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Investigating the Reversible Inhibition Model of Laccase by Hydrogen Peroxide for Bioelectrocatalytic Applications

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The reversible inhibition of laccase by H_2O_2 as a bioelectrocatalyst was studied in mediated- and direct electron transfer-based configurations to understand the differences in mechanism. The reversible inhibition of laccase follows a noncompetitive inhibition model when 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is used as an electron mediator, whereas laccase is inhibited by an uncompetitive inhibition model when anthracene-moieties are used to intelligently "dock" laccase to electrodes (consisting of multi-walled carbon nanotubes, MWCNTs) which afford direct electron transfer (DET). This further confirms the efficient orientation of laccase onto suitably-designed docking moieties for bioelectrocatalysis applications.
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Laccase (*Trametes versicolor*, E.C: 1.10.3.2) is a multi-copper oxidase (MCO) commonly incorporated within O_2-reducing enzymatic biocathodes in enzymatic fuel cells (EFCs), because laccase can efficiently reduce O_2 to H_2O (in most cases, following a 4-electron reduction mechanism) therefore allowing O_2 to be utilized as the oxidant and final electron acceptor in EFCs.[1–5] Along with some other MCOs (such as bilirubin oxidase, BOx), laccase contains 3 different copper centers. A type-1 copper center (T1 Cu) is proximally located within the enzyme structure and is responsible for the single-electron oxidation of phenolic-type substrates, and a further type-2 copper center (T2 Cu) and 2 further type-3 copper centers (T3 Cu) are combined in a tri-nuclear cluster (TNC), which is responsible for the 4-electron reduction of O_2 to H_2O. Following the oxidation of substrates at the T1 Cu site, electrons are quickly and individually transferred to the TNC *via* a His-Cys-His tripeptide chain.[6,7] Although laccase (from *Trametes versicolor*) is reported to optimally reduce O_2 at acidic pH (which may limit its incorporation within implantable EFCs) further complications can be found within a physiological setting, where laccase can be inhibited by Cl^- (approximately 150 mM physiological concentration) *via* a competitive inhibition model whereby Cl^- can compete against certain electron mediators at the T1 Cu site of the enzyme.[8,9]

Laccase can be intelligently orientated (or "docked") onto electrode architectures whereby moieties with structural similarities to the natural substrates of laccase (oxidized at the T1 site) are incorporated on specifically-designed electrode architectures, which subsequently allow direct electron transfer (DET).[2,4,10–14] Efficient mediated electron transfer (MET) has also been demonstrated using mediators such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or osmium-based complexes[1,15–17] and comparison between orientationally-driven DET and MET of laccase (with ABTS) has been reported, contrasting catalytic current densities that can be achieved with both electron transfer methods.[18] Such electrode architectures can be capable of demonstrating increased resistance to Cl^- inhibition, since the moieties used for docking can efficiently out-compete Cl^- at the T1 site and therefore permit electron transfer.[9,13,19] Recent studies, however, determined that certain membrane-less EFC configurations (favorable to lower overall device cost and size) can present further problems for laccase-based biocathodes, whereby oxidoreductases utilized at EFC bioanodes (such as glucose oxidase (GOx)) which utilize O_2 as their natural electron acceptor can produce significant quantities of H_2O_2 (taking place as a side-reaction at bioanodes) to rapidly inhibit laccase and its bioelectrocatalytic reduction of O_2, in both MET and DET.[12,20,21] This loss in bioelectrocatalytic performance at the EFC biocathode can result in an overall loss-of-performance of the EFC. While it appears evident that H_2O_2 can significantly affect the catalytic/bioelectrocatalytic performance of laccase, the mechanism by which H_2O_2 inhibits laccase remains unclear; a single study investigating the inhibition of laccase by F^- previously proposed that H_2O_2 binds to the T2 Cu of laccase.[22]

Although the authors have previously demonstrated the reversible inhibition of laccase by H_2O_2 on DET-type bioelectrodes (incorporating anthracene-modified multi-walled carbon nanotubes (AcMWCNTs)),[12,13] an inhibition mechanism for the reversible inhibition of laccase (by H_2O_2) does not currently exist. Thus, this study investigates and reports the reversible inhibition mechanism by which H_2O_2 inhibits laccase, in both an MET and DET context.

