

Communication

# Kinetic Study of Peroxidase-Catalyzed Oxidation of 2-Hydroxyanthracene and 9-Phenanthrol in Presence of Biosurfactant Escin

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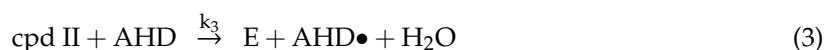
**Abstract:** The kinetics of fungal peroxidase-catalyzed 2-hydroxyanthracene and 9-phenanthrol oxidation was investigated in presence of biosurfactant escin at pH 5.5 and 25 °C. The kinetic measurements were performed using the fluorimetric method and the critical micelle concentration (CMC) of escin was determined using the dynamic light scattering technique. Inactivation of peroxidase was observed in absence of biosurfactant escin. It was shown that escin, used in concentrations lower than CMC, decreases or completely stops the peroxidase inactivation and increases the conversion of 2-hydroxyanthracene as well as of 9-phenanthrol. The environmentally friendly method of peroxidase-catalyzed 2-hydroxyanthracene and 9-phenanthrol oxidation in presence of biosurfactant Escin has an advantage over traditional decontamination methods due to their less environmental impact.

**Keywords:** 2-hydroxyanthracene; 9-phenanthrol; biocatalytic oxidation; biosurfactant; peroxidase

## 1. Introduction

Aromatic hydroxyl derivatives (AHDs) are treated as pollutants that are produced and released into the environment by various industrial processes, and effective methods of their decontamination are very important [1,2]. Bio-utilization methods are usually not very expensive and environmentally friendly. Pollutants can be degraded with the help of enzymes, synthesized by bacteria, fungi or plants [3–5]. Peroxidases and other phenol-oxidizing enzymes have great potential to decrease environmental pollution by bioremediation of waste water containing a broad spectrum of aromatic hydroxyl derivatives [5]. Progress in genetic engineering also provides possibilities for application of recombinant peroxidases that demonstrate an elevated stability compared with native peroxidases [6].

Peroxidases undergoes a cyclic reaction when reacting with AHDs. This sequence is summarized in the following reactions [7]:



Native peroxidase (E) is oxidized by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) with compound I (cpd I) formation. The cpd I in its turn oxidizes AHDs (AHD) via the formation of radical (AHD•) and compound II (cpd II) (reactions 1–3). The resulting radicals can react chemically, forming oligomers (Olig, reaction 4) that are subject to further oxidation by peroxidase to constitute higher polymers until the solubility

limit is reached. Unfortunately, biocatalytic oxidation of AHDs has some limitation, i.e., inactivation of enzymes during the reaction. It was proved that peroxidases and other phenol-oxidizing enzymes are inactivated by the polymer product formed during the reaction due to formation of polymer-peroxidase conjugates [8–10]. In an attempt to decrease the inhibition rate, some additives were used, i.e., non-ionic polymeric compounds and surfactants [8,11,12]. Synthetic surfactants such as SDS, CTAB and Triton X-100 are very poisonous to aquatic organisms, and they can influence the aquatic environment by causing long-term adverse effects [13,14]. Recently, biosurfactants have received a lot of attention due to biodegradability, low toxicity and safety [15].

The task of this investigation was to explore recombinant *Coprinus cinereus* peroxidase-catalyzed oxidation of 2-hydroxyanthracene and 9-phenanthrol in the presence of environmentally friendly biosurfactant escin (Figure 1).

