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SHORT COMMUNICATION

11β-HYDROXYLATION OF STEROIDS BY *COCHLIOBOLUS LUNATUS*

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Summary— The substrate specificity of 11β-hydroxylase of *Cochliobolus lunatus* was studied and a close parallelism to the results obtained with 11α-hydroxylase of *Rhizopus nigricans* was observed. It was found that the cell wall does not differentiate between the steroid substrates used and the absence of the cell wall increases the bioconversion.

INTRODUCTION

We have recently studied the 11α-hydroxylation of steroids with *Rhizopus nigricans* and it seemed desirable to compare our findings on the effect of the structure of steroid substrates on 11α-hydroxylation [1] with the effect of the same structural features, as used in the above study, on the 11β-hydroxylation. *Cochliobolus lunatus*, shown by Hörhold *et al.*[2] to be an excellent 11β-hydroxylator, hydroxylating progesterone under our conditions almost exclusively at the position 11β in good yield (up to 90%) was chosen for this purpose.

EXPERIMENTAL

Chemicals

Alginic acid (sodium salt), cycloheximide and succinic acid were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), steroid substrates and their 11β-hydroxylated products from Steraloids Inc. (Wilton, U.S.A.). Lytic enzyme NOVOZYM TM 234 purchased from Novo Industri A/S (Bagsvaerd, Denmark).

Microorganism

Cochliobolus lunatus m 118 from the strain collection of the Friedrich Schiller University of Jena, G.D.R., was kindly supplied by Dr C. Hörhold. Cultivation conditions were the same as described by Plemenitaš *et al.*[3].

Bioconversion of steroids with induced mycelium

For the induction of 11β-hydroxylase in 24 h old mycelium of *Cochliobolus lunatus*, essentially the same conditions as those used for the induction of 11α-hydroxylase in *Rhizopus nigricans* [4], were chosen. Induced mycelium (2 g, wet wt) was resuspended in 10 ml of osmotically stabilized buffer, pH 5.0 (OS buffer: 0.05 M succinic acid/NaOH, with 1 M NaCl as osmotic stabilizer). Steroid substrates (100 μg/ml final conc.) were incubated in the presence of cycloheximide (100 μg/ml final conc.) at 301 K for 3 h. The reaction products were extracted with chloroform, separated by TLC on pre-coated silica gel 60F-254 plates (Merck, Darmstadt, G.F.R.) with the solvent system $CHCl_3$–CH_3OH–H_2O (94:6:0.5, v/v) and evaluated densitometrically with a CAMAG TLC Scanner II.

Immobilization of Cochliobolus lunatus mycelium

400 mg (wet weight) of 24 h old mycelium were washed with OS buffer and immobilized in 2% Ca-alginate beads as described by Komel *et al.* [5]. After 1 h, the resulting beads were washed with 1 M NaCl, resuspended in OS buffer and used for the bioconversions of steroids.

Formation and immobilization of protoplasts

1 g (wet wt) of 24 h old *Cochliobolus lunatus* mycelium was suspended in 10 ml of OS buffer and incubated overnight with NOVOZYM TM 234 (2 mg/ml final conc.) at 301 K. After separation from undigested mycelium through a No. 1 glass filter, the protoplasts were washed with OS buffer. The resulting protoplasts were sedimented at 1700 *g* and the final concentration of protoplasts was adjusted to 10^7 protoplasts/ml. Protoplasts were immobilized in 2% Ca-alginate beads [5]. Immobilized protoplasts were washed with 1 M NaCl, suspended in OS buffer and tested for 11β-hydroxylase activity.

Bioconversion of steroids with mycelium and protoplasts of Cochliobolus lunatus

The activities of protoplasts and mycelium (both free and immobilized) were tested by the addition of steroid substrates to the mycelium in 10 ml of OS buffer (100 μg/ml final conc.) or an adequate quantity of protoplasts in 5 ml of OS buffer. Adequate quantities of mycelium and protoplasts were established on the basis of protein content [6]. In a parallel assay, equal quantities of progesterone (18 μg/ml final conc.) were added to a quantity of immobilized protoplasts in 5 ml of OS buffer and to an equal quantity of reverted protoplasts, which reverted to the mycelial form in 24 h after the addition of glucose. The hydroxylated products were extracted with chloroform after 4, 6, 8 and 18 h from the reaction mixture and from filtrates, protoplasts and mycelium (both free and immobilized) separately. The hydroxylated products were evaluated densitometrically.

For the repeated semicontinuous procedure, the immobilized protoplasts were filtered, washed with 1 M NaCl and resuspended in 5 ml of fresh OS buffer every 24 h, with the addition of progesterone. The yield of 11β-hydroxyprogesterone in the filtrates was estimated densitometrically after separation on TLC.

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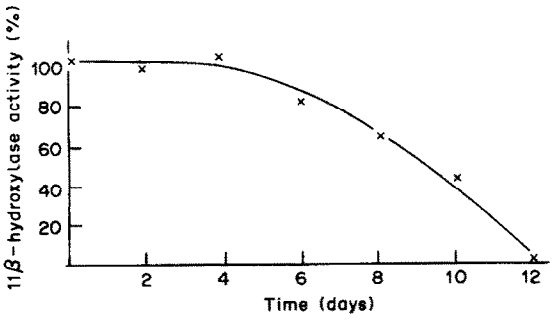


Fig. 1. The maintenance of 11β-hydroxylase activity of immobilized *Cochliobolus lunatus* protoplasts in the bioconversion of progesterone. Initial activity is set at 100%.