Experimental

Chemicals.— Laccase (*Trametes versicolor*, ≥10 U mg^{-1}, EC: 1.10.3.2), citric acid, sodium phosphate (dibasic), paraffin wax, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, Nafion perfluorinated resin solution (5 wt% in lower aliphatic alcohols) and tetrabutylammonium bromide (TBAB) were purchased from Sigma Aldrich and used as received. Toray carbon paper (TGP-H-060, non wet-proofed) was purchased from Fuel Cell Earth and used as received. Water used was taken from a Millipore Type 1 (Ultrapure) Milli-Q system (18.2 MΩ cm). Hydroxyl-modified multi-walled carbon nanotubes (MWCNTs) were purchased from www.cheaptubes.com and used as received.

Instrumentation.— Electrochemical analyses were conducted using a Digi-IVY DY2100-2300 mini-potentiostat (Digi-IVY Inc., USA). Spectroscopic enzymatic activity and inhibition assays were performed with a Thermo Scientific Evolution 260 Bio UV-Visible Spectrophotometer; the specific enzymatic activity of laccase was determined using ABTS as the redox indicator. Pt-mesh electrodes were used as counter-electrodes in three-electrode configurations, using saturated calomel electrodes (SCE) as reference electrodes. Toray paper electrodes were cut and waxed (paraffin) to give an untreated geometric surface area of 1 cm^2. A Clark-type electrode was used to monitor dissolved O_2 concentrations and was calibrated prior to use.

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For chronoamperometric experiments, aliquots of electrolyte that had been purged with O_2 were added to bulk electrolyte that had been purged with N_2 (to partially displace O_2); the resulting increase in dissolved [O_2] was then recorded. Dissolved O_2 concentrations were normalized to the initial dissolved O_2 concentration (due to difficulty with completely displacing O_2 from the electrolyte/buffer); initial dissolved O_2 concentrations were 0.022 ± 0.002 mM. All experiments were performed at 22 ± 1°C.

Spectroscopic activity/inhibition laccase assays.— Spectroscopic UV inhibition assays of laccase were performed in 1.5 mL vials. Citrate/phosphate buffer (1290 μL, pH 4.5, 50 mM) was added to 10 μL of a laccase solution (0.5 mg mL^{-1}, prepared in water) and 200 μL of ABTS (1 M, prepared in water). Final ABTS concentrations were 0.03, 0.13, 0.33, 0.67, 0.83, 1 and 1.33 mM). Stock solutions of H_2O_2 were prepared (100 mM in citrate/phosphate buffer, as above) and substituted within the citrate/phosphate buffer aliquot, giving final concentrations of 1, 2, 5 and 10 mM H_2O_2. The specific activity of laccase was determined to be 8.9 ± 0.5 U mg^{-1} at pH 4.5. Michaelis-Menten non-linear regression analyses were performed with GraphPad Prism.

Preparation of laccase bioelectrodes.— Nafion was hydrophobically modified with TBAB and AcMWCNTs were prepared from hydroxyl-modified MWCNTs, as previously reported.[11] Laccase (1.5 mg) was initially dissolved in 75 μL of citrate/phosphate buffer (pH 4.5, 200 mM) and then added to 7.5 mg of AcMWCNTs. In total this mixture was vortexed for 4 minutes and sonicated for 1 minute, in 4 vortex/sonication steps. TBAB-modified Nafion (25 μL) was then added to this mixture, followed by one final vortex-mixing (1 minute) and sonication (15 second) step. The final mixture was then divided between 3 Toray paper electrodes and applied with a brush (yielding approximately 33 μL per electrode). The resulting electrodes were dried under positive airflow, before use. For ABTS-mediated bioelectrocatalysis, laccase electrodes were prepared using the same methodology as the above DET-type bioelectrodes although AcMWCNTs were not included within the electrode architecture (therefore not affording DET).

Results and Discussion

Bioelectrocatalytic reduction of O_2 by laccase.— The bioelectrocatalytic reduction of O_2 by laccase in both MET- and DET-based configurations was investigated. Figure 1 presents catalytic cyclic voltammograms of laccase when either anthracene-modified multi-walled carbon nanotubes (AcMWCNTs) or ABTS are used to establish DET or MET, respectively. AcMWCNTs have been shown to act as an efficient docking moiety for DET between laccase and an electrode.[4,11–14] The onset potential for the direct bioelectrocatalytic reduction of O_2 to H_2O by laccase was found to begin at approximately +670 mV (vs. SCE) at pH 4.5. In contrast, the onset potential for the mediated bioelectrocatalytic reduction of O_2 to H_2O by laccase *via* MET was found to begin at approximately +640 mV (vs. SCE) at pH 4.5. Purging of the buffer/electrolyte with N_2 significantly lowered the catalytic current (due to O_2 displacement by N_2); however, a small catalytic current is still present and can be explained by the incomplete displacement of dissolved O_2 by N_2 purging. Figures 1A and 1B indicatively demonstrate both MET and DET of laccase bioelectrodes, however the current densities reported for these MET and DET bioelectrodes are not comparable or indicative of the performance of both electron transfer mechanisms (since the ABTS bioelectrode was not optimized).