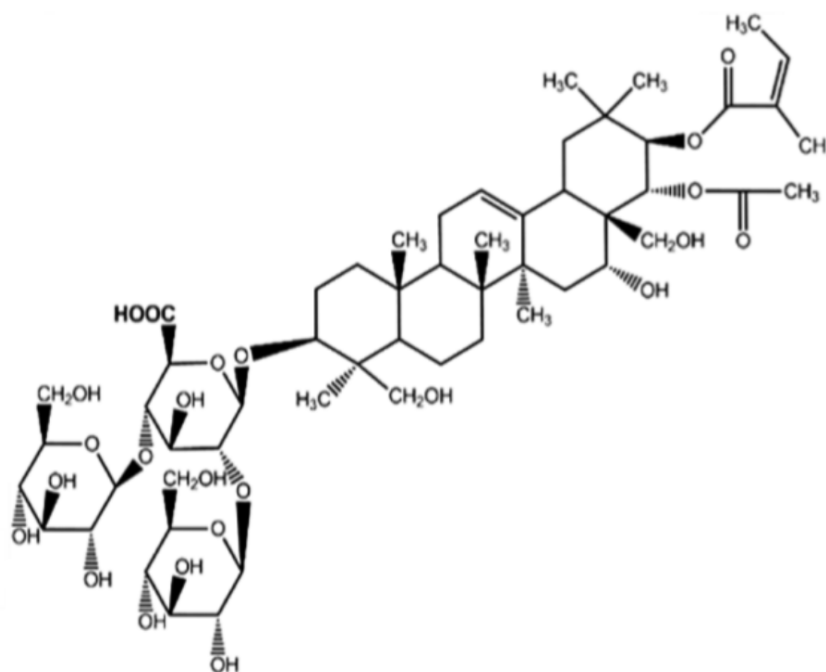


Figure 1. Chemical structure of Escin [16].

2-hydroxyanthracene and 9-phenanthrol (also called 9-hydroxyphenanthrene) are tricyclic aromatic hydrocarbons with hydroxyl group compounds, that being considered persistent, bioaccumulative and toxic for freshwater and marine ecosystems [17,18]. Escin is the main natural constituent of the saponin extract from the seeds of *Aesculus hippocastanum* (known also as the horse chestnut tree) [19]. Horse chestnut seed extract, containing around 20 wt % escin, is widely used in medicine for its venotonic effect, vascular protection and free radical scavenging properties [20]. The experiments of other scientists show that escin is obtained from crude extracts of aesculus plants with high efficiency and it is not relatively expensive [21]; it is also sold as a market product.

## 2. Results and Discussion

### 2.1. Kinetics of Peroxidase-Catalyzed 2-Hydroxyanthracene and 9-Phenanthrol Oxidation in the Absence and Presence of Biosurfactant Escin

The oxidations of 2-hydroxyanthracene and 9-phenanthrol were not complete in the absence of biosurfactants (Figure 2a,b curves 1). During oxidation of these compounds, kinetic curves have saturated. The fluorescence is not noticeable in enzymes, hydrogen peroxide and the products of the reaction. So fluorescence intensity is minimized only due to 2-hydroxyanthracene or 9-phenanthrol concentration decrease. The addition of a new portion of hydrogen peroxide did not affect the reaction.

On the other hand, the addition of a new portion of enzyme has caused further 2-hydroxyanthracene and 9-phenanthrol oxidation. This proves the peroxidase inactivation during the reaction. Other curves on Figure 2a,b (curves 2–6) show the kinetics of peroxidase-catalyzed 2-hydroxyanthracene (a) and 9-phenanthrol (b) oxidation in presence of biosurfactant escin. With small quantities (up to  $1 \mu\text{mol L}^{-1}$ ) of biosurfactant, the conversion of both pollutants has risen up gradually. In presence of  $2 \mu\text{mol L}^{-1}$ – $0.2 \text{ mmol L}^{-1}$  concentration of escin the conversion of both substrates was fully completed, so the enzyme inhibition was stopped. Also, escin (up to  $0.2 \text{ mmol L}^{-1}$ ) did not impact the initial oxidation rate of both pollutants. When the concentration of escin exceeded  $0.2 \text{ mmol L}^{-1}$ , the initial rate of 2-hydroxyanthracene and 9-phenanthrol oxidation gradually decreased as well as the degree of both AHDs' conversion.

