Enzymatic Synthesis of Quinolizidine Alkaloids in Lupin Chloroplasts

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The enzymatic sequence responsible for the biosynthesis of tetracyclic quinolizidine alkaloids could be localized in chloroplasts isolated from *Lupinus polyphyllus* leaves and *L. albus* seedlings by differential centrifugation. Upon feeding of cadaverine to isolated chloroplasts lupanine is produced as the main alkaloid. Chloroplasts treated with digitonine produce sparteine and 17-oxosparteine instead of lupanine, thus indicating that the biosynthetic sequence is interrupted. The intermediacy of 17-oxosparteine could be confirmed since exogeneous 17-oxosparteine is converted into lupanine by intact chloroplasts. 17-Oxosparteine synthase (see Z. Naturforsch. **34 c,** 704 1979) the key enzyme of quinolizidine alkaloid biosynthesis could be solubilized from chloroplasts treated with detergents or osmotic shock.

Introduction

Quinolizidine alkaloids are produced in the green parts of lupin plants [1]. Alkaloid synthesis shows a circadian rhythm which is positively correlated with illumination [2]. Furthermore, histochemical investigations of lupin tissues revealed that the alkaloids are associated with chloroplasts [3]. These results suggest that chloroplasts might be involved in the biosynthesis of quinolizidine alkaloids.

Recently we could demonstrate the presence of the key enzyme responsible for the synthesis of the tetracyclic quinolizidine skeleton in a crude enzyme preparation obtained from cell suspension cultures of *Lupinus polyphyllus* [4]. The enzyme called 17-oxosparteine synthase is assumed to catalyze the following reaction [5]:

$$3 \text{ cadaverine} + 4 \text{ pyruvate} \rightarrow 17\text{-oxosparteine} + 4 \text{ alanine}.$$

In this communication we report on the localization of the integrated sequence of lupin alkaloid biosynthesis in lupin leaf chloroplasts.

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Materials and Methods

Organelle preparations

Leaves of bitter *Lupinus polyphyllus* Lindl. were collected from non-fruiting specimens between April and July and were stored at 4 °C for at least three hours. Leaves of *L. albus* seedlings were taken at the age of 14 days. Organelle isolation was carried out according to Jacobi [6]. The isolation buffer (pH 7.8) consisted of 600 mM mannitol, 10 mM pyrophosphate, 1 mM dithioerythritol (DTE), and 10 mM diethyldithiocarbamate (DIECA). Leaves (50 g fresh weight) were homogenized either in a mortar or a Waring-blendor containing 200 ml isolation buffer. The homogenate was squeezed through 3 layers of cotton wool and cheese-cloth and centrifuged at $500 \times g$ for 5 min. The supernatant was centrifuged at $1000 \times g$ for 10 min. The resulting pellet was suspended in 10 ml buffer and recentrifuged at $1000 \times g$ for 10 min. The pellet was suspended in 5 ml isotonic isolation buffer (pH 7.8) or hypotonic 0.1 M sodium phosphate buffer (pH 7.8) complemented with 1 mM DTE and 10 mM DIECA ("crude chloroplasts"). The supernatant of the $1000 \times g$ centrifugation was recentrifuged at $10000 \times g$ for 10 min. The resulting pellet was resuspended in 5 ml buffers as given above ("crude mitochondria").

For purification of "crude mitochondria" by gradient centrifugation 1 ml "crude mitochondria" (suspended in isolation buffer) was layered on a 4-step (8 ml each) discontinuous sucrose gradient (20–50%) containing 10 mM pyrophosphate (pH 7.8). Centrifugation was carried out in a Sorvall SS 90 vertical rotor at 18000 rpm for 30 min.

Identity and quality of the organelle fractions were checked by phase contrast light microscopy (Zeiss). The chloroplast fraction usually consisted of 70% type I chloroplasts [6]; this percentage decreased however during incubation at 30 °C, thus resulting in type II chloroplasts.

Organelle incubation

The experimental conditions for alkaloid synthesis in organelle preparations were similar to those given by [4]: The standard reaction mixture consisted of 5 ml (radioactive assays 2 ml) suspension of "crude chloroplasts" or "crude mitochondria" complemented with 10 mM cadaverine and 20 mM pyruvate. The reaction vessels were gassed with nitrogen, sealed and incubated at 30 °C for 5 h. The reaction was terminated with trichloroacetic acid. Appropriate blank controls without added substrates were performed for each assay.

Analytical methods

Total alkaloid contents of incubation mixtures were estimated photometrically with Reifer's reagent [4, 7].

For alkaloid extraction the alcalized reaction mixture was applied on an Extrelut column (Merck) and the alkaloids were eluted with methylene chloride. After evaporation of the solvent the alkaloid extracts were separated by thin-layer chromatography according to [4] or subjected to capillary gas-liquid chromatography (GC) according to [8].

The structures of all alkaloids involved in this study were confirmed by gas-liquid chromatography-mass spectrometry (GC-MS) [8].

