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INCORPORATION OF L-[U-^{14}C] LEUCINE INTO ERGOSINE BY CELL-FREE EXTRACTS OF *CLAVICEPS PURPUREA* (FR.) TUL

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SUMMARY

A cell-free system of *Claviceps purpurea* has been described which incorporates [^{14}C] leucine into the peptide-type ergot alkaloid ergosine. Cell-free extracts may be prepared either from protoplasts or lyophilized mycelium. Production was markedly stimulated by addition of agroclavine compared with d-lysergic acid and by agitation of the incubation mixture. The synthesis of ergosine is strongly dependent on the presence of ATP in the reaction mixture.

INTRODUCTION

The peptide-type ergot alkaloids e. g. ergotamine possess an unique cyclol structure. From the biogenetic point of view cyclol alkaloids may be regarded as derivatives of d-lysergyl tripeptides, e. g., ergosine is related to d-lysergyl-L-alanyl-L-leucyl-L-proline. Cyclol formation results from the reactions of an α-hydroxy-α-amino acid adjacent to the lysergyl moiety with the carboxyl group of a C-terminal proline. The latter amino acid is part of a lactam ring with the second amino acid of the peptide moiety.

There is ample evidence from *in vivo* experiments that appropriate amino acids are incorporated into the corresponding amino acid and/or hydroxyamino acid positions in the peptide alkaloids (1). Potential intermediates like di- and tripeptides or lysergyl tripeptides (2) are *in vivo* not incorporated as

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intact units into the cyclol alkaloids. Inhibitor experiments support the view that ergot peptides are formed in a non-ribosomal process (3, 4). Floss et al. (5) have suggested that the peptide moiety of ergot alkaloids is built up on a multienzyme complex similar to the enzyme systems which catalyse the synthesis of peptide antibiotics. The study of the mechanism of cyclol alkaloid formation will be greatly facilitated with cell-free systems. This report describes the first cell-free synthesis of a peptide ergot alkaloid starting with ergolines and an appropriate amino acid.

MATERIAL AND METHODS

Growth of organism. The Claviceps purpurea strain MUT 168/2 was used, which produces an alkaloid mixture of ergosine (80 %) and chanoclavine-I (20 %). The strain was maintained on asparagine/sucrose agar. For alkaloid production cultures were incubated at 24° C in 500 ml round bottle flasks containing 100 ml of a sucrose/ammonium citrate medium, NL 833, and shaken on a rotary shaker at 240 rpm (6).

Preparation of cell-free extracts. A. For preparing protoplasts essentially the method of Stahl et al. (7) was used. 5 - 7 g of fresh mycelium, harvested between 3 - 4 days after inoculation was treated with snail digestive extract. The resulting protoplast pellet was washed twice with 0.9 M KCl solution (6), resuspended in 0.01 M Tris-HCl buffer (pH 7.7) and gently homogenized at 4° C in a homogenizer. 1 x 10^8 protoplasts were used to prepare 1 ml cell-free extract (protoplast lysate). B. The mycelium which was grown for 4 days in NL 833 was separated from the culture broth and then three times washed with 1 M KCl solution. The damp-dry mycelium was immediately lyophilized and afterwards stored in a freezer at -25° C for not longer than 10 days. For routine enzyme preparations lyophilized mycelium was intensively ground in a mortar with dry ice (8) and 6 volumes of 0.2 M Tris-HCl buffer (pH 7.7) containing 40 % glycerol, 20 mM KCl, 10 mM $MgCl_2 \cdot 6H_2O$, 10 mM dithioerythrol, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA. The suspension was centrifuged at 15 000 x g for 45 min. The supernatant fluid was the cell-free extract, containing 14 - 16 mg protein/ml.

Cell-free reaction. The reaction mixture contained 1 ml of extract A or 0.5 ml of extract B, 5.4/µCi of L-[U-^{14}C]-leucine, 1/µmol of clavine alkaloid or d-lysergic acid, 10/µmol of adenosine triphosphate (ATP) 5/µmol of phosphoenolpyruvate (PEP), 200/µg of pyruvate kinase and 0.5 ml of 0.2 M Tris-HCl buffer (pH 7.7) with the above described additives. The final volume amounted to 2.0 or 2.5 ml. The mixture was incubated either in a test tube (17 x 1.5 cm) or a 25 ml erlenmeyer

flask for 2 - 6 h. at 27° C on a shaker at 240 rpm. In control experiments the enzyme extract was held in a boiling-water bath for 10 min and then cooled in ice before addition of the labelled leucine.

