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Artigo

IMMOBILIZATION OF LACCASE ON HYBRID LAYERED DOUBLE HYDROXIDE

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Crystals of Mg/Al layered double hydroxide were synthesized by alkaline precipitation and treated in an aqueous solution of glutamic acid. The glutamate ions were not intercalated into the interlayer space, but were detected in the material by Fourier transform infrared spectroscopy, suggesting that only the external surfaces of crystals were modified with glutamate ions. The resulting hybrid material was tested as a support for immobilization of the enzyme laccase (*Myceliophthora thermophila*). The immobilized enzyme preparation was characterized by electronic paramagnetic resonance spectroscopy and by assays of catalytic activity. The activity of the immobilized laccase was 97% of the activity in the free enzyme. Layered double hydroxide is a suitable support for use in remediation of soil studies.

Keywords: laccase; layered double hydroxide; immobilization.

INTRODUCTION

Laccase (EC 1.10.3.2) is a multi-copper protein produced by many species of bacteria, fungi and higher plants.[1] Laccase has received much attention over the last few decades due to their ability to oxidize both phenolic and nonphenolic lignin-related compounds as well as environmental pollutants like chlorophenols.[2] It has been also used to catalyze key steps during the synthesis of anti-cancer drugs and cosmetic ingredients to remove phenol from olive oil and to detoxify industrial effluents from the pulp, paper, textile and petrochemical industries.[3] In addition, its capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes.

In most of the applications the laccase is immobilized to facilitate recovery, reuse and in some cases to confer thermal stability.[2] Laccase has been immobilized on different supports like polymers and clays, using a wide variety of immobilization methods such as physical adsorption and chemical binding to carrier particles or membranes.[4]

Clays are ideal supports for laccase to be used in remediation of soil or water since they are natural compounds of the terrestrial crust.[2] It is known that pesticides like 2,4-dichlorophenols and simazine can be accumulated in the montmorillonite and chlorite-like clays or repelled, depending on the electrostatic characteristic of the mineral.[5]

Layered double hydroxides (LDH) are synthetic clays where the amount and distribution of positive electrostatic charges are controlled by the synthesis. The structure of a LDH is a modification of brucite ($Mg(OH)_2$), where a fraction of the cationic sites are occupied by trivalent cations, and the resulting excess charge is stabilized by the presence of additional anions in the interlayer space. These compounds are represented by the general formula: $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{x+}A^{m-}_{x/m}.nH_2O$; where M^{2+} and M^{3+} are the cations involved and A is an anion of charge m^-.[6,7] The stacking of the positive layers allows the anions to be exchanged and grafted, creating interesting supports for the immobilization of catalytic species, which could be intercalated within the layers or adsorbed on the external surface.[8-10] Due to their large volume, macromolecules like enzymes are not easily intercalated between the layers of LDHs. However, smaller organic ions can first be intercalated between the layers and adsorbed/grafted onto the external surfaces of the crystallites, in order to produce a hybrid material capable of retaining the enzyme. A proposed model for such retention is the linking of the enzyme to the organic moiety of the hybrid LDH by hydrogen bonding, electrostatic interactions or even covalent bonding when suitable functional groups are available to react with the protein chain,[11] for instance, a LDH has been hybridized with glutamic acid and then the amino group was reacted with glutaraldehyde in order to offer carbonyl groups to form covalent bonds with the enzyme.[12]

Our work presents only the modification with glutamate ions because the amino extremes might also react with the acid groups in the laccase chains. The LDH as a support for laccase immobilization can be used in studies of soil or water remediation since it does not represent further contamination of soils as in the case of non-biodegradable polymer supports.

EXPERIMENTAL

The reagents of analytical grade were used as received and all solutions were prepared with distilled water. The *Myceliophthora thermophila* laccase (EC 1.10.3.2) DeniLite II Base™, was kindly donated by Novozymes, Mexico.

