

---

# Modulation of laccases activity by soluble polymers

---

Juozas Kulys\*,  
Regina Vidžiūnaitė

Institute of Biochemistry,  
Mokslininkų 12,  
LT-2600 Vilnius, Lithuania

The activity of recombinant laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) was studied in presence of soluble polymers. As laccases, *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) and *Rhizoctonia solani* (rRsL) were used. Water-soluble uncharged polymers or polymers carrying negatively and positively charged residues were investigated as modulators. Laccase activity was determined by using N,N'-dimethylamine-4-(4-morpholine) benzene (AMB) at pH 5.5 and 25 °C.

The results acquired demonstrate that 50 ppm of uncharged and negatively charged polymers didn't influence an apparent bimolecular constant of AMB oxidation in presence of rPpL or rMtL. In contrast, positively charged residues decreased the constant 2.5–4.1 times. All types of polymers practically didn't influence the activity of rCcL, whereas the activity of rRsL increased or decreased a little.

The correlation of inhibition and isoelectric points of laccases permitted to conclude that laccases form a complex with positively charged polymers which inhibit the enzyme and substrate interaction.

**Key words:** laccase, *Polyporus pinsitus*, *Myceliophthora thermophila*, *Coprinus cinereus*, *Rhizoctonia solani*, polymer

---

## INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a polyphenol oxidase produced prolifically as an extracellular enzyme by many species of white-rot fungi [1]. Laccases are multi-copper-containing enzymes that catalyze oxidative conversion of a variety of chemicals [2]. Laccase couples 4 one-electron oxidations of the substrates such as phenols, mono-, di-, and polyphenols and aromatic amines to the irreversible four-electron reduction of dioxygen to water. Laccases are widely used in biotechnology, environmental engineering and other areas [3]. The practical application of laccases is associated with complex systems containing surface-active compounds, polymers and specific salts. However, not many systematic and comparative studies are available on the quantitative interaction of laccases with these compounds.

The task of the present investigation was to explore laccase kinetics in presence of water-soluble polymers. As laccases, recombinant laccase *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) and *Rhizoctonia solani* (rRsL) were used. As polymers, water-soluble

neutral, negatively and positively charged high molecular weight compounds were used.

## MATERIALS AND METHODS

### Reagents

Recombinant laccases from *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) and *Rhizoctonia solani* (rRsL) were heterologously expressed in *Aspergillus oryzae* and were the experimental products of Novozymes A/S (Copenhagen, Denmark). Laccases were additionally purified by anion exchange chromatography and were homogeneous as assessed by SDS-PAGE. Isoelectric points of laccases were measured also in Novozymes A/S.

N,N'-dimethylamine-4-(4-morpholine) benzene (AMB) was synthesized and used as described [4]. Polyethylene glycol (PEG; Mw 40 000) was received from Ferak (Berlin). Protasan PG 210 (PRT; chitosan glutamate) was from Pronova (Norway biopolymer), polyvinylpyrrolidone (PVP) and dextran T-500 (D500; Mw 500 000) were from Loba Chemie (Austria), polyvinyl alcohol (PVA; Mw 100 000) and hydroxyethyl cellulose (HEC; medium viscosity) were from Fluka, diethylaminoethyl chloride (DEC) was from Whatman, biopolymer (BIP; copolymer of

---

\* Corresponding author, Tel.: (370-2) 729 176. Fax: (370-2) 729 196. E-mail address: jkulys@bchi.lt

acrylamide, N-vinylpyrrolidone and ethyl acrylate) was a product of "Fermentas" (Vilnius, Lithuania), polyethyleneimine (PEI; Mw 30000–40000) and poly-L-lysine HBr (PLL; Mw 6000–9000, n = 40–60) were received from "Serva" (Heidelberg), diethylaminoethyl-dextran as chloride form (DED; Mw 500 000), alginic acid sodium salt (NaA), ficoll (FIC; Mw 400 000) were from "Sigma", polyacrylic acid (PAA), heparin (HEP), sodium acetate, acetic acid and potassium chloride (KCl) were products of "Reachim" (Russia).

