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17∝ - HYDROXYLATION OF PROGESTERONE BY *CUNNINGHAMELLA ECHINULATA* ON A LABORATORY FERMENTOR SCALE

ISMAIL A. EL-KADY and MOHAMED R. METWALI
Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt.

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ABSTRACT

The mircrobiological transformation of progesterone by a local isolate of *Cunninghamella echinulata* using a laboratory fermentor was studied. Progresterone (10-50 g/l) wetted by Tween 80 was added to 48-hour old culture and the transformation was left to proceed for 72 hours. Thereafter, the different transformation products were resolved chromatographically. The identity of each product was established through the determination of m.p., mixed m.p., optical rotation and ultraviolet as well as infrared absorption spectra. A comparison of the R_f values of each product with that of the corresponding reference using different solvent systems as well as their colour expressed with two spray reagents, was used as a further proof for the identity of the isolated products. With all concentrations of progesterone tested, maximum yield of 17∝ -hydroxyprogesterone was obtained after 48 hours of fermentation Progesterone concentrations of 10 and 20 g/l were almost quantitatively converted to the different transformation products after 72 hours of fermentation. Using a concentration of 20 g/l and incubation period of 48 hours, the transformation product mixture consisted of unchanged progesterone (6%), 17∝ -hydroxyprogesterone (54%),11∝-hydroxyprogesterone (29%) and 11∝,17∝-dihydroxyprogesterone (2.5%).

INTRODUCTION

The importance of the microbial hydroxylation of progesterone by Mucorales fungi (Murrary & Peterson, 1955) led to a new technology for manufacture of adrenocortical hormones and introduced the use of fungi as a source of enzymes for microbiological transformation of steroids. 17∝ -hydroxy group is one of the inportant constituents of all anti-inflammatory steroid molecules. In a previous study (Metwali, 1980), out of 147 fungal isolates tested, an isolate of *Cunninghamella echinulata* was found to be the most active 17∝ -hydroxylator. The role of some culture conditions that influence the 17∝ -hydroxlation of progesterone with the local isolate of *C. echinulata* was studied (Metwali, 1980).

The present study was promoted to extend our investigations to evaluate the potentiality of this local isolate for the production of 17∝ -hydroxyprogesterone on a laboratory fermentor scale.

MATERIALS AND METHODS

Experimental organism:

The local isolate of *Cunninghamella echinulata* (Thaxt.) Thaxt. ex Blakeslee, isolate No. 77, (No. of isolate in culture collection, Mycological Laboratory, Assiut University, Egypt), was used.

Medium:

The medium previously used (Metwali, 1980) as conductive for 17∝ -hydroxlation was used as a nutritive solution. This medium has the following composition (g/l of tap water): glucose, 20; starch, 20; corn-step liquor (solid), 7; $MgSO_4$. $7H_2O$,1; sodium acetate, 1; KH_2PO_4, 2; olive oil, 5 ml/1. Medium was adjusted to pH 6.5.

Description of Fermentor:

The fermentor constructed by Hromatka and Ebner (Hromataka and Ebner, 1951) was employed. This fermentor consists of an 80 cm long glass tube of 12 cm in diameter. To one end of this tube, a sintered glass disc (porosity G 4) was fused. This end was drawn to form funnel shaped part. A delivery tube was made at a distance of 3 cm ober the sintered glass disc and was used for the withdrawal of samples of liquid cultures. Air was passed through the opening of the funnel shaped end of the fermentor. The flowing air passing through the sintered glass disc was divided into small bubbles which were finely dispersed into the liquid culture medium. The air flow passing into the fermentor attained a good mixing of all portions of the culture medium, thus allowing a good distribution of oxygen throughout all the culture medium. The fermentor was sterillized apart from the culture medium and then charged with the sterile medium under aseptic conditions just before inoculation.

Aeration:

A compressor was used to introduce air into the fermentor. The compressed air was passed first into concentrated sulphuric acid followed by cotton wool filter. The dry sterile air was then charged with water vapour by passing it through sterile distilled water, before passing it into the fermentor. Aeration rate was one of air per one litre of medium per minute (vol. / vol. min.).

Inoculum:

The inoculum was prepared by scraping the surface of 10 slants of 72-hour old culture of the experimental organism in 100 ml of the basal medium. Each 100 ml inoculum was used to inoculate 1900 ml culture medium in order to furnish a final volume of two litres in the fermentor.

Cultivation:

The fermentor was shaken at 110 rpm on a 2. inch strocke rotary shaker in a thermostatically controlled incubation room adjusted at 30°C. Progresterone was weighed into a 250 ml Erlenmeyer flask, wetted with Tween 80 solution to a level of 0.01% and shaken. The flask was then exposed to live steam at one atmospheric pressure for 30 min. Progesterone was added to the fermentor after 18 hours of cultivation. One hundred ml samples of fermentation medium drawn and analyzed after 24,48 and 72 hours of the addition of progesterone. All experiments were done in triplicate, and yields were reported as averages.

