatography on a Superdex® 75 column. The peptide sequences recorded by mass spectrometry analysis confirmed the identity of *Mo*POX with other plant peroxidases. The optimum pH and temperature of enzyme activity were 5.2 and 70 °C, respectively. Its enzymatic activity in the presence of metal ions and classical peroxidase inhibitors was also evaluated. *Mo*POX follows Michaelis-Menten kinetics, with specificity, in ascending order, to the substrates ABTS < eugenol < *O*-dianisidine ≅ guaiacol, besides being highly thermostable. The purified peroxidase (0.015, 0.030 or 0.150 mg/mL) degraded different dyes (50 or 100 mg/L), such as Remazol® Blue RGB, Remazol® Navy RGB and Telon® Turquoise M-5G 85%. Decolorization rates varied from 15 to 90% depending on the dye concentration, enzyme concentration and exposure time. *Mo*POX is the first peroxidase purified from *M. oleifera* roots, and the results showed it has biotechnological potential for biodegradation of hazardous compounds.

Keywords: Plant peroxidase, textile dye, *Moringa oleifera*, bioremediation.

RESUMO

As peroxidases são enzimas onipresentes envolvidas na oxidação de uma variedade de substratos aromáticos, incluindo corantes têxteis, prejudiciais à vida aquática e à saúde humana. Portanto, o presente estudo descreve a purificação de uma peroxidase, denominada MoPOX, a partir de raízes de Moringa oleifera usando DEAE-Sephacel e cromatografia de filtração em gel em uma coluna Superdex® 75. As sequências peptídicas registradas por análise por espectrometria de massa confirmaram a identidade de MoPOX com outras peroxidases de plantas. O pH e a temperatura ótimos da atividade enzimática foram de 5,2 e 70 ° C, respectivamente. Também foi avaliada sua atividade enzimática na presença de íons metálicos e inibidores clássicos da peroxidase. O MoPOX segue a cinética de Michaelis-Menten, com especificidade, em ordem crescente, para os substratos

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ABTS <eugenol <O-dianisidina / guaiacol, além de ser altamente termoestável. A peroxidase purificada (0,015, 0,030 ou 0,150 mg / mL) degradou diferentes corantes (50 ou 100 mg / L), como Remazol® Blue RGB, Remazol® Navy RGB e Telon® Turquoise M-5G 85%. As taxas de descoloração variaram de 15 a 90%, dependendo da concentração de corante, concentração de enzima e tempo de exposição. MoPOX é a primeira peroxidase purificada a partir de raízes de M. oleifera, e os resultados mostraram que possui potencial biotecnológico para biodegradação de compostos perigosos.

Palavras-chave: Peroxidase vegetal, corante têxtil, Moringa oleifera, biorremediação.

1. INTRODUCTION

Textile dye manufacturing and processing generate massive volumes of effluents that contaminate the ecosphere [1]. Synthetic dyes are the main recalcitrant molecules found in these effluents [2]. It has been estimated that the disposal of colorants in the environment can reach 10-50% [3]. These compounds decrease the dissemination of daylight, resulting in the reduction of the overall rate of photosynthesis of algae and other aquatic vegetation, the gas solubility and the esthetic condition of contaminated water [4]. Furthermore, some dyes and their breakdown products are highly toxic, carcinogenic and/or mutagenic to living organisms [5].

Various chemical, physical and biological techniques have been applied to remove dyes from industrial wastewater prior to release into the aquatic environment. Biological treatment, especially using microorganisms, offers a cheaper and environmentally friendly alternative for color removal, but it has some drawbacks, such as the susceptibility of toxic dyes and other substances present in the effluent. Therefore, in cases where there is a limitation for organism growth, isolated enzyme systems are preferred [6]. The high specificity of enzymes enables targeted recalcitrant pollutants in wastewater to be removed by precipitation or transformation into innocuous products. Enzymatic treatment processes can occur at extreme pollutant concentrations and over wide ranges of temperature, pH and salinity [7]. Moreover, these treatments are considered efficient because they are not dependent on microbial acclimatization [8].

Peroxidases are a class of enzyme with a potential for decolorize synthetic dyes and decontaminate hazardous pollutants in contaminated water and industrial effluents [9,10].The horseradish peroxidase (HRP), for example, is a versatile enzyme effective in the removal of a wide spectrum of aromatic compounds (phenols, biphenols, anilines) and in the degradation and precipitation of important industrial azo dyes [10–12]. However, the high cost of commercial HRP has led to recent research to discover cheaper sources of plant peroxidases to be used in the treatment of effluents rich in textile dyes [7].

In this context, *Moringa oleifera* is a tree native to India that grows in tropical and subtropical regions of Africa, Asia and the Americas. This plant represents a very effective natural

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agent used for water purification [13]. Previous data acquired by our research group indicated that roots of *M. oleifera* have an elevated peroxidase activity. In the present research, we report the isolation and characterization of a new peroxidase from roots of *M. oleifera* and describe the investigation of this enzyme's potential for the degradation of different classes of textile dyes.

2. MATERIALS AND METHODS

2.1. CHEMICALS

DEAE-Sephacel, Superdex® 75 and molecular mass markers were bought from GE Healthcare (Uppsala, Sweden). Guaiacol, dithiothreitol (DTT), o-dianisidine, eugenol, ascorbic acid, NADH (nicotinamide adenine dinucleotide), 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), aminoantipyrine, syringaldazine, and tryptophan were obtained from Sigma-Aldrich Ltd. (St. Louis, USA). Textile dyes were gently obtained from DyStar.

