BIOSYNTHESIS OF SIDERAMINES IN FUNGI. RHODOTORULIC ACID SYNTHETASE FROM EXTRACTS OF *RHODOTORULA GLUTINIS* *

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1. Introduction

A scheme for the biosynthesis of sideramines in fungi was proposed in 1970 [2]. In a series of experiments we have measured the incorporation of labelled monohydroxamic acids into the products of different fungi [3], but despite may efforts no enzymatic activity for sideramine biosynthesis could be found so far in extracts from the mycelia. As nothing is known on the number, localization and properties of enzymes synthesizing cyclic hexapeptides in fungi, we looked for the enzyme catalyzing the synthesis of the simplest fungal dihydroxamate, rhodotorulic acid [4, 5], in extracts of *Rhodotorula glutinis*. Recently, protonated amino acid precursor studies on rhodotorulic acid biosynthesis in whole cells of *Rhodotorula pilimanae* were reported by Akers et al. [6].

2. Materials and methods

[1-^{14}C]Acetic anhydride was purchased from Amersham Buchler, Frankfurt, [^{32}P]sodium pyrophosphate from NEN Chemicals, Dreiieichenhain, N^5-acetyl-L-ornithine from Pierce Chemical Co., Rockford, Ill.

Rhodotorula glutinis (Fres.) Harrison var. *dairenensis* Hasegawa et Banno CBS 4406 was maintained on yeast extract-malt extract agar slants. Submerse cultivation was done in 150 ml medium (glucose 20 g; L-asparagine · H_2O 5.68 g; $MgSO_4 \cdot 7H_2O$ 1.0 g; K_2HPO_4 1.0 g; $CaCl_2 \cdot 2H_2O$ 0.5 g; 1000 ml aqua dest.) in 500 ml Erlenmeyer flasks with one buffle on a rotary shaker (120 rpm) or in 5 l medium in a New Brunswick Microferm bench top fermentor (230 rpm, 2 l air/min) at 27°. The inoculum was 3% of a 2-day old culture.

The hydroxamic acid test and purification procedures were as previously published [7, 8]. For the calculation of rhodotorulic acid concentrations a value of $A^{1\%}_{436\,nm} = 44$ was used.

Cells were centrifuged after harvesting and resuspended in equal volume of 0.1 M Tris buffer pH 7.2 containing 4×10^{-2} M mercaptoethanol. The suspension was frozen at −20° and pressed four times through a 1.2 mm hole of the X-press (AB Biox, Nacka, Sweden; type X25). After thawing the suspension was centrifuged for 30 min at 30 000 *g*. The protein content of the supernatant was 10 mg/ml.

The incubation mixture for the test of RA synthetase activity contained in a total volume of 3.4 ml: 500 μmoles Tris-HCl pH 8.5; 100 μmoles $MgCl_2$; 20 μmoles mercaptoethanol; 50 μmoles ATP; 0.29 μmoles [acetyl-^{14}C]N^5-acetyl-N^5-hydroxyornithine (= 72 000 dpm); 2.0 ml of the crude extract supernatant. After 10 min of incubation at 37°, 100 μg rhodotorulic acid was added and the protein precipitated by ethanol. The precipitate was separated by centrifugation, the supernatant evapored in vacuo. The residue was dissolved in water, acidified to pH 3.0 and extracted by chloroform/phenol. After reextraction into water the solution was evaporated to dryness. The product was chromatographed on Machery + Nagel No. 214 paper (solvent: n-butanol/ethanol/

* Metabolic products of microorganisms, 112; for preceding publication see [1].

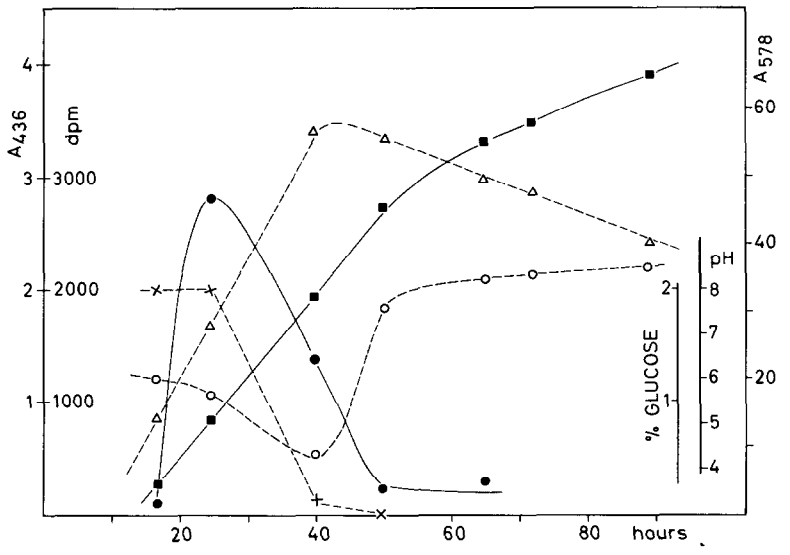

Fig. 1. Fermentation of *Rhodotorula glutinis*. Turbidity (△ — △ - - △); pH (○ - - ○ - - ○), glucose concentration (X- -X- -X); rhodotorulic acid production (A_{436}; ■—■—■) and RA synthetase activity (●—●—●).

water = 4/1/5; upper phase). The paper was dried and sprayed with $FeCl_3$ in methanol. The rhodotorulic acid band was eluted and the radioactivity measured by liquid scintillation counting. To secure identity of radioactivity and rhodotorulic acid, the reaction product was recrystallized twice after paper chromatography with unlabelled rhodotorulic acid. 85% of the radioactivity remained in the crystals.

