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Violacein transformation by peroxidases and oxidases: implications on its biological properties

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Abstract

1,3-Dihydro-2*H*-indol-2-one derivative (violacein) is extracted from *Chromobacterium violaceum* and presents several biological activities as antibiotic, antitumoral, antichagasic and antioxidant. In order to increase its biological activities and decrease its toxicity, one strategy is to slightly transform the molecules through a specific group transformation. Peroxidases, which are hemoproteins, are known as excellent oxidants producing hydroxylation and ring cleavage. Laccases are phenol oxidases produced by fungi as plants and belong to the oxidase group which complexes copper. This enzyme generates phenolates, quinones and also ring cleavage even in the presence of a mediator as 1-hydroxybenzotriazol (HBT). Lignin peroxidase and manganese peroxidase (LiP/MnP) pool from *Phanerochaete chrysosporium*, Horseradish peroxidase (HRP-VI) from horseradish, Lactoperoxidase (LPO) from bovine milk and Laccase (Lac) from *Trametes versicolor* acting on violacein were studied. Kinetics parameters and products distribution indicated a fast and efficient violacein transformation with all of them. HRP-VI acting on violacein was studied in details and spectral evidence indicated that Horseradish peroxidase compound II was formed during the oxidation reaction. Similar behavior with LiP/MnP pool was observed. Laccase, in the presence and absence of mediator (HBT), also showed a rapid violacein transformation. In a more detailed study with the HRP-VI reaction, a hydroxilation in the indol unit and a ring contraction of the pyrrol moiety of violacein molecule occurred.

Keywords: Violacein; *Chromobacterium violaceum*; Transformation; Peroxidases; Oxidase

1. Introduction

Chromobacterium violaceum, a Gram negative bacteria belonging to the Rhizobiaceae family, is a saprophyte found in soil and water in tropical and subtropical areas. In most cases, it is a minor component of the total microflora [1]. Its colonies are lightly convex, not gelatinous, regular and violets, although irregular variants and non-pigmented colonies can also be found [2]. The pathogenic potential of *C. violaceum* was for the first time described in 1905 when the bacteria was identified as the responsible for the death of buffaloes in the Phillipines. In 1927, it was isolated from infections in humans, a case reported by Lesslar, and since then more than 30 further cases, most of them fatal, it has been reported in different countries [3].

Violacein (Fig. 1), the main pigment produced by this bacteria, is constituted by three structural units: 5-hydroxyindol, 2-oxoindol and 2-pyrrolidone. It has

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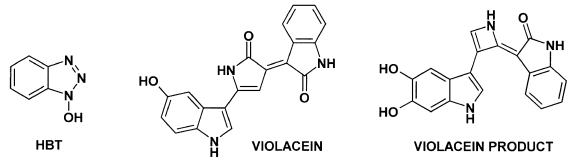

Fig. 1. Chemical structure of violacein, 1-hydroxybenzotriazole and 3-[1,2 dihydro-5(3,5-dihydroxy-1*H*-indol-3-yl)-2-azetine-4-ylidene]1,3 dihydro-2*H*-indol-2-one.

been demonstrated that all skeletal atoms were derived exclusively from L-triptophan, while the oxygen atoms are originated from aerial molecular oxygen [4]. In spite of that, its biosynthetic pathway is not totally clear, neither its physiologic role although some theories have been proposed for its elucidation [5,6]. Besides its antibiotic properties [7,8], violacein presents trypanocide [9,10], tumoricide [11], antiviral [12] and cytotoxic activities [13].

To increase the violacein biological activities and to reduce its toxicity, small alterations in the structure of the molecule were proposed by direct enzymatic treatment. The oxidative enzymes chosen for these transformations were: Horseradish peroxidase (HRP-VI), Lactoperoxidase (LPO), Laccase (Lac) and Lignin/Manganese peroxidase (LiP/MnP).

