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CHARACTERIZATION OF A 5-METHYLTRYPTOPHAN RESISTANT STRAIN OF *CATHARANTHUS ROSEUS* CULTURED CELLS

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Key Word Index—*Catharanthus roseus*; Apocynaceae; suspension cultures; selection; tryptophan biosynthesis; ajmalicine; indole alkaloids.

Abstract—Strains of *Catharanthus roseus* suspension cells resistant to growth inhibition by various tryptophan analogs were selected. Tryptophan synthetase and anthranilate synthetase from the resistant cells differed from the normal cell enzymes by being more resistant to feedback inhibition by tryptophan. Though these altered enzymes allowed the free tryptophan level of the resistant cells to be 30–40 times higher than that of normal cells, the accumulation of tryptamine or ajmalicine could not be detected in the resistant cells.

INTRODUCTION

Following our success [1] in the isolation of a cell-free system for ajmalicine synthesis in *Catharanthus roseus* tissue culture, we have been interested in the induction of the 'alkaloid synthetase' in order to obtain this enzyme for characterization and other studies. A number of enzyme inductions by amino acid analogs are known. Floss and Mothes [2] were successful in inducing alkaloid production in *Claviceps* by addition of tryptophan and tryptophan analogs.

Widholm [3, 4] reported that cultured *Nicotiana tobacum* and *Daucus carota* cells which are resistant to growth inhibition by tryptophan analogs such as 5-methyltryptophan (5-MT) can oversynthesize tryptophan with an altered anthranilate synthetase. Furthermore, it was reported that in the *p*-fluorophenylalanine (PFP) resistant tobacco cell lines, excess phenylalanine did not accumulate but was converted to phenolics [5, 6]. Similar results were reported in a PFP resistant cell line of *Acer pseudoplatanus* by Gathercole and Street [7]. These reports suggest that selection of an amino acid-analog-resistant cell line overproducing the corresponding amino acid might in turn lead to induction of secondary metabolism of the amino acid.

In the present paper, we report the selection of a tryptophan analog-resistant cell line of *C. roseus* tissue culture and the characterization of tryptophan biosynthesis and alkaloid production in this strain.

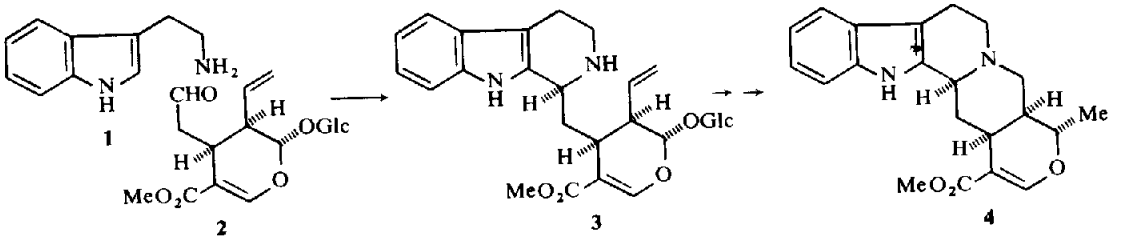
RESULTS

Selection of 5-MT resistant cell lines

The growth of *C. roseus* suspension cultures was completely inhibited by 50 mg/l. 5-MT during a two-week culture period (Fig. 1). Cultured cells were inoculated into the medium containing 50 mg/l. 5-MT and cultured for four weeks. Cultures showing growth were subcultured into the same medium and were selected again after two weeks. These selections were repeated 6–10 times at two-week intervals and three 5-MT resistant strains (CRr1, CRr2, CRr3) established. Cells of strain CRr1 were used for further investigations.

As shown in Fig. 1, resistant cells in the medium containing 50 mg/l. 5-MT showed strong resistance to growth inhibition by 5-MT. The growth of resistant cells was not inhibited even by 100 mg/l. 5-MT. Furthermore, the resistant cells could be grown in the medium containing various tryptophan analogs such as 4-methyl-, 6-methyl-, 7-methyl and 5-fluorotryptophan (Fig. 2).

The growth curve of the resistant cells was compared with that of normal cells by determination of fresh weight and cellular protein content. No difference could be observed in the growth pattern between the resistant cells and normal cells as shown in Fig. 3. The resistance of this strain is stable since such cells which had been cultured in the medium without 5-MT for six generations (twelve weeks) remained resistant.



