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Short communication

Exploitation of neglected horseradish peroxidase izoenzymes for dye decolorization

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ABSTRACT

Horseradish peroxidase (HRP) is enzyme first described more than 200 years ago and yet there are still some aspects of this potent enzyme to be tackled. Researchers were focused on most abundant isoenzyme HRP C1A while remaining, particularly anionic isoenzymes were discarded in purification process. This work describes exploitation of those isoenzymes for removal of recalcitrant pollutants such as reactive dyes. Results demonstrated that not only these enzymes can decolorize dyes but also in some cases anionic forms are more efficient than commercially produced cationic HRP form. Enzyme concentration of 0.14 U ml^{-1} was found to provide maximum dye removal at optimized reaction conditions with dye concentration of 30 mg l^{-1}. Majority of dyes tested were successfully decolorized at pH 5 or 7 while some dyes like Orange 2 and Reactive black 5 are decolorized most efficiently at pH 9. Anionic isoenzymes act by disrupting chromophore of Reactive black 5 while cationic HRP oxidize dye but leaves chromophore present.

Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7) in the presence of hydrogen peroxide can oxidize variety of organic and inorganic compounds. Being seasonal plant horseradish is rather expensive source for enzyme purification yielding up to 100 mg of HRP isoenzyme preparation from 1 kg of roots and hence there are attempts to produce recombinant HRP (Krainer et al., 2014). Despite high cost and due to high activity of HRP it remains indispensible component of vast array of diagnostic kits and research tools. There are many different enzyme isoforms present in the roots of plants, but most of the large scale production uses cationic isoforms (Regalado et al., 2004; Veitch, 2006). Characterization of different isoenzymes is desirable in order to choose one that fits the most for specific application (Krainer et al., 2014). Since anionic isoforms are discarded in purification process of main isoform HRP C1A, they have no commercial values and this opens a new perspective in treatment of colored wastewater. HRP C1A is the only HRP isoform with solved structure, while much less is known about remaining isoforms. It is well known that HRP can polymerize phenolic and bisphenolic compounds, as well as aromatic amines such as dyes (Nicell et al., 1993; Bhunia et al., 2002; Mohan et al., 2005). Previous studies showed promising capability of HRP in decolorization of different textile and non-textile dyes (de Souza et al., 2007; da Silva et al., 2010), but not much data is available on efficacy comparison of anionic and cationic isoforms in dye removal.

The objective of the present study was to compare anionic and cationic HRP isoforms in decolorization of different dyes depending on pH. Optimum conditions regarding concentration of hydrogen peroxide, dye and enzyme were tested in order to achieve best decolorization with minimal inputs.

Material and methods

Reagents

All reagents and solvents were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.

Fractionation of acidic and basic HRP isoforms

Horseradish roots were purchased from local market in village Grabovac near Belgrade (Serbia) directly from producers. Homogenized roots in distilled water were filtered through cheese cloth

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and stored at 4 °C overnight. Homogenate was released from ballast proteins by 40% ammonium sulphate saturation followed by precipitation at 80% saturation. Dissolved precipitate was dissolved in a minimum volume of buffer and desalted on Sepharose G25 Coarse column against 5 mM Na-phosphate buffer pH 7.1.

Desalted sample was fractioned on 60 mL Q-Sepharose FF XK 26/20 column equilibrated in 5 mM Na-phosphate buffer pH 7.1. Unbound fraction containing basic isoforms of HRP was washed with starting buffer and bound proteins containing acidic isoforms of HRP were eluted with 0.5 M NaCl in starting buffer. Collected fractions were assayed for peroxidase activity.

Horseradish peroxidase assay

HRP activity was determined using guaiacol as the substrate at 25 °C by measuring the initial rate of formation tetraguaiacol in the presence of hydrogen peroxide (Tijssen, 1985). The assay mixture contained 2.8 ml of phosphate buffer pH 7.0, 50 μl 0.02 M quaicol, 50 μl 0.008 M hydrogen peroxide and 100 μl HRP. One unit of HRP activity was defined as the amount which catalyzed the conversion of 1 μmol of guaiacol per minute in the assay conditions at 25 °C.

Isoelectric focusing and zymogram detection of HRP

Fractions collected after ion exchange chromatography were analyzed by isoelectric focusing. Isoelectric focusing was performed using Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology) according to manufacturer's instruction. Focusing was carried out on 7.5% acrylamide gel with ampholytes in a pH range 3.0–10.0, at 7 W constant power for 1.5 h at 10 °C. Zymogram detection of HRP was performed after the gel was washed twice (5 min each) with distilled water and then equilibrated with 100 mM phosphate buffer, pH 7.0 twice (each 5 min duration). Thereafter, the gel was dipped into 8.8 mM guaiacol solution in 100 mM phosphate buffer, pH 7.0 with 4.4 mM H_2O_2 and incubated at 25 °C for 10 min until the visualization of dark brown color bands indicating HRP activity.