Extraction:

One hundred ml samples of the fermentation medium were homogenized in a blender (16000 rpm) with 200 ml chloroform. The extraction was repeated three times. The combined chloroform extracts were washed with 100ml of 5% sodium bicarbonate solution, followed by an equal distilled water, dried over anhydrous sodium sulphate, filtered, then distilled to give a semi-solid residue.

Analysis of the mixture of the transformation products:

The semi-solid residue was dissolved in the iminimal volume of benzene and then fractionated on standard activated alumina column (100 g). The following sequence of solvents was used: n-hexane: benzene (1:1); benzene cotaining different concentrations of choloroform and benzene containing different concentrations of methanol. The fractions containing similar products were collected and crystallization from a suitable solvent was carried out whereby determination of m.p., mixed m.p., $(\alpha)_D$, IR and UV absorption spectra were made.

Further proof of the identity of ecah product was attained by the application of thin-layer chromatographic technique (El-Kady and Allam, 1973). The solvent systems used were I: Cyclohexane: acetone: chloroform (75:25:20), II: Ethylene chloride : acetone (80:20), III: benzene : ethyl acetate : acetone (60:20:20) and IV : Chloroform : cyclohexane isopropanol (50:100:20).

The plates were then sprayed with two different colour regents namely: ½/KI (Sallam *et al.*, 1969), and chlorosulphonic acid : acetic acid (3:1) (Waldi, 1965).

RESULTS AND DISCUSSION

Effect of concentration on the microbiological transformation of progesterone:

All progesterone concentrations tested, produced noticeably higher concentrations of

17 ∝ -hydroxyprogesterone than 11 ∝ -hydroxyprogesterone (Tables 1 & 2). With all concentrations investigated, a maximum yield of 17 ∝ -hydroxyprogesterone was obtained after 48 hours of fermentation. The decrease of 17 ∝ -hydroxyprogesterone concentration, which occurred after 72 hours, was associated with a subsequent increase in the formation of 11 ∝ , 17 ∝ -hydroxyprogesterone. At the lower concentrations of the added progesterone (10 and 20 g/l). 11∝ -hydroxyprogesterone reached a maximum concentration after 48 hours of fermentation which remained more or less constant until the end of incubation period. Therefore, it could be concluded that 11 ∝ -dihydroxyprogesterone were derived from 17 ∝-hydroxyprogesterone via 11 ∝ -hydroxylation. In other words 17 ∝ -hydroxyprogesterone, not 11∝-hydroxyderative,is the precursor of 11 ∝ , 17 ∝ -hydroxyprogesterone.

The present conclusion is in accordance with that of Capek *et al.* (1966), who states that 17 ∝ -hydroxylated compound is mainly hydroxlated in the 11 ∝ -position, and 11 ∝ , 17 ∝ -dihydroxy derivative was the principal and final metabolite. Allam and El-Kady (1975), noted that 11 ∝ , 17 ∝ -dihydroxyprogesterone was formed by the action of 11∝-hydroxylase enzyme preparation on 17 ∝ -hydroxyprogesterone, using cellfree extracts of *Rihizopus nigricans* NRRL 1477.

The data given in Table (1) indicate that progesterone concentrations of 10 and 20 g/l can be almost quantitatively converted to the different transformation products, after 72 hours of fermentation. On the other hand, at the higher concentrations of progesterone tested, the rate of hydroxylation decreased. Only about 40 percent conversion was obtained after 72 hours when 50 g/l of progesterone was used. It is of interest to note that very little of the dihydroxylated products (11∝, 17∝ -dihydroxyprogesterone) were formed in the higher concentration experiments. Similar fermentations were conducted at low concentrations (10 and 20 g/l), resulted in extensive formation of the dihydroxyprogesterone. Hence one may conclude that so long as some progesterone remains in the fermentation medium, it will be preferentially hydroxylated to the monohydrocy form and that little or no dihydroxylation takes place, until all or most the untreated progesterone has been monohydroxylated. For this reason, dihydroxylation apparently occurs more readily in fermentation, at low concentrations.

