



## Bioelectrochemical investigations of aryl-alcohol oxidase from *Pleurotus eryngii*

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### ABSTRACT

Aryl-alcohol oxidase (AAO) electrochemistry studies, using graphite-modified electrodes, are presented for the first time herein. The increase in current upon injection of the analyzed substrate was shown to be approximated by Michaelis–Menten type dependence. The calculated kinetic constants were used to characterize the native (non-mutated) recombinant AAO expressed in *Escherichia coli*, as well as the native enzyme and the F501Y and F501A variants expressed in *Emericella nidulans*. Results from cyclic voltammetry experiments conducted with the enzymes adsorbed on graphite electrodes or with the enzymes in solution (using glassy carbon electrode as working electrode) gave information on the redox potential of these enzymes.

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## 1. Introduction

Lignin is a highly irregular biopolymer with an undefined structure. It is composed of oxygenated phenylpropanoid units, which are linked among them through various types of C–C and C–O–C bonds [1–4], the last ones yielding a molecule with a high non-phenolic content. Due to these features, this macromolecule is rather refractory to biodegradation. In fact, in Nature only the white-rot basidiomycetes are able to degrade lignin in lignocellulosic materials. Effective lignin degradation by these fungi is achieved through a ligninolytic system constituted by laccases, lignin peroxidases, manganese peroxidases and versatile peroxidases [5]. Furthermore, some basidiomycetes have the ability to biosynthesize lignin-related compounds that can be used as intermediates in the degradation process.

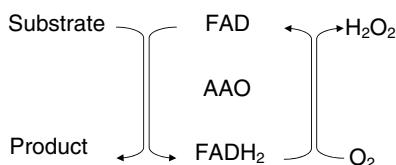
Aryl-alcohol oxidase (AAO) provides hydrogen peroxide necessary for peroxidase activity during lignin biodegradation. This extracellular flavoenzyme is produced by ligninolytic fungi from the genera *Pleurotus*, *Bjerkandera*, *Trametes* and *Phanerochaete* [6–11]. AAO is able to catalyze the oxidative dehydrogenation of a wide range of aromatic and aliphatic primary polyunsaturated

alcohols. During catalysis the non-covalently bound FAD cofactor is reduced by the substrate and subsequently reoxidized by molecular oxygen to yield hydrogen peroxide (Fig. 1) [11,12]. This reaction can be detected amperometrically on the basis of the response achieved due to the generated hydrogen peroxide electrooxidation or due to the electrooxidation of the FADH<sub>2</sub>.

The successful heterologous expression of this oxidase in *Emericella nidulans* (conidial state: *Aspergillus nidulans*) and *Escherichia coli* has permitted to obtain high levels of recombinant AAO (AAO\*) exhibiting similar catalytic properties to those observed for the wild enzyme produced by *Pleurotus eryngii* [13,14], that can be used for a wide biochemical characterization. The molecular model of AAO from *P. eryngii* (PDB entry 1QJN) showed that it is a globular protein with common features with the overall structure topology of the other members of Glucose–Methanol–Choline oxidoreduc-tases (GMC) family, where this oxidase has been included recently [15,16]. AAO substrate-binding pocket is located on the *si side* of the flavin ring and connected to the exposed surface by a hydrophobic substrate access channel. The role of AAO active-site residues have been recently evaluated by site directed mutagenesis [17]. Two putative catalytic histidines (H502 and H546) are essential in AAO activity as a possible general bases in AAO catalysis. Moreover, an aromatic residue (F501), located near of cofactor and the putative catalytic histidines are also involved in substrate oxidation by AAO.

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**Fig. 1.** Schematic representation of the oxidation reaction catalyzed by AAO.

In this study, we used a voltammetric technique to measure the redox potential of native AAO\* and two F501 variants (F501A and F501Y). To better characterize these enzymes, electrochemical measurements were performed in order to determine the kinetic constants. The obtained results are compared with the ones previously obtained from spectrophotometric steady-state kinetics experiments [12,13].