2.2. PLANT MATERIAL AND GROWTH CONDITIONS

Moringa oleifera seeds were harvested from trees naturally growing on the Pici Campus of Federal University of Ceará, Fortaleza, CE, Brazil. The seeds were sterilized with 0.2% active chlorine (hypochlorite) and germinated at 25.0 °C ± 2.0 °C and 100% relative humidity in the dark for 8 days. After this, the seedlings were transferred to plastic pots containing distilled water and concentrated (4x) Hoagland nutritive solution, and maintained in a greenhouse during 30 days [14]. After this period, the plants' roots were collected and frozen. Then they were stored at -80 °C for later analysis.

2.3. EXTRACTION PROCEDURES

The frozen roots (70 g) were ground in liquid N_2 with a mortar and a pestle. After, the frozen powder was placed in contact with 0.1 M sodium acetate buffer, pH 5.2, at the ratio of 1:3 (m/v) for 2 h, under constant stirring at 4 °C. Afterwards, the extract was centrifuged at 12,000 x *g* at 4 °C, 20 min, and the supernatant was dialyzed (12 kDa cutoff) against distilled water for 24 h. The dialysate was designated as the soluble protein extract (SPE). The SPE was concentrated by freeze-drying and posteriorly in the purification process.

2.4. PURIFICATION OF M. OLEIFERA ROOT PEROXIDASE (MOPOX)

The lyophilized soluble protein extract (SPE) was applied in a DEAE-Sephacel column (20.0 x 1.5 cm) which was equilibrated with 0.1 M sodium acetate buffer (pH 5.2). Retained proteins were obtained with 0.1 M sodium acetate buffer containing 0.5 M NaCl. For both retained

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and non-retained proteins, fractions of 2 mL were collected at a flow of 60 mL/h. Absorbances at 280 nm, SDS-PAGE electrophoresis [15] and peroxidase activity were measured [16].

The retained fraction (6.9 mgP), named D2, obtained from the DEAE-Sephacel (20.0 x 1.5 cm) chromatography, presenting high peroxidase activity and low number of protein bands, was loaded into a Superdex® 75 (60.0 x 1.6 cm) column, equilibrated with 0.1 M sodium acetate buffer containing 0.5 M NaCl (pH 5.2). Fractions (5 mL) were obtained under a flow of 24 mL/h, and pressure of 0.5 MPa. The following molecular weight markers were used as calibrators: aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), and conalbumin (75 kDa). The peroxidase activity and the absorbances at 280 nm were monitored [16]. The purified peroxidase, which was named *Mo*POX, was used to further characterization.

2.5. PROTEIN QUANTIFICATION

Protein at each step of the purification procedure was determined according to the bicinchoninic acid (BCA) method [17] or the method described by Bradford [18]. The standard used was bovine serum albumin.

2.6. PEROXIDASE ACTIVITY

Colorimetric assays were performed according to [16] using guaiacol and H_2O_2 as substrates. Aliquots of 0.02 mL of the samples were added to a solution composed of 0.5 mL of 60 mM H_2O_2, 0.5 mL of 20 mM guaiacol, and 0.980 mL of 50 mM Na-acetate buffer, pH 5.2. Reaction was conducted with incubation for 10 min at 30 °C and absorbances were read at 480 nm in 30-second intervals up to 3 min, expressed in UA/mgP or Abs/mL/min.

2.7. PROTEIN PROFILE BY SDS-PAGE ELECTROPHORESIS

Protein profile of chromatography eluted fractions were analyzed by denaturing electrophoresis (SDS-PAGE) using 10% (m/v) polyacrylamide gels (8.5 x 8.0 cm), as previously described [15]. The gel was revealed by the blue silver technique [19]. Molecular mass markers with a range of 14 -225 kDa (ECL™ Rainbow™ Marker - Full Range, Amersham™) were used.

2.8. MASS SPECTROMETRY ANALYSIS

*Mo*POX was submitted to electrophoresis in the above conditions and it was made a digestion with trypsin and peptides were excised from the gel according to [20]. A BEH300 C18 column (100 μm × 100 mm) was used to separate the tryptic peptides using the nanoAcquity™

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(Waters) system. The peptides were eluted at 600 µL/min in a acetonitrile gradient (5-85%) added by 0.1% formic acid. A liquid chromatography was connected to a nanoelectrospray mass spectrometer source (SYNAPT HDMS system, Waters Corp., Milford, MA, USA), which was ran in a positive mode at a source temperature of 90 ºC and 3.5 kV. The calibration was done with phosphoric acid clusters, and a m/z 686.8461 ion was used during acquisition as lock mass. The LC-MS/MS was performed based on data-dependent acquisition (DDA) method, and ion fragmentation was performed by collision-induced dissociation. Data were collected and analyzed using MassLynx 4.1 and Protein Lynx Global Server 2.4, respectively. They were posteriorly converted to peak list text files and loaded into PeroxiBase (UniProt) [21]. The search parameters were: short sequence, E-value of 0.01, and matches ordered by % identity.

2.9. GLYCOPROTEIN NATURE

The glycoprotein nature of *Mo*POX was investigated by periodic acid-Schiff staining (Sigma-Aldrich Co., MO, USA), as described by Zacharius *et al.* [22].

2.10. CARBOHYDRATE CONTENT

The carbohydrate content of *Mo*POX was determined by Dubois *et al.* [23]. The determination was based on a calibration curve plotted with different concentrations of galactose and expressed in percentage (%) of carbohydrate/protein mass.

2.11. KINETIC PARAMETERS

The K_m and V_{max} values of the *M. oleifera* root peroxidase were estimated, using guaiacol and H_2O_2 as substrates, from Lineweaver–Burk plots. The reactions took place at 30 ºC, and the enzyme activity was measured at 480 nm for different concentrations (0.00125 – 0.16 M) of guaiacol, while the H_2O_2 concentration was constant (0.06 M), and for different concentrations (0.00011 - 0.0075 M) of H_2O_2 while the guaiacol concentration was constant (0.02 M).

