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Production of Substituted L-Tryptophans by Fermentation[1]

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Claviceps purpurea has been shown to produce extracellular L-tryptophan from indole in stirred fermentors. The substrate specificity of this conversion was investigated by using substituted indoles, anthranilic acid, and 4-chloro-anthranilic acid. Addition of 2-, 4-, 5-, 6-, and 7-methyl indole or 6-chloroindole to *C. purpurea* C1M produced the corresponding substituted L-tryptophan. In contrast, addition of 1-methyl, 6-trifluoromethyl, 6-nitro-, or 4-benzyloxy-substituted indoles, or anthranilic and 4-chloroanthranilic acids did not produce detectable amounts of the corresponding tryptophan.

Certain substituted tryptophans possess interesting biological activities. Some affect bacterial growth (7) and inhibit viral multiplication (1), and D-6-chloro- as well as D-6-methyl tryptophans have been found to be exceedingly sweet compounds (3). However, the large-scale synthesis and resolution of synthetic tryptophan derivatives are difficult and expensive.

The biosynthesis of substituted tryptophans from indole derivatives has been studied in a variety of microbial systems. Indole and indole derivatives have been added to purified tryptophan synthetase of *Escherichia coli* (6) and *Neurospora crassa* (2) to study the mechanism of tryptophan biosynthesis. Strains of *Claviceps purpurea* possess a tryptophan synthetase which catalyzes the production of L-tryptophan from exogenous indole (8). An addition of indole to a stirred fermentation of *C. purpurea* strain C1M has been reported (4) to produce 1.5 mg of extracellular L-tryptophan per ml. In the following study, we examined the substrate specificity of this strain for the production of substituted L-tryptophans from the corresponding indoles.

MATERIALS AND METHODS

C. purpurea Lilly C1M NRRL 2583 was propagated from lyophilized culture stocks to agar slants of the following composition: glucose, 2%; peptone (Difco), 0.5%; KH_2PO_4, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.025%; $FeSO_4 \cdot 7H_2O$, 0.001%; and agar (BBL), 2.0%. After an incubation at 25 C for 5 to 7 days, a portion of the growth from the slant was used to inoculate 250-ml Erlenmeyer flasks containing 50 ml of a vegetative medium of the following composition: glucose, 1%; NaCl, 0.5%; N-Z-Amine A (Sheffield Chemical, Div. of National Dairy Products Corp., Norwalk, Conn.), 0.5%; peptone (Difco), 0.5%, and beef extract, 0.5%. The vegetative culture was incubated at 25 C on a rotary shaker [250 rev/min, describing a 2-inch (5.08 cm) diameter] for 48 hr. A portion of the vegetative culture was used to provide a 5% (v/v) inoculum for either a synthetic or a semisynthetic production medium. The synthetic medium was composed of glucose, 2%; $(NH_4)_2SO_4$, 0.2%; glycine, 0.15%; KH_2PO_4, 0.27%; $CaCl_2$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.001%; and biotin, 1.0 μg/liter. The semisynthetic production medium contained glucose, 2%; $(NH_4)_2SO_4$, 0.4%; glycine, 0.15%; KH_2PO_4, 0.88%; $Na_2HPO_4 \cdot 12H_2O$, 1.54%; and corn steep liquor, 1.0%. Erlenmeyer flasks (250 ml) containing 50 ml of the production medium were sterilized by autoclaving at 121 C for 20 min. The inoculated production flasks were incubated at 25 C for periods up to 9 days.

The indole substrates were dissolved in a minimal amount of ethanol and added to the culture as described below.