1 Tryptamine
2 Secologanin
3 Strictosidine (isovincoside)
4 Ajmalicine

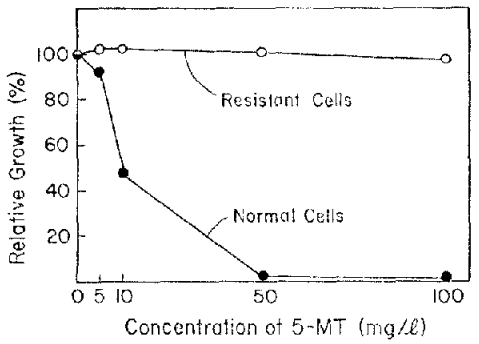


Fig. 1. Effect of 5-MT on growth of normal and the resistant cells of *C. roseus*.

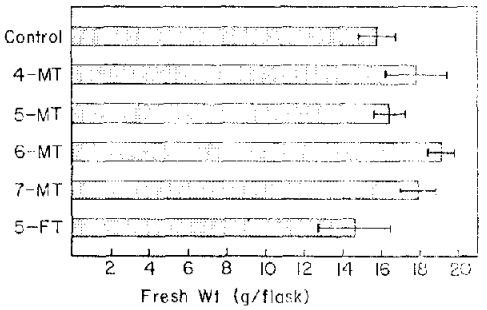


Fig. 2. Effects of various tryptophan analogs (50 mg/l.) on growth of the resistant cells of *C. roseus*. 4-MT: 4-methyltryptophan, 6-MT: 6-methyltryptophan, 7-MT: 7-methyltryptophan, 5-FT: 5-fluorotryptophan.

Tryptophan synthetase and anthranilate synthetase activities

The levels of tryptophan synthetase and anthranilate synthetase of the resistant cells were compared with those of normal cells. Cells which had been subcultured in the medium without 5-MT were inoculated into the medium without 5-MT, harvested on the sixth day after inoculation, when activities of those enzymes were highest, and used for assay of enzymes.

Table 1. Tryptophan synthetase and anthranilate synthetase activity of normal and the resistant cells of *Catharanthus roseus*

	Tryptophan synthetase (units*/mg protein)	Anthranilate synthetase (units†/mg protein)
Normal cells	40.2	0.289
Resistant cells	87.7	0.449

* 1 unit = 1 nmol indole utilized in 60 min.
† 1 unit = 1 nmol anthranilate formed in 30 min.

The resistant cells had about twice the tryptophan synthetase activity and also about 1.5 times the anthranilate synthetase activity of normal cells (Table 1). The inhibitory effects of tryptophan on tryptophan synthetase and anthranilate synthetase activities in crude cell extracts were estimated (Fig. 4). Tryptophan synthetase of normal cells was inhibited *ca* 26% by 5 μM tryptophan, whereas tryptophan synthetase of the resistant cells was inhibited 18% by the same concentration of tryptophan. Furthermore, 100 μM tryptophan inhibited anthranilate synthetase of normal cells completely, whereas anthranilate synthetase of the resistant cells was inhibited only *ca* 55% by the same concentration of tryptophan.

Table 2. Contents of tryptophan, tryptamine and ajmalicine in normal and the resistant cells of *C. roseus*

	Tryptophan (μg/g dry wt)		Tryptamine (μg/g dry wt)	Ajmalicine (μg/g dry wt)
	4 days	9 days		
Normal cells	23.7	6.8	63.6	33.3
Resistant cells	668	293	87.9	—

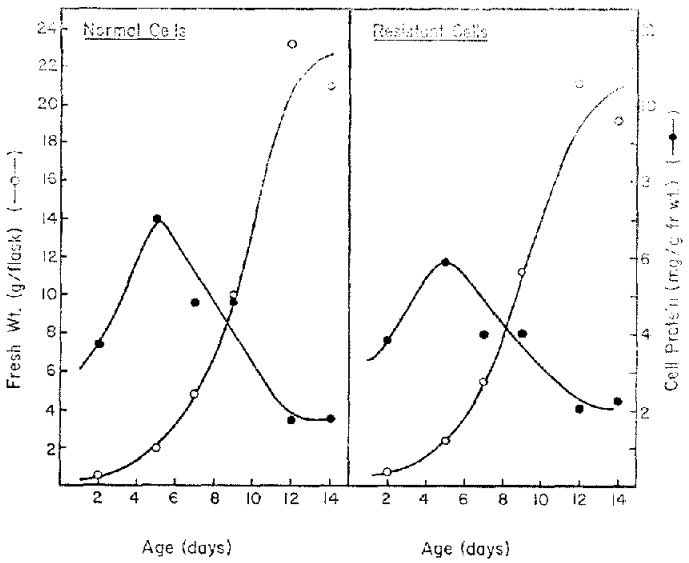


Fig. 3. Growth curves of normal and the resistant cells of *C. roseus*.

