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Laccase-mediated oxyfunctionalization of 3β-hydroxy-Δ^5-steroids

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ABSTRACT

Laccase mediator systems (LMS) were studied as catalysts for steroid oxidation. The fungal laccases from *Lentinus strigosus* 1566 and *Trametes versicolor* were used in the work. Among five mediators screened, 1-hydroxybenzotriasol (HBT) excelled in activity. The LMS effectively catalyzed oxidation of 3β-hydroxy-Δ^5-steroids like DHEA (3β-hydroxyandrost-5-en-17-one) and pregnenolone (3β-hydroxypregn-5-en-20-one), while no activity was observed towards 3-oxo-4-ene-steroids (androstenedione, 9α-hydroxyandrostenedione, testosterone and 20-hydroxymethylpregn-4-en-3-one). The pathway of DHEA oxidation by LMS included the hydroxylation at positions 7α and 7β followed by oxidation of the corresponding 7(α/β)-alcohols to form 3β-hydroxyandrost-5-ene-7,17-dione. Regiospecific oxidation of allylic hydroxyl functions by LMS was confirmed using 3β,7α- and 3β,7β-dihydroxyandrost-5-en-17-ones as substrates. 3β-Hydroxypregn-5-ene-7,20-dione was produced with LMS as an only product from pregnenolone. The yield of crystalline product reached 58.3% yield with a purity of 96%.

The results demonstrate that application of LMS may be a promising approach for steroid oxyfunctionalization.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, E.C.1.10.3.2) are copper-containing oxidoreductases that specifically catalyze the oxidation of a wide range of organic substrates including phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindoles, benzenethiols and inorganic/organic metal compounds, to the corresponding radicals, using molecular oxygen as the final electron acceptor [1–4]. The reactive radical species rapidly react further to various oxidation products forming water as an only by-product. The broad substrate specificity makes laccases interesting "eco-friendly" enzymes with high industrial potential [2]. Laccase applications in biotechnology, e.g., for delignification and bleaching of paper pulp, detoxification and decolorization of textile dyes, bioremediation, detoxification of wastewaters, chemical grafting, polymer surface modifications and others, had been reviewed several times [3,5–7].

Laccase activity has been found in fungi, plants, bacteria, and insects and its significant role was evidenced for synthetic and mineralization processes in nature [3]. Fungal laccases are of particular commercial interest due to their higher redox potential as compared with other enzyme forms isolated from prokaryotes, insects or plants [8]. The main laccase producers are lignolytic basidiomycetes—white-rot fungi, which secrete the enzymes extracellularly, thus providing further simple enzyme purification [6].

Steroids are terpenoid lipids of specific structure that contain a gonane nucleus of four cycloalkane rings. These compounds fulfill essential physiological functions and are widespread in all living systems. Steroid preparations are widely used in different fields of medicine, veterinary, and agrochemistry. Bioconversion is a powerful tool for the production of valuable steroids compounds [9].

The data on laccase action on steroids mainly concern the dimerization, or polymerization of phenolic steroid hormones, such as estradiol, estrone, ethynilestradiol, and their derivatives [10–13]. These reactions can be exploited for the removal of estrogens, which are the endocrine disrupting steroids, from the wastewaters [14,15]. The information on laccase activity towards the non-phenolic steroids is scarce [16,17].

Mechanism of laccase action on phenolic compounds, including estrogens, is often based on the formation of the corresponding phenoxy radicals followed by further condensation. In the case of non-phenolic steroid substrates, the redox potential proves to be

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insufficient for the formation of steroid radicals [16]. It is known that the substrate range of laccases can be expanded to the compounds with a higher redox potential than the enzymes themselves in the presence of small natural (e.g., syringaldehyde or coumaric acid which can be found in the lignocellulosic biomass), or artificial molecules, which are capable to act as electron transfer mediators. Due to the specific mediators, laccases became able to enlarge the substrate spectrum and oxidize non-phenolic compounds. The application of the so-called Laccase Mediator Systems (LMS) for the oxidation of different substrates (e.g., alcohols, sugars, esters, alkenes, amides etc.) had been reviewed [2,4].

LMS composed of *Pycnoporus cinnabarinus* laccase and 1-hydroxybenzotriazole (HBT) as a mediator was applied for the oxidation of the model lipids representative for main paper pulp lipophilic extractives [16]. The unsaturated lipids including phytosterols were shown to be oxidized by the LMS to the corresponding epoxy- and hydroxy fatty acids from fatty acids, and free and esterified 7-ketosterols and steroid ketones from sterols and sterol esters. The treatment of eucalypt kraft pulp by LMS composed of laccase and HBT resulted in the decrease of pulp sterol content with an increase of 7-oxo-sitosterol and stigmasta-3,5-dien-7-one [17]. Detection of 7-ketosterols among the products of phytosterol oxidation indicated the possibility of LMS to catalyze the introduction of an oxygen function at the allylic position of 3β-hydroxy-Δ^5-steroids.

Allylic moiety oxidation of Δ^5-steroids can provide obtainment of valuable 7-keto derivatives with diverse biological properties [18]. Metallic catalysis in combination with hydroperoxides is often used for the reaction. For instance, the allylic oxidation by cobalt (II) alkyl phosphonate modified silica and tert-butyl hydroperoxide resulted in good product yields [19]. The catalyst based on dirhodium caprolactamate was highly effective at the hydroperoxide oxidation of Δ^5-steroids [20]. However, chemical catalysis may not provide regioselectivity of the reaction: e.g., undesirable oxidation at C4 was observed at the application of selenic dioxide [21]. Moreover, hazardous reagents such as chrome trioxide [22], or pyridinium chlorochromate [23] are used as oxidants in stoichiometric, or even higher amounts. Noteworthy, chemical protection of the 3β-hydroxyl function by esterification is required for the preservation of the Δ^5-double bond in steroid molecule at the allylic peroxide oxidation by chemical catalysis. Of importance are new simple methods which exclude the necessity of 3β-hydroxyl function protection, allow reaction performance under mild conditions in aqueous media, and provide effective environmentally friendly synthesis of the valuable steroids.

