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Solvent Effect on Enzymatic Steroid Transformation

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Abstract□As part of our endeavor to increase the productivity of steroid by enzymatic transformation of corticosteroids, attempts have been made to increase the solubility of steroids by using some organic solvents. When the solubility of steroid is the rate limiting factor in the steroid transformation, it was found that the use of solvents significantly improved the yield. Hydrocortisone as a substrate and 3-ketosteroid-Δ^1-dehydrogenase as an immobilized whole cell enzyme were employed as the model system for this study. It was found that the yield of product, prednisolone, goes through a maximum with an increase in the solvent concentration. At a high solvent concentration, the solvent showed a toxic effect and it causes a decrease in the product yield by the second order inhibition mechanism. Among the solvents evaluated, methanol and ethanol were found to be the best. These alcohols are not only good solvents but also showed minimal toxic effect. Based on the experimental results, it was concluded that the productivity of steroid can be increased by using well selected solvents systems for the enzymatic transformation of steroids.

Keywords□Enzymatic transformation of steroids—prednisolone—use of organic solvents—methanol and ethanol—an immobilized whole cell enzyme—3-ketosteroid-Δ^1-dehydrogenase.

The solubility of the corticosteroids including hydrocortisone in water is very low, only about 0.67 mM, and it becomes the rate limiting factor in the enzymatic transformation of steroids. It is thus apparent that the productivity of the steroids can be increased markedly by increasing the solubility of substrate by using proper organic solvent. The intact whole cell enzyme in a fermenting system or the soluble enzyme is usually significantly affected by the organic solvents, and in order to reduce the adverse effect of the solvents the Δ^1–dehydrogenase in *A. simplex* is immobilized in the form of whole cell enzyme and was employed in this study.

The immobilization of enzymes by entrapment in polyacrylamide gel was first proposed by Bernfeld and Wan in 1963[1] and for entrapping the whole cell enzymes in 1966[2]. In 1970, Mosbach *et al.*[2~4] entrapped Δ^1–dehydrogenase partially purified from *A. simplex* in acrylamide gel, and they entrapped the whole-cell of *A. simplex*.

Park and Ryu[5] reported for the first time about the reaction kinetics of the immobilized whole-cell enzyme of *A. simplex*. They found that the Michaelis-Menten constant of the immobilized whole-cell enzyme was 1.57 mM.

We have further investigated the possibility of increasing the productivity of prednisolone by using the immobilized whole cell enzyme and selected organic solvents and carrying out the enzymatic dehydrogenation of hydrocortisone in organic solvent-water mixture.

EXPERIMENTAL METHODS

Culture of Microorganism

Mutant strain of *Arthrobacter simplex* (ATCC 6946), preserved in lyophillized form in an ampule, was used. The inoculum prepared from the agar slant was transferred to the inoculum media. It was cultured, transferred to 50 ml fermentation media[5] in a 500 ml Erlenmeyer flask and cultured at 30°C using a rotary shaker at the speed of 300 rpm. To each flask 10 mg hydrocortisone in dimethyl formamide was added just before the stationary phase. The cell was harvested at the maximum enzyme activity after the inducer addition. The cell concentration was measured with a spectrophotometer at 660 nm.

Assay Method

The assay method by UV spectrophotometer developed by Park and Ryu was used in this experiment[5]. The enzyme reaction was stopped by sodium hydroxide solution, at pH 13, and the sample was centrifuged. The steroid in supernatant was extracted by ethylacetate and washed with alkaline solution at pH 9.0 to prevent the interference. After mixing and centrifugation, and adequate amount of the ethylacetate layer was evaporated at room temperature. The residue was dissolved in 3 ml of ethanol and the OD was measured at 242 nm and 265 nm. The ratio of substrate to the product was obtained from the standard curve made by the OD and the substrate ratio[5].

The enzyme activity was determined from the initial reaction velocity of the enzyme at the given substrate concentration. The enzyme reaction mixture consisted of 0.5% peptone and 0.05 M phosphate buffer at pH 8.0. The enzyme activity was measured after 1 hour reaction at 30°C in 6 mM substrate concentration in 8 ml containing 10% each of organic solvent and 0.5 g wet enzyme gel particles.

Whole-Cell Enzyme Immobilization

The immobilization method reported by Larsson *et al.* was used[3]. 7.2 g of acrylamide monomer was dissolved in 30 ml of 0.05 M phosphate buffer at pH 8.0, and it was added to 20 ml cell suspension containing 2 g dry weight. 100 mg of tetramethylethylenediamine and 150 mg potassium persulfate in 2 ml water was added to the reaction mixture which was equilibrated in ice bath for 5 minutes. The bulk gel formed was granulated to about 30–40 mesh sizes and was washed thoroughly with saline solution to remove the remaining reactants. 5g of the wet gel particle was transferred to a 500 ml Erlenmeyer flask containing 50 ml of fermentation media and 1 mM hydrocortisone and was incubated in a rotary shaker for 48 hours at 30°C, 300 rpm. It was transferred to 0.05 M phosphate buffer (pH 8.0) after washing with saline solution and stored in a refrigerator.