In tracer experiments with [2,5-^{14}C]cadaverine (purchased from NEN) alkaloid extracts were separated by thin-layer chromatography. Radioactive spots were localized by radio-scanning, scraped off the plates, eluted with 70% acidified methanol and measured by scintillation counting.

Total chlorophyll contents were determined according to [9]. Succinate dehydrogenase activity was assayed in samples solubilized with 1% Triton X-100 according to Veeger *et al.* [10].

Synthesis

17-Oxosparteine was prepared by partial synthesis from sparteine according to Schütte *et al.* [11].

Results

Crude chloroplasts and mitochondria prepared from *L. polyphyllus* leaves by differential centrifugation produce quinolizidine alkaloids (Table I). The amount of alkaloids produced is positively correlated with the chlorophyll content but negatively with the activity of succinate dehydrogenase used as mitochondrial marker enzyme. The crude mitochondria which were still contaminated by chloroplasts were further purified by gradient centrifugation. The resulting mitochondrial band was free of alkaloid synthesizing activity. These results strongly point to the chloroplasts as site of alkaloid synthesis.

Similar results were obtained with organelle preparations obtained from *L. albus* seedlings by differential centrifugation. Upon feeding of 10 mM cadaverine or 0.1 mM [^{14}C]cadaverine (2.5 µCi) only 17% (7%) alkaloid synthesizing activity could be measured in the mitochondrial fraction in comparison to chloroplasts (83% and 93%, respectively).

No alkaloid synthesis was detected in the soluble fraction of particle preparations from both lupin species.

The conditions of alkaloid synthesis in isolated chloroplasts (Table II) are similar to those estab-

Table. I. Localization of quinolizidine alkaloid synthesis in organelle fractions isolated from *L. polyphyllus* leaves. A = prepared by differential centrifugation; B = separation of crude mitochondria by gradient centrifugation. The fractions with the highest succinate dehydrogenase activity were classified as "mitochondrial band" and those with the highest chlorophyll content as "chloroplast band". Incubation under standard conditions; total alkaloid content was determined with Reifer's reagent.

Preparation	Alkaloid produced [µg/ml/5 h]	Chlorophyll a + b [mg/ml]	Succinate dehydrogenase activity [nkat/ml]
A Crude chloroplasts	37	1.62	14.9
Crude mitochondria	22	0.59	59.5
B Chloroplast band	12.3	0.16	1.9
Mitochondrial band	0	0.02	17.0

Table II. Conditions of alkaloid synthesis in crude chloroplasts isolated from *L. albus* and *L. polyphyllus*. Alkaloid production was determined photometrically with Reifer's reagent.

Assay	% of alkaloids produced	
	L. albus	*L. polyphyllus*
Standard assay	100	100
minus pyruvate	62	91
minus DIECA	42	4
aerobic	16	0 [a]

[a] Confirmed by GC.

lished for crude enzyme preparations of cell suspension cultures [4]. Anaerobic incubation and addition of DIECA which serves as an inhibitor of diamine oxidase and phenolases are essential for optimal alkaloid synthesis. In the presence of DIECA chloroplast preparations remain green but they turn to a brownish colour when DIECA is omitted. The effect of pyruvate is less pronounced presumably due to a sufficiently high endogenous pool of this compound in chloroplasts.

Lupanine was found to be the main alkaloid produced by crude chloroplasts incubated with cadaverine (Table III). In addition small amounts of 17-oxosparteine were detectable. This result could be confirmed by tracer experiments. When [^{14}C]-cadaverine was fed to crude chloroplasts 1.7% of the radioactivity applied could be detected in lupanine and 0.7% in 17-oxosparteine.

Chloroplasts treated with digitonine synthesize sparteine instead of lupanine as main alkaloid and small quantities of a new alkaloid. The mass spectrum of this compound displays a molecular ion at m/z 246 indicating the presence of a dehydrooxosparteine. The definite structure has still to be established.

To some extent alkaloid synthesizing enzyme activity can be solubilized from isolated chloroplasts treated with detergents or by osmotic shock (Table IV). The soluble enzyme preparation further purified from contaminating low-molecular compounds by gel filtration catalyzes the formation of 17-oxosparteine as the only alkaloid (Table III), indicating its identity with 17-oxosparteine synthase recently detected in crude enzyme preparation of lupin cell suspension cultures [4, 5].

To prove the postulated role of 17-oxosparteine as a key intermediate in lupanine synthesis [5], isolated chloroplasts were incubated with 17-oxosparteine. Lupanine was produced as the main transformation product (Table III). Reduced pyridine nucleotides did not influence this conversion.

Table III. Alkaloid synthesis and alkaloid transformation in preparations of crude chloroplasts upon feeding of different substrates.

