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CYCLODEXTRIN-FACILITATED BIOCONVERSION OF 17β-ESTRADIOL BY A PHENOLOXIDASE FROM *MUCUNA PRURIENS* CELL CULTURES

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Key Word Index—*Mucuna pruriens*; Fabaceae; bioconversion; phenoloxidase; *ortho*-hydroxylation; 17β-estradiol; β-cyclodextrin.

Abstract—After complexation with β-cyclodextrin, the phenolic steroid 17β-estradiol could be *ortho*-hydroxylated into a catechol, mainly 4-hydroxyestradiol, by a phenoloxidase from *in vitro* grown cells of *Mucuna pruriens*. By complexation with β-cyclodextrin the solubility of the steroid increased from almost insoluble to 660 μM. The bioconversion efficiency after 72 hr increased in the following order: freely suspended cells (0%), immobilized cells (1%), cell homogenate (6%), phenoloxidase preparation (40%). Mushroom tyrosinase converted 17β-estradiol, as a complex with β-cyclodextrin, solely into 2-hydroxyestradiol, with a maximal yield of 30% after 6–8 hr. Uncomplexed 17β-estradiol was not converted at all in any of these systems.

INTRODUCTION

Cells of *Mucuna pruriens* L. contain a phenoloxidase (EC 1.14.18.1) that is able to *ortho*-hydroxylate a range of phenolic substrates. It has been shown that L-tyrosine, when fed as a substrate, could be converted into the anti-Parkinson drug L-DOPA [1]. Even the chemically more complex and cell-foreign aminotetralines appeared to be substrates as well [2].

The efficiency and the product yield of bioconversion reactions are, however, often limited by the water solubility of the substrates. Recently cyclodextrins have received considerable attention because of their ability to form inclusion complexes with different organic molecules. By complexation the physical–chemical properties of ligands are changed, including their solubility in aqueous solutions [3]. The aim of the present study was to investigate whether a poorly water soluble compound, like the naturally occurring steroid hormone 17β-estradiol (E_2), could serve as a stubstrate for the phenoloxidase when it was complexed with β-cyclodextrin (β-CD). We compared the capability and efficiency of freely suspended and alginate entrapped cells, a cell homogenate and a phenoloxidase preparation of *M. pruriens*, to convert E_2 into a catechol.

RESULTS AND DISCUSSION

Complexation of E_2 with β-CD resulted in enhanced solubility of E_2, as is illustrated by the phase solubility diagram (Fig. 1). The E_2/β-CD system showed a solubility curve of the so-called B_s-type [4], S_0 represents the solubility of the pure drug and equals 12 μM. Upon addition of β-CD the solubility of E_2 increases linearly, due to the formation of a soluble inclusion complex. At point A the solubility of the complex reaches its limit: *ca* 660 μM (1.7 mg ml^{-1}). Between A and B new solid complex is formed and precipitated from the excess (undissolved) amount of E_2, until all undissolved free drug has been consumed (point B). The subsequent decrease in solubility upon further addition of β-CD is to be ascribed to precipitation of complex from the amount of free dissolved E_2 still present at point B [5]. The K_s value of the complex was calculated to be 20 000 M^{-1}. This relatively high K_s points to a stable complex of E_2 with β-CD.

On a weight basis the complex, used in the experiments described, contained 10% E_2; the molar ratio between E_2 and β-CD was 1:2. In the differential scanning calorimetry (DSC) curve of the complex the melting peak of E_2 (173–179°) had completely disappeared, indicating complex formation. This was in good agreement with the result of the 'ether-wash' procedure, in which an uncomplexed fraction of only 13% was found.

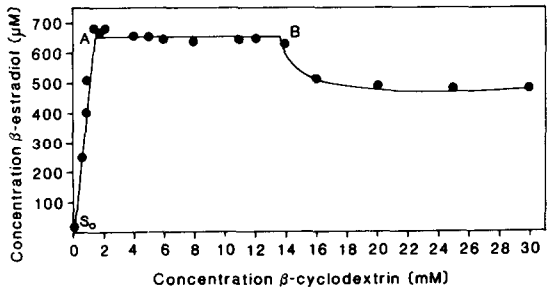

Fig. 1. Phase-solubility diagram of the E_2/β-CD system at 25°.

