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Steroid Modification with *Aspergillus phoenicis* : Effects of Solvents and Glucose

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미생물(*Aspergillus phoenicis*)을 이용한 스테로이드의 변형에 관한 연구 : 유기용매와 포도당의 효과에 관한 고찰

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Abstract: The bioconversion of progesterone by *Aspergillus phoenicis* has been studied. The metabolism in the conditions of experiment gave 11α-hydroxyprogesterone as main product. The concentration of 11α-hydroxyprogesterone increased monotonically and leveled off after 40 hours of incubation. Addition of glucose into the medium reduced considerably the time for attaining limiting concentration of 11α-hydroxyprogesterone. The increase in initial progesterone concentration did not affected the percentage of conversion nor the time required for termination of the reaction. But it could not be represented as first order reaction with respect to progesterone concentration. The degree of inhibition of enzymes by organic solvents depended upon the concentration of solvents. At low solvent concentration, acetone proved to yield the highest conversion.

Keywords: *Aspergillus phoenicis*, Progesterone, 11α-.hydroxyprogesterone.

The filamentous fungi have been used traditionallly for human aliments to flavor, to modify the texture and the nutritional properties of protein of vegetal origin like Doinjang, or animal origin like cheese.

Owing to the develoment of submerged fermentation, recently the applicable biotechnological domain of fungi has been considerably enlarged, notably for the production of organic acids, antibiotics, enzymes and the bioconversion of steroids.

The bioconversion of steroids and especially the hydroxylation reaction, constitutes an important industrial problem.

Hormal steroids are usd as metabolism regulators, antiinflammatory, contraceptive….

Their synthesis needs generally the first chemical step which arrives at the progesterone. The bioconversion play an important role to obtain the final high value products from the progesterone produced.

One uses therefore the whole cell microorganism to preserve the enzyme systems with cofactor regeneration which is indispensable to the transformation.

The culture medium must permet at the same time the growth of microorganisms and the substrate solubilization.

The reaction mediated by immobilized biocatalysts have been so far restricted mainly to the aquous phase or to the aquous solution containing water-soluble organic solvent.

The maximal substrate concentration is limited by the solvent tolerance of the microorganism.

To avoid this phenomenon, the reaction has been performed in a continuous chain of reactors. The

ground substrate is continually added (Kondo *et al.* 1961, Constantinides 1980). Maxon *et al.* (1966) have simulated the transformation of progesterone into 11α-hydroxyprogesterone by *Rhizopus nigricans*, using a mathematical model including substrate solubilization and product precipitation. El-Refai *et al.* (1970) obtained 11α-hydroxyprogesterone, 11β-hydroxyprogesterone and 3 other products with *Cladosporium cladosporides* using progesterone as substrate. Dulaney *et al.* (1955) found that the bioconversion of 11α-hydroxyprogesterone into the non desired products(6β-, 11α-dihydroxyprogesterone) can be controlled by means of the Cu^{2+} concentration.

The microbial transformation of steroids has been thoroughly reviewed by many authors (Eppstein *et al.* 1956, Prescott and Dunn 1959, Shull 1956, Vézina *et al.* 1968).

Aspergillus phoenicis is found to be capable of transforming progesterone into 11α-hydroxyprogesterone(Kim *et al.* 1982).

It is an important product because it could be easily converted at low cost in cortisone (Peterson *et al.* 1955), which is reported to be effective on rheumatoids (Bohak *et al.* 1977).

This study is devoted to the investigations of the effects of glucose and organic solvent concentration on the reaction rate of 11α-hydroxylation of progesterone.

Materials and Methods

Culture conditions and fermentation

Aspergillus phoenicis was inoculated (inoculation 4 : 50 with 14 hours of preculture) into 250ml flask each containing 50ml of the following medium: 40g malt extract, 3g yeast extract per 1*l* of water. The medium was shaken in a rotary shaker (180rpm) at 28°C.

After 10 hours of the culture, the enzyme activity was initiated by the addition of progesterone dissolved in pure ethanol.

The reaction occurred at 28°C, 180rpm.

Analytical methods

The supernatant collected from the the culture medium by centrifugation was acidified with 6ml of 1M HCl and the products were extracted with 20ml of chloroform. The extracts were evaporated to dryness, then redissolved in a small volume of chloroform (200μl) and chromatographed by thin-layer chromatography on a silica gel precoated quartz rod in a system chloroform : cyclohexane : ethanol (46 : 46 : 8 by volume). The quantitative estimation was realized by scanning with frame ionization detector.

Fig. 1 shows chromatogram of a solution with equal concentration (2.5g/*l*) of progesterone and 11α-hydroxyprogesterone. By their RF values, A and B peaks can be identified respectively to be progesterone and 11α-hydroxyprogesterone.

