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Pigmentation of *Claviceps* species after on Tryptophan Media

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Tryptophan 배지상에서의 *Claviceps* species에 의한 색소 생합성

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초 록

Claviceps spp.는 tryptophan함유배지에서 적갈색 색소를 형성한다. D.L.-tryptophan [side chain-3^{14}C]를 배지에 첨가하였을 때 ^{14}C-labeled pigment와 constant specific radioactivity를 갖는 5-hydroxytryptophan을 재결정 분리하였다. 동시에 ^{14}C-labeled 5-hydroxytryptophan으로 같은 실험을 행한 결과, tryptophan 보다 4배 이상, 색소물질로 주입되는 것을 관찰할 수 있었다. 한편, UV-spectrum 및 fluorometric analysis등에 의하여 배지중 5-hydroxytryptophan이 생합성되는 것을 확인할 수 있었다. tryptophan에서 출발하는 또 하나의 ergot pigment 생합성경로는 곰팡이 생육배지중에서 40pmde/mg protein 농도의 cytochrome p-450을 분리 정제할 수 있어 tryptophan이 hydroxylation되어 5-hydroytryptophan으로 전환하는 효소학적 반응으로 검정되어 hydroxylation반응이 우선하는 기작에 의한 것임을 알 수 있다.

Introduction

In addition to such alkaloids as agroclavine and elymoclavine, *Claviceps purpurea* PRL 198 0 produces a reddish brown pigment. The pigment production was reported by Franck et al.[1~3] They reported that the pigments are anthraquinone carboxylic acids and dimeric hydroxanthone derivatives (ergochromes), the structures of which have been completely determined. When grown on media containing tryptophan, many kinds of organisms produce pigments. *Penicillium purpurogenum* W59 forms a red pigment from tryptophan in the culture.[4] *Candida* species produce reddish brown pigments after growing on a tryptophan medium.[5] Some *Phycomycetes*[6], *Ascomycetes*[6], *Basidiomycetes*[7] and *Schizomyectes*[8] produce reddish brown pigments from a tryptophan medium. This paper reports that *Claviceps purpurea* PRL 1980 produces a brown pigment from tryptophan via 5-hydroxytryptophan in the alkaloid production medium.

Experimental

Culture conditions

A piece of mycelium of *C. purpurea* PRL 19

80 incubated on the slant media was inoculated into 100ml of succinic acid-sucrose medium(Table 1. growth medium)[9]. After the medium turned reddish-brown (8~9 days), 25ml was added to 100ml of the same growth medium and shaken until a reddish-brown color began to appear (4~5 days). The resulting mycelia were washed twice with sterile water and suspended to 50ml growth medium was mixed with 100ml mannitol-tryptophan-succinic acid medium (Table 2)[10] in a 500ml flask and shaken at 300 rpm at 25°C. The incubation was continued for 15 days.

Table 1. The composition of growth medium for *Claviceps purpurea* PRL 1980

Sucrose	40g
Succinic acid	4g
Yeast extract	5g
NH_4NO_3	4g
KH_2PO_4	1g
$MgSO_4$ $7H_2O$	0.2g up to 1L H_2O
Adjusted to pH 6.5 with 40% KOH	

Table 2. The composition of alkaloid production medium

Mannitol	65g
Succinic acid	7.2g
KH_2PO_4	250mg
$MgSO_4$ $7H_2O$	300 g
$FeSO_4$ $7H_2O$	5mg
Tryptophan	500mg
Biotin	5μg
Na, Cu, Ca, Mn, Zn, Mo	1mg
NH_4OH	to pH 5.2
Distilled water	to 1l

Separation of a reddish brown pigment and thin layer chromatography[19]

The cultures were homogenized and filtered. The pH of mother liquor was adjusted to pH 11 with 30% NH_3 and extracted with diethyl ether to remove alkaloids. The aqueous layer was neutralized with 20% acetic acid, lyophilized and dissolved with aqueous methanol solution. The pigment mixtures were separated by column chromatography through cellulose powder column (J.T. Baker Chemical Co.) which was washed with n-butanol/0.5M phosphate buffer, pH7.0(1 : 1). The pigment mixtures were suspended in 2.0ml of the phosphate buffer added to the column (2.5/20cm). After separation, the pigment was extracted with 80% cold aqueous methanol and evaporated. The residue was washed twice with 5ml of petroleum ether, evaporated completely, and redissolved with MeOH-H_2O(4 : 1). An aliquot of the extract was subjected to TLC on Silicagel G. The solvent system was BuOH-HAc-H_2O(4 : 1 : 1) (BAW) or MeOAc-iso-PrOH-30%NH_3 (9 : 7 : 4)(MIA) for separation of 5-hydroxytryptophan and brown pigment. The plates with radioactive products were scanned with a Packard 7220 radioactivity scanner and the radioactivity was determined by comparing the area under the curve with plates containing radioactive standards. Radioactivity of brown pigment peak was calculated by the triangulation method[13].