Synthesis of the Mg/Al-Cl LDH and adsorption of glutamate ions

The Mg/Al-Cl LDH was obtained by the co-precipitation method. The salts $MgCl_2{\cdot}6H_2O$ and $AlCl_3{\cdot}6H_2O$, in a molar ratio Mg/Al = 2, were dissolved in decarbonated water and then precipitated by addition of NH_4OH at a final pH of 7.5. The suspension was stirred for 72 h in a nitrogen atmosphere. The solid, LDH-chloride, was recovered by centrifugation, washed with water and dried at 65 °C for 12 h. The modification with glutamate ions was done by adding the LDH to a glutamic acid solution and then adjusting the pH to 9.0 with NH_4OH. The reaction was conducted for 72 h at room temperature and the LDH-Glutamate hybrid product (LDH-G) was separated by centrifugation, washed with water, and then dried at 65 °C for 12 h.

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Immobilization of the laccase

The commercial product DeniLite II Base™ was pretreated in order to eliminate undesired matter such as polysaccharides. One gram of DeniLite II Base™ was added to 50 mL of water and heated at 45 °C under stirring at 5.5 rpm for 20 min. The suspension was centrifuged at 3500 rpm in a Sigma 2-15 centrifuge for 3 min at room temperature to separate solids and the liquid was transferred to a balloon flask and diluted to 200 mL total volume. This suspension, hereafter referred to as laccase suspension, was separated in four samples of 50 mL each.

The immobilization was conducted by mixing three of the above laccase suspension samples with 0.3 g of the LDH-G support in dark flasks. They were shaken at 400 rpm at 5 °C for 12 h. These solids represented the immobilized laccase and were labeled as LDH-GL. A control sample was prepared with the support treated in distilled water to check whether the support catalyzed the reaction in the absence of enzyme. The solids were recovered by centrifugation at 3500 rpm for 3 min. The fourth laccase suspension sample was used as the free enzyme reference.

The three samples of LDH-GL were washed a further two times by resuspension in water and then centrifuged. The liquid phases of washing (LPhW) were separated and subjected to the enzymatic activity test in order to detect laccase released from the support.

The degree of immobilization was calculated based on the difference between the initial activity of the laccase suspension and the total laccase activity in the LDH-GL. The solid samples of LDH-GL were lyophilized at 5 °C for 12 h and then ground for characterization by X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy and electronic paramagnetic resonance (EPR).

Determination of laccase enzymatic activity in LAMU units

The enzymatic activity was determined by the LAMU method.[13] Under anaerobic conditions laccase catalyzes the oxidation of syryngaldizine, producing tetramethoxi-azo-bis (methylene quinone), which can be quantified by UV-vis-spectrometry at 530 nm. One LAMU unit is defined as the amount of enzyme that transforms 1 μmol of syringaldazine per min at pH 7.5 and 30 °C. The assays were conducted by mixing 0.3 mL of a 0.28 mmols of syringaldizine solution in methanol, 3 mL of Tris-HCl buffer 23 mmol/L, pH 7.5, and 0.1 mL of either i) the free enzyme suspension, ii) the liquid phases from the washing (LPhW) steps of the LDH-G and iii) the suspension of the immobilized enzyme in the LDH-GL. This suspension was done by adding 0.3 g of the LDH-GL to 50 mL of water. The activity was followed for 2 min at 530 nm in a UV-Vis Shimadzu spectrometer, model UV-2401PC.

Analysis of the layered double hydroxide

XRD measurements were made in a Shimadzu XRD-6000 diffractometer with Cu-k_α radiation (λ = 1.5418 Å) operated at 40 kV and 30 mA, using films formed by evaporation of the samples onto a glass sample holder. Thermogravimetric (TG) studies were done in a Netzsch equipment (model STA 409 series EP) with 0.065 mL alumina crucibles in static air atmosphere at a heating rate of 10 °C min^{-1}. FTIR spectroscopic studies were carried out in a Bio-Rad spectrometer, model FTS 3500GX, using pellets prepared with KBr. The electronic paramagnetic resonance (EPR) recordings were carried out in quartz tubes containing powdered samples which were placed in a suitable support containing liquid nitrogen (77 K) and analyzed in a BRUKER ESP 300E spectrometer at 9.5 GHz (X band). For registration of the spectra, a microwave power of 2 mW was used, with frequency modulation of 100 kHz and field modulation amplitude of 5 G.