Inhibition of laccase in ABTS-mediated applications.— The inhibition of laccase by H_2O_2 was investigated spectrophotometrically, using ABTS as an electron mediator at different concentrations (in quiescent buffer solutions). Figure 2 presents apparent Michaelis-Menten kinetic non-linear regression fits for the reduction of O_2 by laccase (spectroscopically determined by ABTS oxidation) in different concentrations of H_2O_2. Table I reports apparent Michaelis-Menten

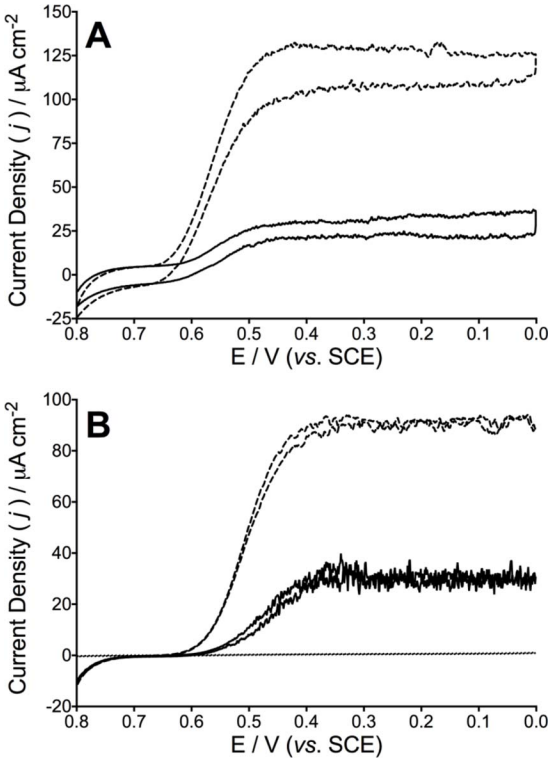

Figure 1. (A) Representative cyclic voltammetry of laccase immobilized on Toray paper electrodes modified with AcMWCNTs in air-purged (dashed) and N_2-purged (solid) citrate/phosphate buffer (pH 4.5, 0.2 M, hydrodynamic (stirred)), performed at a scan rate of 1 mV s^{-1}. (B) Representative cyclic voltammetry of laccase immobilized on Toray paper electrodes in air-purged (dashed) and N_2-purged (solid) citrate/phosphate buffer (pH 4.5, 0.2 M, hydrodynamic (stirred)) – the solid and dashed traces were performed in the presence of 0.2 mM ABTS as an electron mediator, at a scan rate of 1 mV s^{-1}.

constant (K_m) and maximum velocity (V_{max}) values for laccase (as determined by ABTS) at each concentration of H_2O_2.

Although the reversibility of the inhibition of laccase by H_2O_2 has previously been demonstrated by the authors (enzymatic activity recovered by the rapid decomposition of H_2O_2 by catalase), Figure 2 and Table I reveal that H_2O_2 increases the apparent K_m while decreasing the apparent V_{max} of laccase, suggesting that H_2O_2 acts *via* a mixed inhibition model (subcategory of noncompetitive inhibition) with re-

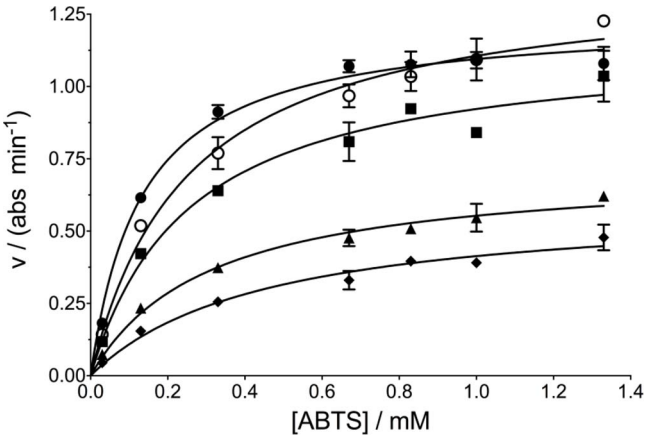

Figure 2. Spectroscopic determination of apparent Michaelis-Menten kinetics for the biocatalytic reduction of O_2 by laccase in 50 mM phosphate-citrate buffer (pH 4.5, 50 mM), containing 0 (●), 1 (O), 2 (■), 5 (▲) and 10 (♦) mM of H_2O_2 (n = 3).

