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NOVEL CATALYTIC ACTIVITY OF IMMOBILIZED SPORES UNDER REDUCED WATER ACTIVITY

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Abstract: Onset of a new catalytic function during transformation of progesterone by immobilized spores of *Aspergillus ochraceus* TS under reduced water activity is reported. The pathway of transformation, which furnished 1,4-androstadien-17β-ol-3-one and 1,4-androstadien-3,17-dione due to cleavage of C_{17}-C_{20} bond, is different from normal reaction sequence. © 1997 Elsevier Science Ltd. All rights reserved.

Alteration of substrate specificity of the enzyme is one of the most challenging targets in the field of biocatalysis. Water activity (a_w = P/Po, where P and Po are vapour pressures over a sample and pure water respectively) is now being considered to be an important physiological parameter responsible for change in product pattern during biotransformation[1,2]. Transformation of progesterone by *Aspergillus ochraceus* TS (*A. ochraceus* TS) exclusively to its 11α-hydroxy derivative both *in vivo* and *in vitro* had been reported from this laboratory[3-6]. Role of water activity on transformation of progesterone by immobilized spores was studied.The present communication, which describes the change in product pattern, seems to be the first report on water activity as an important regulatory determinant in biotransformation of steroid.

The spores of *A. ochraceus* TS grown on SCP-agar (g/L : sucrose 10, cornsteep liquor 5, K_2HPO_4 0.5, pH 7.0-SCP medium) at 30°C for 7 days were immobilized in calcium alginate according to the method described previously[7]. The spores ($3x10^8$ spores/ml) suspended in Tris-maleate buffer (50 mM, pH 7.0) were mixed with sodium alginate solution (2% w/v). The resultant mixture was then dropped into a solution of calcium chloride (0.1 M) to have spherical beads.

Water activity of the biocatalyst was reduced either by freezing at -20°C or by incubation with additives viz., sodium chloride (10%), sucrose (30%), PEG 6000 (25% w/v) separately in buffer at 30°C on a rotary shaker for different time period up to 48 h. Dehydrated preparations were rehydrated in a vacuum desiccator at a relative humidity of 100% for 24 h at 30°C as previously described[8].

The water activity of the biocatalyst was estimated by isopiestic method recommended by Bull and Breese[9]. The water vapour pressure of the sample was allowed to attain equilibrium with that of a standard sulphuric acid solution under vacuum at 30°C for a week. Then the strength of the reference solution was determined by titrating an aliquot against a standard sodium hydroxide solution. The water activity of different samples were calculated since the vapour pressure of standard sulphuric acid and pure water is known.

Immobilized spores (equivalent to 0.065 g free spores, normal, dehydrated and rehydrated) were incubated with progesterone (9.54 μmoles) separately in Tris-maleate buffer (10 ml, 50 mM, pH 7.0)

supplemented with or without glucose (1% w/v) on a rotary shaker at 30°C for 48 h. Parallel controls (free spores, 0.065 g) were incubated with progesterone under identical conditions.

After transformation was over, the clarified filtrate was extracted with chloroform thrice. The pooled chloroform extract was processed and totally evaporated under reduced pressure. The homogeneity of the bioconverted products was tested on silica gel G plate (TLC) using ethylacetate : chloroform : water (60 : 40 : 1) as solvent system and compared with authentic steroids.

The bioconverted products were also resolved on a μ-bondapak C_{18} analytical column (reverse phase, 0.7 x 25 cm) at 30°C in conjunction with programmed solvent gradient (Waters chromatography model 570). The two extremes of the solvent concentration were acetonitrile : water (50 : 50) and (75 : 25) respectively. A convex gradient was employed with a flow rate of 1 ml/min at 1000 p.s.i. The elution was monitored with a UV detector (Waters 484) at 254 nm. The retention time of the bioconverted products were determined using authentic steroids as reference.