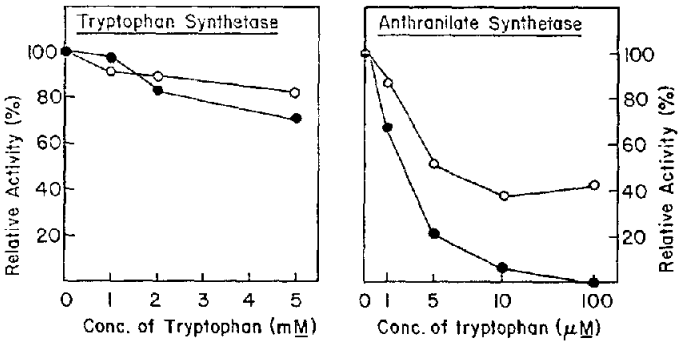


Fig. 4. Effects of tryptophan on tryptophan synthetase and anthranilate synthetase activity of normal and the resistant cells of *C. roseus* (—●—, normal cells; --○--, resistant cells).

Estimation of tryptophan, tryptamine and ajmalicine

The resistant cells which had been cultured in the medium without 5-MT for at least two generations were used for the determination of tryptophan, tryptamine and ajmalicine. The free tryptophan levels of normal and resistant cells were estimated at four days (exponential growth phase) and nine days (linear growth phase) after inoculation. The resistant cells contained thirty and forty times as much tryptophan as normal cells at four and nine days after inoculation, respectively (Table 2).

Analysis by HPLC and TLC showed the presence of tryptamine and ajmalicine in a normal cell extract. The fractions corresponding to tryptamine and ajmalicine were collected from the HPLC column and identified with authentic samples on TLC by using solvents systems (I)–(III). The contents of tryptamine and ajmalicine were estimated by HPLC on the fourteenth day after inoculation (Table 2). Though the resistant cells contained a little more tryptamine than normal cells, ajmalicine was not detectable in the resistant cell extract. The excretion of tryptophan, tryptamine or ajmalicine into the medium was not detected in either normal cells or resistant cells.

DISCUSSION

In higher plants, as in micro-organisms, anthranilate synthetase and also an altered tryptophan synthetase, way specific for the biosynthesis of tryptophan, is controlled by feedback inhibition by the end product [8]. The results shown in this paper suggest that the resistance of CRr1 cells is apparently due to an altered anthranilate synthetase and also can altered tryptophan synthetase, like those from tobacco [3] and carrot [4] resistant cells. The altered enzymes are more resistant to feedback inhibition by tryptophan. In the resistant cells, therefore, the normal feedback control of tryptophan biosynthesis is indeed relaxed and the free tryptophan pool is greatly enlarged.

It has been reported that tryptophan has a stimulatory effect on ergot alkaloid biosynthesis in *Claviceps* mycelium [9, 10]. Recently, Schmauder and Gröger [11] found that dysregulation of tryptophan biosynthesis is a prerequisite for ergot alkaloid production in *Claviceps*. Krupinski *et al.* [12] proved that dimethylallyltryptophan synthetase, the first unique enzyme in ergot alkaloid biosynthesis, is indeed induced by tryptophan. Furthermore, in *Catharanthus roseus* cell cultures Zenk *et al.* [13] reported that tryptophan stimulates ajmalicine and serpentine production in one of their strains, and tryptamine was also reported to stimulate indole alkaloid production by Carew and Krueger [14]. However, CRr1 cells, which were able to overproduce tryptophan, had a tryptamine pool size similar to that of normal cells but, surprisingly, ajmalicine was not detected.

The results of this study indicate that (1) the accumulation of tryptophan not only is unnecessary for alkaloid induction, but actually triggers an inhibitory mechanism to block alkaloid biosynthesis. Success in the ergot fungus cannot be generalized to all other plants; (2) tryptophan decarboxylase is not inducible by excess tryptophan; (3) although tryptamine is present in the 5-MT-resistant cells, it is not converted to the alkaloids as a shunt to prevent overproduction of the auxin, indoleacetic acid. This has been thought to be a possible function of the alkaloid pathway in regulation of plant growth and development.