2.12. OPTIMUM PH

The optimum pH value for *Mo*POX peroxidase activity (0.21 µg) was determined by assaying enzyme activity using the following buffers: 0.1 M glycine-HCl (pH 2.0); 0.1 M sodium acetate, (pH 4.0 and 5.2); 0.1 M sodium phosphate (pH 6.0 and pH 7.0); 0.1 M Tris-HCl (pH 8.0);

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0.1 M glycine-NaOH (pH 9.0); and 0.1 M sodium borate (pH 10.0) . The peroxidase assay conditions were similar to those previously described.

2.13. OPTIMUM TEMPERATURE AND HEAT STABILITY

The optimum temperature value for the peroxidase activity of MoPOX (0.21 µg) was ascertained using guaiacol and H_2O_2 as the substrates at temperatures in a range from 20 to 90 °C at pH 5.2. The heat stability was also evaluated using guaiacol and H_2O_2 as the substrates and incubating the enzyme at 60, 70, 80 and 90 °C for 10, 20, 30 and 60 min in 0.05 M sodium acetate (pH 5.2). After heating, the enzyme solutions were cooled and residual peroxidase activity was immediately evaluated under standard assay conditions.

2.14. INFLUENCE OF METAL IONS ON PEROXIDASE ACTIVITY

The activity of MoPOX in the presence of differente ions was evaluated by adding $MgCl_2$, $CaCl_2$, NaCl or $MnCl_2$ to the reaction at different concentrations: 0.01, 0.05, 0.10, 0.20, and 0.50 M. MoPOX (0.21 µg) activity was measured according to the procedure described previously.

2.15. INFLUENCE OF INHIBITORS ON PEROXIDASE ACTIVITY

The inhibitory effect of different compounds (DTT, sodium dodecyl sulfate - SDS, sodium azide or ethylene diamine tetra acetic acid - EDTA) on MoPOX activity was determined by combining a reaction mixture containing guaiacol (20 mM) as substrate, MoPOX (0.21 µg), inhibitor (1 mM) and 60 mM H_2O_2 in 0.05 M sodium acetate buffer (pH 5.2). The peroxidase activity was measured according to the procedure described previously.

2.16. DETERMINATION OF SUBSTRATE SPECIFICITY

The specificity of MoPOX (0.21 µg) was tested using some compounds as substrates instead of guaiacol: ABTS, o-dianisidine, eugenol, ascorbic acid, NADH, aminoantipyrine, syringaldazine, Coomassie brilliant blue, tryptophan, and tyrosine. These compounds were used at a final concentration of 0.02 M dissolved in 0.05 M sodium acetate buffer, pH 5.2, plus 0.06 M H_2O_2 [24]. The specific activity was defined as an increase in absorbance after a period, excepting for ascorbic acid, for which a decrease in the absorbance denoted the activity.

2.17. DECOLORIZATION OF TEXTILE DYES

The dyes Remazol® Blue RGB (RB), Remazol® Navy RGB (RN), Levafix® Orange E-3 GA (LO), Telon® Turquoise M-5G 85% (TT), Astrazon® Yellow 5GL 200% (AY), and Astrazon® Red FBL (AR), at different concentrations (10, 25, 50 and 100 mg/L), were used in decolorization assays to determine if they could be used as reducing substrates of *Mo*POX. The assays were performed in polystyrene flat-bottom 96-well microtiter plates. Each well received 120 µL of dye solution or 80 µL of 0.05 M sodium acetate, pH 5.2, for the control. The treatment consisted in a reaction mixture as cited above added by 60 µL 0,001 M hydrogen peroxide and 20 µL of *Mo*POX (0.015, 0.03 and 0.15 mg/mL). A microplate spectrophotometer (Epoch, Biotek) was employed to obtain the absorbance measurements at a specific wavelength for each substrate: 595 nm (RB), 600 nm (RN), 490 nm (LO), 610 nm (TT), 555 nm (AY), 725 nm (AR) up to 48 h of incubation. Percentage of dye decolorization was determined as follows:

$$\% \text{ decolorization} = \frac{\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}}{\text{Abs}_{\text{initial}}} \times 100$$

3. RESULTS AND DISCUSSION

3.1. PURIFICATION OF MOPOX

The crude extract obtained from *M. oleifera* roots (specific activity 23.55 UA/mg of protein) was submitted to dialysis (12 kDa cutoff). After dialysis, the resulting soluble protein extract (SPE) exhibited specific peroxidase activity of 261.4 UA/mg of protein. Then it was concentrated by freeze-drying and solubilized at a minimum volume of 0.1 M sodium acetate, pH 5.2, for analysis by anion-exchange chromatography (DEAE-Sephacel) at a flow of 60 mL/h. Peroxidase activity was distributed into two peaks: one of them was obtained from non-retained pooled fractions (D1) (213 UA/mg of protein) while the other was eluted with 0.1 M sodium acetate, pH 5.2, containing 0.5 M NaCl (Fig. 1A). The retained fractions (D2) presented a small number of protein bands in electrophoresis gel, besides high peroxidase activity (278 UA/mg of protein). Thus, the D2 fraction was chosen to proceed with the purification process (data not shown). After peroxidase isolation, the D2 fraction was loaded into a molecular exclusion chromatograph, and only one peak (S1) was obtained, with specific activity of 2109.1 UA/mg of protein (Table 1), concentrated at the peak decline (Fig. 1B). The *Mo*POX purification process resulted in 89.56-fold purification with 0.13% protein yield.