The water activity of the different preparations varied between 0.98-0.86 ranging from 0.98 (sucrose), 0.98 (freeze-thaw), 0.97 (PEG-6000), 0.97 (glycerol), 0.96 (NaCl), 0.90 (n-butyl acetate), 0.89 (amylacetate), to 0.86 (ethylacetate). The transformation of progesterone by both free and immobolized spores of *A. ochraceus* TS, which underwent freeze-thaw treatment, furnished 11α-hydroxy progesterone and C_{19} steroids indicating onset of a new catalytic function. Similar results were obtained with both free and immobilized spores pretreated with and in presence of various additives, viz., electrolyte, disaccharide and polyhydric alcohol (data not shown). A significant change in product profile was noticed when the transformation was tried with the above catalyst(fixed amount) pretreated with varying volue of aliphatic esters (100%), which furnished 1,4-androstandien-17β-ol-3-one (10) and 1,4-androstadien-3,17-dione (11) characterized with the help of high pressure liquid chromatography using authentic steroids as reference. There was lysis of C_{17}-C_{20} bond and complete loss of 11α-hydroxylase activity (Fig.1, Table 1).

Apparently the lysis of C_{17}-C_{20} bond in progesterone involved a typical Baeyer-Villiger type oxidation furnishing a 17β-acetate which was hydrolysed by an esterase to produce the corresponding alcohol. The product 8 and 9 further underwent dehydrogenation to furnish finally 10 and 11(Fig.2). The pathway of this bioconversion appeared to be different from the normal reaction sequence already reported[10,11]. The Δ^1- dehydrogenation was found to be independent of substrate structure because the products 10 and 11 were also obtained with progesterone (1), Δ^1-progesterone (4) and testosterone (9) used individually as substrate. In contrast, 11α-hydroxylase exhibited substrate specificity towards progesterone only since no 11α-hydroxylated C_{19} steroid was detected in the bioconverted mixture. Furthermore, the presence of 17α-hydroxyprogesterone and absence of testosterone acetate in the bioconverted mixture clearly showed that lysis of C_{17}-C_{20} bond in progesterone by the biocatalyst pretreated with ethylacetate followed a different route (Fig.2 path way b). In fact, the compound 5 on incubation with the biocatalyst treated similarly furnished both 10 and 11. It is suggested that some of the enzymes (Δ^1-dehydrogenase and C_{17}-C_{20} lyase) in the biocatalyst become functionally on only under reduced water activity.

In the present study the change in catalytic function of the biocatalyst really depends on its water activity (Table 1). It is very difficult to determine the critical water activity of the preparation *in vivo* exhibiting such change. The change is irreversible in respect of loss in 11α-hydroxylase activity and/or onset of new catalytic function and it sees to be unique. The transformation by different enzymes under reduced water activity exhibiting altered product pattern are reported to be reversible in nature[12]. Presumably, the change in

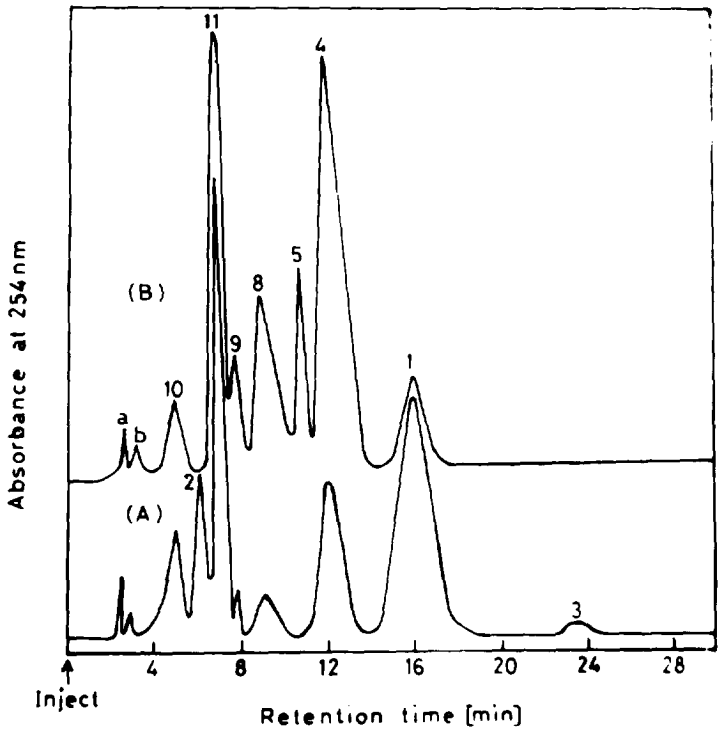

Figure 1. HPLC elution profile of the transformed products of progesterone by immobilized spores of *A. ochraceus* TS (A) freeze-thaw (B) ethylacetate.
Peaks : (a, b) control ; (10) 1,4-androstadien-17β-ol-3-one ; (2) 11α-hydroxyprogesterone ; (11) 1,4-androstadien-3,17-dione ; (9) testosterone ; (8) 4-androsten-3,17-dione ; (5) 17α-hydroxyprogesterone ; (4) 1,4-pregnadien-3-20-dione; (1) progesterone ; (3) testosterone acetate.

