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Inhibition of Over-oxidation of 11β-17α, 21-trihydroxy-pregna-1, 4-diene-3, 2-dione in Fermentative Process.

Moo Bae
Applied Microbiology Lab , Korea Institute of Science and Technology
Seoul, Korea
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Prednisolone 발효중의 산화분해 저지법

배 무
한국과학기술연구소
응용미생물연구실

ABSTRACT

Inhibition of over-oxidation of prednisolone in the fermentation has been studied by using vegetative cells as enzyme source.

Firstly, A. simplex (ATCC 6946) was demonstrated to degrade prednisolone in the vegetative culture of the microorganism.

Over 72% of hydrocortisone was transformed into prednisoloneby 3 hours of the fermentation. However, the prednisolone produced was considerably oxidized forming over-oxidation product in 8hours of fermentation period with the intact cells.

Secondly, in order to depress the over-oxidation and the breakdown of the steroid skeleton of prednisolone, chelating agents such as $\alpha\alpha'$-dipyridyl, o-phenanthroline and 8-hydroxyquinoline were added to the fermentation broth. Consequently, the breakdown of prednisolone by the intact cells was able to be remarkably retarded and an intermediate regarded as an oxidized product derived from prednisolone was accumulated, by the addition of $\alpha\alpha'$-dipyridyl in the fermentation.

INTRODUCTION

Vischer et al[1] and Nobile[2] reported that it was possible to introduce a 1, 2 double bond into ring A of the steroid nucleus to transform hydrocortisone to $\Delta^{1,4}$-pregnadiene-11β 17α, 21-triol-3, 20-dione (prednisolone) through the use of *Corynebacterium simplex*.

Since then, a large number of microorganisms have been found to carry out 1-dehydrogenation of steroids.[3,4] And, as practical application of most important 1-dehydrogenation of steroids, prednisolone can be prepared from hydrocortisone by fermentative process or with the acetone-dried cells of *Arthrobacter simplex*. For the purpose of the optimal application of 3-ketosteroid-Δ^1-dehydrogenase, two stage fermentation can be employed for the production of 3-keto-$\Delta^{1,4}$-steroids. The first fermentation is run on as cell growth and as the enzyme production and the

second fermentation is run on as the enzyme reaction of the substrate. In second fermentation the acetone-dried cell enzyme and the vegetative cell enzyme can also be used as the reaction enzyme source. When the growing culture of *Arthrobacter simplex* was employed in preparation of the 1-dehydrosteroids, [9] a considerable amount of the steroid was found to yield over-oxidation products.

Fried et al[4] and Vischer et al[1] reported that the some molds cleaved the 17β-acetyl side chain while introducing a 1, 2 double bond into the ring A of 17-deoxysteroids, and that $\Delta^{1,4}$-steroid could be produced without over-oxidation by using selected species of *Alternaria*, *Calonectria* and *Ophiobolus*, and by *Didymella lycopersia*.

Schubert et al[5] and Dodson group[6] showed by the use of *Mycobacterium smegmatis* and *Arthrobacter* sp. respectively that the initial steps of the microbiological degradation of steroid skeleton might involve a 1-dehydrogenation and a 9α-hydroxylation with the cleavage of the cleavage of the steroid ring B.

Trufitt[7] isolated Proactinomyces sp. from soil capable of utilizing steroid which yield Windaus's keto acid as an interrmediate by the disruption of ringA at C_2-C_4.

Bae et al[8] previously reported that sterols were degraded ultimately to carbon dioxide and water by many soil bacteria and to accumulate androsta-1, 4-diene-3, 17-dionè and androst-4-ene-3, 17-dione in the presence of $\alpha\alpha'$-dipyridyl in medium.

Sih[10] reported that 9α-hydroxyprogesterone and 9α-hydroxy-androstene-dione accumulated in the progesterone fermentation with Nocardia restricts in the presence of KCN.

The methods of inhibiting the breakdown of the steroid skeleton were demonstrated by the use of chelating agent[8] and by the modification of steroids skeleton such as 6β, 19-oxido steroids resistant against the microbial attack. [10]

This paper reports that *A. simplex* was able to degrade prednisolone in the growing culture, and that chelating agents would be able to prevent the breakdown of the steroid skeleton in the process of preparing prednisolone ($\Delta^{1,4}$-steroids) from (Δ^4-steroids) hydrocortisone by fermentative process.

