CONVERSION OF RETICULINE INTO SCOULERINE BY A CELL FREE PREPARATION FROM *MACLEAYA MICROCARPA* CELL SUSPENSION CULTURES

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Received 6 November 1974

1. Introduction

One of the key reactions leading to the wide range of benzylisoquinoline derivatives is the conversion of reticuline into scoulerine, a tetrahydroprotoberberine alkaloid [1], by the conversion of the *N*-methyl group of the former into the so called berberine bridge of the latter (fig. 1) [2,3]. Our interest in this important step arose from the finding that an interruption of alpinigenine biosynthesis takes place in young thebaine (e^h) types of *Papaver bracteatum* [4]. The reaction affected is probably the formation of scoulerine from reticuline [5]. While our experiments with alpinigenine producing *P. bracteatum* plants have not resulted so far, in the isolation of an enzyme responsible for this step, investigations with cell suspension cultures of the Papaveracee *MacLeaya microcarpa* have been more successful. This system is one of the few cell cultures capable of synthesizing alkaloids. In the present paper we report the isolation and partial characterization of an enzyme catalyzing the conversion of reticuline into scoulerine (fig. 1).

2. Materials and methods

2.1. *Cell suspension cultures*

Cells of *M. microcarpa* (3–5 g fresh weight inoculum) were shaken in Erlenmeyer flasks (200 ml) containing 45 ml medium (mineral salts according to [6]; vitamins and amino acids from [7]; sucrose 20 g/litre; 2,4-dichlorophenoxyacetic acid 2 mg/litre; kinetin 0.2 mg/litre; gibberellic acid 0.1 mg/litre at 80 rev/min on rotatory shakers. The cultures were kept at 24–26°C alternately 12 hr in the dark and 12 hr illuminated with white light (approx. 400 lux). After 6 days grown the cells were harvested by vacuum filtration.

2.2. *Enzyme preparation*

By the following procedures at 0–4°C a crude enzyme preparation was obtained. A definite amount of wet cells was homogenized with an equal volume of 0.1 M borate buffer, pH 8.8, ('standard buffer') for 5 min in a prechilled mortar with quartz sand. The supernatant from centrifugation at 20 000 *g* for 20 min, final protein concentration of approximately 1 mg per ml, was generally used for the experiments (crude extract). Protein was determined by the Lowry method [8]. It was necessary to precipitate the protein with trichloroacetic acid (final concentration 5%) and to redissolve it in 0.1 M NaOH.

2.3. *Purification*

For some investigations the crude enzyme extract was further purified. The protein precipitated with solid $(NH_4)_2SO_4$ (60% saturation) was centrifuged off and resuspended in a minimal amount of standard buffer. The solution was passed through a Sephadex G-25 column (1 cm × 20 cm). After a second $(NH_4)_2SO_4$ precipitation (60% saturation) the redissolved solution was chromatographed with the standard buffer on a column (2 cm × 70 cm) of Sephadex G-100.

2.4. *Synthesis of (±) N [$^{14}CH_3$] reticuline*

Starting with the conversion of *O*-benzylvanillin and O-benzylisovanillin into the corresponding alcohols, (±) *N* [$^{14}CH_3$] reticuline was synthesized by the methods of Battersby's group [9] (steps up to

the cyclization after acylation) as well as of Barton et al. [10] (further reactions). The product was radiochemically pure (TLC systems I and IV, Dragendorff reaction, scannogram). The specific radioactivity of (±) $N[^{14}CH_3]$reticuline was 3.51 mCi/mmol.

2.5. *Standard assay*

The incubation mixture in a total volume of 2 ml containing 235 nmoles of (±) $N[^{14}CH_3]$reticuline hydrochloride, 0.3–1.5 mg protein, and standard buffer was incubated at 30°C for 30–120 min. The reaction was stopped and the alkaloids extracted by the addition of one drop of 12% NH_4OH followed by intensive shaking with 3 portions (5 ml) of chloroform. The combined extracts were evaporated in vacuo and chromatographed (TLC system I). The radioactive substances were detected with a TLC scanner (LB 2723, Berthold, Wildbad). The amount of [^{14}C]scoulerine formed was determined by integration of the radioactive scoulerine peak after scanning, and was expressed in nmoles scoulerine per mg protein. This integration is possible, because the specific radioactivity of the scoulerine formed is identical with that of the reticuline. Therefore one unit of the scoulerine peak corresponds to one unit of the reticuline peak.

2.6. *Chromatography*

Thin-layer chromatography was performed on silica gel G (Merck, Darmstadt) with the following solvent systems:

I Benzene:methanol, 8:2 [11]
II Benzene:acetone, 8:2
III Chloroform:methanol, 9:1 [12]
IV Benzene:methanol, 9:1 / Chloroform:methanol: acetone:ethylacetate, 6:2:1:1 [13]

Gas chromatography: Pye panchromatograph, glass column, 1.5 × 4 mm (i.d.), 3% QF1 on Gaschrom Q (125–150 μm); Ar as carrier gas (100 ml/min); t = 245°C; FID.

3. Results and discussion

Like the intact plant, cell suspension cultures of *M. microcarpa* also produce the alkaloids allocryptopine and sanguinarine beside the main alkaloid protopine [14]. This finding confirms earlier information about the alkaloid production in primary calli of *Macleaya cordata* [15]. The biosynthetic pathway of the predominant alkaloids belonging to the protopine and benzophenanthridine type, respectively, includes as one of the first steps the conversion of reticuline into scoulerine (fig. 1) [1].