Quantitative determination of 5-hydroxytryptophan

The amount of 5-hydroxytryptophan was determined as described by Kiepert and Voigt[11,12]. The 5-hydroxytryptophan was eluted and reacted with Van Urk's reagent.

Recrystalliztion of 5-hydroxytryptophan

C. purpurea PRL 1980 was incubated in the production medium containing DL-tryptophan [side chain-3-^{14}C] (90μCi, 3.7mCi/mmol), the sample was adjusted to pH10.5-11 with 30% NH_3, extracted with diethyl ether, the ether layer was evaporated to dryness, and the residue was dissolved in MeOH-H_2O(4 : 1). 1.0ml of water was added to 0.3ml of the MeOH-H_2O solution. The solution was heated to boiling, filtered through a preheated funnel, cooled in an ice bath and centrifuged. The crystals were

collected and dissolved in a minimum volume of hot water. An aliquot was removed for determination of radioactivity and amount of 5-hydroxytryptophan. The remainder was cooled. The crystals were collected and the procedure repeated.

Fluorometric analysis

All fluorometric studies were carried out using a recording Farrand spectrofluorometer. Flat-bottomed tubes made from glass tubing (5-mm, o.d.; 3-mm i.d.×50mm in length) served as cuvettes. A Corning filter No. 5840 was used between the light source and the first monochromator and a No. 3387 filter was placed between the sample and the second monochromator. The fluorescence spectra of 5-hydroxytryptophan isolated from cultures and 5-HT plus HCl solution were determined respectively using an excitation wavelength of 295nm. All spectrofluorometric work was done at room temperature.

Conversion of ^{14}C-labeled tryptophan or ^{14}C-labeled 5-hydroxytryptophan to brown pigment

10m*l* of growth medium was washed with distilled water and transfered to 10m*l* of production medium with tryptophan (Culture No. 1, 3, 5) or without tryptophan (Culture No. 2, 4) in 50m*l* erlenmeyer flask. Culture No. 1 and 2 were incubated directly for 12 days. On the other hand, after 2 days of incubation, the mother liquor of Culture No. 3, 4 & 5 was removed by centrifugation and suspended in 10m*l* production medium without tryptophan plus 5m*l* DL-tryptophan [side chain-3-^{14}C] (10μCi, specific activity: 50.6mCi/mmol) or DL-5-hydroxytryptophan [side chain-3-^{14}C] (10μCi, specific activity: 59.2 mCi/mmol). The cultures were incubated for an additional 10 days. The kind of the ^{14}C-labeled compounds used and the times at which they were added are summarized in Table 3.

Table 3. Supplements added to cultures of *C. purpurea* PRL 1980

Culture No.	Supplement	Weight added (μmol)	Time added (days)
1	^{14}C-Try	0.17	0
	Try	24.5	0
2	^{14}C-Try	0.17	0
3	Try	24.5	0
	^{14}C-Try	0.17	2
4	^{14}C-Try	1.17	2
5	Try	24.5	0
	^{14}C-5-HT	0.20	2

Try: Tryptophan, 5-HT: 5-Hydroxytryptopnha

Results

Pigmentation

When cultured in a chemically defined medium having tryptophan as a major nitrogen source, *C. purpurea* PRL 1980 produced a reddish brown water-soluble pigment. The brown pigment was also soluble in methanol. Under opimum conditions the reddish brown pigment was produced after 1-3 days of incubation in the production medium. The pigment production increased linearly up to 10 days of incubation and thereafter the production rate decreased gradually. After 12 days of incubation it seemed to remain constant. (Fig. 1).