## METHODS

Spectrophotometric measurements were performed by using a computer-controlled UV/Visible Ultraspec II LKB Biochrom spectrophotometer [5]. Concentrations of laccases were determined spectrophotometrically. The extinction coefficient of rMtL was  $134 \text{ mM}^{-1} \text{ cm}^{-1}$  at 276 nm [6], whereas for rPpL, rRsL and rCcL it was assumed  $78 \text{ mM}^{-1} \text{ cm}^{-1}$  at 280 nm [7]. Laccase activity was determined by measuring the cation radical of AMB formation at 604 nm [4]. The kinetics of AMB oxidation was measured in 0.05 M acetate buffer solution pH 5.5 at 25 °C. The concentration of rPpL was 300 nM, rMtL – 75–80 nM, rCcL – 300 nM, rRsL – 100 nM. The concentration of polymers was 50 ppm by weight. This corresponded to 2.5  $\mu\text{M}$  of a polymer with the molecular weight 20 kDa.

The initial reaction rate was calculated by linear approximation of an absorbance change during 0–30 s. The dependence of the initial rate on AMB concentration was fitted by monosubstrate Michaelis-Menten equation since the AMB concentration in the experiments was less than the oxygen concentration. The calculated  $V_{\text{max}}$  and  $K_{\text{m}}$  were used for the apparent rate constants ( $k_{\text{cat}}$  and  $k_{\text{ox}} = k_{\text{cat}}/K_{\text{m}}$ ) calculations. In the case when an initial rate ( $V$ ) was measured at an AMB concentration less than  $K_{\text{m}}$ , the apparent bimolecular constant was calculated as  $k_{\text{ox}} = V/[E][\text{AMB}]$ .

## RESULTS AND DISCUSSION

Catalyzed by rPpL AMB oxidation rate started to saturate at 0.05 mM of AMB (Fig. 1). An apparent  $K_{\text{m}}$  was 81  $\mu\text{M}$  and  $k_{\text{ox}}$  was  $1.2 \mu\text{M}^{-1}\text{s}^{-1}$ . These values are similar to wild type PpL [5]. Addition of 50 ppm of DED decreased  $K_{\text{m}}$  as well as the maximal rate (Fig. 1). The calculated  $k_{\text{ox}}$ , however, decreased only by 6%.

Saturation of AMB oxidation rate catalyzed by rMtL started at a higher substrate concentration (Fig. 2). The calculated  $K_{\text{m}}$  was 1.3 mM and  $k_{\text{ox}}$   $41 \text{ mM}^{-1}\text{s}^{-1}$ . Addition 50 ppm of DED significantly

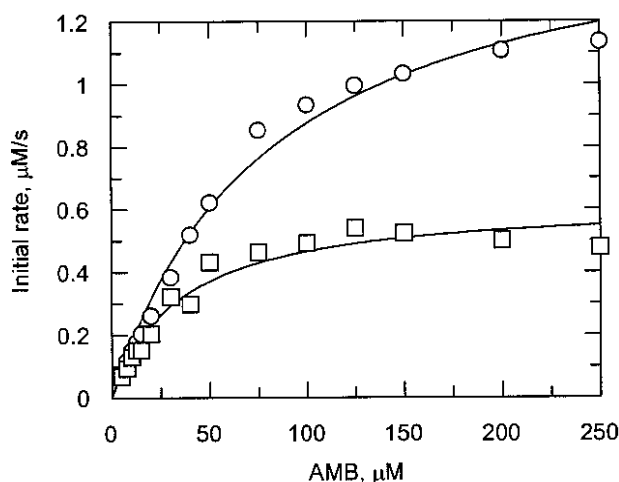


Fig. 1. Dependence of rPpL-catalyzed AMB oxidation on substrate concentration in absence (O) and presence (□) of 50 ppm of DED. 16 nM of rPpL

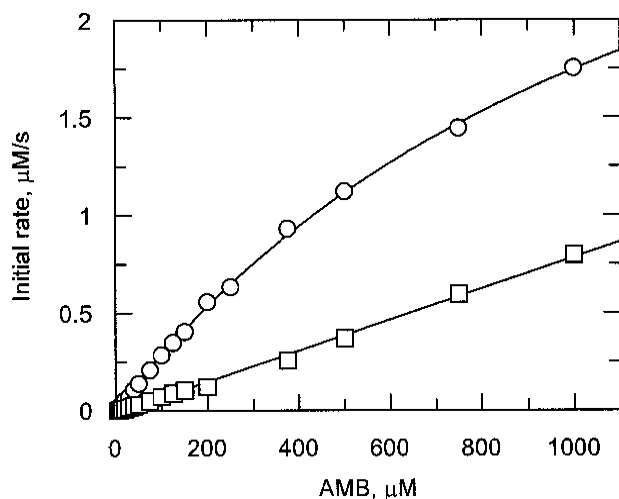


Fig. 2. Dependence of rMtL-catalyzed AMB oxidation on substrate concentration in absence (O) and presence (□) of 50 ppm of DED. 75 nM of rMtL