RESULTS AND DISCUSSION

X-ray diffraction

The XRD pattern of the initial Mg/Al-Cl LDH (Figure 1a) has low intensity and broad peaks, associated with low crystallinity. The calculated basal distance, 7.6 Å is typical for a LDH containing chloride ions between the layers.[14] The LDH treated with the glutamate solution (LDH-G) has a narrow basal reflection with two harmonic reflections (marked with asterisk), indicating an increase in crystallinity (Figure 1b). However, it would appear that there is no intercalation of the glutamate ions, since there is no increase in the basal space. This point will be discussed later in relation to the FTIR results. In addition, two peaks appeared between 18 and 21 (2θ) degrees, corresponding to planes distances of 4.8 and 4.3 Å, indicating the formation of $Al(OH)_3$ (gibbsite, marked with g in Figure 1) with the treatment with the glutamate solution.

The XRD pattern of the LDH-G after treatment in the buffer (Figure 1c) and enzymatic solutions show still higher crystallinity, indicating that the time in the solution enabled the layered structure to achieve higher organization (Figure 1d). However, the basal spaces of these materials are not modified. In other words, no glutamate ions were intercalated and thus the interlayer space did not change.

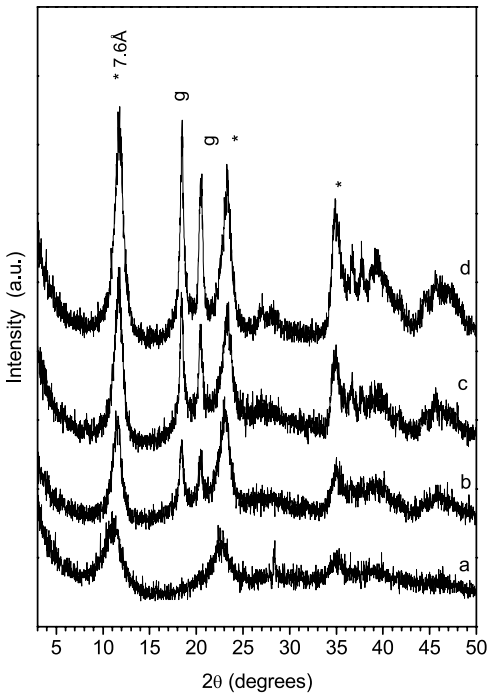

Figure 1. *X-ray diffraction (XRD) pattern of (a) the Mg/Al-Cl layered double hydroxide LDH, (b) LDH treated in glutamate solution (LDH-G), (c) LDH-G treated in buffer and (d) LDH-G treated in laccase solution (LDH-GL). * = LDH phase and g = gibbsite phase*

FTIR spectroscopy

The FTIR spectrum of the initial Mg/Al-Cl LDH shows a wide band in the 3400-3600 cm^{-1} region due to vibrations of hydroxyl groups in the LDH layers with different degrees of hydrogen bonding with interlayer water molecules (Figure 2a).[9,15] The water bending mode forms a typical intense and broad signal at 1637 cm^{-1}.[7,9] Vi-

brations of residual NH_4^+ ions absorbed/adsorbed from the NH_4OH solution that was used in the synthesis could also appear at this wavenumber.[16] The carbonate ion is a common contaminant in LDH samples that appears when the synthesis or reactions are conducted in air atmosphere.[10,17] In this LDH, the ν_3 vibrational mode of CO_3^{2-} is weak and broad at 1360 cm^{-1}.[15,18]

The spectrum of the hybrid LDH-G (Figure 2b) has a new weak band at 3527 cm^{-1}, corresponding to OH groups in the gibbsite structure which is narrow because they do not form hydrogen bonds.[19] The formation of gibbsite was showed in the XRD patterns in the previous section. The stretching modes the M-O bonding (M= Mg or Al) are present in near to 455 cm^{-1}.[18]