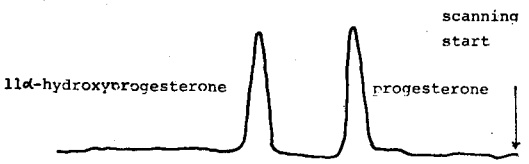

Fig. 1. Chromatogram of progesterone and 11α-hydroxyprogesterone in ethanol solution with 2.5g/*l* respective concentration.

Comparing the values of the peak area devided by the molarity of each substrate, the response of FID is almost same for these two materials.

Results and Discussion

It has been checked that the three most important products produced from the transformation reaction of progesterone were 11α-hydroxyprogesterone, 15β-hydroxyprogesterone and dihydroxyprogesterone, among which only 11α-hydroxyprogesterone was produced with important guantity at the early stage of the reaction.

It has also been verified that there were no spontaneous degradation of progesterone in the medium (Kim *et al.* 1982).

Fig. 2 represents time course for transformation of progesterone to 11α-hydroxyprogesterone with different progesterone concentrations.

The percentage of conversion of progesterone seems to be almost independent of initial progesterone

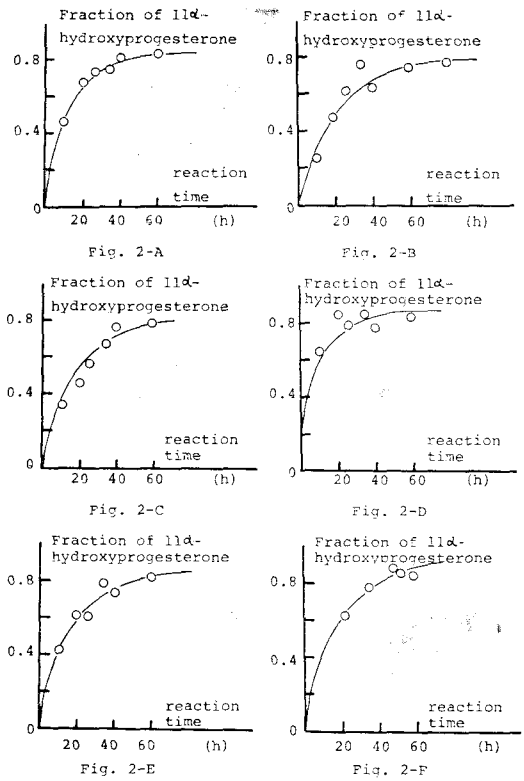

Fig. 2. Time course for transformation of progesterone to 11α-hydroxyprogesterone with different progesterone concentrations. A: 0.005, B: 0.01, C: 0.05, D: 0.1, E: 0.2, F: 0.5 g/l

concentration. However, the transformation reaction can not be abridged to be a first-order reaction form, since logarithmic concentration of 11α-hydroxyprogesterone is not a linear function of time.

It is seen in Fig. 2 that the concentration of 11α-hydroxyprogesterone increases monotonically until 40 hours of reaction time and then levels off afterward. This behavior indicates that 11α-hydroxyprogesterone produced is converted into another substances, since the transformation reaction is an irreversible one. Schleg *et al.* (1962) found also that after 40 hours of incubation, the 11α-hydroxylation reaction was almost terminated. However it can be thought that subsequent transformation reaction of 11α-hydroxyprogesterone is not important since dihydroxylation of 11α-hydroxyprogesterone favors alkaline side (Sallam *et al.* 1976) and the reaction medium becomes more acidic as the transformation of progesterone proceeds. Vézina *et al.* (1963) reports that the dihydroxylation reaction becomes less important with increase in progesterone concentration.

It is expected that the addition of glucose into the reaction medium accelerates the hydroxylation reaction rate, since cofactor regeneration, which is indispensable to the hydroxylation reaction of prog-

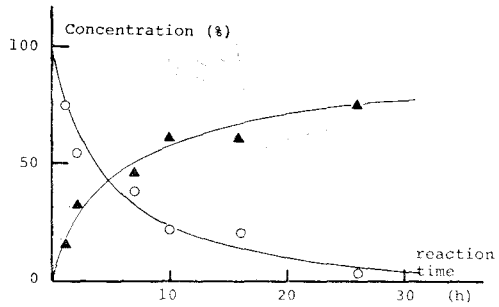

Fig. 3-A. Effect of glucose on the 11α-hydroxylation reaction. glucose concentration: 0.01g/l open circle: progesterone, filled triangle: 11α-hydroxyprogesterone.

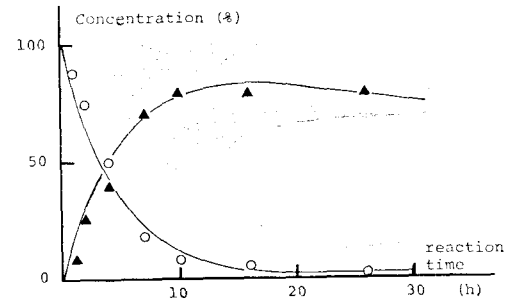

Fig. 3-B: Effect of glucose on the 11α-hydroxylation reaction. glucose concentration: 0.05 g/l open circle: progesterone, filled triangle: 11α-hydroxyprogesterone.