Peroxidases, hemoproteins with 35 to 100 kDa molecular weight, are known as excellent oxidants producing hydroxylation and ring cleavage of the substrate. The classical catalytic cycle of the peroxidases is [14]:

$$E + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + SH \rightarrow \text{Compound II} + S^{\bullet} \quad (2)$$

$$\text{Compound II} + SH \rightarrow E + S^{\bullet} + H_2O \quad (3)$$

HRP-VI is one of the less specific peroxidases and for that reason it catalyzes a great number of different reactions with several kinds of substrate. LiP, initially called as ligninase, was obtained for the first time from cultures of *Phanerochaete chrysosporium* [16,17]. This enzyme presents multiple forms that depends on the strain, culture conditions, age and separation type. LiP generates the oxidation of benzyl alcohols, lateral aromatic chains cleavage, demethylation, etc. [18]. MnP is an enzyme very similar to LiP and was also discovered in cultures of *P. chrysosporium* [19]. LPO, another peroxidase, can be found in the saliva, milk and tears of mammals. It is a glycoprotein with 78 kDa of molecular weight. Its intermediated metabolites behavior is very different from the other peroxidases [15,20]. Lac is a phenol oxidase that complexes copper. It differs from the peroxidases because it does not need hydrogen peroxide to become active. Lac range of primary substrates encompasses phenolic compounds, including lignin model dimers; these are oxidized, as in the case of peroxidases, by electron-abstraction mechanisms. Chlorinated phenols, guaiacols and cathecols are at least partially dechlorinated [21]. The capabilities of Lac can be greatly enhanced via the inclusion of suitable mediator compounds. In the presence of some primary substrates, such as ABTS (2,2′-azinobis-(3-etilbenzetiazoline)-6-sulfonato) or 1-hydroxybenzotriazole (HBT) (Fig. 1), Lac can catalyze the oxidation of non-phenolic compounds, including veratryl alcohol [22].

The aim of this work was to study violacein reaction with different enzymes, giving some special attention to the transformation with HRP-VI, by characterizing both the product chemical structure and its biological activities.

2. Results and discussion

Violacein treatment with the enzymes HRP-VI, LPO, LiP/MnP and Lac was monitored spectrophotometrically in visible range (200–700 nm) for an hour. The maximum absorption band of violacein in a buffer medium solution was at 558 nm (Fig. 2A). The initial violet solution changed to a pale yellow, indicating that a transformation took place with each enzyme.

The treatment using HRP-VI was initiated by adding an aliquot of HRP-VI into phosphate buffer solution at pH 7.6 containing 250 μmol l^{-1} of violacein. The enzyme showed a maximum absorption at 400 nm referring to Compound I (Fig. 2B). Hydrogen peroxide was added to the mixture cuvette and Compound II formation was observed by the change in the absorption wavelength to 414 nm (Fig. 2C).

HRP needs hydrogen peroxide to catalyze one-electron oxidation of chemicals to free radicals, thus

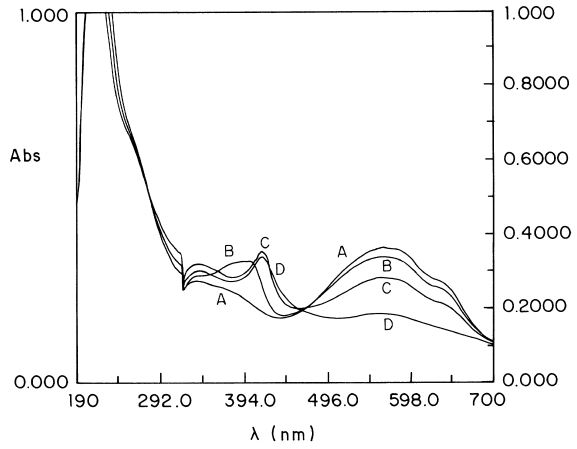

Fig. 2. UV–Vis spectrum of 250 μmol l^{-1} violacein in 50 mmol l^{-1} phosphate buffer (-A-), after 15 U/ml HRP-VI addition (-B-), after 4 μmol l^{-1} hydrogen peroxide addition (-C-), and after 30 min of reaction (-D-).