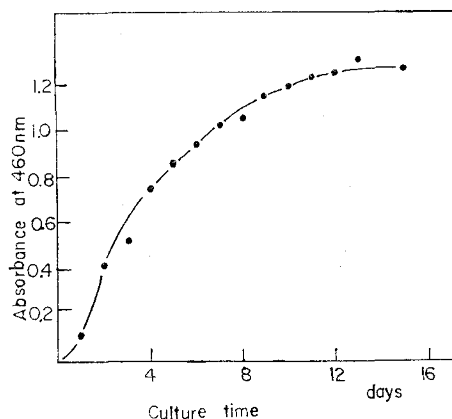

Fig. 1. Time course study of pigment production of *Claviceps purpurea* PRL 1980

Radioactivity scan of pigment mixture

C. purpurea PRL 1980 converted ^{14}C-labeled tryptophan to five radioactive metabolites. The separated thin layer chromatogram was all located by the characteristic fluorescence under a ultraviolet light. (Fig. 2. A: yellowish red, B-F: pinkish blue) By comparison with reference they were identified as brown pigment (A), 5-hydroxytryptophan(B), tryptophan(C), Dimethylallyltryptophan (D), Clavicipitic acid (E), and N-acetyltryptophan. In the aqueous layer tryptophan, 5-hydroxytryptophan, and pigment were heavily labeled.

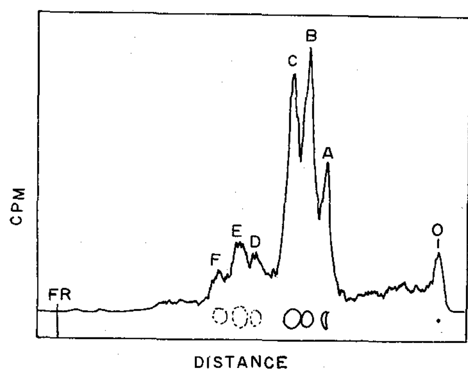

Fig. 2. Radioactivity scan of aqueous methanol extract of *C. purpurea* PRL 1980 cultures O: origin, FR: front, A: reddish brown pigment, C: tryptophan, D: dimethylallyltryptophan (DMAT) E: clavicipitic acid, F: N-acetyl tryptophan

Recrystallization and Identification of 5-hydroxytryptophan isolated from cultures

To give evidence for the existence of 5-hydroxytryptophan in the cultures it was purified by crystallization. The results were shown in Fig. 3. 5-hydroxytryptophan isolated from the aqueous layer of the cultures contained an appreciable radioactivity and was recrystallized to constant specific radioactivity. Conversion of tryptophan to 5-hydroxytryptophan was shown by this isotopic trapping procedure and was identified by UV-spectrum and fluorometric analysis. (Fig. 4 & Fig. 5).

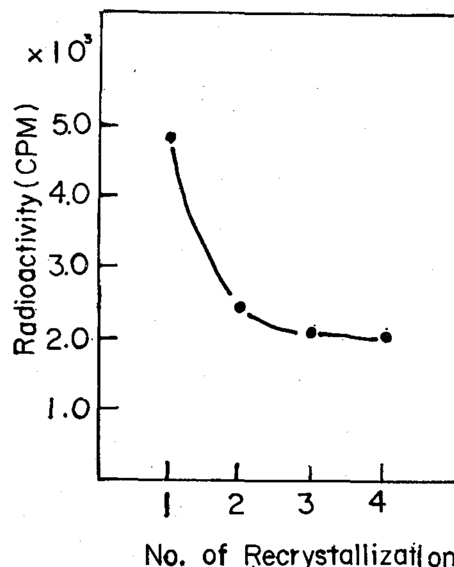

Fig. 3. Recrystallization of 5-hydroxytryptophan contained in the cultures incubated with ^{14}C-labeled tryptophan

