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ONSET OF NEW CATALYTIC ACTIVITY IN IMMOBILIZED SPORES OF ASPERGILLUS OCHRACEUS TS DUE TO IN SITU GERMINATION: C_{17}-C_{20} LYSIS ACCOMPANIES 11α-HYDROXYLATION OF STEROID

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Summary. The change in product pattern during transformation of progesterone by calcium alginate entrapped spores of Aspergillus ochraceus TS (A. ochraceus TS) due to germination in situ is reported. While progesterone was transformed exclusively to its 11α-hydroxy derivative by both free and immobilized spores of A. ochraceus TS, the latter germinated in situ by yeast extract furnished 11α-hydroxyprogesterone and C_{19} steroids during transformation under identical conditions. The characterization of metabolite(s) and the pathway proposed demonstrated that Δ^1-dehydrogenation and lysis of C_{17}-C_{20} bond are apparently two independent reactions. The change in product profile due to activation of immobilized spores is believed to be caused by accumulation of compatible solutes in the biocatalyst which had relatively low water content.

The C_{19} steroids with Δ^4-3-keto function can act as precursor for the synthesis of either oestrogens or adrenocorticosteroid which are anti-rheumatic and anti-allergic in character. The Δ^1-adrenocorticoids were observed to surpass the parent hormones in above respects (1,2). The introduction of a double bond between C_1 and C_2 in steroid by microbes is preferred to the chemical method because of poor yield and purity (3). Again, C_{19} steroids are obtained from naturally abundant sterols both by chemical and microbial selective side chain cleavage. In fact, process for commercial conversion of sterols to C_{19} steroids by microorganism had been developed (4).

Immobilized cells are currently used for the transformation of organic compounds including commercially important molecules. During our work on

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11α-hydroxylation of progesterone by Aspergillus ochraceus TS it had been shown that the calcium alginate entrapped spores of A. ochraceus TS transformed progesterone exclusively to its 11α-hydroxy derivative and there was no side product (5). In the present communication we report the transformation of progesterone by immobilized spores germinated in situ which showed an altered product pattern.

MATERIALS AND METHODS

Chemicals. Progesterone, 11α-hydroxyprogesterone, 1,4-pregnadien-3,20-dione, testosterone, testosterone acetate, 4-androsten-3,17-dione, 1,4-androstadien-17β-ol-3-one, 1,4-androstadien-3,17-dione, sodium alginate and the high performance liquid chromatographic (HPLC) solvents viz., acetonitrile and water were purchased from Sigma, USA and Spectrochem, India respectively. All other chemicals were of analytical grade and were used without further purification.

Cultivation and immobilization of Aspergillus ochraceus TS. The test organism was grown in sterile medium (g/L: sucrose 10, cornsteep liquor 5, K_2HPO_4 0.5, pH 7.0-SCP medium) at 30°C under liquid culture condition. The spores were harvested from the culture grown on SCP-agar surface at 30°C for seven days. Immobilization of spores in calcium alginate was carried out as previously described (6). The spores suspended in Tris-maleate buffer (50 mM, pH 7.0) was mixed with sodium alginate solution. The resultant mixture was then dropped into a magnetically stirred solution of calcium chloride (0.1 M) through a hypodermic syringe to have spherical beads. The spore and alginate concentration in immobilization mixture was maintained at 20 and 2% (w/v) respectively.

Activation by yeast extract. Both free and calcium alginate entrapped spores of A. ochraceus TS were incubated respectively in sterile potassium phosphate and Tris-maleate buffer (50 mM, pH 7.0) in presence of yeast extract 1% (w/v) for 24 h at 30°C under shake culture condition. The activated preparations were then tested for 11α-hydroxylase activity.

Reaction conditions. Transformation of progesterone (9.54 μmoles) by activated and non-activated free and calcium alginate entrapped spores (equivalent to 0.065 g free spores) was carried out in potassium phosphate and Tris-maleate buffer (10 ml, 50 mM, pH 7.0) respectively at 30°C. The bioconversion was attempted both in presence and absence of glucose 1% (w/v). In case of transformation with mycelia, the reaction was carried out by incubating vegetative cells (0.3 g) with progesterone (12.72 μmoles) in phosphate buffer (20 ml, 50 mM, pH 7.0) under above mentioned conditions.

Analysis. Steroids were extracted from the reaction buffer thrice with half of its volume of chloroform. The solvent extract was washed with water, dried over sodium sulfate (anhydrous) and finally evaporated to dryness under reduced pressure. The bioconverted products were resolved by thin layer chromatography (TLC) on silica gel G plate using a solvent system of ethylacetate : chloroform : water (60:40:1) and analysed by HPLC using a C_{18}-μbondapak column (reverse phase). The authentic steroids were used as reference.

RESULTS AND DISCUSSION

Transformation of progesterone exclusively to its 11α-hydroxy derivative by A. ochraceus TS both under in vivo and in vitro conditions had been reported in earlier communications (7-9) from this laboratory. Moreover, the spores of the test organism had been shown to have 11α-hydroxylase activity both in presence or absence of glucose. On the other hand,

calcium alginate entrapped spores of A. ochraceus TS activated by yeast extract when incubated with progesterone furnished 11α-hydroxyprogesterone and C_{19} steroids but exhibited some decrease in 11α-hydroxylase activity against the control (Table 1). This product pattern remained unaltered even when the reaction was carried out either in presence or absence of glucose. The bioconverted products were characterized by TLC and HPLC. The hRf and retention time of the transformed products were compared with the authentic steroids used as reference. The bioconverted products were identified as 11α-hydroxyprogesterone [II], testosterone acetate [III], 1,4-pregnadien-3,20-dione [IV], testosterone [V], 4-androsten-3,17-dione [VI] 1,4-androstadien-17β-ol-3-one [VII], 1,4-androstadien-3,17-dione [VIII] (Fig. 1). It may be mentioned that the cleavage of acetyl moiety in progesterone by Rhizopus nigricans had been reported (10) to be associated with 11-oxygenase activity.