Decolorization studies

The studied dyes (Table 1) were dissolved in distilled water giving a stock solution of 200 mg/L in water. A stock solution of each dye was diluted in buffer (0.1 M acetate buffer pH 3.0 and 5.0, 0.1 M phosphate buffer pH 7.0, 0.1 M bicarbonate buffer pH 9.0). Dye solution was scanned over a wavelength range 380–800 nm using UV–VIS spectrophotometer Shimadzu UV-1800. Characteristic wavelength maximum was determined for each dye tested and concentration that corresponds to absorbance at λ_{max} of 1.0 is given in Table 1.

One mL of each dye was treated with equal amount of acidic or basic HRP isoforms of final concentration 0.14 U/mL and in the presence of 0.44 mM H_2O_2. The reaction mixture was vortexed, and stored in a dark place at constant temperature 25 °C. After 1 h spectra were recorded in a wavelength range 380–800 nm by using Shimadzu UV-1800 spectrometer.

Concentration of substrate present in reaction mixture can be a very important parameter in dye removal, since decolorization by HRP is an enzyme-mediated process (Mohan et al., 2005; de Souza et al., 2007). Increasing dye concentrations of RG15 (0.06–1.0 mg ml^{-1}) were removed by the same concentration of acidic and basic HRP isoforms (0.14 U ml^{-1}) and H_2O_2 (0.44 mM) in 0.1 M acetate buffer pH 5.

HPLC analysis of dye degradation by HRP

Reversed-phase chromatography was performed using Dionex Ultimate 3000 with photodiode array detector (DAD) using Hypersil Gold C8 column (250 × 3mm, 5 μm) as described recently (Loncar et al., 2013). Acetate buffer pH 5 (0.1 M) as component A and acetonitrile as component B were used as mobile phase. The column was set at 25 °C and components were eluted at flow rate 0.5 ml min^{-1}. Injection volume was 10 μl. Gradient elution from 10 to 95%B in 9 min, and 95-10%B in 0.2 min. Run time was 25min.

Statistical analysis

All experimental results reported in the next sections were based on averaging results of repeated experimental runs (triplicates), with the SD ranging from 2 to 6% of the reported average. Statistical significance is confirmed by Student's t-test.

Table 1
Effect of pH on decolorization of dyes with anionic and cationic HRP isoforms. Studied dyes and their concentration in aqueous solutions with absorbance at λ_{max} of 1.0 as used in the experiments. λ_{max} were determined by recording UV/Vis spectra.

Dye	HRP isoform	% decolorization*				Final conc (mg l^{-1})	λ_{max}
		pH 3	pH 5	pH 7	pH 9		
Amido black 10b (AB)	Acidic	19 ± 1.2	92 ± 5.0	91 ± 4.4	88 ± 3.8	30	620
	Basic	12 ± 1.5	92 ± 4.5	93 ± 3.1	93 ± 4.6		
Comassie brillant blue R250 (CBB)	Acidic	25 ± 2.1	58 ± 3.1	68 ± 2.2	48 ± 3.5	45	550
	Basic	27 ± 1.5	85 ± 6.1	51 ± 4.3	46 ± 3.5		
Orange 2 (OR2)	Acidic	9 ± 1.1	15 ± 3.6	89 ± 3.5	98 ± 5.1	20	490
	Basic	5 ± 2.0	11 ± 2.5	18 ± 2.6	91 ± 5.0		
Ponceau S (PS)	Acidic	6 ± 2.5	3 ± 1.5	2 ± 1.0	76 ± 4.2	20	515
	Basic	4 ± 2.5	2 ± 1.0	2 ± 1.0	52 ± 3.7		
Procion dark blue (PDB)	Acidic	47 ± 3.1	98 ± 3.0	85 ± 3.5	14 ± 3.0	60	603
	Basic	47 ± 3.6	95 ± 4.6	11 ± 2.3	9 ± 1.3		
Reactive blue 52 (RB)	Acidic	65 ± 4.5	99 ± 3.9	99.6	63 ± 4.0	60	605
	Basic	73 ± 6.3	99 ± 4.5	99.6	86 ± 3.9		
Reactive green 15 (RG)	Acidic	26 ± 3.5	98 ± 3.7	39 ± 3.0	15 ± 2.0	80	625
	Basic	26 ± 2.5	78 ± 3.1	48 ± 4.2	20 ± 3.2		
Reactive black 5 (RB5)	Acidic	8 ± 3.2	6 ± 2.1	4 ± 1.6	74 ± 4.5	80	605
	Basic	5 ± 1.5	7 ± 3.3	4.5 ± 1.5	38 ± 3.6		
Realan blue (RLB)	Acidic	35 ± 3.3	92 ± 5.5	85 ± 5.0	18 ± 2.4	70	600
	Basic	35 ± 4.1	82 ± 3.4	43 ± 3.5	12 ± 1.6		
Remazol brilliant blue (RBBR)	Acidic	3 ± 1.1	91 ± 6.6	91 ± 4.0	13 ± 1.7	100	595
	Basic	10 ± 1.7	3 ± 1.0	9 ± 1.5	9 ± 1.2		

*Statistical significance is confirmed by Student's *t*-test.