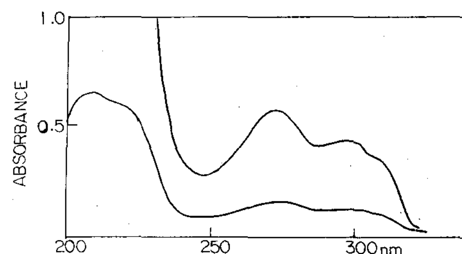

Fig. 4. UV-spectrum of 5-hydroxytryptophan isolated from the cultures

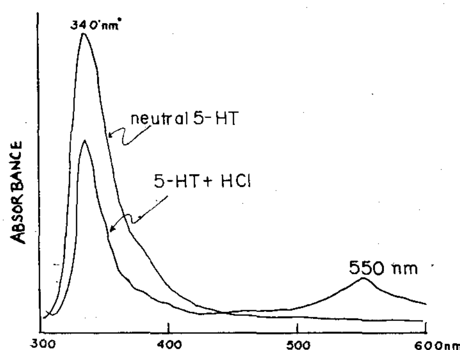

Fig. 5. Fluorescence spectrum of 5-hydroxytryptophan isolated from the cultures

Conversion of tryptophan or 5-hydroxytryptophan to brown pigment

A radioactive brown pigment was obtained in

the cultures after addition of ^{14}C-labeled tryptophan or ^{14}C-labeled 5-hydroxytryptophan. With the latter compound the percent incorporation into the brown pigment was 4 times that obtained when ^{14}C-labeled tryptophan was added (24.0% & 5.7%, Table 4). This result showed that the addition of 5-hydroxytryptophan gives an incorporation of radioactivity into brown pigment significantly higher than tryptophan. Initial addition of ^{14}C-labeled tryptophan resulted in lower radioactivity in the pigment compared to additon after two days incubation in production medium (3.6% & 5.7%; 2.1% & 2.9%) presumably because some of the tryptophan was utilized for growth. Addition of unlabeled tryptophan to the culture medium increased the conversion of ^{14}C-labeled tryptophan to brown pigment (3.6% & 2.1%; 5.7% & 2.9%). This indicated that tryptophan induces the enzymes involved in the formation of the brown pigment.

Table 4. Radioactivity of the brown pigment peak in culture fractions

Culture No.	% Incorporation*
1	3.6
2	2.1
3	5.7
4	2.9
5	24.0

* Expressed as % of total radioactivity added to cultures

Discussion

Through feeding of tryptophan and analysis of the occurring decomposition products, for instance, through the examination of the conversion of the intermediate products of tryptophan metabolism, we have tried to clarify which way could be involved in the pigment formation by *Claviceps purpurea* PRL 1980 and what significance the conversion of tryptophan could have. Even though the incorporation of tryptophan into alkaloids represents a substantial demand upon the capacity of the organism for its synthesis, the only other major pathway by which tryptophan is metabolized appears to be the formation of a reddish brown pigment. Visconitini and Mattern[14,15], studying hydroxylation of ^{14}C-tryptophan by means of tetrahydropterin in the presence of ferrous ions, observed that radioactive dark brown pigment, melanin, was formed via 5-hydroxytryptophan. Their research on the action of polyphenol oxidase showed that only some indole compounds (5-hydroxytryptophan, 5-hydroxytryptamine, and tryptamine) give a formation of black-brown pigments. Udenfriend et al[16,17] also reported that 5-hydroxytryptophan and violaceen, a pigment containing 5-hydroxyindole have been found in *Chromobacterium violaceum*. And they reported that some of the pigments loosely referred to as "melanins" may be derived from tryptophan through 5-hydroxyindole intermediates. Anyway the hydroxylation of indole compounds is the first step for many pathways to an oxidative tryptophan degradation by microorganisms. Taking all these results and references into consideration, there seem to be two pathways through which tryptophan is involved in the pigment biosynthesis: the 'via tryptophan→ 5-hydroxytryptophan[14,15,17,18] and the 'via tryptophan→ kynurenine→ 3-hydroxykynurenine'. No tryptamine, 5-hydroxytryptamine, or 5-hydroxyindoleacetic acid was found in the cultures. Cultures grown with 5-hydroxyindoleacetic acid have a bright red color rather than the normal reddish brown color. These results suggest that 5-hydroxytryptophan is not converted to the brown pigment via 5-hydroxytryptamine or 5-hydroxyindolacetic acid. The side chain C-1 carbon is probably retained and the 5-hydroxyindole moiety of 5-hydroxytryptophan undergoes oxidation and condensation reactions to form the pigment.

A major metabolite of tryptophan by *C. purpurea* PRL 1980 is 5-hydroxyptophan. The formation of 5-hydroxytryptophan could com-

pete significantly with formation of DMAT for alkaloid production.

Conclusive proof of the structure and further information concerning physical properties of the brown pigment must await a pure isolation of the pigment.

Abstract

Claviceps purpurea PRL 1980 produces a fluorescent reddish brown pigment in the alkaloid production medium. When D,L-tryptophan [side chain-3-^{14}C] was administered into the production medium, the radioactive pigment and 5-hydroxytryphan were isolated from the cultures. Conversion of tryptophan to 5-hydroxytryptophan in vivo was shown by an isotopic trapping procedure. 5-hydroxytryptophan isolated from the cultures contained appreciable radioactivity and was recrystallized to constant specific radioactivity. The injection of the ^{14}C-labelled 5-hydroxytryptophan showed an incorporation of radioactivity into brown pigment significantly higher than that of tryptophan. The brown pigment produced by *Claviceps purpurea* PRL 1980 seems to be derived from tryptophan through 5-hydroxytryptophan.

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