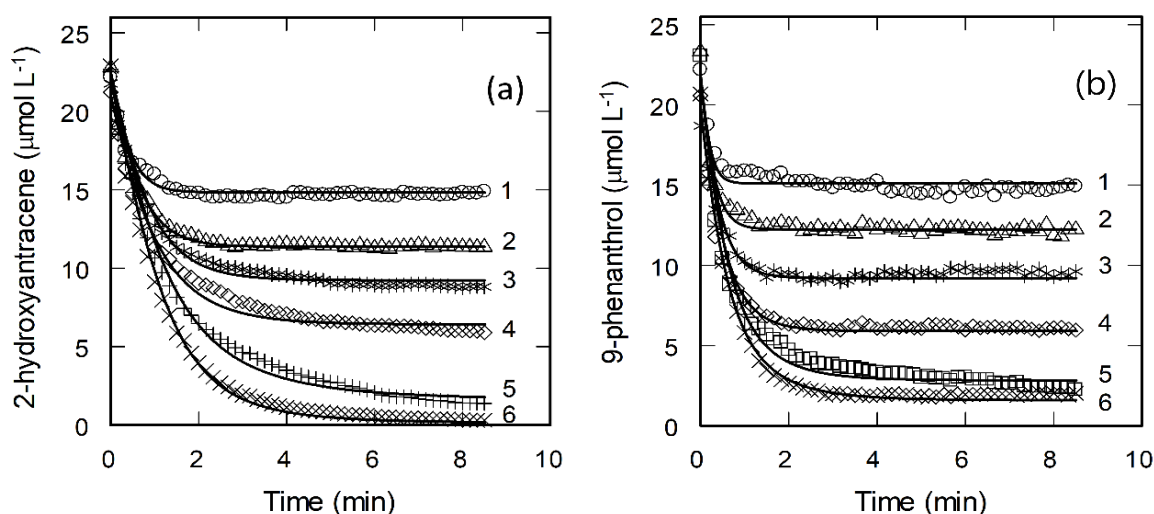


**Figure 2.** Kinetics of peroxidase-catalyzed 2-hydroxyanthracene (a) and 9-phenanthrol (b) oxidation in presence of Escin. The reaction mixture contained  $25 \mu\text{mol L}^{-1}$  2-hydroxyanthracene (a) or  $25 \mu\text{mol L}^{-1}$  9-phenanthrol (b),  $1 \text{ nmol L}^{-1}$  rCiP,  $100 \mu\text{mol L}^{-1}$   $\text{H}_2\text{O}_2$ , Escin:  $0 \mu\text{mol L}^{-1}$  (1)  $0.2 \mu\text{mol L}^{-1}$  (2)  $0.5 \mu\text{mol L}^{-1}$  (3)  $0.7 \mu\text{mol L}^{-1}$  (4)  $1 \mu\text{mol L}^{-1}$  (5)  $2 \mu\text{mol L}^{-1}$ – $0.2 \text{ mmol L}^{-1}$  (6) in  $50 \text{ mmol L}^{-1}$  acetate buffer pH 5.5,  $25^\circ\text{C}$ . Curves marked by signs represent experimental data; solid curves were drawn following a model (Reactions (1)–(6)).

## 2.2. Enhancing Effect of Escin Monomers on Biocatalytic Oxidation of 2-Hydroxyanthracene and 9-Phenanthrol

Escin is the amphiphilic molecule. At low concentration, it forms as monomers. At high concentration, it may form a micelle. The concentration of the biosurfactant above which micelles are formed is described as the critical micelle concentration (CMC, Figure 3).

The CMC of escin at  $25^\circ\text{C}$  in  $50 \text{ mmol L}^{-1}$  sodium acetate buffer (pH 5.5) employing the dynamic light scattering method was determined. Figure 3 presents the variation of the hydrodynamic diameter with the escin concentration at  $25^\circ\text{C}$ . It shows that the CMC of escin was  $0.2 \text{ mmol L}^{-1}$  in the sodium acetate buffer. As shown in Figure 2, the conversion of 2-hydroxyanthracene and 9-phenanthrol was high in the escin concentration range  $2 \mu\text{mol L}^{-1}$ – $0.2 \text{ mmol L}^{-1}$ . Within this concentration range, escin exists as a monomer. This shows that the monomer receives a better augmenting effect on the conversion of 2-hydroxyanthracene and 9-phenanthrol than micelle.