Similarly to the pristine LDH, the spectrum of the LDH-G presents bands due to carbonate and water. The band of water in 1630 cm^{-1} becomes broader since the asymmetric vibration of COO^- groups, expected from the glutamate ions, appears between 1500 and 1600 cm^{-1} and overlaps with the water mode.[16,20] The symmetric stretching mode of COO^- appears at 1400 cm^{-1}. Between these two bands, around 1450 cm^{-1}, one expects to see a narrow band of the C-H stretching in the glutamate ion. The intensity of this band increases with the number of methylene ($-CH_2-$) groups in the molecules. In a previous work, the intensity of this band increased gradually in the spectra of layered compounds intercalated with succinate (C4), glutamate (C5) and adipate (C6) ions.[16] Higher intensity was detected with long chain ions like laurate (C12).[20] The asymmetric and symmetric $-CH_2-$ stretching form intense bands near to 2900 cm^{-1}, but in the LDH-G spectrum this bands are not seen because of the small amount of glutamate in the LDH.

The main information from this spectrum is that glutamate ions are present in the sample. Although they are not intercalated, according the X-ray diffraction patterns, they could be adsorbed on the external surfaces of the crystals.[7-10]

The spectrum of the LDH-GL (Figure 2c) does not shows different bands comparing with the LDH-G, making difficult to confirm the immobilization of the laccase by FTIR.

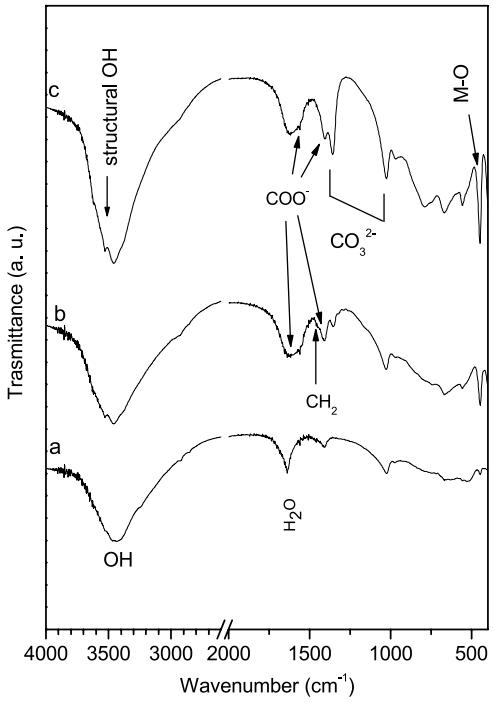

Figure 2. *FTIR spectra of the (a) Mg/Al-Cl layered double hydroxide, LDH, (b) LDH treated in glutamate solution (LDH-G) and (c) LDH after mixing in laccase solution (LDH-GL)*

Electronic paramagnetic resonance

The EPR measurements helped to prove the immobilization of the laccase enzyme. LDH is silent in the EPR analysis (Figure 3a). The spectrum for the free laccase sample showed typical absorption lines of the outer sphere of the Mn^{2+} ion complex, $[Mn(OH_2)_6]^{2+}$-Laccase (Figure 3c). For these absorption lines A = 87.3×10^{-4} cm^{-1} and g = 2.00**.**

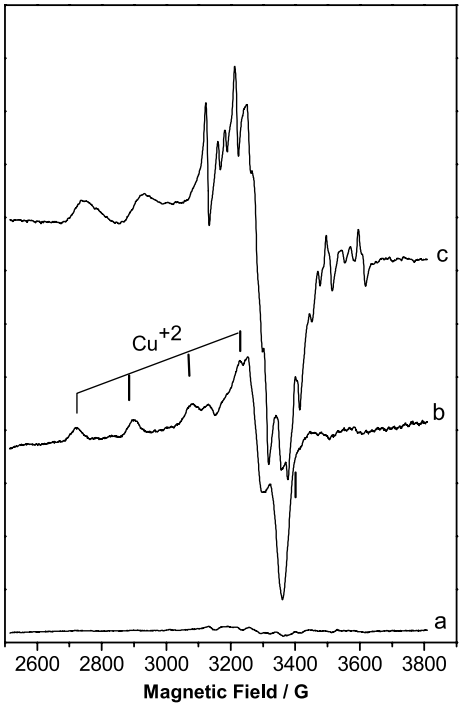

Figure 3. *EPR spectrum in the X band for (a) the hybrid support LDH-G, (b) the immobilized laccase, LDH-GL and (c) the free laccase*