## 2. Experimental

### 2.1. Chemicals

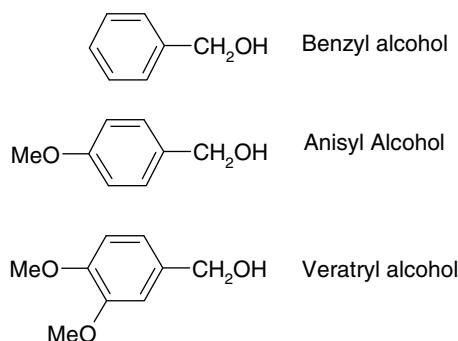
Phosphate buffer salts were of analytical grade and obtained from Merck, Darmstadt, Germany. The enzyme substrates (Fig. 2) veratryl (3,4-dimethoxybenzyl) alcohol, anisyl (4-methoxybenzyl) alcohol, and benzyl alcohol were purchased from Sigma, St. Louis, MO, USA. All aqueous solutions were prepared using distilled water.

### 2.2. AAO expression and site directed mutagenesis

Native AAO\* was produced in *E. nidulans* and *E. coli* according to the protocols described by Varela et al. [16] and Ruiz-Dueñas et al. [13]. AAO\* variants, F501A and F501Y, were obtained by PCR with the Quick-change site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) and used to transform *E. nidulans* [17].

### 2.3. Electrode preparation

The AAO modified electrodes were prepared using rods of solid spectroscopic graphite (SGL Carbon, Werke Ringsdorff, Bonn, Germany, type RW001, 3.05 mm diameter). The graphite rods were first polished on wet fine-structured emery paper (grit size: P1200) and then additionally polished on paper to obtain a mirror-like surface. The electrode rods were carefully rinsed with deionised water and allowed to dry at room temperature. A 10  $\mu$ l aliquot of the enzyme solution was added to each of the polished ends of the graphite rods and the adsorption was allowed to proceed for 1 h at 4 °C. The enzyme electrodes were then thoroughly



**Fig. 2.** Chemical structure of the three aromatic alcohols used as AAO substrates in this study.

rinsed with 0.1 M phosphate buffer, pH 5.5, and if not immediately used, they were stored in the same buffer at 4 °C. Weakly adsorbed enzymes were desorbed before measurements, by rotating the electrode in buffer for at least 30 min.

### 2.4. Electrochemical measurements

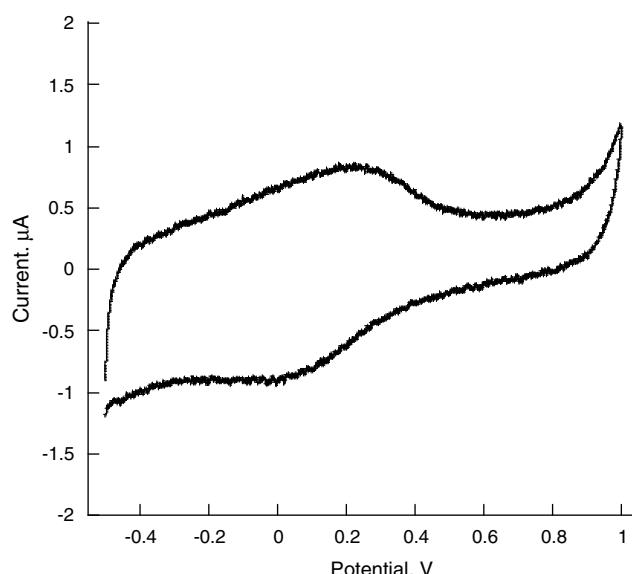
The enzyme modified electrode was placed in a three-electrode cell with an Ag/AgCl (3 M NaCl) reference electrode (BAS, Bioanalytical Systems, West Lafayette, IN, USA) and a platinum wire auxiliary electrode. The electrodes were connected to a Voltalab 30 Potentiostat (Radiometer Analytical) controlled by the Voltamaster 4 (version 5.6) electrochemical software. All amperometric measurements were performed at an applied potential of –50 mV vs. Ag/AgCl [18,19] with an electrolyte of 0.1 M phosphate buffer, pH 5.5. The current was registered at a constant rotation speed of 500 rpm.

Cyclic voltammetry experiments were performed at a scan rate of 5 mV/s. The redox potential of the enzymes was determined with the enzymes adsorbed onto graphite electrodes and with the enzymes in solution. In the latter case as working electrode was used a glassy carbon electrode. Prior to experiments, the solution was bubbled with nitrogen for 15 min. Nitrogen was passed over the solution during the experiments. To evaluate the possible contribution of the enzyme physically adsorbed on glassy carbon electrode surface the blank test was made. The glassy carbon electrode was immersed in an enzyme solution for about 2 h, then thoroughly rinsed and tested for the enzymatic activity using veratryl alcohol. No activity was observed. Therefore, it was considered that the adsorbed enzyme does not have any contribution.