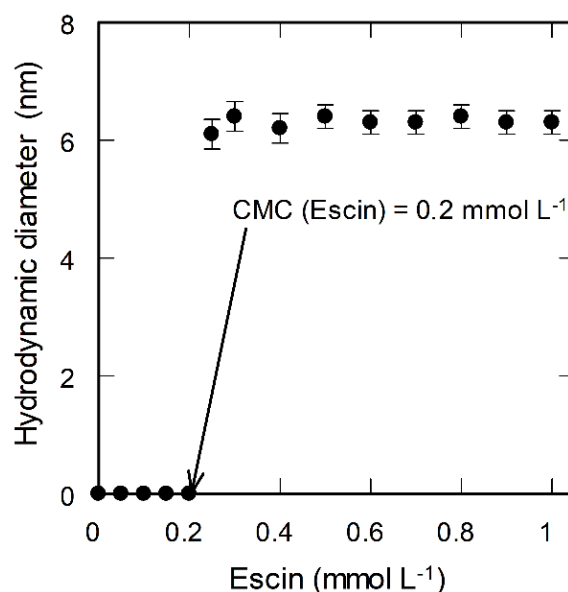


**Figure 3.** Dependence of hydrodynamic diameter on various concentrations of Escin prepared in 50 mmol L<sup>-1</sup> acetate buffer solution pH 5.5, 25 °C with defined CMC. Vertical lines with cap indicate error bars.

### 2.3. Mechanism of Escin Action

The saturation reaction rate during 2-hydroxyanthracene and 9-phenanthrol oxidation indicates that enzyme is inhibited during the reaction (Figure 2a,b curves 1). The prevention of inhibition by addition of biosurfactant escin indicates that the inhibition proceeds by enzyme interaction with oxidation products. The results are accounted for by a scheme, which contains a stage of enzyme inhibition by oligomers (Reactions (1)–(6)), using KinFitSim program. The second order constant of hydrogen peroxide reaction with the native enzyme (Reaction (1)) was taken as  $k_1 = 7.1 \times 10^6 \text{ mol}^{-1} \text{ L s}^{-1}$  at 25 °C [22]. The fitting of data gave constants of 2-hydroxyanthracene or 9-phenanthrol reactivity with the ferryl compounds of rCiP (with compound I ( $k_2$ ) and with compound II ( $k_3$ )), which were similar at different biosurfactant concentrations (Table 1).

**Table 1.** Kinetic parameters of biocatalytic oxidation of 2-hydroxyanthracene and 9-phenanthrol in presence of escin, when concentrations of biosurfactant are less than CMC, in 50 mmol L<sup>-1</sup> acetate buffer pH 5.5, 25 °C.

| AHDs                | Escin, $\mu\text{mol L}^{-1}$                        | $k_2, \text{mol}^{-1} \text{ L s}^{-1}$ | $k_3, \text{mol}^{-1} \text{ L s}^{-1}$ | $k_{in}, \text{mol}^{-1} \text{ L s}^{-1}$ |
|---------------------|------------------------------------------------------|-----------------------------------------|-----------------------------------------|--------------------------------------------|
| 2-hydroxyanthracene | 0                                                    | $(1.0 \pm 0.3) \times 10^8$             | $(1.0 \pm 0.2) \times 10^8$             | $(2.2 \pm 0.1) \times 10^4$                |
| 2-hydroxyanthracene | 0.2                                                  | $(1.0 \pm 0.2) \times 10^8$             | $(1.0 \pm 0.3) \times 10^8$             | $(9.5 \pm 0.3) \times 10^3$                |
| 2-hydroxyanthracene | 0.5                                                  | $(1.0 \pm 0.3) \times 10^8$             | $(1.0 \pm 0.2) \times 10^8$             | $(5.3 \pm 0.2) \times 10^3$                |
| 2-hydroxyanthracene | 0.7                                                  | $(9.2 \pm 0.5) \times 10^7$             | $(9.0 \pm 0.5) \times 10^7$             | $(4.2 \pm 0.2) \times 10^3$                |
| 2-hydroxyanthracene | 1                                                    | $(9.0 \pm 0.6) \times 10^7$             | $(9.5 \pm 0.4) \times 10^7$             | $(1.6 \pm 0.3) \times 10^3$                |
| 2-hydroxyanthracene | $2 \mu\text{mol L}^{-1}$ – $0.2 \text{ mmol L}^{-1}$ | $(1.0 \pm 0.2) \times 10^8$             | $(1.0 \pm 0.2) \times 10^8$             | $(9.0 \pm 0.2) \times 10^2$                |
| 9-phenanthrol       | 0                                                    | $(3.0 \pm 0.1) \times 10^7$             | $(3.0 \pm 0.1) \times 10^7$             | $(5.0 \pm 0.2) \times 10^4$                |
| 9-phenanthrol       | 0.2                                                  | $(3.0 \pm 0.1) \times 10^7$             | $(3.1 \pm 0.1) \times 10^7$             | $(3.3 \pm 0.4) \times 10^4$                |
| 9-phenanthrol       | 0.5                                                  | $(3.0 \pm 0.1) \times 10^7$             | $(3.0 \pm 0.1) \times 10^7$             | $(1.5 \pm 0.3) \times 10^4$                |
| 9-phenanthrol       | 0.7                                                  | $(3.2 \pm 0.2) \times 10^7$             | $(3.3 \pm 0.2) \times 10^7$             | $(7.4 \pm 0.2) \times 10^3$                |
| 9-phenanthrol       | 1                                                    | $(3.0 \pm 0.1) \times 10^7$             | $(3.0 \pm 0.2) \times 10^7$             | $(3.3 \pm 0.1) \times 10^3$                |
| 9-phenanthrol       | $2 \mu\text{mol L}^{-1}$ – $0.2 \text{ mmol L}^{-1}$ | $(3.1 \pm 0.2) \times 10^7$             | $(3.0 \pm 0.1) \times 10^7$             | $(1.5 \pm 0.1) \times 10^3$                |