Isotopic exchange between pyrophosphate and ATP was measured as described by Calendar and Berg [9] in the modification of Walker et al. [10]. The reaction mixture was incubated for 10 min at 37°. The protein concentration was 2.4 mg/ml.

N^5-Hydroxyornithine was prepared by hydrolysis of fusigen and purified by ion-exchange chromatography [11]. Labelled acetyl-hydroxyornithine was synthesized from hydroxyornithine and [1-^{14}C]acetic anhydride in water and purified by chromatography on Whatman 3MM paper.

3. Results

When washed cells of *Rhodotorula glutinis* were incubated for 2 hr with 50 000 dpm of [1-^{14}C]N^5-hydroxyornithine (I) or [acetate-^{14}C]N^5-acetyl-N^5-hydroxyornithine (II), incorporation of 27.6% of I (dilution factor 222) and 18.6% of II (dilution factor 57) was observed. After disintegration of the cells, I was no longer incorporated in the test system described.

In order to obtain a cell-free extract with high activity of the rhodotorulic acid synthesizing enzyme (RA synthetase), growth, enzyme activity and some other parameters were determined in a fermentor. Cells were harvested at various time intervals and extracts for the enzyme assay were prepared as described. It is shown in fig. 1, that the specific activity of RA synthetase increases rapidly during the early phase of growth and decreases in the second half of the logarithmic growth phase. The slight increase in the concentration of rhodotorulic acid after 70 hr may be due to lysis of cells.

The importance of the components of the test system was determined. The results are given in table 1.

RA synthetase in the extracts could be precipitated by ammonium sulfate, and most of the nucleic acids were separated by addition of 1% streptomycin sulfate. Centrifugation at 100 000 *g* for 1 hr left 93% of the enzyme activity in the supernatant. 1 μMole of PCMB in the test inhibited the enzyme activity completely,

Table 1
Characteristics of RA synthetase.

Test system	Incorporation of II in rhodotorulic acid (%)
Complete	100
– $MgCl_2$	73.5
– Mercaptoethanol	86.1
– ATP	6.8
– ATP + 50 μmoles GTP	7.1
– ATP + 50 μmoles CTP	7.0

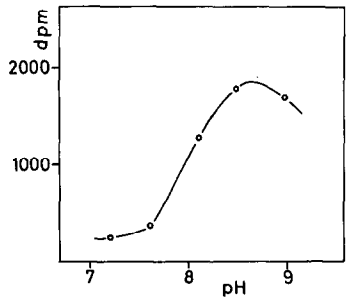

Fig. 2. pH dependence of RA synthetase activity in 0.1 M Tris-HCl buffer.

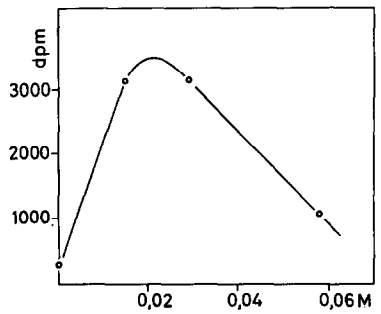

Fig. 3. Dependence of RA synthetase activity on ATP concentration.

while 500 μg of RNAase were without any effect.

A 3-fold purified preparation was tested for activity/pH relationship (fig. 2), dependence on ATP concentration (fig. 3) and pyrophosphate-ATP exchange (table 2).

Table 2
Substrate dependence of ATP-pyrophosphate exchange by crude RA synthetase.

Substrate added (2 mM)	(dpm)	Exchange (nmoles)
None	14 500	82
Acetylhydroxyornithine*	22 000	124
Hydroxyornithine*	10 600	60
N^5-acetyl-L-ornithine	12 600	71
L-ornithine	12 100	69
Acetylhydroxyornithine*, no enzyme	780	

* Presumably L-configuration as in fusigen [8].

No RA synthetase activity could be found in extracts from cells grown for 36 hr in a medium containing 20 mg $FeCl_3 \cdot 6\ H_2O$/l.

4. Discussion

Numerous investigations were initiated to study the mechanism of biosynthesis of cyclic peptides, first of all of peptide antibiotics [12]. A new mechanism of peptide synthesis on protein templates (for a review see [13]) emerged from the study of the biosynthesis of gramicidin S and other polypeptides from bacteria and actinomycetes. As far as we know, this is the first report on the cell-free synthesis of a diketopiperazine in extracts from fungi. The labilization of the α hydrogen during the incorporation of acetylhydroxyornithine reported by Alkers et al. [6] and the exchange of pyrophosphate into ATP (table 2) seem to indicate that a mechanism similar to that of gramicidin S biosynthesis is operative. The rapid increase and decrease of enzyme activity during the fermentation resembles the biosynthesis and degradation of gramicidin S synthetase [14], except that the highest specific activity was found in the early – and not in the late – phase of logarithmic growth. It is remarkable that no RA synthetase activity could be detected in cells grown on a medium containing iron, while Ong and Emery [15] found no dependence on iron concentration with the biosynthesis of hydroxyornithine: acetyl CoA transacetylase from *Ustilago sphaerogena*. Further work

on the purification and investigation of molecular properties of RA synthetase and the regulation of its biosynthesis is in progress.

Acknowledgements

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