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3.2. MOLECULAR WEIGHT AND PROTEIN IDENTIFICATION

Based on molecular exclusion chromatography (Superdex), *Mo*POX presented a molecular mass of 45.6 kDa (Fig. 1B). The purity of *Mo*POX was also analyzed by SDS-PAGE, in which only one band under non-denaturing conditions was visualized (Fig. 1B-insert). In comparison to other molecular weights of purified plant peroxidases, *Mo*POX is similar in weight to peroxidases from *M. oleifera* leaves (43 kDa) [25], cress (*Lepidium sativum*) (44 kDa) [26], and *Vigna radiata* roots (50 kDa) [27].

The *Mo*POX tryptic digestion generated three peptides, which were identified by mass spectrometry analysis as GGTKEEFFEEK, LKDDWTDTER, and EVIAINQDPLGVQGR. The peptides were analyzed in the PeroxiBase database and showed high identity with plant peroxidases (Table 2).

3.3. GLYCOPROTEIN NATURE AND CARBOHYDRATE CONTENT

Since several class III peroxidases have been described as glycoproteins, the presence of carbohydrate in *Mo*POX was examined. *Mo*POX exhibited a pink color on SDS-PAGE, after the treatment with periodate-Schiff, which is a characteristic of glycoproteins (Fig. 1B-insert). The carbohydrate content was 3.4%, determined by the phenol-sulfuric acid reaction, which is less than that reported for other species such as *Vanilla planifolia* (15.0%) [28], *Viscum angulatum* (12.6%) [29] and *Borassus flabellifer* (22%) [30]. Though, this value was comparable to that of a peroxidase from wheat germ (4.1%) [31].

3.4. KINETIC PARAMETERS

*Mo*POX exhibited characteristic Michaelis-Menten kinetics using guaiacol and H_2O_2 as substrates (Fig. 2). In order to investigate the specificity of the enzyme concerning these substrates, kinetic parameters (V_{max} and K_m) were determined by Lineweaver-Burk plots. The effect of the substrates on the peroxidase activity was determined by varying the concentration of guaiacol while keeping a fixed concentration of H_2O_2 or the opposite. *Mo*POX presented K_m values of 46.8 mM and 0.70 mM for guaiacol and H_2O_2 substrates, respectively, and V_{max} values of 376 Abs. mL^{-1} min^{-1} for guaiacol and 65.34 Abs. mL^{-1} min^{-1} for H_2O_2. The K_m values obtained in the present work suggest that *Mo*POX has high affinity for both substrates, with a higher specificity for H_2O_2. *Mo*POX presented a k_{cat} value of 129,65 s^{-1} for guaiacol and 22,53 s^{-1} for H_2O_2. Comparison of the obtained data with those reported in other studies endorses the high specificity of *Mo*POX for H_2O_2, such as the peroxidase purified from *Ficus carica* latex, which showed K_m values of 0.34 mM for

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H_2O_2 [32]. Regarding the affinity for guaiacol, *Mo*POX presented higher affinity for this substrate compared to other peroxidases. For instance, in a study of a lactoperoxidase immobilized on graphene oxide nanosheets and copper oxide nanoparticles, the free enzyme presented a K_m value for guaiacol of 53,19 mM [33]. Catalytic activities of peroxidases vary depending on different characteristics such chemical structure and composition of the substrates [34].

3.5. OPTIMUM PH

*Mo*POX showed optimum activity at pH 5.2 using guaiacol as substrate (Fig. 3A). Under extreme pH conditions it was observed a decrease in its enzymatic activity. In general, plant peroxidases present different optimum pH values, which vary from 3.6 to 8.0. Our results, therefore, are similar to those reported by other researchers. For example, two peroxidase isoenzymes from *F. carica* latex and jackfruit peroxidase presented optimum pH value of 5.5 [32,35]. pH is an important parameter in enzyme activity, since it changes the ionization states of amino acid side chains, which can modify the conformation of the active site, enzyme-substrate binding or reaction catalysis [36]. Al-Bagmi *et al*. [37] observed that under extreme acidic pH (pH 2-3), palm and horseradish peroxidases lost their tertiary structures.

3.6. OPTIMUM TEMPERATURE AND THERMAL STABILITY

The optimum temperature of *Mo*POX was determined at the optimum pH (5.2) and with the temperature ranging from 20 to 90 °C. Fig. 3B shows that *Mo*POX presented maximum activity at 70 °C. Afterwards, the thermal stability of *Mo*POX was investigated. The peroxidase retained approximately 80% of its activity after incubation at 70 °C for 60 min and about 50% at 80 °C for 60 min, indicating a very high thermostability (Fig. 3C). A similar result was reported for a date palm peroxidase, which maintained its stability during 60 min at 75 °C [37]. The optimum temperature of activity and thermostability vary greatly among different plant peroxidases depending on features such as their amino acid composition and three-dimensional conformation [38]. The low thermostability if one of most limiting factor for the use of plant peroxidases in bioremediation strategies [39]. Since *Mo*POX presented a high thermostability temperature may not be a limitation for its application in environmental biotechnology.

3.7. EFFECT OF METAL IONS

The effects of metal ions (Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}) on the catalytic activity of *Mo*POX, at pH 5.2, is shown in Fig. 4A. Mg^{2+} and Mn^{2+} had no effect on *Mo*POX activity while Na^+ and Ca^{2+} at

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the concentration of 0.5 M increased the catalytic activity (30%), indicating their possible role as cofactors. Different metal cations may have an influence peroxidase activity. For instance, the activity of a peroxidase from jackfruit bulbs was enhanced by K^+, Zn^{2+} and Ba^{2+}, but reduced by Cu^{2+} and Ca^{2+} [35]. In other study, the peroxidase from *Leucaena leucocephala* had its activity increased by Ca^{2+} and Mn^{2+} at lower concentration (up to 50 mM), while being decreased at higher concentrations [40]. A considerable increment in enzyme activity was observed for the peroxidase isoenzymes purified from white and red cultivars of kola nut (*Cola nitida*) in the presence of Ca^{2+} and Na^+. In addition, Mg^{2+} had no effect on isoenzyme A of peroxidase from red *C. nitida* but inhibited white *C. nitida* peroxidase [41]. Peroxidases are metalloproteins due to the presence of a heme group in their structure, which is stabilized by metallic ions such as Ca^{2+}, Na^+, Mn^{2+} and Mg^{2+}, among others [40].