On the terms of 2-hydroxyanthracene, a mean value of  $k_2$  was  $(9.7 \pm 0.4) \times 10^7 \text{ mol}^{-1} \text{ L s}^{-1}$  and  $k_3$  was  $(9.8 \pm 0.3) \times 10^7 \text{ mol}^{-1} \text{ L s}^{-1}$  if escin concentrations had varied between 0 and 0.2 mmol L<sup>-1</sup> (Table 1). In the event of 9-phenanthrol, a mean value of  $k_2$  as well as  $k_3$  was  $(3.1 \pm 0.1) \times 10^7 \text{ mol}^{-1} \text{ L s}^{-1}$

if escin concentrations had varied between 0 and 0.2 mmol L<sup>-1</sup> (Table 1). The results show that  $k_2$  and  $k_3$  values did not change at different biosurfactant concentrations for both derivatives. This means that biosurfactant escin does not interact with peroxidase. The calculated inhibition constant  $k_{in}$  value has decreased for 2-hydroxyanthracene and 9-phenanthrol if biosurfactant escin concentration was increased (Table 1). The decline of  $k_{in}$  when biosurfactant concentration was increased could be illustrated by reversible oligomeric aromatic hydroxyl derivatives oxidation products interaction with biosurfactant escin.

Demonstration of the mechanism of peroxidase inactivation by the molecular clothing of active center by oligomeric derivatives has been submitted previously [10]. Docking and molecular dynamics computations have indicated that oligomeric AHDs have interacted with the enzyme better than the substrates. Opposed to the substrate, the binding of oligomeric derivatives did not form effective complexes, and closed the active center. With small quantities of biosurfactant (less than critical micelle concentrations), the enzyme inhibition was prevented. This may be defined by the fact that monomers of biosurfactant connect with oligomeric compounds, which show strict hydrophobicity [12] and interact with biosurfactant's monomers.

### 3. Materials and Methods

#### 3.1. Materials

Recombinant peroxidase from fungus *Coprinus cinereus* (rCiP) was obtained from Novozymes A/S (Denmark). 2-hydroxyanthracene was obtained from Santa Cruz Biotechnology (Canada) and 9-phenanthrol was obtained from Sigma-Aldrich (USA). Hydrogen peroxide (30% H<sub>2</sub>O<sub>2</sub>) was received from Polskie Odczynniki Chemiczne S.A. (Poland). Sodium acetate was purchased from Chempur (Poland). Biosurfactant escin was received from AppliChem (Germany). The rCiP and H<sub>2</sub>O<sub>2</sub> solutions were prepared in deionized aqua and concentrations of these materials were measured spectrophotometrically by using Nicolet evolution 300 spectrophotometer (Thermo Electron Corporation, USA). For rCiP and H<sub>2</sub>O<sub>2</sub>, the extinction coefficients are  $\lambda_{\max}$  (H<sub>2</sub>O)/nm 405 ( $\epsilon/\text{dm}^3 \text{ mmol}^{-1} \text{ cm}^{-1}$  109) [22] and  $\lambda_{\max}$  (H<sub>2</sub>O)/nm 240 ( $\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  39.4) [23], respectively. 2-hydroxyanthracene and 9-phenanthrol were weighted and dissolved in methanol; the final concentration of methanol in the solutions for kinetic measurements was 2% (v/v). The escin solutions were dissolved in 50 mmol L<sup>-1</sup> acetate buffer pH 5.5.