RESULTS AND DISCUSSION

The Effect of Organic Solvents

Several good organic solvents for the steroid were chosen. Among several solvents tested,

Table I: Effect of solvents on the enzyme activity of immobilized whole-cells

Solvents*	Activity** (μ mole/hr/g-gel)	%Activity
Methyl alcohol	11.0	100
Benzyl alcohol	9.4	85
Propylene glycol	8.4	76
Dimethylsulfoxide	10.6	96
n-propyl alcohol	8.6	78
Allyl alcohol	9.1	83
Dimethylformamide	9.9	90
Ethyl alcohol	11.2	102

* 10% (v/v) solvents were used.

** The initial enzyme reaction velocity at 6 mM substrate concentration. A half gram wet enzyme gel was used.

methanol, ethanol, dimethylsulfoxide, and dimethylformamide were effective at high substrate concentration(Table I).

For methanol and ethanol solvent system, the enzyme reaction rates were determined by varying the concentrations of substrate and organic solvents. The initial reaction rate of the enzyme decreased with increasing solvent concentration in the low substrate concentration range where the solvent could not contribute to the increase of the solubility of the substrate, whereas the initial reaction rate went through a maximum and then decreased with increasing solvent concentration when the substrate concentration was high as shown in Figs. 1 and 2. From this result, one can find that the organic solvent not only increase the solubility of the steroids but also inhibit the enzyme reaction. Up to a certain concentration range of organic solvent the increase in the available substrate concentration for enzyme reaction may overcome the inhibitory effect of the solvent. This result shows a significant advantage of using a solvent in enzymatic transformation of steroids. At a higher concentration of organic solvent the inhibitory effect becomes dominant, and there is no advantage of using a solvent. The inhibitory effect of the solvent on the enzyme could be partially overcome by the increment of the substrate concentration

Inhibition Kinetics

To elucidate the inhibition pattern of the

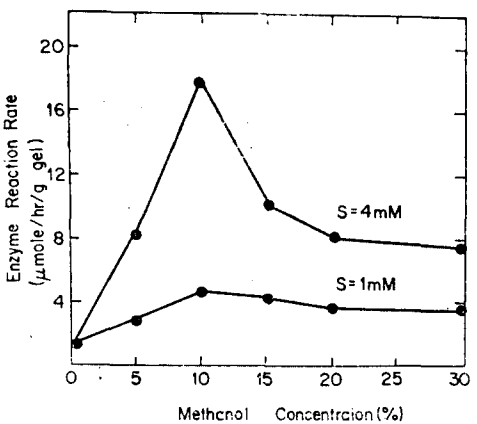

Fig. 1: The Effect of Methanol Concentration of the Δ^1-Dehydrogenase.

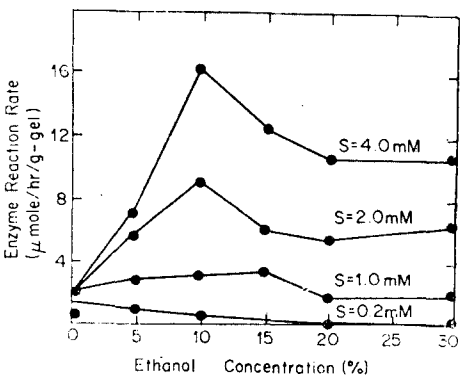

Fig. 2: The Effect of Ethanol Concentration on the Δ^1-Dehydrogenase at Varying Steroid Concentration.

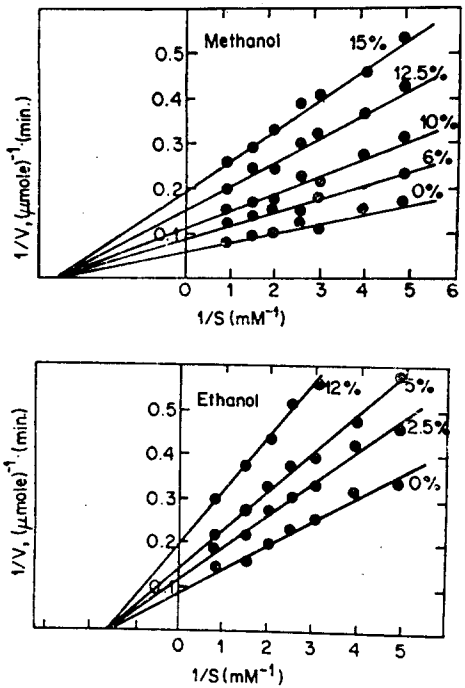

Fig. 3: Lineweaver-Burk Plots for the Enzymatic Reaction with Methanol (3-a) and Ethanol (3-b) Solvent System.