3.8. EFFECT OF INHIBITORS

The influence of some compounds (at the concentration of 1 mM) on *Mo*POX activity is reported in Fig. 4B. SDS and EDTA did not affect *Mo*POX activity. On the other hand, sodium azide inhibited the peroxidase activity of *Mo*POX by 23%. This compound is a sequester of heme groups, so this result is an indication of the importance of Fe in the action of the enzyme. Sensitivity to this inhibitor is common in a large number of heme-catalyzed reactions. For example, sodium azide decreased the activity of VanPrx, a cationic peroxidase of *Viscum andulatum*, by more than 90% [29], whereas the peroxidase from *Vigna radiata* roots was more resistant to sodium azide, reducing the peroxidase activity by only 48% [27]. In addition, this peroxidase, did not suffer inhibition of EDTA, similar to what was observed for *Mo*POX. Regarding the activity in the presence of DTT, *Mo*POX was completely inhibited. It is possible that intramolecular disulfide bonds contribute to stabilization of protein structure and consequently to the maintenance of its catalytic activity.

3.9. SUBSTRATE SPECIFICITY

The specific activity of *Mo*POX in other substrates is shown in Fig. 5. There was no difference between the oxidation rates of guaiacol and *O*-dianisidine by *Mo*POX, with these two compounds being the best substrates for the enzyme. Similarly to *Mo*POX, guaiacol and *O*-dianisidine were the best substrates for a peroxidase from *M. oleifera* leaves and for a peroxidase from *M. megalantha* latex [25,42]. *Mo*POX was also able to catalyze ABTS and eugenol oxidation with activity rates of 28% and 49%, respectively, compared to the activity in the presence of guaiacol as substrate (Fig. 5). In fact, many plant peroxidases use ABTS as substrate, which in

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some cases is the main substrate for these enzymes [43,44]. In addition, one study showed that eugenol, an allyl chain-substituted guaiacol, can be oxidized by some peroxidases, although this reaction can result in the formation of cytotoxicity products [45]. When other chemicals were used as substrates (ascorbic acid, tyrosine, Coomassie brilliant blue, tryptophan, aminoantipyrine, NADH and syringaldazine), there was no detectable activity of *Mo*POX (Fig. 5). In particular, the absence of ascorbic acid oxidation by *Mo*POX corroborates other works reporting this behavior as characteristic of peroxidases (Alneyadi et al., 2017; Baumer et al., 2018). Similar results were reported for a peroxidase from *Withania somnifera*, which readily catalyzed the oxidation of phenolic substrates like guaiacol and *o*-dianisidine, but not ascorbic acid [24].

The ability of *Mo*POX to interact with different types of substrates suggest its potential for being used in environmental applications such as the detoxification of contaminated effluents.

3.10. DECOLORIZATION OF DYES

*Mo*POX decolorized dyes of different classes (Fig. 6A-F)). The greatest decolorization rates were achieved for Remazol® Blue RGB (RB), Remazol® Navy RGB (RN) and Telon® Turquoise M-5G 85% (TT) (Fig. 6). On the other hand, Levafix® Orange E-3 GA (LO), Astrazon® Yellow 5GL 200% (AY) and Astrazon® Red FBL (AR) were only slightly degraded by *Mo*POX (Data not shown). The decolorization rate after the treatment with *Mo*POX (0.15 mg/mL) for RN at the concentrations of 50 mg/L and 100 mg/L was around 30% and 15.3%, respectively (Fig. 6A and 6B). Regarding RB (50 mg/L), 64.1% of decolorization was obtained after 48 h of exposure to *Mo*POX (0.15 mg/mL) (Fig. 6C) while for a higher concentration of RB (100 mg/L), the degradation rate was 73.2% (Fig. 6D). The results were even more promising for TT at the concentration of 50 mg/L as the decolorization rates were higher than 85% after the treatment with *Mo*POX (0.015, 0.03 and 0.15 mg/mL), after 48 h of incubation (Fig. 6E). Moreover, for TT (100 mg/L), the decolorization rates remained high (> 75%) using the same enzyme concentrations (0.015, 0.03 and 0.15 mg/mL) (Fig. 6F).

The different structures of the tested dyes may explain the different decolorization rates observed. These dyes present different chromophores attached to aromatic rings, which confer the color of each dye. A number of these functional groups are located in *ortho* or *para* positions, and this difference in positions may accelerate or reduce the decolorization rate, or even inhibit it [9,46]. Moreover, the decolorization rate may depend on dye concentration, environmental conditions (such as pH and temperature), enzyme specificity and concentration etc. [4,47]. As an example, the Remazol dyes have a complex structure containing at least one aromatic group and as a result, the decolorization rates achieved by different enzymes vary greatly.

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The most studied peroxidase, horseradish peroxidase, discolored Reactive Red, Reactive Black, Reactive Blue 21, Reactive Blue 19, and dye at rates of 17%, 87%, 90%, and 96%, respectively [48]. In other study, Souza *et al.* [49] observed that horseradish peroxidase was able to degrade 59% of Remazol Turquoise Blue G 133% while a soybean peroxidase degraded more than 95% of the same dye [50]. Low degradation rates (3.3 to 8.7%) were observed for the acid Telon® Turquoise dye by a free polyphenol oxidase from *Cydonia oblonga* leaves [51]. Nevertheless, *Mo*POX was able to degrade more than 90% of this dye. As mentioned before, many factors can affect the success of an enzyme in degrading a specific dye or a mixture of them. Thus, the best conditions must be defined in each specific case.