MATERIALS AND METHODS

Microorganism

The growing culture of *Arthrobacter simplex* (ATCC 6946) was used in the fermenitaton.

Fermentation

The seed culture of the microorganism was prepared in a culture medium consisting of 1% beef extract, 1% peptone and 0.5% sodium chloride by incubating at 28°C for 24 hours on a recipocal shaker. The pH of the medium is adjusted to 7.0 before sterilization. An aliquot of the vegetative culture coresponding to 5% was used to inoculate 100 ml Erlenmeyer flasks containing 20 ml of the medium. The culture was incubated at 28°C on a reciprocal shaker operating at 200 rpm for 72 hours and then followed by the addition of several 2-400 mg of finely powdered hydrocortisone and a given amount of a test chelating agent to the culture flasks. The fermentation was run on for subsequent 7-24 hours under the same conditions as mentioned above. A test compound in the inhibition experiment was added on the initial stage of fermentation at the final concentration of 10^{-3} and 10^{-4} M.

Extraction and Separation of Steroids

At the intervals of 7, 9 and 24 hours, the fermention broth was filtered with the aid of a diatomaceous earth at pH 5.0 and then steroids were extracted with an equal vloume of ethyl acetate twice. The extract of a broth sample was dried over anhydrous sodium sulfate and concentrated. Prednisolone in the extract was separated from the residual hydrocortisone and over-oxidation products by thin layer chromatography of kiesel gel G using benzene-dioxane (2:1,) as solvent system. The sterods on the plate were detected with conc. sulfuric acid heating at 110 °C for 10 min. or by UV light.

Detection and determination of hydrocortisone and perednisolone

The progresses of microbial conversion of Δ^4-steroid was detected and determined quantita-

tively. For the quantitative determination, an aliquot of the ethyl acetate extract of a fermentation broth was subjected to thin layer of kieselgel GF 254 nach stahl and chromatographed with the same solvent system. Prednisoloone was recognized as distinctive UV absorbing spot and hydrocortisone as a fluorescent spot on the thin layer.

The spots of prednisolone and hydrocortisone were scraped out under UV light and eluted out with dioxane and ethyl ether. The eluants were quantitatively determined in spectrograde methanol by spectrophotometer at 242 mμ. The standard curves of prednisolone and hydrocortisone were prepared using authentic samples by spectrophotometer and were linear over the range 0-20 μg /ml at 242 mμ.

Results and Discussion

1) Decomposition of hydrocortisone by A. simplex

Δ^1-3-ketosteroids decomosing ability of *A. simplex* has been recognized as mentioned above. And Naito et al[12] reported that the side chain degrading products of hydrocortisone were 11β-hydroxyandrost-4-ene-3, 17-dione and 11β-hydroxyandrosta-1, 4-diene-3, 17-dione as intermediates by the use of *Pseudomonas chloraphis* IAM 1511. In preparation of predrisolone in this experiment as shown in Table 1, it was also demonstrated that in the conversion of hydrocortisone into prednisolone, it did not reach 100% of the product, even if hydrocortisone as substrate completely disappeared from the culture broth. The result of Table I shows that the predrisolone, once transformed up to over 75% of hydrocortisone by 3 hours of fermentation, was considerabley decomposed in 8 hours of fermentation period when substrate was added to the vegetative culture at the concentration of 0.05 %. Thereafter, both substrate and the product have gradually disappered according to the progress of fermention. Thus, *A. simplex* ATCC 6946 was proved to degrade prednisolone in a vegetative culture of the microorganism. In the process to use the growing culture as the enzyme source, relatively large amount of hydrocortisone as substrate could be converted into prednisolone by a given amount of the growing culture, compared with the reaction by the aceton-dried cells as the enzyme source. The decomposition by over-oxidation could be reduced by the regulation of fermentation period and by the increase of the amount of hydrocortisone supplemented in powderey state up to 0.5%.

Table 1. The Yield of Prednisolone from Hydrocortisonoe and its Over-oxidation by the Action of *A. simplex.*

Fermentation periods	Concentations of substrate	Transformation	
		Recovery of Hydrocortisone	Prednisolone
2hr	0.05%	37.5%	56.3%
3	〃	20.0	72.5
4	〃	16.0	49.5
6	〃	14.5	46.8
8	〃	trace	31.3
3hr	0.2%	35.5	62.5
24	〃	10.0	77.0

Before the supplment of substrate for the fermentation, *A. simolex* was cultivated for 72 hours.