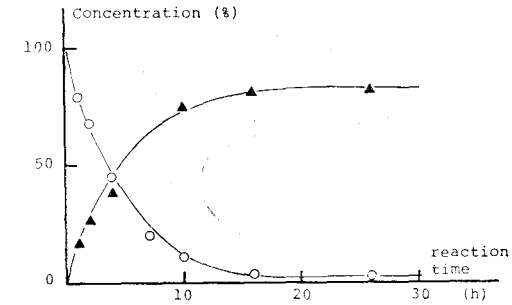

Fig. 3-C: Effect of glucose on the 11α-hydroxylation reaction. glucose concentration: 0.2 g/l open circle: progesterone, filled triangle: 11α-hydroxyprogesterone.

esterone, occurs in the glucose metabolic pathways.

The variation of concentration of progesterone and 11α-hydroxyprogesterone as function of time with different glucose concentrations in the reaction medium is shown in Fig. 3.

Comparaison of Fig. 2 and Fig. 3 indicates that, at a low value of glucose concentration, glucose effect on the reaction rate is negligeably small and the limiting value of 11α-hydroxyprogesterone concentration is not achieved until 30 hours of reaction time.

The concentration of 11α-hydroxyprogesterone ceases to increase after 10 hours of reaction at higher glucose concentrations, while without glucose in the reaction medium, this phenomenon appears after 40 hours. These results can be interpreted as saying that increase in glucose concentration in the reaction medium accelerates the hydroxylation reaction rate. However, the glucose effect levels off above a certain level of glucose concentration. Vézina *et al.* (1973) and Clark *et al.* (1983) reported also favorable effects of glucose on the transformation reaction rate.

One of the most important factors having great influence upon bioconversion reaction rate of steroid is the solubility of substrate.

Only small quantity of steroid (about 10^{-5} mol/*l*) can be dissolved in water, and it is inevitable to use organic solvents to increase the concentration of substrate.

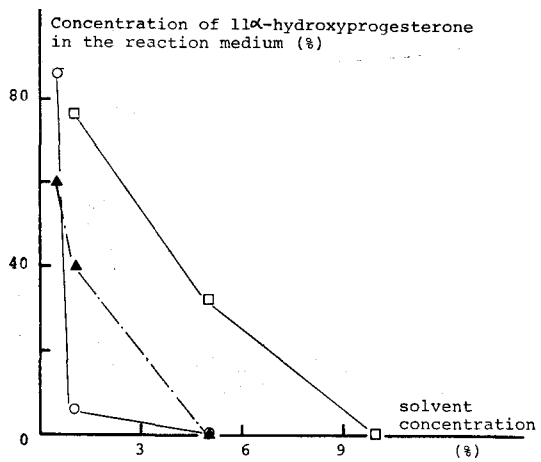

Fig. 4: Effect of organic solvent concentration on the 11α-hydroxylation reaction. reaction time: 20h, reaction temperature: 28°C, initial progesterone concentration; 0.5g/l
○—○ Acetone, ▲—▲ THF, □—□ Ethanol

However, the quantity of solvents should be limited, since they inhibit the activity of enzymes of the reaction. Fig. 4 shows the effect of organic solvent concentration on the 11α-hydroxylation reaction. The reaction was carried out at 28°C for 20 hours with 0.5 g/*l* of initial progesterone concentration.

When the concentration of organic solvent was above 1% level, acetone inactivated enzymes most, followed by tetrahydrofuran (THF) and ethanol in the order cited. At 0.4% level of organic solvent concentration, which can not be attained with ethanol maintaining 0.5g/*l* of progesterone concentration, acetone gave the highest level of conversion.

摘 要

*Aspergillus phoenicis*에 의한 progesterone의 전환반응에 대하여 고찰하였다. 본 실험조건에서의 물질대사 결과 11α-hydroxyprogesterone이 주생성물로 생성되었다. 11α-hydroxyprogesterone의 농도는 반응시간에 따라 서서히 증가하였으나 40시간 이후부터는 점근값에 도달하였다. 반응액에의 glucose 첨가는 progesterone의 전환반응 속도를 증가시켜 점근값의 농도에 도달하는 시간이 괄목할 만큼 앞당겨졌다.

Progesterone의 초기 농도를 증가시키는 것은 전환율이나 반응완결에 소요되는 시간에 큰 변화를 주지 않았으나 전환반응은 progesterone 농도에 대하여 1차 반응 형태로 요약될 수는 없었다.

유기용매에 의한 효소 활성의 저하정도는 유기용매의 종류 및 그 농도에 의존하였으며, 낮은 유기용매의 농도에서는 acetone이 가장 높은 전환율을 나타내었다.

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