4. CONCLUSIONS

To the best of our knowledge, this is the first report of a peroxidase purified from *M. oleifera* roots. Our results indicate that *Mo*POX is highly thermostable, and it presents a great capacity to degrade the dyes Remazol® Blue RGB, Remazol® Navy RGB, and Telon® Turquoise M-5G 85%. Therefore, *Mo*POX has a great potential to be used in bioremediation strategies.

4.1 COMPETING INTEREST

The authors declare no competing financial interest.

4.2 FUNDING

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FIGURES

Fig. 1. Purification steps of *Mo*POX. (A) Ion-exchange chromatography in DEAE-Sephacel column equilibrated with 0.1 M Na-acetate buffer, pH 5.2. The elution of retained proteins was done using 0.5 and 1.0 M NaCl. (B) D2 was loaded in Superdex® 75 column equilibrated with 0.1 M Na-acetate buffer added by 0.5 M NaCl, pH 5.2. Dashed lines denote the peroxidase activity. Insert: SDS-PAGE of *Mo*POX and polyacrylamide gel stained with periodic acid-Schiff reagent.

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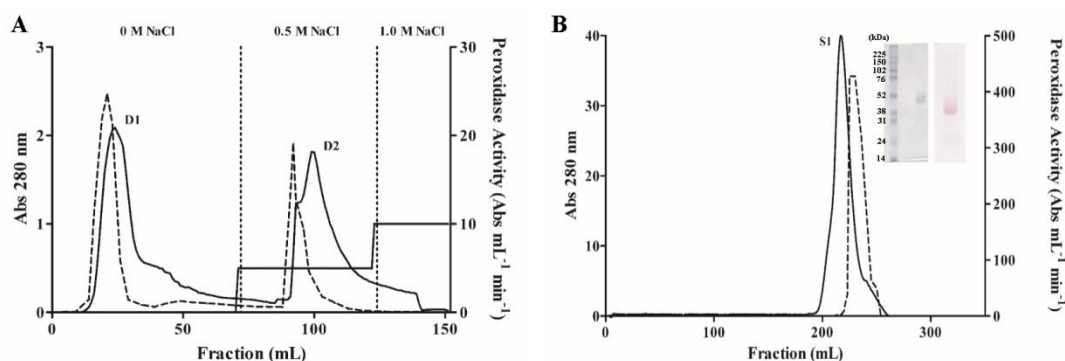
Fig. 2. Michaelis-Menten and Lineweaver-Burk graphics indicating the *Mo*POX activity using as substrates guaiacol (A and B) and H_2O_2 (C and D).
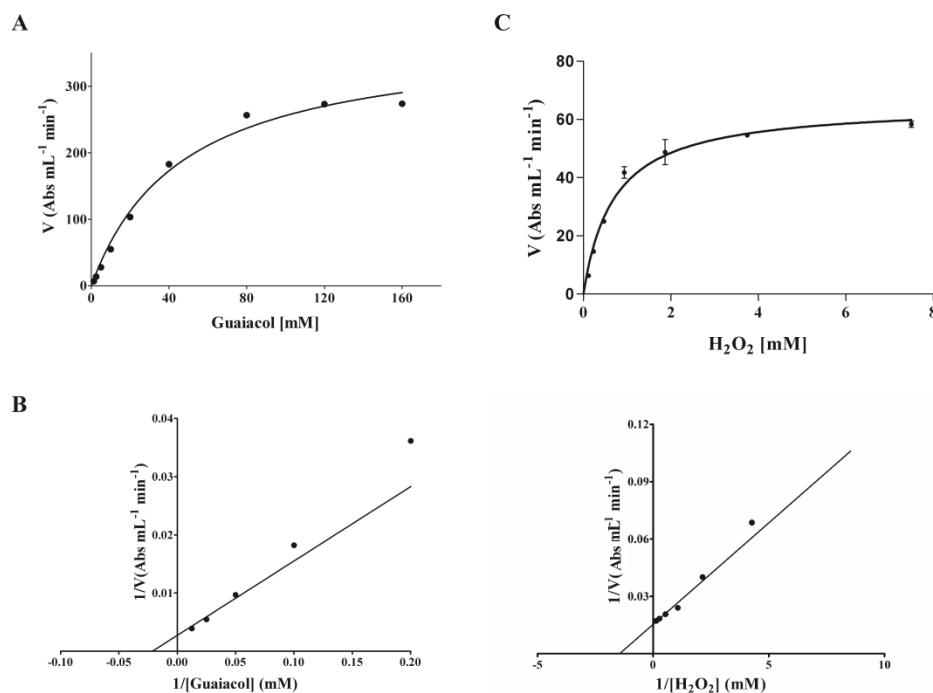

Fig. 3. Optimum pH (A), optimum temperature (B), and thermostability (C) of *Mo*POX. The optimum pH was determined by measuring enzyme activity in a pH range from 2.0 to 10.0. Peroxidase activity was measured from 20 to 90 °C to determine the optimum temperature. Thermal stability was assessed by incubating *Mo*POX for 0-60 min at temperature varying from 60 to 90 °C. Each point represents the mean of three biological replicates (± SD). The asterisks indicate no significant differences (p < 0.05) compared to the specific activity under the standard conditions. Different letters indicate differences between groups (p < 0.05), as calculated by two-way ANOVA.