#### 3.2. CMC Determination

The CMC of biosurfactants in 50 mmol L<sup>-1</sup> sodium acetate buffer at pH 5.5 and 25 ± 0.1 °C was determined by using a Zetasizer Nano-ZS analyzer (Malvern Instruments, UK). This gadget contains a 4 mW He-Ne laser operating at a wavelength of 633 nm and an avalanche photodiode detector. The scattered light is analyzed at an angle of 173° and optics arrangement maximizes the detection of scattered light while maintaining signal quality. For dynamic light scattering measurements, buffer solution was filtered (pore size = 200 nm). The escin concentration in the solutions was ranged to 0.2 mmol L<sup>-1</sup> with an step of 0.02 mmol L<sup>-1</sup> and up to 1 mmol L<sup>-1</sup> with an step of 0.1 mmol L<sup>-1</sup>. All prepared solutions were left to equilibrate for 15 to 20 min then they were analyzed.

#### 3.3. Kinetic Analysis

The kinetic analysis was done by using a Aminco Bowman luminescence spectrometer (Thermo Electron Corporation, USA). The fluorescence of 2-hydroxyanthracene was determined at 526 nm and at excitation 333 nm. The fluorescence of 9-phenanthrol was determined at 470 nm and at excitation 340 nm. The kinetic analysis was done in 50 mmol L<sup>-1</sup> sodium acetate buffer solution at pH 5.5 and 25 °C and the reaction mixture contained 25  $\mu\text{mol L}^{-1}$  2-hydroxyanthracene or 9-phenanthrol, 0.3–1 mmol L<sup>-1</sup> rCiP, 100  $\mu\text{mol L}^{-1}$  H<sub>2</sub>O<sub>2</sub>, 0–1 mmol L<sup>-1</sup> of escin. The reactions began when adding the

enzyme solution. All the experiments were carried out 3 times. Out of 3 kinetic curves the best curve was chosen and it was marked using signs in Figure 2.

### 3.4. Mathematical Calculations

The fluorescence 2-hydroxyanthracene and 9-phenanthrol was normalized to the initial concentrations. Computer programs Grafit 7 and Mathcad 15 were employed for data processing. The kinetics of the process were interpreted according to the Dunford scheme (reactions 1–4), adding the steps 5 and 6 corresponding to the enzyme inhibition by oligomers [12]:



According to the scheme (reactions 1–6), all the best kinetic curves (Figure 2) were fitted 3 times and kinetic parameters ( $k_2$ ,  $k_3$ ,  $k_{\text{in}}$ ) were calculated 3 times also using the KinFitSim 2.1 program [24]. The fitting of data gave chi-square value ( $\chi^2$ ). According to the best  $\chi^2$  value, the solid lines were marked in the Figure 2. The mean values of kinetic parameters and standard errors were marked in Table 1.

## 4. Conclusions

The performed investigations of biocatalytic oxidation of 2-hydroxyanthracene and 9-phenanthrol in presence of escin can be used for further intensification of peroxidase-catalysed biotechnological processes that can be applied while developing environmentally friendly decontamination methods. It was shown that the biosurfactant escin, used with concentrations lower than the CMC, decreased or completely stopped the peroxidase inactivation and increased the conversion of 2-hydroxyanthracene as well as of 9-phenanthrol. Environmentally friendly methods has an advantage over traditional decontamination methods due to the lesser environmental impact.

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**Conflicts of Interest:** The author declares no conflict of interest.

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