BIOSYNTHESIS OF SIDERAMINES IN FUNGI. FUSIGEN SYNTHETASE FROM EXTRACTS OF *FUSARIUM CUBENSE**

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1. Introduction

Fusigen, a cyclic triester possessing three hydroxamic acid functions [2], is synthesized by many fungi grown under iron-deficient conditions [3, 4]. Labelled δ-*N*-hydroxyornithine and *cis*-fusarinine are readily incorporated into fusigen in washed mycelia from *Fusarium cubense* and *Penicillium chrysogenum* [1]. In a previous publication we reported the detection and partial characterization of rhodotorulic acid synthetase from extracts of *Rhodotorula glutinis* [5]. Following a similar experimental procedure, we succeeded in finding enzymatic activity for fusigen synthesis in cell-free extracts from *Fusarium cubense*.

2. Materials and methods

Fusarium cubense Smith (Tü 133) was cultured, its mycelial weight and sideramine content measured, and the radioactivity counted as previously described [2, 3, 6]. For preparation of cell-free extracts, the mycelium was filtered through a Büchner funnel, washed with 0.1 M Tris-buffer (pH 7.2), resuspended in twice the amount of buffer (containing 0.04 M mercaptoethanol) and sonicated for 1.5 min (Branson Sonifier B-12). After centrifugation at 30 000 *g*, the sediment was discarded, the supernatant serving as a crude extract. Nucleic acids were separated from the extract by precipitation with 1.0% streptomycin sulphate.

The incubation mixture for the fusigen synthetase test consisted of (in a total volume of 3.5 ml): 500 μmoles Tris–HCl pH 7.2; 100 μmoles $MgCl_2$; 50 μmoles ATP; 2 μmoles mercaptoethanol; 0.45 μmoles *cis*-fusarinine (= 32 000 dpm) and approx. 20 mg protein of the crude extract. After incubation for 10 min at 37°C, 1 mg $FeCl_3 \cdot 6H_2O$ and 1.3 mg sideramine (containing 80% fusigen and 20% fusigen B) were added to the incubation mixture and the protein precipitated by the addition of 50 ml ethanol. The sideramines were then extracted by chloroform–phenol and the fusigen purified by electrophoresis on gelatinized cellulose acetate strips [2].

Isotopic exchange between pyrophosphate and ATP was measured according to the Walker et al. [7] modification of the method described by Calendar and Berg [8] (incubation conditions: 10 min. 37°C).

[^{14}C]*cis*-fusarinine was prepared by fermentation of *Fusarium cubense* in the presence of [2-^{14}C]DL-mevalonic acid, purification of the fusigen by CM-cellulose chromatography followed by careful hydrolysis at pH 13 [1, 9, 10]. Labelled mevalonic acid was purchased from Buchler-Amersham, Frankfurt, and [^{32}P]sodium pyrophosphate from NEN Chemicals, Dreieichenhain.

3. Results

During in vivo experiments with *Fusarium cubense*, it was shown that double-labelled *cis*-fusarinine was incorporated into fusigen without splitting of the

* Metabolic products of microorganisms, 127; for preceding publication see [1].

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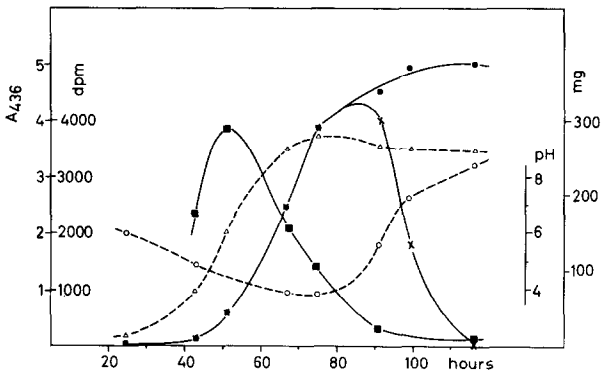

Fig. 1. Fermentation of *Fusarium cubense*. Mycelial weight (△---△---△); pH (○---○---○); total sideramines (●—●—●) and fusigen (X—X—X) production; fusigen synthetase activity (20 mg protein/test; ■—■—■).

hydroxamic acid bond [1]. Therefore, we incubated [^{14}C]*cis*-fusarinine with the crude extract supernatant after disruption of the mycelium by ultrasonic treatment and centrifugation at 30 000 *g*. When we detected incorporation of the radioactivity into fusigen, we developed the test procedure described in Materials and methods, and followed the enzyme activity during the course of fermentation. The results are shown in fig. 1.