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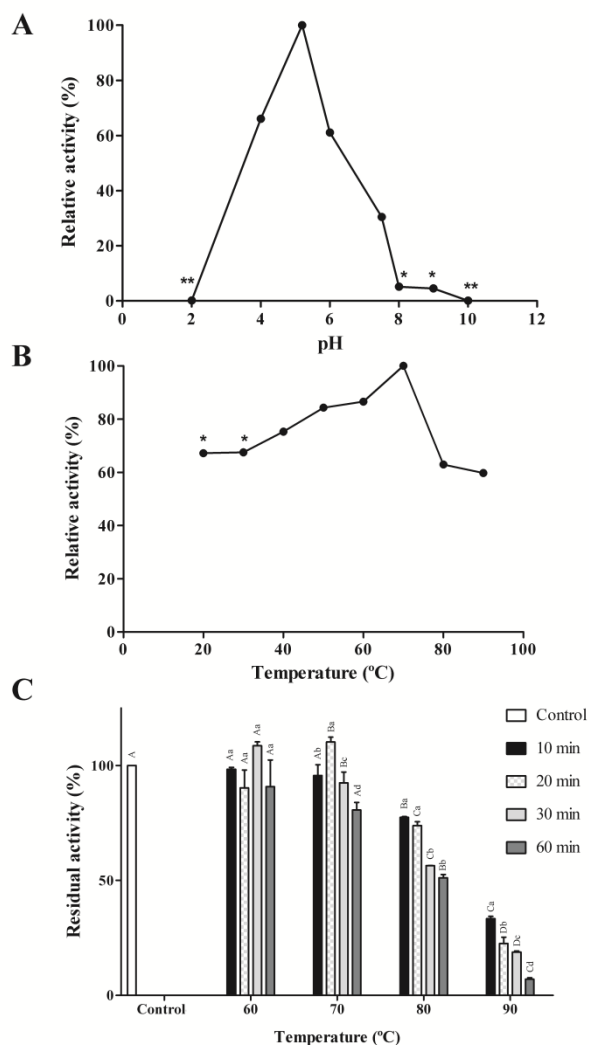
Fig. 4. Influence of metal ions and inhibitor compounds on *Mo*POX activity. (A) Peroxidase activity was measured after adding to the reaction mixture, $CaCl_2$, $MgCl_2$, $MnCl_2$, and NaCl (0.01, 0.05, 0.10, 0.20 and 0.50 M). (B) Each chemical was tested at 1 mM. The asterisk indicates significant differences ($p < 0.05$) compared to the specific activity under the standard conditions. Different letters indicate differences between groups ($p < 0.05$), as calculated by the two-way ANOVA.

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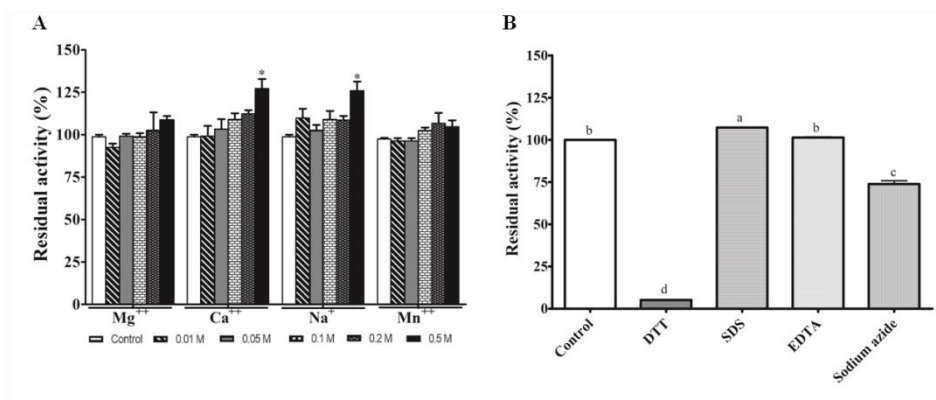
Fig. 5. *Mo*POX specificity towards different substrates. A concentration of 20 mM of each substrate was used. Significant differences between groups are represented by different letters (p < 0.05).
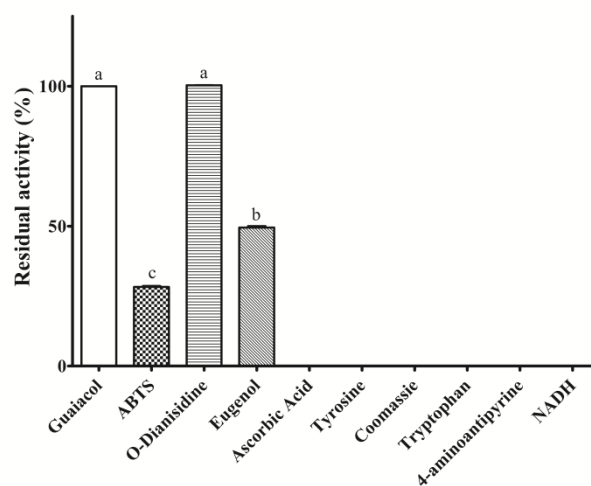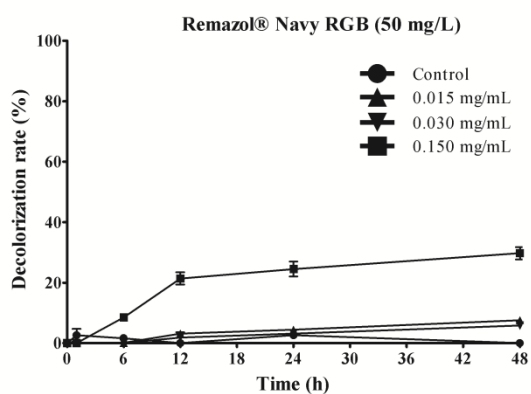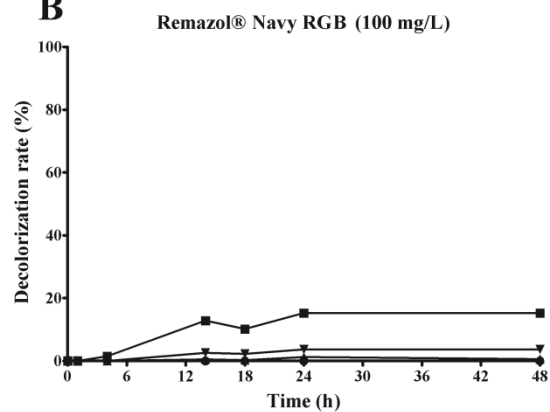