TABLE 1. Transformation of progesterone by immobilized spores of *A. orchraceus* TS

Preparation		Bioconverted products (μmoles/g spores)							
		2	3	4	5	8	9	10	11
Immobilized spores		27.2							
Dehydrated immobilized spores									
(a)	Freeze-thaw	14.0	5.0	7.6	-	14.7	5.5	5.8	14.4
(b)	Ethylacetate	-	-	23.6	20.8	58.7	3.9	4.2	22.2
(c)	Amylacetate	-	-	16.1	13.8	42.6	2.7	2.9	16.1
(d)	n-Butylacetate	-	-	13.8	12.2	40.4	2.6	2.7	15.8

2. 4-Pregnen-11α-ol-3,20-dione
3. 4-Androsten-17β-ol-3-one acetate
4. 1,4-Pregnadien-3,20—dione
5. 4-Pregnen-17α-ol-3,20-dione
8. 4-Androsten-3,17-dione
9. 4-Androsten-17β-ol
10. 1,4-Androstadien-17β-ol-3-one
11. 1,4-Androstadien-3,20-dione

catalytic function of immobilized spores *A. ochraceus* TS under reduced water activity may possibly be due to either activation or denaturation of the membrane bound enzyme or alteration in protein-protein interaction with the reorganization of water molecules around the enzyme surface.

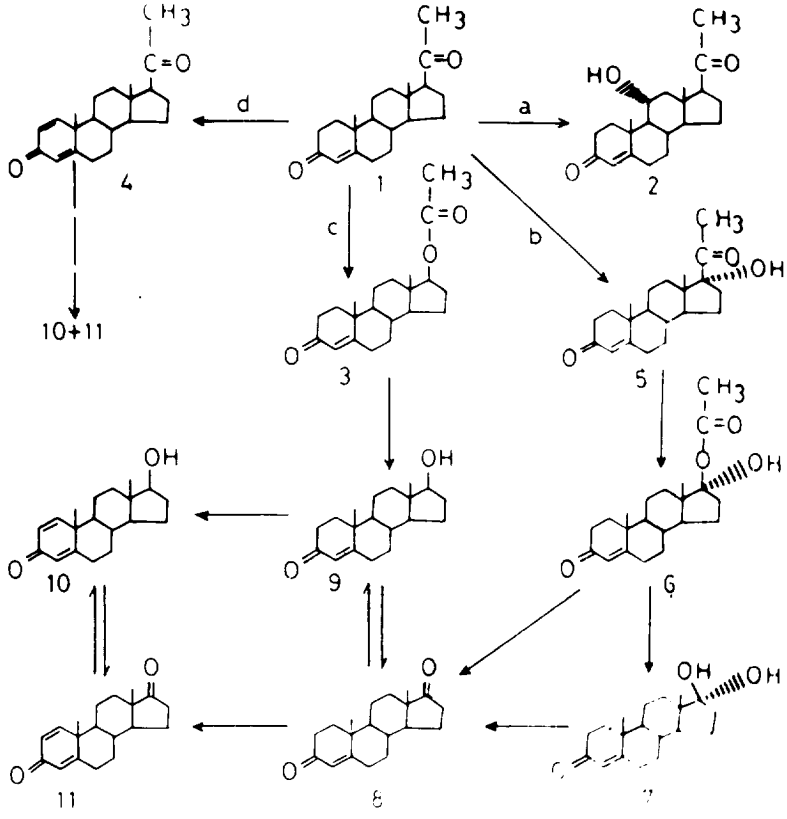

Figure 2. Proposed pathway of transformation of progesterone by immobilized spores of *A. ochraceus* TS. a, normal water activity; b-d, reduced water activity.

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