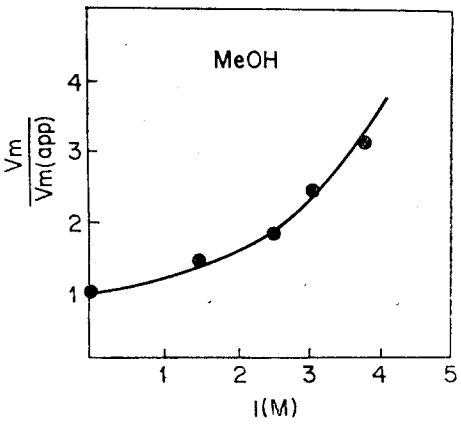

Fig. 4: Test for the Second order Inhibition V_m/V_m (app) vs. Inhibitor Concentration.

enzyme by the solvent, the apparent enzyme kinetic constants, K_m (app) and V_m (app), for methanol and ethanol were obtained from

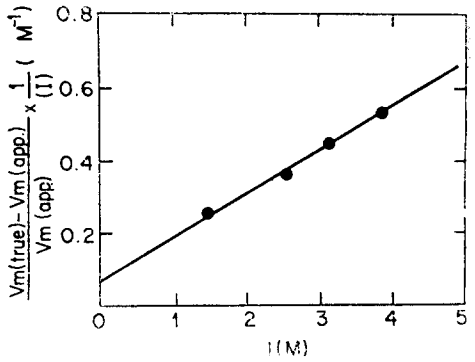

Fig. 5-a: Determination of Inhibition Constants for Methanol System.

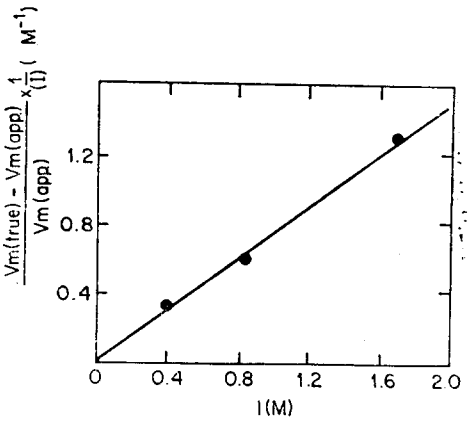

Fig. 5-b: Determination of Inhibition Constants for Ethanol System.

the Lineweaver-Burk plots in the low substrate concentration (Fig. 3). The V_m (app) value decreased with increasing methanol and ethanol concentrations while the K_m (app) values remained constant, which implies that the inhibition follows the noncompetitive type.

The second order inhibition constant was hypothesized as shown in Equation (1) since the conventional V_m/V_m (app) vs. (I) plot no longer yields a straight line (Fig. 4).

$$V_m(app)=\frac{V_m}{1+(I)/K_i+(I)^2/K_{ip}} \quad (1)$$

Equation (1) can be transformed to

$$\frac{V_m-V_m(app)}{V_m(app)}\times\frac{1}{(I)}=\frac{1}{K_i}+\frac{(I)}{K_{ip}} \quad (2)$$

The experimental data were plotted using the Equation (2) and an excellent straight linear correlation was shown (Fig. 5-a and 5-b). Each inhibition contant was determined from the intercept and slope of the graph. The first order inhibition constants determined were 14 M for methanol and 2.4 M for ethanol and the second order inhibition constants found were 83.3 M^2 for methanol and 27.0 M^2 for ethanol.

The exact inhibition mechanism by organic solvent is not certain as yet from our experimental data since the reaction velocity of the pure Δ^1-dehydrogenase cannot be determined from the multi-enzyme system such as *A. simplex* whole cells. However, one possible explanation is that such solvents may influence the enzyme reaction through the formation of enzyme-solvent complex or the electrostatic effect of the solvent to the enzyme-substrate complex as suggested by Scatchard[6] and Clement[7] . Partition model[8] and extraction model[9] were also proposed for the effect of organic solvents on the electrostatic interaction between enzyme and on the binding of the solvent molecules to enzymes. Regardless of the exact inhibition mechanism, our kinetic studies showed that non-competitive inhibition model agree very well with experimental results when the second order inhibition constant was introduced into the kinetic equation.

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