Fig. 6. Decolorization rate of the textile dyes Remazol® Navy RGB (A-B) Remazol® Blue RGB (C-D), and Telon® Turquoise M-5G 85% (E-F) by *Mo*POX over time.
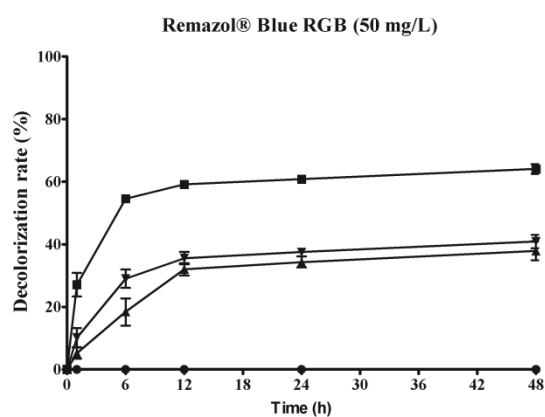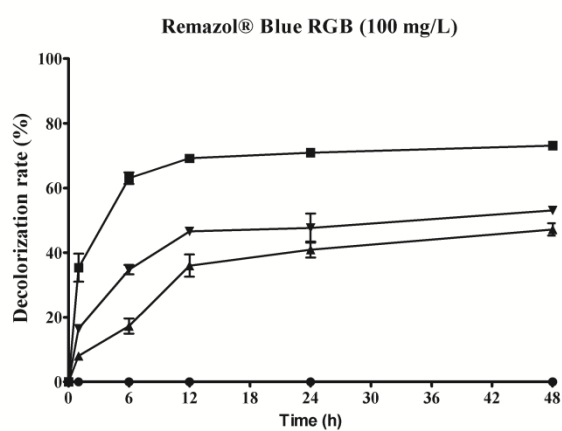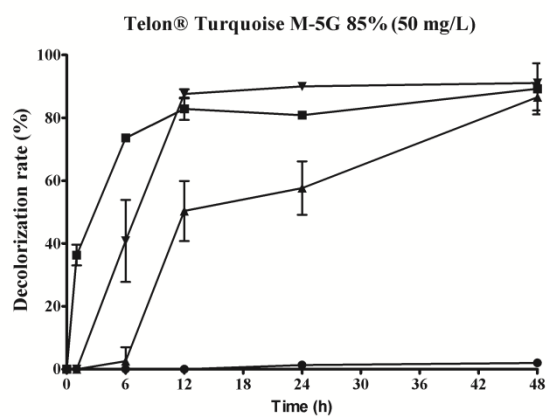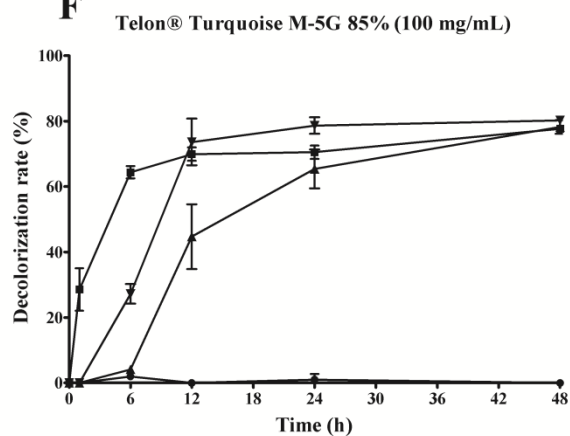
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TABLES

Table 1. Purification steps of a peroxidase from *M. oleifera* roots

Step	Total protein[a] (mg)	Total activity[b] (UA)	Specific activity (UA/mg of protein)	Yield[c] (%)	Purification[d] (fold)
SPE[e]	13.80 ± 0.90	325.66 ± 4.80	23.55 ± 0.22	100	1
DEAE-Sephacel	0.34 ± 0.03	94.62 ± 8.50	278.00 ± 0.4	2.46	11.82
Sephadex-75	0.02 ± 0.00	36.46 ± 2.31	2109.09± 0.51	0.13	89.56

[a]The total amount of protein recovered from 70 g of *M. oleifera* roots

[b]One unit of peroxidase activity (UA) was defined as the change of an absorbance unit per min at 480 nm.

[c]The recovery of protein at each purification step (SPE, 100%).

[d]Purification index is calculated as the ratio between the specific activity obtained at each purification step and that of the SPE taken as 1.0.

[e]SPE – Soluble protein extract.

Table 2. Characterization of the peptides obtained by tryptic digestion of *Mo*POX. Peptides were identified by LC-MS/MS and had their identityes analyzed by RedoxiBase database

Peptide sequence	Mass (Da)		Peroxidase with identity (specie)	ID RedoxiBase	Identity (%)
	Experimental	Calculated			
GGTKEEFFEEK	1299.61	1299.59	AtMDAR03 (*Arabidopsis thaliana*)	13878	85
			ThasMDAR03 (*Tarenaya hassleriana*)	15763	85
			TaPrx100 (*Triticum aestivum*)	443	70
LKDDWTDTER	1277.62	1277.58	MpPrx117 (*Marchantia polymorpha*)	14649	75
EVIAINQDPLGVQGR	1607.93	1607.86	HaPrx08 (*Helianthus annuus*)	1478	63