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Catalytic efficiency of natural and synthetic compounds used as laccase-mediators in oxidising veratryl alcohol and a kraft lignin, estimated by electrochemical analysis

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ABSTRACT

The electrochemical properties of eighteen natural and synthetic compounds commonly used to expand the oxidative capacity of laccases were evaluated in an aqueous buffered medium using cyclic voltammetry. This clarifies which compounds fulfil the requisites to be considered as redox mediators or enhancers. Cyclic voltammetry was also applied as a rapid way to assess the catalytic efficiency (CE) of those compounds which oxidise a non-phenolic lignin model (veratryl alcohol, VA) and a kraft lignin (KL). With the exception of gallic acid and catechol, all assayed compounds were capable of oxidising VA with varying CE. However, only some of them were able to oxidise KL. Although the oxidised forms of HBT and acetovanillone were not electrochemically stable, their reduced forms were quickly regenerated in the presence of VA. They thus act as chemical catalysts. Importantly, HBT and HPI did not attack the KL via the same mechanism as in VA oxidation. Electrochemical evidence suggests that violuric acid oxidises both substrates by an electron transfer mechanism, unlike the other N–OH compounds HBT and HPI. Acetovanillone was found to be efficient in oxidising VA and KL, even better than the synthetic mediators TEMPO, violuric acid or ABTS. Most of the compounds produced a generalised increase in the oxidative charge of KL, probably attributed to chain reactions arising between the phenolic and non-phenolic components of this complex molecule.

1. Introduction

It is well known that laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are enzymes with broad industrial applications, which are gaining interest from the pulp and paper industry [1], biosensor technology [2,3], in the organic synthesis of useful compounds [4] and offer great interest in environmental biotechnology [5]. They are multicopper oxidases capable of oxidising lignin, a complex aromatic polymer synthesised by higher plants, with a broad target substrate range (mostly phenolic compounds). These enzymes present low redox potentials (0.45–0.8 V vs. NHE [5]), permitting only phenolic compounds to be oxidised, which represent only about 14–25% of the total functional groups in lignin [6,7]). Up to 70% of the lignin residues consist of other non-phenolic groups, which due to their higher redox potential (>1.5 V vs. NHE [8]) cannot be oxidised by laccases. For this reason, non-phenolic compounds hinder the pulp-bleaching process in paper-making [9]. However, it is well known that using appropriate substrates (low molecular-weight compounds, so-called laccase-mediators) enables laccases to indirectly oxidise even non-phenolic groups, through a redox cycle [10]. Thus, the compound itself oxidised by the laccase is capable of oxidising other un-attacked non-phenolic substrates, thus regenerating its reduced form.

As indicated by Morozova et al. [11], most compounds described as laccase-mediators are not strictly redox mediators, since their oxidised intermediates are electrochemically unstable. Consequently, only a small number of redox cycles would occur during catalytic oxidation of non-phenolics. The compounds thus have to be continually replenished in the media and the term *'laccase-enhancer'* should be accepted as a more precise definition for them. Whether mediators or enhancers, they notably widen the substrate range of laccases.

The proper selection of such compounds plays a key role in further applications of laccase-mediator systems [12] and requires knowledge of the stability of the radicals generated, their redox potential and ability to oxidise substrates such as lignin or other aromatic compounds. Cyclic voltammetry is a powerful technique in electrochemical analysis that provides this information. Tech-

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niques used up to now to select mediators are mainly based on tracking dye decolourisation by spectrophotometry [13], mass analysis of the oxidation products of lignin-model compounds by GC–MS [9] or HPLC [14]. However, these latter approaches take longer since they require enzymatic reactions and further characterisation of the reaction products.

In the light of the above, one aim in this work is to discriminate between redox mediators and enhancers by cyclic voltammetry in an aqueous medium employed in lignin biotransformation [15,16]. In addition, we will apply cyclic voltammetry as a quick and efficient way to determine the catalytic efficiency of the selected compound for oxidation of (1) veratryl alcohol (VA, as non-phenolic lignin model) and (2) a kraft lignin (KL, from paper industry effluents). In this way, catalytic efficiency was studied against a high redox potential compound (VA), a widely used monomer to simulate oxidation of xenobiotic pollutants [17], and a fully complex industrial lignin (KL). This enabled clearer conclusions to be reached in selecting compounds for use in industrial processes such as pulp delignification, and evaluate the catalytic efficiency of the assayed compounds, providing a ranking which facilitates their selection for future applications in combination with laccases.