## 3. Results and discussion

### 3.1. Cyclic voltammetry studies

Cyclic voltammograms of the studied AAOs were recorded in 0.1 M phosphate buffer, pH 5.5, at a scan rate of 5 mV/s between the extremes –400 and 700 mV vs. Ag/AgCl. Fig. 3 presents a cyclic voltammogram of the native AAO\* expressed in *E. nidulans* in solu-



**Fig. 3.** Cyclic voltammogram of the native AAO\* from *E. nidulans* in solution, 0.1 M phosphate buffer pH 5.5 at 5 mV/s (glassy carbon as working electrode).

tion using glassy carbon as working electrode. From the cyclic voltammograms obtained, the AAO redox potential was calculated, and the results are presented in Table 1. As it can be seen from this table, when the enzymes are adsorbed onto the graphite electrodes the redox potentials are higher than in the solution. In the case of the *E. coli* recombinant AAO the increase is much smaller than in the other cases. This can be justified by the lack of glycosylation of the enzyme expressed in a prokaryotic organism. It is also interesting to observe that the F501A variant showed lower redox potential than the native (non-mutated) enzyme, while the redox potential of the other variant (F501Y) was higher. This result confirms the role of Phe501 in AAO redox potential suggested in previous steady-state kinetic studies by Ferreira et al. (2006) [11]. This can be explained by considering the mutations that were done: a phenylalanine residue was changed to alanine in F501A and a phenylalanine residue changed to tyrosine in F501Y

(the former change being more drastic than the second one, where one aromatic residue is substituted by other aromatic residue).

In the literature was found that for other oxidases the redox potential for the oxidized-fully reduced free flavin is  $-207\text{ mV}$  in aqueous solution [20]. However, the redox potential of flavo-oxidases is strongly modulated by cofactor environment. In fact, protein-bound flavins exhibit wide range redox potentials from  $-400\text{ mV}$  to  $+60\text{ mV}$ . Thus, vanillyl-alcohol oxidases from *Penicillium simplicissimum* has a high redox potential ( $+55\text{ mV}$ ) [21]. In the opposite way, we can find the reduction potential of nitroal-kane oxidase from *Fusarium oxysporum* ( $-365\text{ mV}$ ) [22]. For both cases, and in general for other flavoenzymes, the determinations of their redox potentials were done spectrophotometrically with the enzyme in solution.

### 3.2. Kinetic constants for AAOs oxidation of different substrates

In this work the amperometric measurements were performed using the enzyme modified graphite electrode as working electrode, rotating at 500 rpm. The responses are dependent on the concentration of the substrate in the solution of interest. At higher substrate concentrations the current-concentration dependence gradually reached saturation (Fig. 4). The apparent Michaelis-Menten constants ( $K_m^{app}$ ) and maximal currents ( $I_{max}$ ) have been calculated by fitting the variation of current-concentration dependencies of the analyzed compounds to the electrochemical Michaelis-Menten equation (Table 2) [23]. The kinetics of the reactions are firstly affected by the affinity between enzyme and the mediator. An estimation of this influence can be done by amperometric measurements in terms of  $I_{max}/K_m^{app}$  ratio. These parameters are often calculated in the design of enzymatic sensors to evaluate the sensitivity of the system proposed, which is related to the low or high affinity of the enzyme towards a specific substrate.

Comparing these results with the ones obtained from the spectrophotometric measurements by Ferreira et al. [17] it can be observed that  $K_m^{app}$  values obtained from the amperometric measurements vary in the same way as the ones from spectrophotometric measurements when *p*-anisyl alcohol is used as a substrate. Regarding the affinity of the F501A and F501Y for veratryl alcohol or benzyl alcohol I used as substrate, the obtained results can be explained based on the different behavior of the immobilized mutants on the electrode surface than when in solution.