2) Inhibition of microbial over-oxidation of prednisolone

The microbial degradation of hydrocortisone might be presumably clssified to the following steps; (i) Δ^1-dehydrogenation, (ii) the cleavage of the 17β-acetyl side chain, forming C_{19}-steroids,[12] and the lactonization at the ring D, (iii) the breakdown at the ring B of the steroid skeleton,

forming 9α-hydroxy-steroid, followed by the formation of a phenolic A-ring and 9-keto-9, 10-seco-steroids.[13] αα′-dipyridyl, ortho-phenanthroline and 8-hydroxyquinoline as cheltating agents of mainly Fe^{2+} were added to the fermentation broth to depress the breakdown of the steroid skeleton of prednisolone. As it was estimated from the previous experiment[8] with cholesterol, αα′-dipyridyl was found to be the most favorable inhibitor for the depression of breaking the steroid skeleton of prednisolone in this fermentation, but did not depress the Δ^1-dehydrogenation of hydrocortisone by *A. simplex* as shown in Table 2. And hydrocortisone was mostly transformed into

Table 2. Effects of αα′-dipyridyl on the Depression of breaking Prednisolone in the Vegetative Culture of *A. simplex*.

Fermentation periods	Concentration	Hydrocortisone	Prednisolone	Oxidation product
3hr	none	18%	64%	−
〃	1×10^{-3}M	21	76	+
5hr	none	11	58	±
〃	1×10^{-3}M	9	66	+
7hr	none	trace	41	+
〃	1×10^{-3}	trace	60	++
10hr	none	trace	24	+
〃	1×10^{-3}M	trace	55	++
24hr	1×10^{-3}M	trace	53	+
48hr	1×10^{-3}M	trace	49	++

Concentration of substrate: 0.01%

1) Oxidation product: spot in Rf=0.07, using benzene dioxane (2:1) as solvent system of TLC. (+) detected, (−) not detected
2) Hydrocortisone: Rf=0.42 color with H_2SO_4: Yellow green
3) Prednisolone : Rf=0.29 color with H_2SO_4: Red brown

prednisolone in 8 hours with or without αα′-dipyridyl. The metabolite formed from prednisolone was detected at 0.07 of Rf value (Rf of prednisolone: 0.29) on the thin-layer plate developed with benzene-dioxane (2:1) as solvent system. The oxidation product, presenting red brown color with conc. H_2SO_4 spraying, seems to be an intermediate with the cleavage of 17-acetyl side chain of prednisolone[12] or certain oxidized intermediate. Although the oxidation product temporary formed without αα′-dipyridyl, it was steadily accumulated on the addition of αα′-dipyridyl in the growing culture. The reaction step of the inhibition by the agent appears to be estimated from the result that androst 4-ene-3, 17-dione, and 21-hydroxy-prega-4-ene-3, 20-dione were good substrates for the 9α-hydroxylase from *Nocardia restrict.*[14]

요 약

Hydrocortisone 에서 Prednisolone 을 발효학적으로 제조함에 있어서 발생하는 산화분해 현상을 방지하기 위하여 저해제를 이용한 방법이 연구되었다.

첫째, A. simplex (ATCC 6946)의 생균을 사용하여 발효시키는 과정에서 Prednisolone이 분해된다는 현상을 밝혔다. 발효후 3시간만에 기질인 hydrocortisone의 72% 이상이 prednisolone으로 전환되었으나 시간이 경과하여 8시간이 지나면 이균의 생균세포는 상당량의 prednisolone을 산화 분해하였으며 한편으로는 분해중간산물을 생성하였다.

두째로는, prednisolone의 산화분해를 억제하기 위하여 αα′-dipyridyl과 같은 chelating agents를 발효액에 첨가하였다. 그 결과 이균의 생균세포에 의한 steroid 핵의 산화분해는 현저히 억제되었으며 또한 산화중간산물이 축적되었다. 이중간산물은 αα′-dipyridyl의 첨가에 의하여 한층 현저하게 나타났다.

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