2. Experimental

Most of the substrates used as mediators or enhancers were commercially available from Aldrich Chemical Co. (N-hydroxyphthalimide, HPI; 2,2′,6,6′tetramethyl piperidine N-oxyl, TEMPO; 1-(3′-sulphophenyl)-3methyl-pyrazolone-5, SPP-m; 1-(4′-sulphophenyl)-3 methyl-pyrazolone-5, SPP-p; 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone, acetosyringone; 3,4,5-trihydroxybenzoic acid, gallic acid; 4-hydroxy-3,5-dimethoxybenzoic acid, syringic acid; 4-hydroxy-3,5-dimethoxybenzaldehyde, syringaldehyde; 4-hydroxy-3-methoxy-acetophenone, acetovanillone; 3-hydroxy-anthranylic acid, HAA; 1,2-dihydroxybenzene, catechol and 2,6-dimethoxyphenol, DMP). 5-isonitrosobarbituric acid (violuric acid; VLA) was purchased from Across. 4-hydroxy-3-methoxy-benzyl alcohol (vanillyl alcohol) was obtained from EGA-CHEMIE. 4-hydroxy-3-methoxy-benzaldehyde (vanillin) and 3-(4-hydroxyphenyl)-2-propenoic acid (*p*-coumaric acid) were purchased from Aldrich-Chemie (Milwaukee, WI). 2,2′-azino-bis (3-ethylbenzothiazoline-6 sulphonic acid (ABTS) and 1-hydroxy benzotriazole (HBT) were obtained from Sigma (St. Louis, MO). Citric acid monohydrate, di-sodium hydrogen phosphate dihydrate and sodium citrate were supplied by Scharlau Chemie. All these reagents were analytical grade and used without further purification. Ultra-pure water was obtained from a Milli-Q purification system and used to prepare all the solutions. The lignin preparation used throughout the study was a kraft pine lignin, Indulin AT, supplied by Westvaco Co., Charleston, S.C., USA. The electrochemical solution used here was previously employed to modify industrial lignins, using a laccase-mediator system under different assay conditions [15,16].

The electrochemical measurements were conducted in a potentiostat–galvanostat (EG&G PARC mod. 273A) and a thermostatted three-electrode cell at room temperature or 37 °C. A glassy carbon electrode (GCE) was used as working electrode, a platinum ring as counter-electrode and a saturated calomel electrode (SCE) as reference electrode. All the data in this work are referred to this reference electrode. The surface of GCE was built from a 3 mm diameter rod (Tokai GC-20) sealed in glass tubing. Before each measurement the disk was activated as follows. Firstly, it was polished using different diamond pastes (Struers 0.25 and 1 μm) on a diamond-polishing pad. The electrode was immersed in ethanol, ultrasonicated for 5 min and dried. It was then electrochemically activated in the background solution by means of several voltammetric cycles at 200 mV s^{-1} (more details in [18]). The voltammetric responses of each compound were first assayed alone at different sweep-rates (5–200 mV s^{-1}) and different concentrations (0.2 and 1 mM). For each of the voltammograms shown in the present work, each compound, except ABTS, was added at 1 mM final concentration to the electrolytic solution made up of McIlvaine buffer (20 mL; 0.1 M citric acid–0.2 M disodium hydrogen phosphate) adjusted to pH 6. The ABTS was added at 0.2 mM final concentration to the solution indicated above and also to a 0.1 M citric acid–sodium citrate buffer at pH 6. Ethanol (2%) was added to the electrochemical cell when HPI or TEMPO were assayed in order to ensure they were completely dissolved. The voltammetric responses of either veratryl alcohol (added at 0.2 M final concentration) or kraft lignin (0.4 mg mL^{-1} added from a 10 mg mL^{-1} stock KL in 50 mM NaOH), were also registered separately at the same scan rate potential (5 mV s^{-1}). Finally, the voltammograms of the compounds in the presence of either VA or KL were studied as indicated above. Potential was scanned from −200 to 1100 mV vs. SCE. Standard redox potentials (E°) of different compounds were determined at 100 and 200 mV s^{-1} as the mean values of the anodic peak potential (E_{pa}) and the cathodic peak potential (E_{pc}). All experiments were performed in Ar atmosphere at room temperature and 37.0 ± 0.2 °C.

The catalytic efficiency is expressed by the ratio i_k/i_c, where i_k is the anodic peak current (catalytic current) of the compound acting as catalyst in the presence of VA or KL and i_c is the diffusion-controlled peak current of the catalyst alone. Catalytic efficiency was also determined by the oxidation potential shift given by the difference between VA or KL potential oxidation in the presence or absence of the assayed compounds (ΔE), as well as by the potential shift of the compound itself.

All the experiments were performed three or more times, and the measurements were highly reproducible. Before each voltammetric measurement, we ensured electrode surfaces were properly activated as described above, in order to rule out mediator or enhancer polymerisation.

3. Results and discussion

3.1. Selection of compounds that indirectly increase the substrate range of laccase

The natural compounds: acetosyringone, gallic acid, vanillyl alcohol, vanillin, syringic acid, *p*-coumaric acid, syringaldehyde, acetovanillone, HAA and catechol were chosen, since they are: (1) secreted extracellularly by fungi (e.g. HAA [19]), (2) present *in situ* as common secondary plant metabolites (e.g. acetosyringone, syringaldehyde and *p*-coumaric acid [20]) or (3) released in large amounts during the microbial degradation of lignocellulose (vanillin [21], syringic acid, *p*-coumaric acid, syringaldehyde, acetovanillone [22,23]). Catechol was selected because it is a recurring phenolic structure in lignin [24].

The synthetic compounds assessed, described in the literature as mediators, belong to different families like those included in the >N–OH group: HPI, VLA and HBT; TEMPO as the only mediator included in the –N–O• species group; two phenyl-methyl-pyrazolones: SPP-m and SPP-p, and two substrates widely used as laccase targets: ABTS and DMP. The latter (DMP), since found as a lignocellulose degradation derivative product is considered a natural mediator. We have previously checked that these compounds are target substrates of laccases [9,11,13,19,25–29], and therefore with potential use in the so-called 'laccase-mediator/enhancer systems'. Thus, they can mediate lignin or veratryl alcohol oxidation only after being oxidised by laccase, or as in the present study by an electrode replacing it, which greatly simplifies the experimental design. In any case, the interaction between the oxidised media-