Tryptophan and indole assays. The production of substituted L-tryptophans and residual indole was monitored by both colorimetric assays and paper chromatography. After an incubation of the substrate in the presence of the culture, samples of the synthetic medium fermentations were filtered and extracted with equal volumes of toluene. Both the toluene and the aqueous phases were colorimetrically assayed with *p*-dimethylaminobenzaldehyde (9) between 525 and 645 nm with an L & N Spectronic 20 spectrophotometer. The toluene extract was assayed for residual indole, and the aqueous solution was tested for tryptophans by paper chromatography. Broth samples were applied to paper chromatograms and chromatographed in 2-butanol-NH_4OH (3:1). Tryptophan was detected by spraying the chromatograms with a ninhydrin or a *p*-dimethylaminobenzaldehyde spray (9).

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Isolation of tryptophans. The substituted tryptophans were isolated from the fermentation after filtering off the solids and extracting the resulting filtrate with 0.5 volume of toluene to remove the residual indole. The aqueous portion was passed over a Pittsburg carbon column (12 by 40 mesh). The carbon was washed with an excess of water to remove residual glycine from the medium. A 1-butanol-ethanol-water mixture (4:1:1) was used for elution of the substituted tryptophans. For further purification, the crude tryptophan obtained from the carbon column was chromatographed on a cellulose (Avicel, tech grade) column with acetonitrile-water mixtures. The substituted tryptophans were usually eluted in relatively pure form with a water-acetonitrile mixture (1:6).

Comparison of the infrared and mass spectra of the samples crystallized from ethanol and water with authentic tryptophans provided proof of their identity.

RESULTS

The specificity of *C. purpurea* strain C1M in the conversion of substituted indoles is shown in Tables 1 and 2. In Table 1, the indole derivatives converted to the corresponding tryptophans are reported. The indoles were added to a 48-hr culture grown in synthetic medium. Three additions (2.5 mg/50 ml) of each substrate were made at 24-hr intervals. The colorimetric assay performed 48 hr after the last substrate addition indicated the production of the substituted tryptophans. Indole conversion was confirmed by paper chromatography of the fermentation broths and physical data of the isolated tryptophans.

Table 2 shows the indole substrates which did not produce detectable levels of tryptophans.

TABLE 1. *Production of substituted L-tryptophan by Claviceps purpurea C1M grown in synthetic medium*

Substrate[a]	Colorimetric assay[b]	
	Residual substrate (mg/50 ml)	Substituted tryptophan[c] produced (mg/50 ml)
2-Methyl indole	0.35	6.00
4-Methyl indole	2.00	2.70
5-Methyl indole	2.10	6.15
6-Methyl indole	0.17	7.00
6-Chloroindole	0.20	7.75
7-Methyl indole	0.27	5.75

[a] Three additions of 2.5 mg per 50-ml culture per 24-hr interval.
[b] Colorimetric readings taken 48 hr after last addition.
[c] Confirmed by paper chromatography, infrared, mass spectroscopy, and optical rotation.

TABLE 2. *Substrates which did not produce detectable levels of substituted tryptophans by Claviceps purpurea C1M*

Substrate	Amt of substrate[a] added per 24 hr (mg/50 ml)	Per cent[b] mycelium	Residual substrate[c] (mg/50 ml)
Anthranilic acid	2.5	17.6	0.00
	1.25	18.0	0.00
	0.625	19.0	0.00
4-Chloroanthranilic acid	2.5	8.4	0.00
	1.25	18.8	0.00
	0.625	21.0	0.00
4-Benzyloxyindole	2.5	3.0	1.25
	1.25	2.9	1.14
	0.625	15.0	0.18
1-Methyl indole	2.5	5.2	5.20
	1.25	8.8	2.75
	0.625	12.6	1.05
6-Nitroindole	2.5	4.7	
	1.25	10.7	
	0.625	22.7	
6-Trifluoromethyl indole	2.5	4.0	2.0
	1.25	4.4	0.94
	0.625	13.3	0.24
C1M culture control	None	16.7	

[a] Total of three additions starting at 48 hr.
[b] Taken 48 hr after last addition.
[c] Assayed colorimetrically. Taken 48 hr after last addition.