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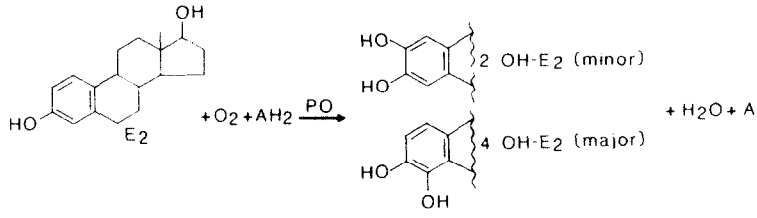

Fig. 2. Reaction scheme for the bioconversion of E_2 into 2OH-E_2 and 4OH-E_2 by *Mucuna*-phenoloxidase (PO). Na-ascorbate (AH_2) serves as a co-factor and an anti-oxidant, to prevent quinone formation.

Table 1. The bioconversion of E_2 into 2OH-E_2 and 4OH-E_2 in the different biocatalytic systems, expressed as nmol product formed per mg protein (mean ± s.d.; $n=3$) after 72 hr

	2OH-E_2	4OH-E_2
Freely suspended cells	0	0
Immobilized cells	6.5 ± 1.2	37.6 ± 6.9
Cell homogenate	12.6 ± 3.2	62.1 ± 9.4
Phenoloxidase preparation*	60.8 ± 8.7	688.3 ± 83.5
Mushroom tyrosinase	5476.5 ± 21.4	0

*Purification fold *ca* 185.

Some solubility of the substrate appeared to be an absolute prerequisite for the bioconversion into a catechol. The solubility (and dissolution rate) of uncomplexed E_2 was so low, that no bioconversion could be measured.

When E_2 was fed as a complex with β-CD, the efficiency of the bioconversion, after a 72 hr incubation period, increased in the following order: free cells (0%), immobilized cells (1% 4OH-E_2), cell homogenate (6% 4OH-E_2, 1% 2OH-E_2), phenoloxidase preparation (40% 4OH-E_2, 3% 2OH-E_2). A reaction scheme for these bioconversions is given in Fig. 2. The bioconversions of E_2 into 2OH-E_2 and 4OH-E_2 are compared for the different biocatalytic systems in Table 1, in terms of nmol product formed per mg protein after 72 hr.

It is known that the phenoloxidase from *M. pruriens* possesses a broad bioconversion spectrum [1, 2]. Our finding, that even a steroid structure can serve as a substrate for this enzyme, emphasizes its low substrate specificity.

The difference between bioconversion efficiency of free and immobilized cells on one side and cell homogenate and enzyme preparation on the other side, is most likely due to bad accessibility of the enzyme for the substrate. As the phenoloxidase occurs intracellularly [6], the substrate has to penetrate through the plant cell wall and cell membrane, being an apparent barrier for the steroidal structure. The Ca-alginate matrix itself was proven not to be a barrier for the E_2/β-CD complex. The diffusion behaviour of E_2 was equal to earlier tested phenolic substrates, which could diffuse freely into and out of the alginate matrix [7].

We attempted to enhance the bioconversion by free and immobilized cells by adding 10% (v/v) *iso*-propanol to the reaction medium. This treatment permeates the cell wall but, unfortunately, a convincing effect on the bioconversion efficiency was not found.

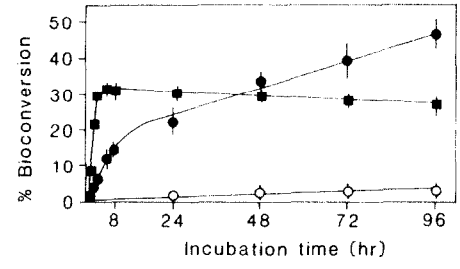

Fig. 3. The bioconversions of E_2 into 2OH-E_2 (○) and 4OH-E_2 (●) by the *Mucuna*-phenoloxidase preparation and of E_2 into 2OH-E_2 by mushroom tyrosinase (■) at pH 5.0 and 25° (100% bioconversion corresponds with 660 μm product). Given is the mean value ± s.d. (bars) of at least triplicate experiments.

With respect to the hydroxylation of E_2, highest activity of the phenoloxidase preparation of *M. pruriens* was found under (slightly) acid conditions (pH 3–6). No real pH optimum was found for this bioconversion. Preliminary experiments in our laboratory revealed clear pH optima for the hydroxylation of simple monocyclic monophenols: acids at pH 4 and amines at pH 7.5 (unpublished results). Because E_2 is a neutral compound, its hydroxylation is probably less sensitive to pH changes in the above range. In alkaline solutions (pH 8–10) hardly any bioconversion occurred. At higher pH values the enzyme may become deactivated. Moreover, E_2 then exists in its phenolate form and, being negatively charged, most likely possesses different affinity for the enzyme.

The optimal temperature for the bioconversion of E_2 was 25°. At higher temperatures the initial bioconversion was slightly enhanced, but after 24 hr the produced catechol decomposed and no high product yield could be obtained. At lower temperatures less bioconversion was measured.