The magnitude of the EPR hyperfine A parameter is inversely related to the degree to which the Mn^{2+} ion is involved in covalent bonding within its complexes.[21] This value indicates an electrovalent interaction of Mn^{2+} in the laccase structure. The EPR spectra of the two Cu^{2+} coordination sites for the free laccase (Figure 3c) and LDH-GL (Figure 3b) indicate axial symmetry. The Hamiltonian parameters for the free laccase are $g\perp = 2.0700$, $g_{\|} = 2.2310$, $A\perp = 30\times10^{-4}$ cm^{-1} and $A_{\|} = 190\times10^{-4}$ cm^{-1} while for the immobilized laccase (LDH-GL) they are $g\perp = 2.0580$, $g_{\|} = 2.2580$, $A\perp = 30\times10^{-4}$ cm^{-1} and $A_{\|} = 185\times10^{-4}$ cm^{-1}.

For the two materials, $g_{\|} > g\perp > 2$, which suggests copper(II) sites with distorted tetragonal, square-pyramidal or square-planar geometry. Moreover, the g_{II} and A_{II} values of copper in the free laccase and in the laccase immobilized in the hybrid material (Table 1) are found in regions characteristic of CuN_4 and CuN_3O chromophores in the $g_{\|}$ versus $A_{\|}$ diagram.[22]

Table 1. Parameters g and A ($\times10^{-4}$ cm^{-1}) and $g_{\|}/A_{\|}$ (cm) ratio for the free and immobilized laccase in the hybrid material (LDH-GL)

Compound	$g_{\|}$	$A_{\|}$	$g_{\|}/A_{\|}$
Free Laccase	2.2310	190	117
Immobilized laccase (LDH-GL)	2.2580	185	122

The $g_{\|}/A_{\|}$ ratio can be used as a convenient empirical index of tetrahedral distortion in CuN_4 units. This value ranges from about 105

to 135 cm for square-planar structure and increases with the tetrahedral distortion of the chromophore. Further, tetrahedral distortion of a square-planar chromophore is observed when any of the biomimetic donors (N, O, S) reduce $A_{\parallel}$ and increase $g_{\parallel}$. Using this relationship, both of the Cu^{2+} sites have a slightly tetrahedral distortion, that is, they seem to be square-pyramidal. The increased $g_{\parallel}$ values and decreased $A_{\parallel}$ values from the free laccase and immobilized laccase (LDH-GL) samples show that the ligand field strength for the Cu^{2+} ion decreases in these materials in the same order. The free laccase has a stronger ligand field than does LDH-GL: the $A_{\parallel}$ values are 190 and 185×10^{-4} cm^{-1}, respectively, while the $g_{\parallel}$ values are 2.231 and 2.258, respectively.[23] Thus the copper coordination has a slightly higher tetrahedral distortion after immobilization.

These results, when combined with the FTIR information, suggest that the laccase was immobilized onto the LDH-G powder and, since the basal space of the layered material did not change, it can be concluded that the laccase was immobilized onto the external surface of the layered support, probably interacting with the pendant glutamate ions.

Thermal analysis

The TG profile of the free laccase shows that the sample starts to decompose at 230 °C (Figure 4a). The amount of copper from the laccase is not enough to be detected by this technique, since there are only four copper centers in the active site each laccase unit of about 50-70 kDa. The hybrid support, LDH-G, lost 48.3% of its original mass when heated to 885 °C (Figure 4c) while the LDH-GL preparation lost 51.5% of its original mass at the same temperature (Figure 4b). This result is to be expected since immobilization of the laccase adds organic matter to the system.