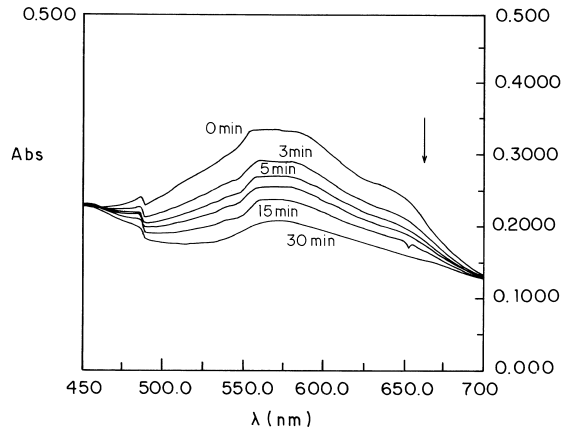

Fig. 3. Kinetics of the UV–Vis spectrum of violacein absorption decrease at 558 nm with HRP-VI (50 mmol l^{-1} phosphate buffer) treatment in variated intervals.

it oxidizes the heme enzyme by two electrons to Compound I, which is a ferryl (Fe(IV)) π-porphyrin cation radical of the enzyme. A chemical molecule can then be oxidized by one electron to a radical, and Compound I is reduced by one electron to Compound II. A subsequent oxidation of another chemical molecule by Compound II returns the peroxidase to its ferric resting state. The presence of much hydrogen peroxide or the slow reduction of Compound II to resting state may lead to the oxidation of Compound II to Compound III, an inactivated enzyme form [15].

The absorbance of violacein solution was continuously monitored with time as shown in Fig. 3. No further reaction was observed after 30 min at room temperature (around 24°C). Violacein absorbance intensity decreased almost 40% of its initial value. Control tests with HRP-VI being replaced by distilled water indicated negligible bleaching effect of H_2O_2 on violacein ($< 2\%$ absorbance change).

The reaction mixture was extracted with ethyl acetate and evaporated under reduced pressure. The product obtained was dissolved in ethanol and submitted under thin layer chromatography. Salkowsky test that consists of the identification of indolic compounds, was applied on the layer and the appearance of a yellow spot indicated a positive test meaning that the obtained product still presents an indolic moiety. The MS spectrometry fragmentation showed that the indol moiety exhibited 2 hydroxyl groups and the parent peak 331 m/z was detected (not showed). The structure of this compound was defined as 3-[1,2 dihydro-5(3,5-dihydroxy-1*H*-indol-3-yl)-2-azetine-4-ylidene]1,3 dihydro-2*H*-indol-2-one (Fig. 1) and was obtained in 40% crude yield.

The preliminary cytotoxicity test following the methodology of De Conti et al. [23] showed that the biotransformed violacein exhibited four folds less cytotoxicity in fibroblasts Chinese hamster V79 cell line at 20 μmol l^{-1} concentration (results not showed). The use of LiP/MnP pool acting on violacein was also studied but the reaction showed a lower conversion rate than with the use of HRP-VI (Table 1). The constant rate obtained was also too low. Compounds I and II of LiP and MnP act by

Table 1
Kinetics parameters obtained from violacein transformation with different enzymes

Enzyme	Kinetics parameters
HRP-VI	$k_R = 0.0377$, CR = 37.57%
LPO	$k_R = 0.1112$, CR = 37.27%
LiP/MnP	$k_R = 0.0045$, CR = 21.60%
Lac: HBT presence	$k_R = 0.0210$, CR = 24.20%
Lac: HBT absence	$k_R = 0.0164$, CR = 28.80%

k_R = Rate constant (min^{-1}).
CR (Conversion Rate) = Decrease in violacein absorption after 30 min.

abstracting a single electron from aromatic rings of the target compound, generating cation radicals which are then decomposed chemically. The substrate is not required to bind to the enzyme; instead oxidation occurs through simple electron transfer [24].