Preparation	Substrate	% of total alkaloids produced [a]		
		Lupanine	Sparteine	17-Oxosparteine
Crude chloroplasts	Cadaverine (10 mM)	93	–	<5
Crude chloroplasts plus 2% digitonine	Cadaverine (10 mM)	–	87	<5
Solubilized chloroplasts enzyme [b]	Cadaverine (10 mM)	–	–	95
Crude chloroplasts	17-Oxosparteine (2 mM)	88	<5	
Crude chloroplasts	17-Oxosparteine (1 mM) NADH (1 mM)	87	<5	
Crude chloroplasts	17-Oxosparteine (1 mM) NADPH (1 mM)	82	<5	

[a] Alkaloids were extracted from the incubation mixtures and analyzed quantitatively by GC; the respective structures were confirmed by GC-MS as in [8].
[b] Solubilized from crude chloroplasts by digitonine treatment as given in Table IV, and further purified by gel filtration (Sephadex G-25).

Table IV. Solubilization of the alkaloid synthesizing enzyme activity from crude lupin chloroplasts by osmotic shock and treatment with detergents. Crude chloroplasts were incubated in hypotonic 0.1 M phosphate buffer (pH 7.8) containing 1 mM DTE and 10 mM DIECA and detergents as indicated. The incubation mixture was stirred at 4 °C for 45 min and centrifuged at 20000×*g* for 20 min. Supernatant and resuspended pellet were incubated under standard assay conditions. Alkaloid production was determined photometrically with Reifer's reagent.

Preparation	Alkaloid produced (%) [a]		
	Osmotic shock	Digitonine (2 % w/v)	Triton X-100 (2% v/v)
L. polyphyllus chloroplasts			
pellet	40	37	24
supernatant	60	63	76
L. albus chloroplasts			
pellet		60	
supernatant		40	

[a] The total amount of alkaloid produced in the pellet and respective supernatant, corrected by appropriate blank controls, was set as 100%.

Discussion

The experiments with isolated organelle preparations of lupins prove that the biosynthesis of tetracyclic quinolizidine alkaloids is associated with chloroplasts, thus confirming former physiological and histochemical results [2, 3]. There is increasing evidence that the biosynthetic pathways of the aspartate-family amino acids (methionine, threonine, isoleucine, lysine) are located in the chloroplasts [12, 13]. Several enzymes of this pathway including diaminopimelate decarboxylase [14], the final enzyme in lysine biosynthesis, could be isolated from chloroplasts. Thus the biosynthetic pathways of lysine and quinolizidine alkaloids share the same subcellular compartment. Preliminar studies using isolated lupin chloroplasts revealed a significant conversion of [^{14}C]lysine into cadaverine; therefore, the total sequence from lysine via cadaverine to the alkaloids can be established in lupin chloroplasts (Schoofs, Hartmann, and Wink, unpublished).

The pathway of lupin alkaloid biosynthesis observed in chloroplasts may be summarized in the sequence illustrated in Fig. 1. Since lupanine, the main alkaloid of *Lupinus polyphyllus*, is produced almost exclusively by isolated intact chloroplasts, an enzymatic sequence must be postulated. The first step is the synthesis of the tetracyclic quinolizidine skeleton catalyzed by 17-oxosparteine synthase [5]. This enzyme could be solubilized from chloroplasts. The intermediacy of 17-oxosparteine was demonstrated by its conversion to lupanine by isolated chloroplasts whereas exogenous sparteine is not converted into lupanine under these conditions (Wink and Hartmann, unpublished). Cho *et al.* [15] found that lupanine and sparteine are synthesized independently and proposed a compound related to $\Delta 1(2)$-dehydrosparteine as the common precursor for both alkaloids. This assumption is in agreement with our results as membrane altered chloroplasts produce sparteine instead of lupanine. Furthermore a dehydrosparteine though not detected in chloroplasts could be identified from *L. polyphyllus* cell suspension cultures (Wink, Hartmann, and Witte, unpublished).

The complete interruption of lupanine synthesis in chloroplasts with experimentally altered membranes resulting in a release of sparteine and 17-oxosparteine suggests that the intactness of membranes is important for lupanine biosynthesis. Membrane association is indicated for 17-oxosparteine synthase which could be at least partially solubilized from chloroplasts but was found to be insoluble in acetone powder preparations obtained from cell suspension cultures [4, 5]. It might be speculated that the biosynthesis of quinolizidine alkaloids in chloroplasts constitutes a channelled process with a sequence of membrane associated enzymes.

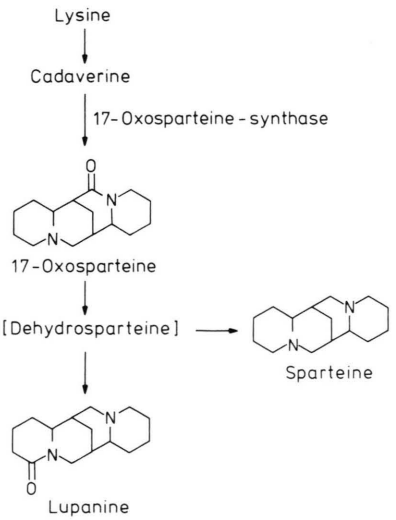

Fig. 1. Possible pathway of lupanine biosynthesis.

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