The bioconversion of E_2 into 4OH-E_2 and 2OH-E_2 between 0 and 96 hr incubation at 25° at pH 5.0 is shown in Fig. 3. From the first linear part of the curve the initial rate of bioconversion (V_0) was calculated. For 4OH-E_2 the V_0 was 9.2 pkat mg^{-1} protein and for 2OH-E_2 0.2 pkat mg^{-1} protein. After 8 hr incubation, the rate of bioconversion of E_2 into 4OH-E_2 decreased, probably due to product inhibition. The amount as well as the rate of 2OH-E_2 formed during the incubation period was minor as compared with the 4OH-E_2 production.

We compared with activity of the phenoloxidase preparation of *M. pruriens* with the commercially available mushroom tyrosinase (E.C. 1.14.18.1). Mushroom tyrosinase specifically converted E_2 (as a complex with β-CD)

into 2OH-E_2 with a maximal yield after 6–8 hr incubation (Fig. 3). The calculated V_0 for this bioconversion was 561 pkat mg^{-1} protein. In contrast with the phenoloxidase from *M. pruriens*, longer incubation did not result in increased product formation. Comparing these results, it is obvious that both enzymes convert the substrate regiospecifically.

The property of mushroom tyrosinase, to react with steroid estrogens to produce 2-hydroxylated derivatives, has been used to develop methods for the synthesis of catechols [8]. Because of its bioconversion efficiency and regiospecificity, the phenoloxidase of *M. pruriens* may render a suitable candidate for the production of 4-hydroxylated derivatives of steroid estrogens. The catechol estrogens are a group of naturally occurring and biologically active estrogenic compounds, formed by aromatic hydroxylation in tissues of mammals [9]. Therefore, these compounds should be available as reference compounds for analytical as well as pharmacological purposes.

To our knowledge, this is the first report dealing with the bioconversion of a steroid by an enzyme of a higher plant, i.e. a member of the Spermatophyta. Williamson *et al.* [9] described the formation of 2OH-E_2 and 4OH-E_2 from E_2 as precursor, by the fungus *Aspergillus alliaceus* (UI 315). These authors found 45% 4OH-E_2 and 16% 2OH-E_2 after 48 hr incubation. Comparing these results with our findings, it seems that the positional specificity of the phenoloxidase from *M. pruriens* is better than from *A. alliaceus*.

As most steroidal substrates are water-insoluble and thus less easily converted by enzymatic reactions, several approaches have been used to circumvent this problem, for example the addition of organic solvents to the reaction medium [10]. Recently, cyclodextrins have been applied successfully to enhance the bioconversion rate of steroidal substrates by microorganisms [11]. The results, obtained from the current study, strengthen our view that cyclodextrins may also be applied successfully in plant cell biotechnology, because of their solubilizing action. By increasing the water solubility of substrates, increasing bioconversion efficiencies can be obtained. During the growth cycle of a culture the β-CD concentration remained unchanged, indicating that it was not broken down or used as a carbon source by the cells. The use of β-CD to increase the concentration of poorly water soluble precursors in culture medium offers the possibility to obtain higher yields of secondary plant products, formed in *in vitro* cell cultures. The latter subject is currently under investigation in our laboratory.

EXPERIMENTAL

Cell culture. Cell suspensions of leaf explants of *Mucuna pruriens* L. DC f. utilis (Wall ex Wight) cv Back, line 1.7, were grown in MS medium, supplemented with 4% sucrose, 1 $mg\,l^{-1}$ IAA and 1 $mg\,l^{-1}$ BAP, pH 5.9, at 25° and 2000 lux. The cultures were maintained by weekly subculturing [1].

E_2/β-CD complex. β-CD (AVEBE, Veendam, The Netherlands) is a cyclic oligosaccharide consisting of 7 glucose units, linked by α(1→4) glycosidic bonds. The solubility of the E_2/β-CD complex and its stability constant (K_s) were determined using the phase solubility method of ref. [4]. An excess of E_2 was weighed into 10 ml H_2O containing increasing concns of β-CD (0–30 mM) and shaken at 25° until equilibrium was reached. The solutions were filtered (0.2 μM) and the concentration of dissolved E_2 was determined by measuring the A_{280}. K_s was calculated from the initial straight line portion of the phase-solubility diagram (Fig. 1), using the equation $K_s = \text{slope}/S_0(1 - \text{slope})$, in which S_0 is the solubility of E_2 alone [12].