Table I. Apparent Michaelis-Menten kinetics for the reduction of O_2 by laccase, as determined by ABTS (n = 3).

$[H_2O_2]$ / mM	$K_m^{app.}$ / mM	V_{max} / (abs / min)
0	0.13 ± 0.01	1.24 ± 0.02
1	0.25 ± 0.03	1.38 ± 0.04
2	0.26 ± 0.04	1.16 ± 0.05
5	0.30 ± 0.03	0.72 ± 0.03
10	0.45 ± 0.07	0.60 ± 0.04

spect to ABTS.[13] Michaelis-Menten non-linear regression analysis reports an overall coefficient of determination (r^2) of 0.96 for a pure noncompetitive inhibition model.

Lineweaver-Burk double-reciprocal and Eadie-Hofstee (Figure 3) linearizations further reveal that although H_2O_2 acts as an apparent noncompetitive (mixed competitive and uncompetitive) inhibitor on laccase, H_2O_2 inhibition more closely resembles that of a competitive inhibitor than an uncompetitive inhibitor.[23,24] For Lineweaver-Burk double-reciprocal plots, the convergence of the data points on the ordinate axis is typical of a competitive inhibition model, whereas convergence on the abscissa is characteristic of a pure noncompetitive inhibition model; the location of convergence (if located between the abscissa and the ordinate) reflects the ratio of competitive and noncompetitive inhibition models in mixed inhibition. Similarly, a set of parallel linear-fits on Eadie-Hofstee plots are characteristic of a noncompetitive inhibition model.[23] Lastly, a noncompetitive inhibition model suggests that the inhibitor binds to a different site than the substrate, although it is envisaged that both the substrate and inhibitor influence each other's binding.[25]

While the largely pure noncompetitive nature of H_2O_2 inhibition suggests that H_2O_2 binds with close affinity to either the enzyme (E) or the enzyme-substrate (ES) complex, a slight preference of competitive inhibition over uncompetitive inhibition suggests that H_2O_2 binds with slightly larger affinity to E than ES, which reflects the minimal apparent competitive inhibition nature of laccase.[23,24] Since the inhibition of laccase by H_2O_2 is observed using an artificial electron source (ABTS), the binding affinity of H_2O_2 to E or ES, in this case, may not accurately reflect the actual binding affinity for H_2O_2 to laccase, but only reflect affinities in this specific experimental configuration and MET-based laccase bioelectrodes that utilize ABTS as an electron mediator.

Inhibition of laccase in DET applications.— The reversible inhibition of laccase by H_2O_2 was investigated on DET-based bioelectrodes. DET was established using AcMWCNTs to orientate laccase *via* its T1 Cu site. For this investigation the concentration of $O_{2\,dissolved}$ was monitored (using a Clark-type O_2 electrode) and plotted against the catalytic current produced as a result of the direct bioelectrocatalytic reduction of O_2 by the laccase bioelectrode. Figure 4 presents a chronoamperometric trace for the determination of apparent Michaelis-Menten kinetics for DET-based laccase bioelectrodes and non-linear regression fits for the reduction of O_2 by laccase (on DET-based bioelectrodes) in different concentrations of H_2O_2 (note:

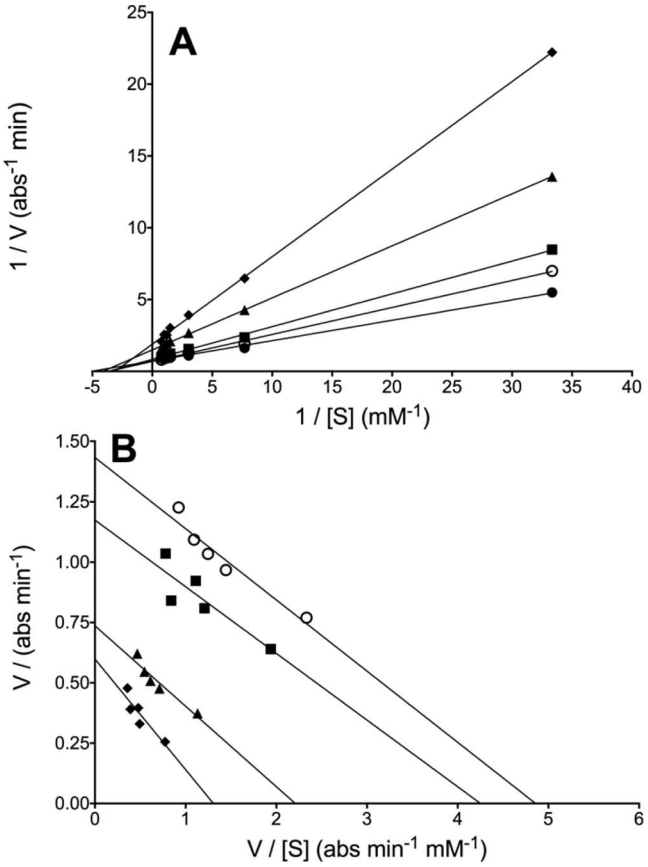

Figure 3. (A) Lineweaver-Burk double reciprocal plot and (B) Eadie-Hofstee plot for the reduction of O_2 by laccase, monitored using ABTS in citrate/phosphate buffer (50 mM, pH 4.5) containing 0 (●), 1 (O), 2 (■), 5 (▲) and 10 (♦) mM of H_2O_2. Data are presented as the mean values (n = 3).

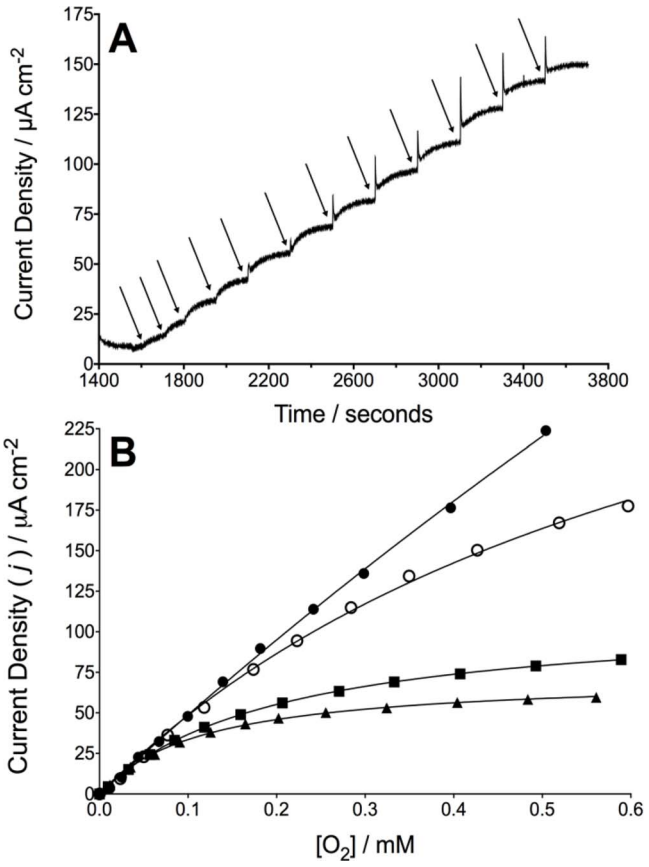

Figure 4. (A) Representative chronoamperometric trace for a laccase/AcMWCNTs bioelectrode (n = 3), with an applied potential of +0.2 V (vs. SCE) in hydrodynamic citrate/phosphate buffer (0.2 M, pH 4.5) containing 5 mM H_2O_2. (B) Resulting representative apparent Michaelis-Menten nonlinear regression plots (n = 3) of laccase/AcMWCNTs bioelectrodes in hydrodynamic citrate/phosphate buffer (0.2 M, pH 4.5) containing 0 (●), 5 (O), 10 (■) and 15 (▲) mM H_2O_2, determined at an applied potential of +0.2 V (vs. SCE).

Table II. Apparent Michaelis-Menten kinetics for the reduction of O_2 by laccase, as determined at a DET-based bioelectrode (n = 3).