Chromatographic resolution of the transformation mixtures:

The early fraction removed with n-hexane : benzene (1:1) after evaporation and crystallization from chloroform: methanol mixture yieled a crystalline product (0.12 g), m.p. 128-129°C, $(\alpha)_D$+195°. This substance was found to be unchanged

progesterone. Elution with 5% choloroform in benzene gave a solid residue, which after crystallization from chloroform: methanol yielded 0.95 g crystals m.p. 217-219°C, $(\alpha)_D$+120.3°, $\lambda_{max.}^{alc.}$ 239 mμ(ε =12.920). The product did not depress the m.p. of an authentic sample of 17∝ -hydroxyprogesterone (reported m.p. 210-214°C) (McAleer & Dulaney, 1956). Changing the polarity of the solvent, using 20% chloroform in benzene, eluted a residue which upon crystallization from methanol gave 0.58 g of crystalline material, m.p. 165-167°C, $(\alpha)_D$+175°, $\lambda_{max.}^{alc.}$ 242 mμ, (ε =12.960). The product did not depress the m.p. of an authenic sample of 11 ∝ -hydroxyprogesterone (reported m.p. 166-168°C, $(\alpha)_D$+176° (Peterson *et al.* 1952)). The combined fractions, eluted with 5% methanol in benzene when crystallized from methanol contributed 0.05 g crystals, m.p. 216-218°C, $(\alpha)_D$+72°, $\lambda_{max}^{alc.}$ mμ(ε =10.100). No depression in the m.p. was observed, when the product was mixed with an authentic sample of 11 ∝, 17 ∝ -dihydroxyprogesterone (reported m.p. 220-222°C, $(\alpha)_D$+76° (Peterson *et al..*, 1953).

This markedly high yield of17∝-hydroxyprogesterone using *C. echinulata* (isolate No. 77) at laboratory fermentor scale, strongly recommends the employment of this isolate for the technical production io 17 ∝ -hydroxyprogesterone.

Table 1

Transformation of Progesterone with *Cunninghamella echinulata* Isolate No. 77

Progest-erone (g/l)	Time (hr)	Residual progest-erone (g/l)	11 ∝-Hydroxy progestrone (g/l)	17 ∝ -Hydroxy-progesterone (g/l)	11 ∝, 17∝Dihydroxy-progesterone (g/l)
10	24	4.2	1.5	3.5	—
10	48	1.0	3.6	4.5	0.5
10	72	—	3.8	2.6	2.7
20	24	8.8	3.2	7.4	—
20	48	1.7	5.8	10.7	0.5
20	72	—	5.8	7.3	6.3
30	24	12.2	4.5	7.2	—
30	48	8.6	7.7	9.8	—
30	72	5.3	9.8	9.4	0.9
50	24	39.0	4.3	5.7	—
50	48	32.8	5.9	9.4	—
50	72	29.5	8.4	9.8	—

Table 2

The R_f values of colours of the different transformation products obtained from progesterone bioconversion by *C. echinulata.*

Products	R_f × 100 with solvent system				I_2/KI	Chlorosulphonic/acetic acid	
	I	II	III	IV		Day light	UV
17 ∝ -hydroxyprogesterone	88	83	97	85	weak pink	brown	bluish violet
11∝-hydroxyprogesterone	75	61	74	72	deep blue	brown	greenish yellow
11 ∝ , 17 Dihydroxyprogesterone	48	46	67	57	deep blue	brown	yellow

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إنتاج مادة ١٧ ألفا هيدروكسي البروجستيرون على مستوى المخمر المعملي بواسطة فطرة كاننجهاميلا ايكينولاتا

إسماعيل عبد الرزاق القاضي و محمد رضا متولي

تم استخدام مخمر صناعي سعة ٢ لتر لاختيار مقدرة الفطرة على تكوين هذه المادة في ظروف تشبه تلك المطبقة في الصناعة . وبدراسة تركيزات متعددة من مادة البروجستيرون تتراوح ما بين ١٠ جرام/لتر إلى ٥٠ جرام/لتر ، وجد أن أنسب التركيزات المختبرة هو تركيز ٢٠ جرام من البروجستيرون لكل لتر من الوسط الغذائي ، حيث تم تحويل كل البروجستيرون المضاف إلى المشتقات المختلفة خلال ٧٢ ساعة من بدء الاضافة . ووجد أن أعلى معدل لتكوين مادة ١٧ ألفا ـ هيدروكسي البروجستيرون كان بعد ٤٨ ساعة من بدأ إضافة البروجستيرون .

عند فصل المواد الناتجة من تحول البروجستيرون بواسطة الفطرة المستخدمة وذلك بواسطة اعمدة الفصل باستخدام مادة الالومينا وجد أن البروجستيرون يتحول إلى :

١٧ ألفا ـ هيدروكسي البروجستيرون (٥٤٪)

١١ ألفا ـ هيدروكسي البروجستيرون (٢٩٪)

١١ ألفا ، ١٧ ألفا ـ ثنائي هيدروكسي البروجستيرون (٥ر٢٪) .