Analytical methods. After incubation 0.5 ml of a 2 % tartaric acid solution containing 480/ug of ergosine as carrier. was added to each tube and the material frozen at -20° C. The extraction of alkaloids was performed according to Maier et al. (6, 9). The ergosine was separated by thin-layer chromatography (TLC) in solvent system I and subsequently rechromatographed in system II. Aliquots of the twice purified ergosine were used for quantitation of alkaloids and counting of radioactivity using a liquid scintillation counter (Tricarb, model 2660, Packard Instruments). TLC was carried out on silica gel, PF_{254} "Merck" plates using the following solvent systems. System I: chloroform:methanol:acetic acid (85:10:3); system II: ethylacetate:methanol (95:5); system III: chloroform: methanol (9:1); system IV: chloroform:methanol:acetic acid (85: 12:2). Protein was determined by the procedure of Bradford (10).

Purification of ergosine synthesized by cell-free extracts: Eight 2 ml batches each containing 2.7/uCi of labelled leucine and agroclavine were incubated at 27° C at 240 rpm for 2 h. In control experiments boiled cell-free extracts were used. The alkaloid extracts of each set of eight incubation mixtures were combined and 30 mg non-labelled ergosine added as carrier. The alkaloid was chromatographed on silica gel using different solvent systems. After each TLC separation the ergosine was heated for 30 min in methanol and kept (12 h) at room temperature for a partial conversion into ergosinine. The ergosinine fractions were combined and three times recrystallized from methanol. After different treatments the specific radioactivity was determined (Table 2).

Materials. ATP (disodium salt), pyruvate kinase, and phosphoenolpyruvate (monopotassium salt) were obtained from Boehringer, Mannheim. Dithioerythrol was purchased from Reanal, Budapest. PMSF was obtained from Serva, Heidelberg. L-[U-^{14}C]-leucine (240 mCi/mmol, 8880 MBq/mmol) was a product of UVVVR, Praha.

RESULTS AND DISCUSSIONS

The ability of cell-free extracts to incorporate L-[U-^{14}C]-leucine into ergosine is shown in Table 1. For this studies the ergosine producing Claviceps purpurea strain MUT 168/2 was found especially useful. We have tested a large variety of cofactors and enzyme-protecting substances to obtain active cell-free systems. Eventually a Tris-HCl buffer containing 40 % of glycerol, dithioerythrol and phenyl methylsulfonyl fluoride proved to be suitable.

Table 1. Incorporation of L-[U-^{14}C] leucine into ergosine by cell-free extracts of Claviceps purpurea

Culture batch No	Cell-free extract from	Ergoline added	Other conditions	^{14}C Ergosine formed (dpm)
1	protoplasts	elymoclavine	a)	8850
1	protoplasts	lysergic acid	a)	1835
140	mycelium	elymoclavine	b)	12203
140	mycelium	elymoclavine	c)	4615
140	mycelium	lysergic acid	c)	1850
144	mycelium	agroclavine	b) in erlen-	7025
144	mycelium	elymoclavine	b) meyer	3490
144	mycelium	lysergic acid	b) flasks	2478

a) 12 h incubation on a shaker; b) 6 h incubation on a shaker; c) 6 h incubation without shaking. The counts of control experiments (boiled enzyme extracts) amounting to 180-230 dpm were deducted. All experiments were performed in duplicate.

Recently it has been shown (6, 11) that intact protoplasts of Claviceps purpurea synthesize de novo peptide-type alkaloids. We found that protoplasts of this particular strain as well as lyophilized mycelium can be used for preparation of a cell-free extract. There is a loss of enzyme activity of 50 - 70 % after 10 days storage at -25° C of lyophilized mycelium.