$[H_2O_2]$ / mM	$K_m^{app.}$ / mM	V_{max} / (μA cm^{-2})
5	0.61 ± 0.15	352 ± 71
7.5	0.26 ± 0.07	209 ± 39
10	0.26 ± 0.07	120 ± 21
15	0.15 ± 0.03	84 ± 9

the K_m and V_{max} for uninhibited laccase bioelectrodes is not reported since enzymatically-saturating concentrations of dissolved O_2 could not be measured within the calibration range of the dissolved O_2 electrode). Table II reports apparent K_m and V_{max} values for laccase (on DET-based bioelectrodes) at each concentration of H_2O_2.

In contrast to the effect of H_2O_2 on laccase when mediated/monitored with ABTS, the apparent K_m of laccase at a DET-based bioelectrode decreases with increasing concentration of H_2O_2 suggesting increasing affinity for O_2. Interestingly, the V_{max} also decreases. This initially suggests that the DET-based laccase bioelectrode is uncompetitively inhibited by H_2O_2, whereby H_2O_2 exclusively binds only to the ES complex (as per an uncompetitive inhibition model).[23,24] This in itself provides evidence to further support that laccase efficiently orientates (docks) on to anthracene moieties introduced onto the bioelectrode architecture to afford DET, since the absence of ABTS is unlikely to result in a change in inhibition mechanism and the presence of AcMWCNTs is shown to significantly enhance electron transfer between laccase and the electrode (Figure 1).[4,11] The bioelectrocatalytic reduction of O_2 by laccase, in this case, can only be measured via DET since no electron mediators are present.

The application of a Michaelis-Menten non-linear regression uncompetitive inhibition model yields an overall coefficient of determination (r^2) of 0.96. Further analysis with Lineweaver-Burk and Eadie-Hofstee linearizations (Figure 5) further confirmed the nature of laccase inhibition by H_2O_2, in this DET application, to follow the model of an uncompetitive inhibitor. A series of characteristic parallel lines are observed for the Lineweaver-Burk double-reciprocal linearization plot, which is characteristic of an uncompetitive inhibition model, whereas lines that approach convergence at the abscissa of an Eadie-Hofstee plot are also characteristic of an uncompetitive inhibition model. An uncompetitive inhibition model suggests that while the inhibitor affects the catalytic activity of the enzyme it should not affect binding of the substrate to the enzyme.[23,24]

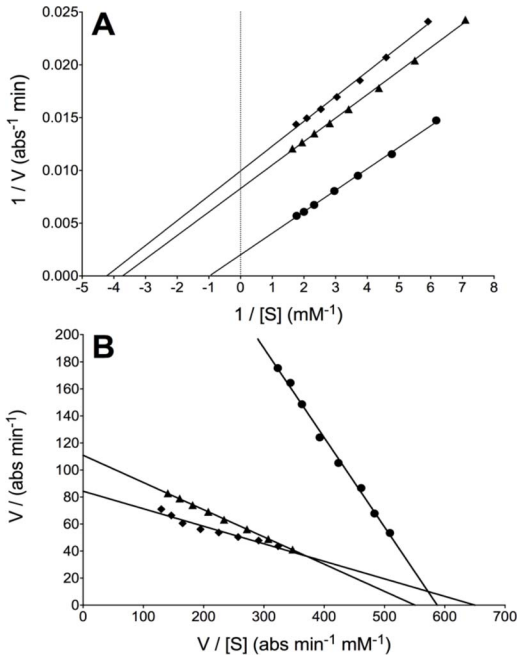

Figure 5. (A) Representative Lineweaver-Burk double reciprocal plot and (B) Eadie-Hofstee plot for the direct bioelectrocatalytic reduction of O_2 by laccase on AcMWCNTs in citrate/phosphate buffer (50 mM, pH 4.5) containing 5 (●), 7.5 (■), 10 (▲) and 15 (♦) mM of H_2O_2. Data are presented as the mean values (n = 3).

Conclusions

The inhibition of laccase by H_2O_2 has been shown to closely resemble a noncompetitive inhibition model, when ABTS was used as a redox-indicator (which is also commonly used as an electron mediator for laccase cathodes in EFCs). These results suggest that H_2O_2 binds, with similar affinity, to free laccase (E) and laccase already bound to its substrate (ES, O_2). In contrast, laccase immobilized on a DET-based bioelectrode (using AcMWCNTs to facilitate DET) was found to follow an uncompetitive inhibition mechanism, whereby H_2O_2 only binds to laccase that has already bound to its substrate (ES). Since laccase was docked onto anthracene-functionalities to undergo DET, it is envisaged that this inhibition mechanism reflects the efficiency with which laccase docks to the AcMWCNTs.

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