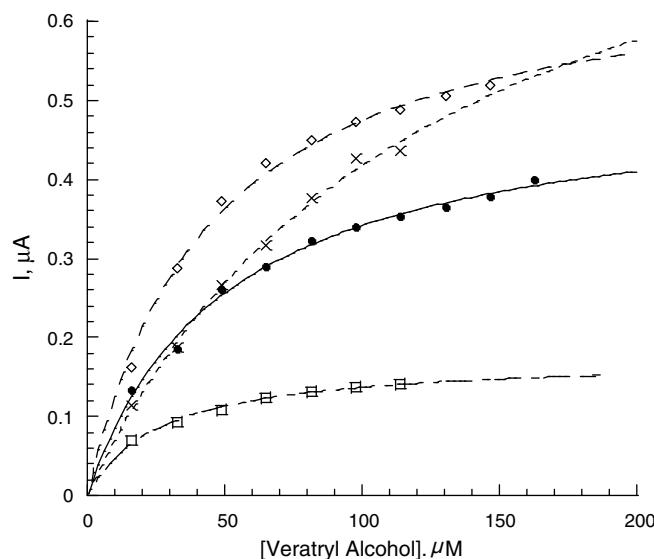
### 4. Conclusion

From the cyclic voltammetry experiments it was possible to calculate the redox potential of recombinant AAO produced in *E. nidulans* and *E. coli*, as well as the F501A and F501A enzyme variants expressed in the former system. The redox potentials of these enzymes were obtained for the enzymes adsorbed on graphite electrodes or with the enzymes in solution. Based on the kinetic values it can be concluded that these enzymes can be also used

**Table 1**

Redox potential of native AAO\*, expressed in *E. nidulans* and *E. coli*, and F501A and F501Y variants, expressed in *E. nidulans*, measured in solution (using a glassy carbon electrode) or adsorbed on a graphite electrode (mV vs. Ag/AgCl)

	AAO* ( <i>E. nidulans</i> )	AAO* ( <i>E. coli</i> )	F501A ( <i>E. nidulans</i> )	F501Y ( <i>E. nidulans</i> )
Enzyme in solution	$13.6 \pm 3.8$	$28.8 \pm 4.7$	$-40.8 \pm 5.9$	$52.9 \pm 6.1$
Adsorbed enzyme	$223.4 \pm 12.4$	$64.4 \pm 5.3$	$124.7 \pm 8.4$	$145.9 \pm 9.8$



**Fig. 4.** Calibration graph obtained for the oxidation of veratryl alcohol using graphite electrodes modified with: ● – AAO\* from *E. nidulans*; □ – AAO\* from *E. coli*; ◇ – F501A from *E. nidulans*; × – F501Y from *E. nidulans*.

**Table 2**

Michaelis-Menten constants obtained for the graphite modified electrodes

	Anisyl alcohol			Benzyl alcohol			Veratryl alcohol		
	$I_{max}$ ( $\mu\text{A}$ )	$K_m$ ( $\mu\text{M}$ )	Sensitivity ( $\text{nA}/\mu\text{M}$ )	$I_{max}$ ( $\mu\text{A}$ )	$K_m$ ( $\mu\text{M}$ )	Sensitivity ( $\text{nA}/\mu\text{M}$ )	$I_{max}$ ( $\mu\text{A}$ )	$K_m$ ( $\mu\text{M}$ )	Sensitivity ( $\text{nA}/\mu\text{M}$ )
AAO* ( <i>E. nidulans</i> )	$2.30 \pm 0.20$	$794 \pm 24$	$2.90 \pm 0.28$	$0.70 \pm 0.26$	$390 \pm 10$	$1.70 \pm 0.13$	$0.50 \pm 0.04$	$50 \pm 4$	$10.20 \pm 0.11$
AAO* ( <i>E. coli</i> )	$1.20 \pm 0.10$	$836 \pm 14$	$1.40 \pm 0.12$	$1.40 \pm 0.49$	$947 \pm 16$	$1.60 \pm 0.16$	$0.20 \pm 0.03$	$27 \pm 3$	$6.40 \pm 0.08$
F501A ( <i>E. nidulans</i> )	$0.20 \pm 0.01$	$108 \pm 10$	$2.00 \pm 0.18$	$1.00 \pm 0.36$	$51 \pm 4$	$19.10 \pm 1.32$	$0.70 \pm 0.05$	$44 \pm 4$	$15.50 \pm 0.27$
F501Y ( <i>E. nidulans</i> )	$0.30 \pm 0.01$	$120 \pm 13$	$2.70 \pm 0.24$	$0.70 \pm 0.31$	$189 \pm 12$	$3.50 \pm 0.47$	$0.90 \pm 0.04$	$123 \pm 16$	$7.60 \pm 0.32$

for construction of biosensors for detection of different substrates of interest.

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