Since cell suspension cultures of *M. microcarpa* during the first days of each subcultivation produce considerable amounts of alkaloids the search for the enzyme responsible for the aforementioned reaction seemed promising. Indeed it was found that crude enzyme preparations from 6 day old subcultures catalyze the conversion of (±) $N[^{14}CH_3]$reticuline into a radioactive alkaloid. Rechromatography (TLC systems II-IV) of the isolated product with several authentic substances demonstrated coincidence with scoulerine. In addition, the u.v. spectrum of the reaction product shows a bathochromic shift in alkaline solution (0.1 N ethanolic NaOH) indicating a phenolic property. Methylation with diazomethane led to tetrahydropalmatine, the identy of which was demonstrated by chromatography with reference samples (TLC systems I–IV), and recrystallization after addition of nonlabelled tetrahydropalmatine to a constant specific radioactivity.

After a crude enzyme extract was kept for 30 min at 100°C no scoulerine formation could be detected after subsequent incubation. The enzymatic reaction shows absolute oxygen requirement. After a 5 min period of nitrogen flow before addition of the substrate, and under exclusion of air during the incubation, no scoulerine was formed from reticuline.

Under standard conditions as well as in nitrogen treatment the following cofactors (concentration: 1 mM) showed no influence on scoulerine formation: NAD, NADP, NADH, NADPH, glutathion (reduced and oxidized), FAD, ascorbic acid.

Purification of the crude extract by $(NH_4)_2SO_4$ precipitation and gel chromatography on Sephadex

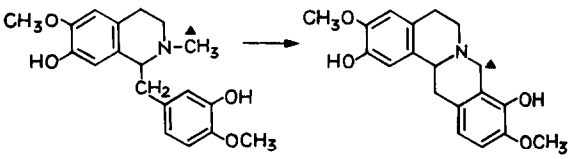

Fig. 1. Conversion of reticuline into scoulerine.

Table 1
Purification of the enzyme

Procedure	Purification step	Fraction no.	Protein (mg/ml)	nmoles Scoulerine formed per mg protein	Relative enzyme activity (%)
Enzyme extraction			0.85	18	100
1. $(NH_4)_2SO_4$ and Sephadex G-25	I		12.00	27	150
2. $(NH_4)_2SO_4$ and Sephadex G-100	II	1–4	0.10–2.55	0	0
		5	2.55	10	55
		6	2.30	23	128
		7	2.00	25	139
		8	1.40	38	210
		9	1.25	49	272
		10	1.15	109	605
		11	0.83	130	722
		12	0.73	109	605
		13	0.65	83	460
		14	0.60	72	400

The incubation was carried out in a final volume of 2 ml for 120 min at 30°C.

G-100 resulted in a more than 7-fold increase of specific enzyme activity (table 1). Using partially purified enzyme extracts (step I) under standard conditions a linear dependence of scoulerine formation on the amount of added protein was found (fig. 2). As shown in fig. 3 the most favourable pH range for the enzymatic scoulerine formation was observed between pH 7.5 and 8.2.

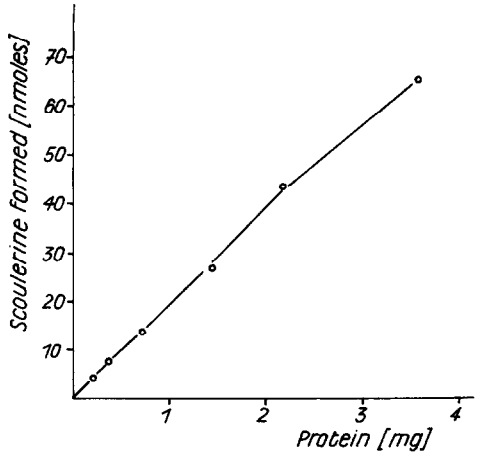

Fig. 2. Dependence of scoulerine formation on protein concentration. Incubation time: 30 min.

Acknowledgements

We thank Professor E. Brochmann-Hanssen, San Francisco, for the gift of (−)–scoulerine, Dr. H. Koblitz, Gatersleben, for samples of tissue cultures of

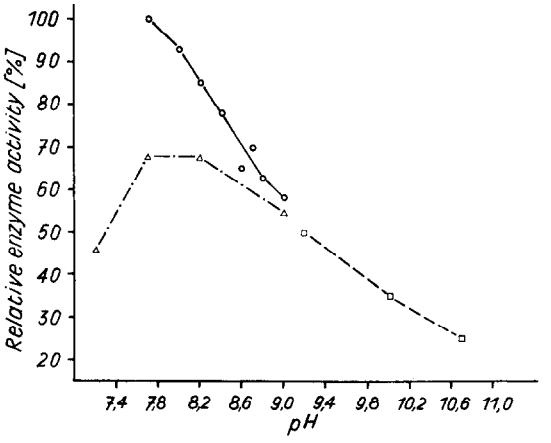

Fig. 3. Effect of pH on scoulerine formation. Assay conditions: 0.2 mg protein (step I); incubation time: 60 min; (○—○—○) 0.1 M boric acid/borax buffer (standard buffer); (□—□—□) 0.1 M Na borate/NaOH buffer; (△—△—△) 0.05 M Tris/HCl buffer.

Macleaya microcarpa, and Dr H. Rönsch for his helpful participation in the synthesis of (±) $N[^{14}CH_3]$-reticuline. The skilled technical assistance of Miss H. Meyer and Mrs H. Nixdorf is gratefully acknowledged.

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