Crystalline complex was obtained by heating 250 mg E_2 (Sigma) and 5 g β-CD in 100 ml H_2O. These amounts were based on the molar ratio of E_2 and β-CD in the complex and derived from the phase-solubility diagram. Upon cooling the complex precipitated and was subsequently separated from the mother liquor and dried. Complex formation was checked by DSC and an 'ether-wash' method, as described earlier [13].

Bioconversion experiments. All bioconversion experiments were carried out at 25°. Na-ascorbate was always added to a final concn of 20 mM. For experiments with freely suspended cells, alginate entrapped cells and a cell homogenate, 7-day-old cell cultures, in the continuous phase of their growth, were used.

For experiments with freely suspended cells, 1 vol. of cell suspension was diluted with 2 vols of fresh culture medium to a final vol. of 210 ml, in 500 ml conical flasks. For experiments with immobilized cells, they were entrapped in 2.5% (w/v) Ca-alginate as described previously [1]. The procedure resulted in beads with 33% (w/v) cell loading and an average vol. of 27 μl. In 500 ml conical flasks 30 g beads were suspended in 180 ml incubation medium, consisting of 25 mM Na-barbital, 25 mM Na-acetate, 3.4 mM $CaCl_2 \cdot 2H_2O$ and 4% (w/v) sucrose, pH 5.8. The E_2 concentration was 0.17 $mg\,ml^{-1}$ (660 μM), dissolved, as a complex with β-CD. To achieve complex formation, the appropriate amounts of E_2 and β-CD were added to the incubation medium before autoclaving (120°, 20 min). For comparison, undissolved E_2 was added at 0.17 $mg\,ml^{-1}$ in parallel experiments. Analogous experiments were carried out in the presence of 10% *iso*-PrOH. The conical flasks were stoppered with a cotton plug, to allow sufficient air supply and gently shaken during the time of the experiment. Samples were taken between 0 and 72 hr.

A cell homogenate was prepared by disrupting a cell suspension by pulsed sonification for 2 × 2 min under ice-salt cooling. To 3 ml homogenate, 3 ml 50 mM Tris–HCl buffer (pH 5.0) were added. Of the E_2/β-CD complex 1.7 $mg\,ml^{-1}$ was dissolved in the reaction mixture. The mixture was saturated with O_2.

A phenoloxidase preparation from suspension grown cells of *M. pruriens* was prepared by $(NH_4)_2SO_4$ pptn as described earlier [14] and stored in 1 ml portions at −20°. The purification fold was about 185 [7]. The reaction mixture was composed of 50 mM Tris–HCl buffer (pH 5.0), 0.7 ml of the phenoloxidase preparation and 1.7 $mg\,ml^{-1}$ of the E_2/β-CD complex in a final vol. of 6 ml. The reaction mixture was satd with O_2. The pH-dependency and temperature optimum for the bioconversion were determined according to this procedure. For comparison, analogous experiments were carried out with mushroom tyrosinase (Sigma). A vol. of 0.4 ml, containing 0.1% (w/v) enzyme suspension with tyrosinase activity of 2200 IU mg^{-1}, was added to the reaction mixture.

Analytical procedure. Samples of 1 ml bioconversion medium were extracted twice by vortexing with 2 ml EtOAc. The combined EtOAc extracts were evapd and the residue taken in 1 ml MeOH and subjected to TLC and/or HPLC analysis. The recovery of the extraction procedure exceeded 95%.

TLC analysis was done on GF_{254} silica gel plates, with toluene–EtOH 96% (9:1) as the mobile phase. The position of E_2, 2OH-E_2 and 4OH-E_2 on the TLC sheets were visualized under UV 365 nm and day-light, after spraying with 20% H_2SO_4 in EtOH 96%, followed by 10 min heating at 110°. E_2, R_f 0.48, orange-yellow; 2OH-E_2, R_f 0.38, pink; 4OH-E_2, R_f 0.43, red-brown.

HPLC analysis was performed on a Microspher C_{18} column (Chrompack, Middelburg, The Netherlands), particle size 3 μm, 200 × 4.6 mm i.d., provided with an RP18 guard column, particle size 30–40 μm, 10 × 3 mm i.d. Mobile phase: MeCN–H_2O–0.1 M H_3PO_4 (350:650:1 w/w). Flow rate 1.5 ml min^{-1}; UV detection at 280 nm.

The identity of the catechol estrogens, formed during the bioconversions, was controlled by comparison with reference substances (Sigma) in the TLC and HPLC system. In addition, peak UV-spectra were compared with reference UV-spectra using diode array detection. The superimposed spectra and retention times were identical and peak purity was verified, confirming the identity of the bioconversion products.

Protein determinations were carried out according to the method of ref. [15], with bovine serum albumin as the standard. β-CD determinations were carried out according to the method of ref. [16].

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