The specific activity maximum appears 50 hr after inoculation: from 50–70 hr the decrease in specific activity is compensated by further increase in mycelial weight which results in rapid fusigen synthesis. The total enzyme activity drops sharply after 75 hr of fermentation, and soon thereafter fusigen is hydrolyzed.

In all further studies, the mycelium was harvested approximately 50 hr after inoculation. Because we had found that an unusually high concentration of ATP is

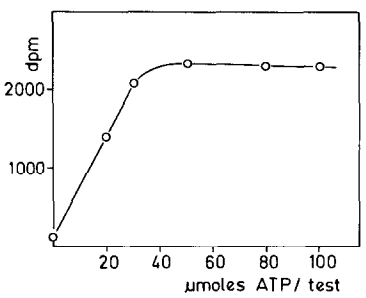

Fig. 2. Dependence of fusigen synthetase on the concentration of ATP in the test.

Table 1
Characteristics of fusigen synthetase.

Test system	Incorporation of *cis*-fusarinine in fusigen (%)
Complete	100
$-MgCl_2$	43
–ATP	7
–ATP + 50 μmoles UTP	9
–ATP + 50 μmoles GTP	7
–ATP + 50 μmoles CTP	0

necessary for optimal activity of the rhodotorulic acid synthetase, we tested the fusigen synthetase for ATP-dependency (fig. 2). Whereas the test without added ATP exhibited almost no activity, maximal enzyme activity was obtained with 50 μmoles ATP/3.5 ml test volume.

Another experiment showed the dependence of the enzyme on Mg^{2+}. ATP could not be substituted by UTP, GTP, or CTP (table 1).

Results on the specificity of pyrophosphate-exchange are given in table 2.

The exchange of approximately 40 nmoles may be compared with the formation of 17 nmoles of fusigen by 20 mg protein of crude supernatant which may be calculated from the test for specific activity in fig. 1. That means a more than 10-fold rate for exchange compared with product formation.

The reaction was inhibited by 1 μmole pCMB in the test to 16% of its original activity. Coenzyme A had no effect in stimulating, nor did either 200 or 500 μg

Table 2
Substrate dependence of ATP-pyrophosphate exchange by ammonium sulphate-(*s* = 0.8) precipitated protein from *Fusarium cubense* (crude fusigen synthetase). The test con-
The test contained 4 mg protein and 76 120 dpm (= 2 μmoles) sodium pyrophosphate.

Substrate added (2 mM)	dpm	Exchange (nmoles)
None	6850	179
δ-*N*-Hydroxy-L-ornithine	6700	176
trans-Fusarinine	6350	166
cis-Fusarinine	8400	220

RNAase have an effect in reducing the enzyme activity.

No fusigen synthetase activity could be detected when *Fusarium cubense* was grown in a medium containing 20 mg $FeCl_3 \cdot 6H_2O$/l.

4. Discussion

The reaction that was studied, namely the cyclization of three molecules of *cis*--fusarinine to the triester, is similar to the biosynthesis of enterochelin (enterobactin) [11, 12], the cyclic triester of dihydroxybenzoylserine [13, 14].

Bryce and Brot concluded from their experiments, that all components of the ring system are bound to the same enzyme-complex either as adenylates or thioesters.

From our experiments in vivo and in vitro, it may be concluded, that fusigen is synthesized from the monohydroxamic acid *cis*-fusarinine without the appearance of either a linear dimer or trimer. These compounds, which can be found in later stages of the fermentation [2, 15, 16], are mostly products of the hydrolysis of fusigen. However, there are indications that smaller amounts of the linear dimer or trimer may be synthesized under special conditions.

cis-Fusarinine is formed from hydroxyornithine and the coenzyme A-derivative of *cis*-anhydromevalonic acid [9, 17] in a reaction comparable to that between hydroxyornithine and acetyl-CoA studied by Ong and Emery [18].

The characteristics of the fusigen synthetase reaction resemble the biosynthesis of other cyclic peptides and esters, e.g. gramicidin S [19] and enterochelin. Further work on the purification, and investigation of molecular properties of fusigen synthetase and regulation of its biosynthesis is in progress.

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