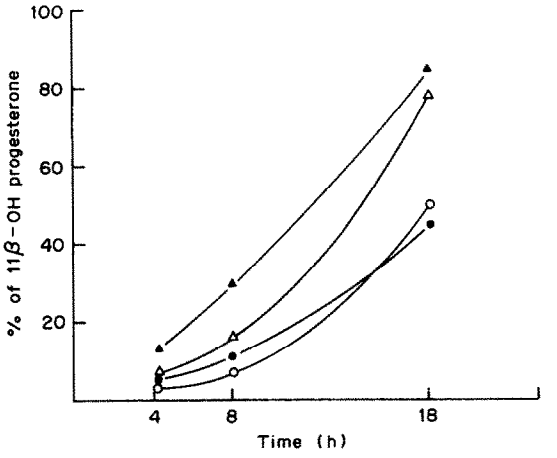


Fig. 2. Bioconversion of progesterone by free and immobilized systems of *Cochliobolus lunatus*. (▲) Immobilized protoplasts, (●) immobilized mycelium, (△) free protoplasts, (○) free mycelium.

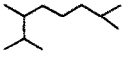
RESULTS AND DISCUSSION

Quantitative results of bioconversions with mycelium, previously induced with progesterone, are presented in Table 1 (column 1). The bioconversion of progesterone gives the highest yield of a 11β-hydroxylated product and its yield was, therefore, taken as 100%. If the polarity of the side chain increases, the yields of 11β-hydroxylated products decrease and at the same time the amount of byproducts increases. On the other hand, if the side chain is nonpolar, no products can be detected. When these results are compared with those obtained with the 11α-hydroxylase of *Rhizopus nigricans* [1], an exact parallelism becomes evident.

Possible factors besides the side chain structure that might affect the hydroxylation of steroid substrates were discussed in our previous paper [1], In addition, the assumption that the cell wall might play a role as a limiting barrier, was supported by the findings of Sedlaczek *et al.*[7], who found that the cell wall of *Cunninghamella elegans* affects the bioconversion of cortexolone.

The obvious choice for an investigation of the effect of the cell wall on the bioconversion of steroids is the use of protoplasts. By entrapping them in Ca-alginate gel a system suitable for manipulation was obtained. To test whether the immobilization affects the 11β-hydroxylation in any way, the bioconversions were carried out with both free and immobilized protoplasts. Since the immobilization was carried out under mild conditions, the 11β-hydroxylase activity in immobilized protoplasts remained at the same level as in

Table 1. Bioconversion of steroids by *Cochliobolus lunatus*

R	Substrate	Yields[a] (%) of 11β-hydroxylated products relative to 11β-hydroxyprogesterone		
		Induced mycelium*	Mycelium**	Protoplasts**
	Progesterone	100	100	200
	21-Hydroxyprogesterone	72	52	122
	17α-Hydroxyprogesterone	40	44	130
	17α, 21-Dihydroxyprogesterone	28	22	46
	4-Cholesten-3-one	0	0	0

[a]Results are the means of 3 experiments. *Bioconversion of steroids (3 h) in the presence of cycloheximide with mycelium previously induced with progesterone in 3 h. **Bioconversion of steroids (6 h) used as inducers and substrates of 11β-hydroxylase.

free protoplasts. At 301 K, immobilized protoplasts preserved their 11β-hydroxylating activity over a period of 8 days, 5 days at the same level and gradually decreasing thereafter (Fig. 1). When the protoplasts were stored at 277 K for 3 weeks, regeneration to mycelial form did not occur and their viability declined only after that time. Progesterone as the best substrate for the induced 11β-hydroxylase was used simultaneously as the inducer and substrate of the hydroxylating enzymes. The product of the bioconversion 11β-hydroxyprogesterone was detected almost exclusively in the medium. In the immobilized protoplasts a small retention of 11β-hydroxyprogesterone in the beads indicated a slower release of the product throughout the beads.

Protoplasts of *Cochliobolus lunatus*, both free and immobilized, were found to be more active in 11β-hydroxylation than the free and/or immobilized mycelium (Fig. 2). This was proven by two different approaches: 11β-hydroxylase activity was tested with 24 h old mycelium and an adequate quantity of protoplasts prepared from mycelium of the same age. In an alternative approach, a quantity of immobilized protoplasts, prepared from 24 h old mycelium, were reverted to the mycelial form in 24 h after the addition of glucose to the incubation medium. Immobilized reverted protoplasts and intact protoplasts were tested for 11β-hydroxylase activity. The yield of 11β-hydroxyprogesterone with free and/or immobilized protoplasts was twice as great as with free and/or immobilized mycelium. These results prove unequivocally that under our conditions the cell wall has a negative effect on the bioconversion of progesterone by *Cochliobolus lunatus*.

There is of course the possibility that the cell wall can differentiate between various steroids. Therefore, the same steroids used as substrates for the induced 11β-hydroxylase of *Cochliobolus lunatus* mycelium (Table 1), were simultaneously used as inducers and substrates of the hydroxylating enzyme in the protoplasts and mycelium of *Cochliobolus lunatus*. The yields of 11β-hydroxylated products in Table 1 are expressed relative to that of 11β-hydroxyprogesterone. Protoplasts were found to be 2–3 times more active in 11β-hydroxylation than the mycelium for all the substrates used, with the exception of 4-cholesten-3-one, which is neither an inducer nor a substrate for the protoplasts or mycelium of *Cochliobolus lunatus*.

From all the above mentioned results it can be concluded that the cell wall as a limiting barrier negatively affects the bioconversion of steroid substrates, but the cell wall seems not to differentiate between the steroid substrates used. Greater changes in the structure of the side chain seem to be necessary for the cell wall to recognize them, as demonstrated by Sedlaczek *et al.* [7].

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