increased an apparent  $K_{\text{m}}$ , resulting in  $k_{\text{ox}}$  decrease 3.8 times.

The ratio of AMB oxidation constants measured at a fixed (50  $\mu\text{M}$ ) AMB concentration is depicted in Table. It is possible to notice that uncharged polymers such as HEC, PAA, PVP, PVA, and FIC practically didn't change the rPpL-catalyzed AMB oxidation rate. 50 ppm of PEG little decreased the rate.

Anionic polymers, *i.e.* HEP and BIP, didn't influence the rate, either. Among the cationic polymers, DEC didn't change the rate, but PLL and PEI inhibited the rate 2.5 and 4.1 times. DED, PLL, PEI inhibited the rMtL activity stronger than rPpL, but other polymers acted as rPpL (Table). In contrast to rPpL and rMtL, all polymers practically didn't

Table. Modulation of laccases activity with water-soluble polymers. Concentration of polymers 50 ppm, pH 5.5, 25 °C. Standard error of  $k_{rel}$  determination was 8–17%

Polymer	Laccase/ pI	$k_{rel}$	Laccase/ pI	$k_{rel}$	Laccase/ pI	$k_{rel}$	Laccase/ pI	$k_{rel}$
–	rPpL/3.5	1.0	rMtL/4.2	1.0	rCcL/3.8	1.0	rRsL/7.5	1.0
HEC	rPpL/3.5	1.0	rMtL/4.2	1.1	rCcL/3.8	1.0	rRsL/7.5	1.1
PAA	–	–	–	–	rCcL/3.8	1.0	rRsL/7.5	1.5
PVP	rPpL/3.5	1.0	rMtL/4.2	1.0	–	–	–	–
PVA	rPpL/3.5	1.0	rMtL/4.2	1.1	–	–	–	–
D500	–	–	–	–	rCcL/3.8	1.0	rRsL/7.5	1.3
FIC	rPpL/3.5	0.94	rMtL/4.2	0.9	rCcL/3.8	1.05	rRsL/7.5	1.7
PEG	rPpL/3.5	0.78	rMtL/4.2	1.0	rCcL/3.8	1.2	rRsL/7.5	1.4
NaA	–	–	–	–	rCcL/3.8	1.1	rRsL/7.5	1.4
HEP	rPpL/3.5	1.0	rMtL/4.2	1.0	–	–	–	–
BIP	rPpL/3.5	1.0	rMtL/4.2	1.0	rCcL/3.8	1.1	rRsL/7.5	1.8
DEC	rPpL/3.5	1.0	rMtL/4.2	1.0	–	–	–	–
DED	rPpL/3.5	0.9	rMtL/4.2	0.26	rCcL/3.8	1.0	rRsL/7.5	0.8
PRT	–	–	–	–	rCcL/3.8	1.0	rRsL/7.5	1.1
PLL	rPpL/3.5	0.40	rMtL/4.2	0.22	rCcL/3.8	0.9	rRsL/7.5	0.8
PEI	rPpL/3.5	0.25	rMtL/4.2	0.13	–	–	–	–

influence rCcL activity. The inhibition of rRsL activity with polymers was more complex. PAA, D500, FIC, PEG, NaA and BIP increased the rate on average by 61%. DED and PLL decreased the rate on average by 24%.