Several questions are raised by these results: (1) Is the anthranilate synthetase from the 5-MT-resistant cells different structurally from that of the normal cells? (2) Why is tryptophan inductive in normal cells while inhibitory in the 5-MT-resistant cells in terms of alkaloid biosynthesis? (3) If the control does not lie on the tryptophan pool, where is the bottleneck? Would the mevalonoid pathway be the limiting factor for alkaloid biosynthesis? In spite of the negative outcome of this experiment for increased alkaloid production, the 5-MT-resistant cell lines may eventually provide answers to the control of alkaloid biosynthesis. Further experiments are under way.

EXPERIMENTAL

Plant material and culture method. Callus tissues were induced from seedlings of *Catharanthus roseus* (L.) G. Don on SH medium [15] containing 0.8% agar and have been subcultured on the same medium. Suspension cultures were initiated by inoculating callus tissues into liquid SH medium and have been subcultured in the same medium every 2 weeks. Suspension cells were incubated on a rotatory shaker (100 rpm) under dim light at *ca* 27°. Tryptophan analogs were added into the medium before autoclaving.

Growth experiments. Cultured cells were inoculated by

pipeting a cell suspension (5 ml) into 40 ml fresh media. After 14 days cells were harvested, weighed and homogenized with 0.1 M K-Pi buffer (pH 7.0) for the estimation of cell protein.

Tryptophan synthetase assay. Cells harvested at 6 days after inoculation were homogenized with 0.4 M K-Pi buffer (pH 8.5) containing 40 μg/ml pyridoxal phosphate and 10 mM 2-mercaptoethanol with Sorvall Omni-Mixer for 2 min at full speed. The homogenate was centrifuged at 37000 *g* for 5 min at 1–4° and the supernatant was used for incubation. The crude enzyme (0.7 ml) was added to 0.3 ml of 0.07 M K-Pi buffer (pH 8.5) containing 40 μmol L-serine, 0.2 μmol indole and 40 μg pyridoxal phosphate, and was incubated for 60 min at 30°. The indole remaining was extracted into 4 ml of toluene and was determined according to the method of ref. [16].

Anthranilate synthetase assay. Cells harvested 6 days after inoculation were homogenized with 0.2 M HEPES buffer (pH 7.5) containing 60 (v/v)% glycerol, 0.2 mM Na_2EDTA and 0.2 mM dithiothreitol. After centrifugation at 37000 *g* for 5 min, the supernatant was desalted with Sephadex G-25 column. The enzyme was eluted with 0.1 M HEPES buffer (pH 7.5) containing 10 (v/v)% glycerol, 0.1 mM Na_2EDTA and 0.1 mM dithiothreitol. One ml of the enzyme soln was added to 1 ml of aq. soln containing 0.1 μmol chorismic acid, 40 μmol L-glutamine and 8 μmol $MgCl_2$-$6H_2O$ and was incubated for 30 min at 30°. The anthranilate formed was extracted into 2 ml of EtOAc and was determined spectrofluorometrically (excitation 345 nm, emission 400nm).

Determination of free L-tryptophan levels. Cells collected at 4 and 9 days after inoculation were extracted by the method of ref. [17]. The medium and the cell extract were partially purified with Amberlite IR-120 column and free tryptophan levels were determined using tryptophanase [18].

Analysis of alkaloids. Cells were collected at 14 days after inoculation. The medium was adjusted at pH 10 with 10% K_2CO_3 soln and was extracted with EtOAc. Cells were extracted with MeOH and the cell extract was condensed to small vol., adjusted to pH 3 with 0.5 N HCl and extracted with Et_2O. The aq. layer was adjusted to pH 10 with 10% K_2CO_3 soln and extracted with EtOAc. The EtOAc from both the cells and the medium were analysed by using TLC and HPLC. For TLC analysis, Si gel plates were used with the solvent systems (I) $CHCl_3$–MeOH (9:1), (II) $CHCl_3$–MeOH–NH_3 (70:18:2) and (III) Me_2CO–petrol–Et_2NH (20:70:10). HPLC analysis was performed using a 0.39 × 30 cm stainless steel column packed with μC_{18} (Waters). The solvent was delivered at the rate of 2.0 ml/min by a Waters 6000 M solvent delivery system. Compounds were monitored at 254 nm. Elution was with MeOH–0.01 M Na-Pi buffer (pH 7.2) (70:30). The quantitative assay of tryptamine and ajmalicine was performed with the same system.

Determination of protein. Protein concn was estimated by the method of ref. [19] with BSA as standard after cell protein was precipitated with TCA.

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