The mechanism of side chain cleavage and the enzymes involved were ascertained from the results of transformation of various steroid substrates in vivo by the activated immobilized spores of the test organism. Presumably cleavage of C_{17}-C_{20} bond involved a Bayer-Villiger type chemical oxidation (11) to furnish 17β-acetoxytestosterone which was then hydrolysed by an esterase to the corresponding alcohol. The introduction of the double bond between C_1 and C_2 was possibly effected by a Δ^1-dehydrogenase. Apparently, Δ^1-dehydrogenation and lysis of C_{17}-C_{20} bond are independent of each other because transformation of progesterone, 1,4-pregnadien-3,20-dione, testosterone, testosterone acetate and 4-androsten-3,17-dione, by activated immobilized spores of the test organism furnished identical products [VII and VIII]. It was interesting to note that 11α-hydroxyprogesterone so produced did neither undergo Δ^1-dehydrogenation nor lysis of C_{17}-C_{20} bond and remained unchanged even when it is used as a substrate. Moreover, there was no 11α-hydroxy C_{19} steroid isolated from the mixture of bioconverted products. Presumably, the conformation of this

Table 1. Transformation[a] of progesterone by A. ochraceus TS

Biocatalyst	μmoles of product(s)/g biocatalyst	
	11α-Hydroxylation	C_{17}-C_{20} lysis
Whole cell	2.63	-
Free spores	33.73	-
Immobilized spores	27.20	-
Immobilized spores activated by yeast extract	13.0	46.81

[a]Transformation was carried out in presence of glucose and the products obtained were expressed as μmoles per g of cells and spores both free and entrapped in calcium alginate.

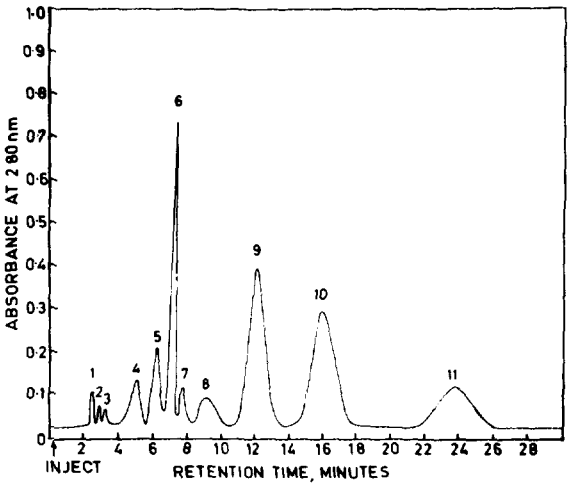

Fig. 1. HPLC profile of the bioconverted products of progesterone [I] by immobilized spores of A. ochraceus TS activated by yeast extract. Peaks: (1,2,3) control; (4) 1,4-androstadien-17β-ol-3-one [VII]; (5) 11α-hydroxyprogesterone [II]; (6) 1,4-androstadien-3,17-dione [VIII]; (7) testosterone [V];(8) 4-androsten-3,17-dione [VI]; (9) 1,4-pregnadien-3,20-dione [IV]; (10) progesterone [I]; (11) testosterone acetate [III].
Conditions: Mobile phase, acetonitrile-water convex gradient from 50:50 to 75:25 in 40 min; flow rate, 1 ml/min; pressure, 1000 psi; flow mode, constant pressure; column temperature, 38°C; detection, UV 280 nm (Waters 484, Tunable absorbance detector).

molecule (11α-hydroxyprogesterone) did not permit either Δ^1-dehydrogenation or lysis of C_{17}-C_{20} bond under the conditions used. Thus the pathway (Fig. 2) of this bioconversion appeared to be different from the normal reaction sequence already reported (12) and seems to be novel. The product [VIII] was isolated as a stable compound which did not undergo further degradation to furnish seco-steroid and finally the lactone.

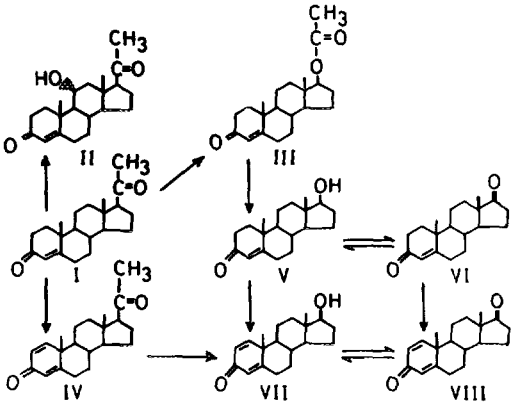

Fig. 2. Proposed pathway of transformation of progesterone by immobilized spores of A. ochraceus TS germinated in situ by yeast extract.

In the present study the side chain degradation was not typical of the 'hungry' fungus (10) because the immobilized spores of A. ochraceus TS activated by yeast extract effected 11α-hydroxylation and lysis of C_{17}-C_{20} bond in progesterone during transformation even in presence of glucose. Alternatively, the accumulation of compatible solutes (13) due to the restricted growth of the biocatalyst inside the matrix may be the factor responsible for onset of new catalytic activity. This was substantiated by the fact that the free spores activated by yeast extract under identical conditions did not exhibit C_{17}-C_{20} lyase and Δ^1-dehydrogenase activity. In addition, the water content (per g of the dry mass) of the immobilized spores activated by yeast extract was found to be less than the free spores activated identically. Thus water appears to play an important role on catalytic activity of the biocatalyst but remains to be precisely defined.

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