Calculated at 50  $\mu$ M of AMB,  $k_{ox}$ , in fact, is a not “true” bimolecular constant, but it represents the laccase reaction rate with AMB. The reason for  $k_{ox}$  change is a change of an apparent  $K_m$ . It decreased, if  $K_m$  increased, and *vice versa*. The decrease of  $k_{ox}$ , as in the case of rPpL and rMtL, indicates a competitive inhibition with PLL and PEI. It is possible to suggest that the mechanism of the inhibition is associated with enzyme/polymer complex formation. The formation of the complex retards substrate interaction with the enzyme. The isoelectric points (pI) of both laccases are 3.5 and 4.2. Therefore at pH 5.5 the enzymes carry total negative charge and the complex is formed by electrostatic interaction between cationic polymers and a negatively charged globule. Uncharged or negatively charged polymers do not form a complex. The effect of polymers on rCcL activity indicates that other factors than pI determine the complex formation. rCcL carries out some glucosylated residues [8]. The glucosylation may prevent polymer interaction with the enzyme globule, therefore all the polymers studied were inert (Table).

The isoelectric point of rRsL is 7.5. For this reason the action of polymers was quite different in comparison to the other laccases. Little activation and inhibition of laccase activity is possibly associat-

ed with formation of weak complexes not determined by electrostatic interaction.

Little influence of DEC on rPpL and rMtL as well as DED on rPpL activities need special discussion. In water PLL and PEI forms an open structure due to a strong electrostatic repulsion between the charges. DEC, in contrast, forms a compact structure due to hydrogen bondings of backbone and due to a relatively low content of charged groups. The compact structure possibly prevents the complex formation between the enzyme globule and the polymer. The stability of the compact structure of DED is less in comparison to DEC. As a consequence, it forms a complex with rMtL but not with rPpL, because the rMtL interaction with cationic polymers is stronger.

## ACKNOWLEDGEMENTS

Authors express sincere thanks to Palle Schneider (Novozymes A/S, Denmark) for laccases supply, isoelectric points determination and for permanent interest and useful discussions during this work.

## References

1. Arora DS, Gill PK. *Biores Tech* 2001; 77: 89–91.
2. Setti L, Giuliani S, Spinozzi G, Pifferi PG. *Enzyme Microb Technol* 1999; 25: 285–9.
3. Ullah MA, Bedford CT, Evans CS. *Appl Microbiol Biotechnol* 2000; 53: 230–4.
4. Krikstopaitis K, Kulys J, Pedersen AH, Schneider P. *Acta Chem Scand* 1998; 52: 469–74.

5. Berges JA, Virtanen C. *Comput Biol Med* 1993; 23: 131–41.
6. Xu F, Berka RM, Wahleithner JA, Nelson B A, Shuster JR, Brown SH, Palmer AE, Solomon EI. *Biochem J* 1998; 334: 63–70.
7. Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dalbuge H. *Appl Environ Microbiol* 1996; 62: 834–41.
8. Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ustergaard P, Schneider P, Yaver DS, Pedersen AH, Davies GJ. *Nat Struct Biol* 1998; 5: 310–6.

**J. Kulys, R. Vidžiūnaitė**

**LAKAZIŲ AKTYVUMO MODULIAVIMAS TIRPIAIS POLIMERAIS**

**S a n t r a u k a**

Tirtas rekombinantinių lakazių (benzendiol:deguoninė oksidoreduktazė, EC 1.10.3.2) aktyvumas esant tirpiems poli-

merams. Naudotos *Polyporus pinsitus* (rPpL), *Myceliophthora thermophila* (rMtL), *Coprinus cinereus* (rCcL) ir *Rhizoctonia solani* (rRsL) lakazės. Vandenyje tirpūs neutralūs polimerai ar polimerai, turintys liekanas su neigiamu ar teigiamu krūviu, tirti kaip modulatoriai. Lakazių aktyvumas buvo nustatomas naudojant N,N'-dimetilamino-4-(4-morfolino)benzeną (AMB), kai pH 5,5 ir 25 °C.

Gauti rezultatai rodo, kad 50 ppm polimero, turinčio neutralų ar neigiamą krūvį, nedaro įtakos tariamajai bimolekulinei AMB oksidacijos konstantai esant rPpL ar rMtL. Priešingai, teigiamą krūvį turinčios liekanos mažina konstantą 2,5–4,1 karto. Visų tipų polimerai praktiškai neveikia rCcL aktyvumo, tuo tarpu rRsL aktyvumas truputį padidėja ar sumažėja.

Koreliacija tarp inhibicijos ir lakazių izoelektrinių taškų reikšmių leidžia daryti prielaidą, kad lakazės sudaro kompleksus su teigiamą krūvį turinčiais polimerais, kurie ir inhibuoja fermento ir substrato sąveiką.