11α-Hydroxylation of Progesterone by Gel-Entrapped Living *Rhizopus stolonifer* Mycelia

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**Summary.** Spores of *Rhizopus stolonifer* were immobilized aseptically by entrapment with photo-crosslinkable resin prepolymers, urethane prepolymers or several kinds of polysaccharides. The entrapped spores were allowed to germinate and develop in situ. The immobilized living mycelia so obtained were induced for the steroid 11α-hydroxylation system and examined for their activity to hydroxylate progesterone at 11α-position in a buffer system containing 2.5% of organic cosolvent. Of various water-miscible organic cosolvents, methanol was found to be most effective in terms of the activity of the entrapped mycelia and the solubility of the product, 11α-hydroxyprogesterone. Though all the living mycelia entrapped in different gels exhibited the hydroxylation activity, mycelia entrapped in photo-crosslinked gels showed the maximum activity which was rather higher than that of the free mycelia. The network size of the photo-crosslinked resins, namely the chain length of the photo-crosslinkable resin prepolymers, affected markedly the mycelial growth in gels, and subsequently, the hydroxylation activity of the entrapped mycelia. Entrapment significantly enhanced the operational activity and stability of the 11α-hydroxylation system in the mycelia, and permitted the intermittent reactivation of the system by incubating the entrapped mycelia in potato-dextrose broth.

**Introduction**

We have developed two novel and convenient methods to immobilize enzymes, microbial cells and organelles by the use of photo-crosslinkable resin prepolymers and water-miscible urethane prepolymers (Fukui et al. 1980b). Enzymes and microbial cells immobilized by these methods have been successfully applied to the dehydrogenation of various steroids (Fukui et al. 1980a; Sonomoto et al. 1980), hydrolysis of a terpene ester (Omata et al. 1981), trans-esterification of triglyceride (Yokozeki et al. 1982a) and transglycosylation to form adenine arabinoside from uracil arabinoside and adenine (Yokozeki et al. 1982b) in organic solvent systems or in water-organic cosolvent systems.

Recently, immobilized living or growing cells attract a world-wide interest because of the self-regeneration of catalytic systems (Fukui and Tanaka 1982). If a large part of or whole metabolic pathways in cells are required for the production of various useful compounds, it might be preferable to use microorganisms in gels in living or growing state. In the sense, hydroxylation of steroids requires a sequence of pathway including activation of molecular oxygen and continuous supply of reducing power.

Ohlson et al. (1980) described 11β-hydroxylation of cortexolone (Reichstein's Compound S) by immobilized living mycelia of *Curvularia lunata* in calcium alginate gels. Recently, Maddox et al. (1981) used *Rhizopus nigricans* immobilized in agar for 11α-hydroxylation of progesterone, and Chun et al. (1981) reported 16α-hydroxylation of dehydroepiandrosterone with *Streptomyces roseochromogenes* entrapped in photo-crosslinked resins. However, the hydroxylation systems of these cells were not enough stable in repeated use.

In the previous papers (Sonomoto et al. 1981, 1982), we demonstrated that the immobilized living *C. lunata* mycelia, developed from spores entrapped with photo-crosslinkable resin prepolymers, had a high and more stabilized steroid 11β-hydroxylation activity which can be reactivated under appropriate conditions. This paper describes 11α-hydroxylation of progesterone by immobilized *Rhizopus stolonifer* mycelia.
buffer (pH 8.0) containing 2.5% of methanol at 30 °C for 12 h. Reaction (total reaction mixture, 20 ml) was carried out in 50 mM potassium phosphate buffer (pH 7.0 or 8.0) and 20 mg of progesterone in 0.3-0.5 ml of methanol or dimethyl sulfoxide. Since calcium alginate gels were not stable in the buffer, 0.5 M CaCl₂ solution was used instead of the buffer. The reaction was initiated by adding the immobilized or free mycelia, and carried out at 30 °C with shaking (150 strokes/min). Reaction rate was calculated for the initial phase of reaction (2-9 h) and expressed as µg of 11α-hydroxyprogesterone produced in the reaction solution per h per system (20 ml). Conversion ratio was molar percent of 11α-hydroxyprogesterone formed based on progesterone added.

**Analytical Methods.** Aliquots (each 0.2 ml) of the reaction solution were added to 0.3 ml of methanol containing a known amount of 4-androstene-3,17-dione as internal standard, and filtered through a 0.22 µm teflon membrane filter. Progesterone and 11α-hydroxyprogesterone were determined by high performance liquid chromatography (Waters Co. ALC/GPC 204) equipped with a data module (Waters Co. type 730) at 254 nm under the following conditions: Column, Radial-Pak A (μBondapak C₁₈, d = 10 µm) liquid chromatography cartridge; mobile phase, water-methanol-acetic acid (10:90:0.02, by volume); flow rate, 2 ml/min; pressure, 70 kg/cm². Under these conditions, the respective retention times of 11α-hydroxyprogesterone, 4-androstene-3,17-dione and progesterone were 2.30, 3.00 and 3.85 min.

**Results and Discussion**

**Effect of Gels on Activity of Immobilized Mycelia**

Mycelia of *Rhizopus stolonifer* ATCC 6227b are known to hydroxylate progesterone at 11α-position (Peterson et al. 1952). As fungal mycelia immobilized directly and homogeneously in gels had only low hydroxylation activity, so the spores of *R. stolonifer* were entrapped in different kinds of gels, and the mycelia were allowed to develop in situ. Although 11α-hydroxylation system in mycelia was induced by progesterone (Hanisch et al. 1980), the inducer inhibited the germination of spores and development of mycelia at a concentration of 0.9 mg/ml. Therefore, the entrapped spores were allowed to germinate and grow at first, followed by induction of 11α-hydroxylation system with progesterone.

Table 1 shows the effect of gels on 11α-hydroxylation activity of immobilized living *R. stolonifer*. The mycelia...