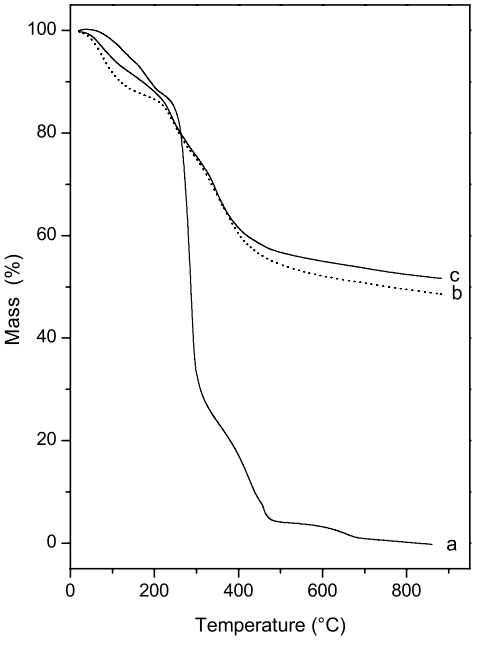

Figure 4. *Thermogravimetry profiles of (a) the free laccase, (b) the immobilized laccase, LDH-GL and (c) the hybrid support, LDH-G*

Activity of the immobilized enzyme

The results above indicate that the laccase was immobilized onto the solid phase. The catalytic activity of this immobilized laccase preparation was then tested. The activity assays were done on the LDH-GL samples as well as in the liquid phases of the washing (LPhW) during the immobilization in order to detect laccase activity of desorbed enzyme from the support.

The activity in the 50 mL laccase suspension was 204.7 LAMU. It represents the total laccase units according the LAMU method.[24] It could be also expressed as 818.8 LAMU per gram of the commercial DeniLite II Base™, in concordance with the specifications given by the provider, since 1 g of DeniLite II was used to prepare the 200 mL of the laccase suspension. For our evaluation, we consider the absolute LAMU units in the 50 mL of the free laccase sample and in the 0.3 g of LHD-GL. By quantifying the activity in the three samples of LDH-GL, it was calculated the mean activity, which was 198.94 LAMU, representing 97.2% of the activity of the free laccase.

The enzymatic activity in the washing water separated after the laccase immobilization (LPhW) was 1.70 LAMU units, suggesting that the laccase is not significantly desorbed during washing of the layered support LDH-GL.

After the first analysis the LDH-GL was lyophilized and the activity measured again. This time the activity decreased to 92.3% of the value obtained with the free laccase.

We propose the LDH-G as a support for laccase immobilization, which could be used in studies of soil or water remediation. This support is synthesized in mild conditions. In this report we use the molar ratio $Mg^{2+}/Al^{3+} = 2$, although the electrostatic charges of the layers can be controlled by the amount of Al^{3+} if required. LDH-G does not require further purification like natural clays and do not represent further contamination of soils like non-biodegradable polymer supports. Immobilizations were also performed in other materials like layered hydroxide salts (LHS),[25,26] whose results will be published elsewhere.

CONCLUSIONS

Although the intercalation of the glutamate ions in the LDH was not accomplished, the external modification of LDH layered crystals was successful by mixing them in a glutamate solution. The resulting hybrid LDH-G was capable of adsorbing laccase from an aqueous medium in the pendant glutamate anions at the surface. This absorption induces a slightly tetrahedral distortion in the symmetry of the Cu^{2+} located in the catalytic site. The percentage of immobilized laccase was 92% of the initial activity offered to immobilization. This fact shows that simple chemical modification of the surface with a molecule compatible with enzyme is sufficient to act as an enzyme support. The immobilization of laccase in hybrid derivatives of LDH is a promising strategy in the designing of bioremediation processes for soils and polluted waters, since do not represent further contamination like non-biodegradable polymer supports and unlike natural clays, the LDH does not requires further purification.

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