Anthranilic, 4-chloroanthranilic acid, and the four indole derivatives listed in Table 2 were added at three concentrations to 48-hr cultures grown in synthetic medium. Culture growth in the fermentations was measured by packed-cell volumes taken 48 hr after the last substrate addition. In comparing per cent mycelium of the substrate fermentations with the culture control, the lowest concentration of the indoles was not inhibitory to the growth; however, slight inhibition was produced by the 1-methyl and 6-trifluoromethyl derivatives. Even at this low substrate level, however, no conversion was detected.

Since we were not able to obtain good color reactions and reproducible standard colorimetric assay curves for 1-methyl, 6-nitro, and 6-trifluoromethyl tryptophans, conversion was de-

TABLE 3. *Production of L-6-chlorotryptophan by Claviceps purpurea C1M grown in synthetic medium with continuous addition of 6-chloroindole in shake flasks*

Substrate	Time of first addition (hr)	Amt of substrate[a] added per 24 hr (mg/50 ml)	Colorimetric assay	
			Residual substrate (mg/50 ml)	6-Chlorotryptophan (mg/50 ml)
6-Chloroindole...	24	5	0.00	26.2
6-Chloroindole..	24	10	39.5	0.6
6-Chloroindole...	48	5	0.00	24.0
6-Chloroindole...	48	10	0.55	35.0

[a] Total of five additions.

TABLE 4. *Production of substituted L-tryptophans from indoles by Claviceps purpurea C1M in 5-liter, stirred fermentors*[a]

Substrate	Amt of substrate added per 24 hr (mg/fermentor)	No. of additions	Isolated substituted L-tryptophan (mg/fermentor)	Optical rotation
6-Chloroindole..	500	5	534	−24.1°
6-Methyl indole..	500	5	654	−31.8°
6-Methyl indole..	750	5	670	
2-Methyl indole..	500	4	869	−25.0°

[a] Five-liter fermentors were stirred at 400 rev/min at 26 C with an aeration rate of 0.5 (v/v).

termined by paper and thin-layer chromatography. Chromatograms of the concentrated eluates from the carbon column did not contain detectable quantities of tryptophan.

Due to the interest in 6-chlorotryptophan as a sweetening agent, an experiment was conducted to determine if L-6-chlorotryptophan production was adversely influenced by a high substrate concentration. The substrate, 6-chloroindole, was added at two levels to a 24-hr and a 48-hr culture grown in the synthetic medium. The results shown in Table 3 indicate that the addition of 5 mg of 6-chloroindole to a 24-hr culture produced about the same yield as 5 mg added to the 48-hr culture. However, addition of 10 mg of the substrate at 24 hr severely affected the production of 6-chlorotryptophan.

To determine if the production in shaken flasks of the substituted tryptophans could be duplicated in larger volumes, three of the indoles were added individually to 5-liter, stirred fermentations of the culture grown in semisynthetic medium.

The substrates were added to a 24-hr culture with four subsequent additions at 24-hr intervals. The incubations were continued for 48 hr after the last substrate addition. The isolated tryptophans were characterized by infrared, mass spectral analysis, and by optical rotation (Table 4).

DISCUSSION

C. purpurea C1M may possess a derepressed tryptophan synthetase. Although exogenous anthranilate depressed tryptophan synthesis, considerable L-tryptophan was synthesized from indole. The substrate specificity of tryptophan synthetase, especially to methyl indoles in the C1M strain, is quite general, which is in contrast to the tryptophan synthetase system of *N crassa* which converted 4- and 7-methyl indole to the corresponding tryptophan but converted the 5- and 6-methyl indole at very low concentrations (2).

Mutschler, Rochelmeyer, and Wölffling have recently reported (5) that addition of 5-hydroxyindole to *C. purpurea* 47A produced 5-hydroxytryptophan.

Although no effort was made to optimize tryptophan yields, the C1M strain grown in semisynthetic medium in stirred equipment gave yields of isolated tryptophans high enough to be considered for laboratory preparation of substituted tryptophans of high optical purity.

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