The specific oxidation of allylic hydroxyls represents another relevant problem [24,25]. Allylic steroid alcohols may be oxidized chemically to the corresponding aldehydes and ketones using different co-oxidant systems in the presence of saturated alcohols. Bioconversion could provide alternative promising approach.

Herein, we studied the possibility of regioselective oxygen functionalization of 3β-hydroxy-Δ^5-steroids and oxidation of the corresponding allylic 7-alcohols using LMS oxidation.

2. Experimental

2.1. Substrates and reagents

Sinapic acid, acetosyringone, syringaldehyde, 1-hydroxybenzotriazole (HBT), N-hydroxyacetanilide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS], (2,2,6,6-tetramethylpiperidin-1-yl) oxyl [TEMPO], 3β-hydroxypregn-5-en-20-one (pregnenolone), 3β-hydroxyandrost-5-ene-17-one (dehydroepiandrosterone, DHEA), 17β-hydroxyandrost-4-ene-3-one (testosterone), 2,6-dimethylphenol, Tween-80, Tween-20 and *Trametes versicolor* laccase were purchased from Sigma–Aldrich Co. (USA). 9α-Hydroxyandrost-4-ene-3-one (9α-OH-AD, purity—98%, m.p.—217–220 °C, [E, 240 nm (0.01% in ethyl alcohol)—14780 M^{-1} cm^{-1}], 20-hydroxymethylpregn-4-en-3-one (purity—98%, m.p. 181–183 C; [E, 240 nm (0.01% in methyl alcohol)—16100 M^{-1} cm^{-1}] were obtained from G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms (RAS, Russia). 3β,7α-Dihydroxyandrost-5-ene-17-one (7α-OH-DHEA) and 3β,7β-dihydroxyandrost-5-ene-17-one (7β-OH-DHEA), 3β-hydroxyandrost-5-ene-7,17-dione (7-keto-DHEA), 3β-hydroxypregn-5-ene-7,20-dione (7-keto-pregnenolone) were provided by Steraloids (USA). Other materials and solvents were of analytical grade and purchased from domestic companies (Russia).

2.2. Strain and cultivation

The strain of *Lentinus strigosus* 1566 was obtained from Basidiomycetes Collection of the Komarov Institute of Botanics (Moscow, Russian Federation), and was grown on a soya–glycerol medium (medium 1) composed of (g/l): NH_4NO_3–0.2; KH_2PO_4–0.2; K_2HPO_4–0.02; $MgSO_4 \times 7H_2O$—0.01; peptone—0.5; soybean flour—0.5; glycerol—2 ml (pH 5.0). The cultivation was carried out in 750 ml rotary flasks containing 100 ml of the Medium 1 for 7 days aerobically, on a rotary shaker at 200 rpm. The biomass obtained was fragmented with glass beads while stirring on a rotary shaker at 200 rpm for 10 min. The fragmented biomass (5 ml) was used for inoculation of 100 ml of Medium 2 composed of (g/l): glucose—20; yeast extract—5.0; peptone—5.0; $MgSO_4 \times 7H_2O$—1.0; and polycaproamide fibre—1.0. The cultivation was carried out aerobically under the same conditions. After 5 days of the growth, 2,6-dimethyl phenol (final concentration—1 mM) and $CuSO_4 \times 5H_2O$ (2 mM) were added for the induction of laccase activity. After 18 days of growth, the cultivation broth was separated by filtration and used for the obtainment of the enzyme preparation.

2.3. Enzyme preparation from L. strigosus

The filtered cultivation broth (700 ml) obtained as described above was applied to a column with DE-52 (carrier volume—350 ml) previously equilibrated with 20 mM Na-acetate buffer (pH 5.0) (Buffer A). The column was washed with an equal volume of the same buffer. Elution was carried out using linear gradient 0–0.5 M NaCl in 1800 ml of Buffer A at a rate of 1.5 ml/min. The fractions with laccase activity were combined, desalinated and concentrated in an ultrafiltration cell (Amicon, USA) with membrane UM-10. The laccase preparation obtained was applied on a column with Q-Sepharose (carrier volume of 60 ml) equilibrated with Buffer A. The column was washed with one volume of the same buffer. The elution was carried out using linear gradient 0–0.4 M NaCl in 1000 ml of Buffer A at a rate of 1 ml/min. The fraction volume was 5.6 ± 0.2 ml. The active fractions were combined, desalinated and concentrated in an ultrafiltration cell (Amicon, USA) with membrane UM-10. The activity of the enzyme preparation obtained was determined spectrophotometrically with ABTS as substrate [17].

2.4. Laccase activity assay

Laccase activity was determined spectrophotometrically on UV-1700 (Shimadzu, Japan). ABTS was used as a substrate (0.5 mM). It was dissolved in 100 mM acetate buffer, pH 5.0 at 25 °C. The enzyme activity was calculated with the absorption coefficient for ABTS $\varepsilon = 29300\ M^{-1}\ cm^{-1}$ [17] at $\lambda = 420$ nm. One unit of laccase activity was defined as the amount of laccase that oxidizes 1 μmol of ABTS per min under the selected assay conditions.