Biosynthesis of Stizolobic and Stizolobinic Acids in *Amanita pantherina**

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^{14}C-labelled 3,4-dihydroxyphenylalanine (DOPA) was fed to the fruit bodies of *Amanita pantherina*. About 0.02% of radioactivity was incorporated into stizolobic and stizolobinic acids after 24 hours of the incubation. Radioactive stizolobic acid was crystallized after the addition of the authentic unlabelled material and the specific radioactivity was shown to be constant after repeated recrystallization.

These results indicate that DOPA is metabolically active as a precursor of stizolobic and stizolobinic acids in *A. pantherina*, as in *Stizolobium hassjoo*.

Introduction

Stizolobic and stizolobinic acids occur only in some specific leguminous plants, *Stizolobium* and its closely related genus, *Mucuna*, all of which contain a large amount of 3,4-dihydroxyphenylalanine (DOPA), suggesting a close biosynthetic relation between the above α-pyronyl amino acids and DOPA.

Previously we reported the formation of stizolobic and stizolobinic acids from DOPA in etiolated seedlings of *S. hassjoo* [1, 2]. Subsequently we extracted and purified two different enzymes, stizolobic acid synthase and stizolobinic acid synthase, which catalyze the conversion of DOPA into stizolobic acid and stizolobinic acid, respectively [3, 4].

In 1974, Chilton and his co-workers [5] isolated stizolobic and stizolobinic acids from the fruit bodies of *Amanita pantherina* (Basidiomycetes), a well-known poisonous mushroom. Since DOPA is reported to be present at levels too low to be detected by $FeCl_3$, ninhydrin and Pauly tests on appropriate fractions of *A. pantherina* [5], it is interesting to know whether or not stizolobic and stizolobinic acids are synthesized from DOPA also in *A. pantherina*.

In this paper, the results obtained from tracer experiments with the fruit bodies of *A. pantherina* are described, indicating the conversion of DOPA into stizolobic and stizolobinic acids in the mushroom.

Materials and Methods

Materials

DL-[β-^{14}C]DOPA (58 mCi/mmol) was purchased from CEA (Gif-sur-Yvette, France). The fruit bodies of *Amanita pantherina* were collected at Karuizawa, Nagano-ken, Japan in September, 1977 and used on the same day for the feeding experiment.

Determination of radioactivity

Radioactivities were determined with a Beckman liquid scintillation (counter spectrometer). Radioactivities of stizolobic acid and stizolobinic acid separated by two-dimensional paper chromatography were estimated after combustion of the spots corresponding to stizolobic acid and stizolobinic acid with a Packard sample oxidizer.

Feeding of radioactive DOPA

Two fruit bodies (162 g in total fresh wt) were used in this study. Labelled DOPA (0.3 μCi/g fresh wt) dissolved in 0.1 ml of distilled water was spotted on a strip of Whatman No. 3 MM paper inserted into a slit made on the stipe. Deionized water was given to the paper strip continuously from a vial. The mushrooms were held in Petri dishes ($3\times$ 14 cm) containing deionized water and kept for 24 and 72 hours under diffused light at 25–27 °C.

Isolation and analysis of radioactive stizolobic and stizolobinic acids

At the end of the incubation period each fruit body was cut into small pieces and put into boiling 80% ethanol. The extraction was repeated five times with the same solvent (each time with 260 ml for 30–40 min). The combined extracts were passed through 15 ml Amberlite IR-120 (H^+-form). Amino acids were eluted with 250 ml 2 M NH_4OH and the eluate was concentrated to a small volume with a rotary evaporator at 35–37 °C. The residue was dissolved in a minimum amount of distilled water

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and fractionated on a column of Dowex-1 (CH_3COO^--form, 0.7×30 cm) with 330 ml 1 M acetic acid and 65 ml 1 M HCl, successively. Radioactive stizolobic acid and stizolobinic acid were separated from the concentrated HCl-eluate by paper chromatography with *iso*-propanol/formic acid/water (20:1:5, v/v/v).

Radioautographic procedures

Radioautograms were prepared by placing double-coated X-ray films in direct contact with paper chromatograms.

Recrystallization of radiolabelled stizolobic acid

Purified stizolobic acid was cocrystallized repeatedly with an authentic specimen (30 mg) from *n*-propanol/water (6:4, v/v). The crystallization was repeated until the crystals showed a constant specific radioactivity.

Results and Discussion

Table I shows the radioactivities found in stizolobic and stizolobinic acids after 24 hours and 72 hours incubation with labelled DOPA. After 24 hours incubation, 0.020% and 0.021% of the radioactivity, which had been taken up by the mushrooms, were found in stizolobic acid and stizolobinic acid, respectively. After 72 hours incubation, the radioactivities incorporated into stizolobic and stizolobinic acids increased 5 – 8 times compared with those after 24 hours incubation, but as a percentage of the total radioactivity taken up the increase was only slightly.

At the end of 72 hours incubation, stizolobic and stizolobinic acids were extracted from the fruit body and enriched by Amberlite IR-120 and Dowex-1 column chromatography. The positions of the radioactive spots on a radioautogram prepared from the concentrated HCl-eluate from Dowex-1 were identical with those of the authentic stizolobic and stizolobinic acids. Incorporation of radioactivity into stizolobic acid was further confirmed by the recrystallization of radiolabelled stizolobic acid after the addition of an unlabelled sample. The stizolobic acid crystals showed a constant specific radioactivity after four or five recrystallizations (Table II).

Table II. Recrystallization of radiolabelled stizolobic acid.

Recrystallization No.	Stizolobic acid wt [mg]	Total act. [$\times 10^3$ dpm]	Specific act. [$\times 10^2$ dpm/μmol]
1	24.21	8.5	0.8
2	19.03	8.4	1.0
3	17.01	6.7	0.9
4	12.20	6.5	1.2
5	9.24	4.7	1.2

Stizolobic acid (30 mg, 0.9×10^2 dpm/μmol) was dissolved in a minimum amount of hot *n*-propanol/water (6:4, v/v) and recrystallized repeatedly. Radioactivity in stizolobic acid was determined as described in the text.

It was shown in previous papers that α-pyronyl amino acids are synthesized in *S. hassjoo* from DOPA by extradiol cleavage of the aromatic ring [1, 2]. Though detailed investigations for the biosynthetic mechanisms of stizolobic and stizolobinic acids have not been done in *A. pantherina*, they may be synthesized by the same route as in *S. hassjoo*. Several reports have appeared recently concerning betalamic acid and muscaflavin in *Amanita* and *Hygrocybe* species [6 – 10] and their possible biosyntheses from DOPA by extradiol ring fission [9]. In *A. pantherina*, DOPA may be converted quickly after its synthesis into other compounds including stizolobic and stizolobinic acids, leading to little accumulation within the fungus.

Table I. Incorporation of the radioactivity from DL-[β-^{14}C]DOPA into stizolobic and stizolobinic acids in the fruit bodies of *A. pantherina*.

Metabolic period	Dose	Radioactivity incorporated into			
		Stizolobic acid		Stizolobinic acid	
[hours]	[μCi/g fr. wt]	[$\times 10^3$ dpm]	[% of uptake]	[$\times 10^3$ dpm]	[% of uptake]
24	0.31	0.71	0.021	0.67	0.020
72	0.31	3.72	0.024	4.64	0.030

DL[β^{14}C]DOPA (58 mCi/mmol) was fed to the freshly collected fruit bodies of *A. pantherina* and allowed to be metabolized for given incubation times. Radioactivities in stizolobic and stizolobic acids isolated were determined as described in the text.

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