The formation of ergosine takes place under static conditions but shaking at high frequencis increases remarkably alkaloid synthesis. The incorporation of radioactive amino acids into alkaloids by protoplasts of Claviceps is stimulated by the addition of d-lysergic acid. Therefore we used in our cell-free system the same ergoline precursor. Surprisingly much more effective proved to be elymoclavine and agroclavine. We performed various experiments with mycelia of different origin and found always that the stimulation of alkaloid synthesis is increased in the following order: d-lysergic acid, elymoclavine, agroclavine. In order to show unequivocally that

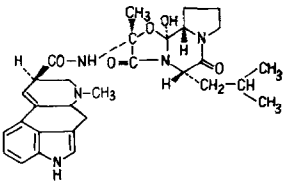

Fig. 1. Ergosine

cell-free extracts of Claviceps are able to synthesize ergosine we diluted in one set of experiments the labelled alkaloid with non-labelled ergosine and purified it by TLC. This product was converted to the isomeric ergosinine and recrystallized to a constant specific radioactivity (Table 2).

In control experiments with boiled cell-free extracts only traces of radioactivity were detectable. These results strongly indicate that the cell-free system catalyzes the formation of ergosine.

The production of ergosine was linear for about 1 h and increases afterwards slightly (Fig. 2). The presence of ATP was required for the incorporation of [^{14}C] leucine into ergosine. Without ATP a slight production of the alkaloid was

Table 2. Authenticity of enzymatically formed ergosine

Treatment	Radioactivity (dpm/mg) ergosine/ergosinine	
	cell-free reaction	boiled cell-free reaction
1. TLC in system I	1783	181
2. TLC in system III	650	59
3. TLC in system II	629	24
4. TLC in system IV	660	24
5. ergosinine	660	15
6. ergosinine	636	8
7. ergosinine	630	12

5-7 recrystallized from methanol

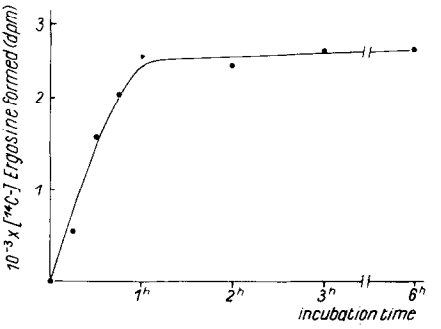

Fig. 2. Ergosine synthesis by cell-free extracts of Claviceps purpurea. Time course of L-[U-^{14}C] leucine (administered 2.7 µCi) incorporation in the presence of agroclavine. The lyophilized mycelium was stored 7 days at -25° C prior to enzyme preparation.

observed, but its addition resulted in a marked increase in the synthesis of the peptide ergot alkaloid (Fig. 3). The incorporation of [^{14}C] leucine into the alkaloid was decreased varying from one cell-free preparation to another between 10 - 50 %, when the energy-generating system was omitted from the incubation mixture.

In recent years the biosynthesis of an important group of amino acid derived fungal metabolites, the ß-lactam antibiotics has been investigated on the enzyme level (12-16). Using protoplast lysats some steps of ß-lactam antibiotic formation e. g. ring expansion of penicillin N to cephalosporin (s) are re-

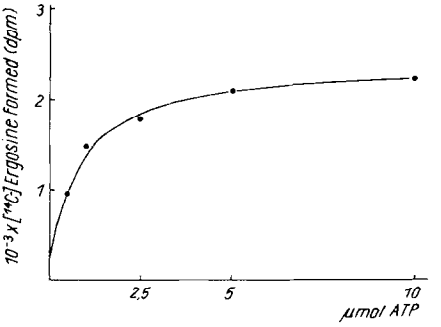

Fig. 3. Dependence of ergosine synthesis on ATP concentration in the reaction mixture containing 2.7 µCi L-[U-^{14}C]-leucine and agroclavine. The lyophilized mycelium was stored 5 days at -25° C prior to enzyme preparation.

markably influenced by agitation and the presence of an energy-generating system. We adopted this conditions which proved to be favorable also for cyclol alkaloid synthesis.

The mechanism of α-hydroxy-α-amino acid formation in the biosynthesis of peptide-type ergot alkaloids was studied by Floss' group (17, 18). A direct hydroxylation of the α-position of the amino acid was found. Furthermore the results of the ^{18}O-experiments strongly indicate a pathway in which an activated derivative of lysergic acid e. g. the coenzyme A ester is generated directly by dehydrogenation of an appropriate precursor (elymoclavine) of the aldehyde oxidation stage rather than by activation of the free acid. Our results that agroclavine and elymoclavine stimulate cyclol alkaloid synthesis on the enzyme level much more than d-lysergic acid favor this assumption.

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