The reaction of violacein with LPO was faster than that of HRP-VI. An important feature that distinguishes LPO from other peroxidases is the behavior of the enzymatic intermediates. LPO reacts with hydrogen peroxide to give rise initially to Compound I, a ferryl porphyrin radical cation [CompI(IV,$P^{\bullet+}$)], that decays quickly to form species that contain an oxidizing equivalent on the polypeptide chain [CompI(IV,$R^{\bullet+}$)]. The latter compound reacts with ferrocyanide using the iron-oxo oxodizing equivalent (at acidic pH) or to the amino acid radical oxidizing equivalent (at neutral and basic pH) [15]. Since the heme structure and the characteristics of LPO intermediates differ from those of other peroxidases such as HRP, it was of some interest to investigate how these differences affected the reactivity of violacein. From the values given in Table 1, it is clear that the difference between the rate constants of these enzymes is due to individual mechanisms of action of the enzymatic intermediates, although the conversion percentage of the substrate did not vary significantly.

Violacein transformation by Lac was also observed in the presence of a mediator such as HBT (1-hydroxybenzotriazole). The rate constant obtained with the presence of HBT was higher than with the absence of the mediator but the conversion rate was less efficient (Table 1). A general mechanism of Lac acting on phenol substrates was proposed [24]:

$$2(\mathrm{Cu[II]})\text{-Laccase} + \mathrm{Phenol} \rightarrow 2(\mathrm{Cu[I]})\text{-Laccase} + \mathrm{Phenol}^{\bullet} + 2\mathrm{H}^{+}$$

$$2(\mathrm{Cu[I]})\text{-Laccase} + \mathrm{oxygen} + 2\mathrm{H}^{+} \rightarrow 2(\mathrm{Cu[II]})\text{-Laccase} + \mathrm{H_2O}$$

Lac reduces oxygen to water and simultaneously performs one-electron oxidation of many aromatic substrates. The substrate range of Lac can be extended to non-phenolic compounds by inclusion of a mediator such as HBT. There are several hypotheses to explain why a Lac mediator system can oxidize a non-phenolic compound: (1) Lac mediator is a redox mediator; (2) active intermediates are generated during the oxidation of the mediator by Lac; (3) the mediator acts as a co-substrate or activator; (4) active oxygen species such as hydroxyl free radicals ($HO^{\bullet}$) are generated during the reaction; besides all these hypotheses, the exact role of mediators is still not clear [25].

3. Experimental

3.1. Materials

The violacein was extracted from *C. violaceum* CCT 3496 [26]. The enzyme HRP-VI, LPO and the mediator HBT were obtained from Sigma. Hydrogen peroxide and veratryl alcohol were purchased from Aldrich.

3.2. Enzymes

LiP/MnP pool was obtained from the extracellular fluid of nutrient nitrogen limited cultures of *P. chrysosporium* ATCC 24725 and the crude Lac from the extracellular fluid of *Trametes versicolor* CCT 4521, with subsequent lyophilization.

3.3. Enzymes assays

The enzyme mixtures consisted of 15 U/ml (HRP-VI and LPO), 65 U/ml (LiP), 1,5 U/l (MnP), 2500 U/l (Lac), and 250 μmol l^{-1} violacein in a buffer medium. Reactions with the peroxidases were initiated by the addition of 4 μmol l^{-1} hydrogen peroxide. The total volume was 1000 μl. The transformation of violacein (in buffer solutions at differents pHs) was monitored at 558 nm every 5 min until 30 min of reaction. The linear coefficient obtained from the equation of a $\ln A_i - \ln A_f$ vs. time/min graph gave the rate value for each enzyme reaction.

3.4. Salkowsky test

A mixture of 3 ml of 1.5 mol l^{-1} aqueous iron (III) chloride solution with 100 ml of water and 60 ml of sulfuric acid was spread over the layers which were heated for 5 min at 60°C [27].

4. Conclusions

This work reports the transformation of violacein with direct use of peroxidases and oxidase. All the enzymes studied showed a significant reduction of the maximum absorbance wavelength of violacein and a change on the color solution reaction. Rate constants obtained showed the following order: LPO > HRP-VI > Lac (+HBT) > Lac > LiP/MnP and the conversion rate indicated that HRP-VI > LPO > Lac > Lac (+HBT) > LiP/MnP. The positive Salkowsky test for the product obtained from the reaction of violacein with HRP-VI, determined a hydroxylation at the indolic group and a ring contraction in the pyrrol group occurred to the violacein molecule. The biotransformed violacein appeared to be less cytotoxic than violacein itself, indicating a decrease in its antitumoral activity.

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