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Subcellular Localization of Spermidine Synthase in the Protoplasts of Chinese Cabbage Leaves[1]

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ABSTRACT

Previous studies on the presence of spermidine synthase (EC 2.5.1.16) in the protoplasts of Chinese cabbage (*Brassica pekinensis* var Pak Choy) leaves had detected a small but significant fraction of the enzyme in a crude chloroplast fraction (Cohen, Balint, Sindhu 1981 Plant Physiol 68: 1150–1155). To establish whether this enzyme is truly a chloroplast component, we have isolated purified intact chloroplasts from protoplasts by density gradient centrifugation in silica sols (Ludox AM). Such chloroplasts contained all of the diaminopimelate decarboxylase (EC 4.1.1.20) of the protoplasts, but were essentially devoid of spermidine synthase. Control experiments showed that the latter had not been inactivated under conditions of isolation, purification, and assay of the intact chloroplasts. Isolation and assay of protoplast vacuoles in a further examination of the supernatant fluid containing the enzyme revealed a significant fraction of the enzyme in the vacuole fraction. However this fraction was found to contain similar proportions of a soluble enzyme, glucose 6-phosphate dehydrogenase. It has been concluded that vacuolar fractions are difficultly separable from soluble cytoplasmic material, which is probably the only compartment containing spermidine synthase.

The spherical RNA virus, TYMV,[2] contains fairly large amounts of spermidine and markedly less spermine (2, 6, 14). The spermidine is nonexchangeable in the intact virus (6) and appears to have become associated with the RNA before the RNA was encapsulated within the virus. It is possible that, as in the condensation of MS2 RNA by spermidine, which increases the penetrability of this RNA during infection of pilus-bearing male bacteria (16), the triamine facilitates the condensation of the TYMV RNA during packaging within the viral protein subunits.

The affinity of RNA for the tetramine, spermine, is greater than that for the triamine, spermidine. Within a common pool of spermidine and spermine, it would be expected that the virus would be relatively rich in the tetramine, but it is not, suggesting that the two polyamines may exist in relatively separate compartments. During virus multiplication TYMV, as a tymovirus, accumulates entirely within pockets between aggregated chloroplasts. It was asked then if the chloroplasts, as possible homologs of Cyanobacteria, which synthesize spermidine but do not synthesize spermine (11, 22), may be the metabolic compartment generating viral spermidine (7).

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[2] Abbreviations: TYMV, turnip yellow mosaic virus; SAM, *S*-adenosylmethionine; DAP, diaminopimelate; G-R, grinding and resuspension; PCA, perchloric acid; PLP, pyridoxal phosphate.

A crude chloroplast fraction isolated from disrupted protoplasts derived from leaves of Chinese cabbage was found to contain a significant fraction of spermidine synthase (5). Furthermore, it had been stated by other laboratories that chloroplasts can probably synthesize methionine (19, 24) and SAM (15). However, it was not clear that these activities were truly those of chloroplast components, since the contamination of chloroplasts by cytoplasmic or other enzymes is well known (13, 23). It was important, therefore, to determine if the enzyme, spermidine synthase, could be found in purified intact chloroplasts. We have found that of the various procedures devised to obtain such intact organelles, gradients of purified Ludox (20) have been effective, permitting the demonstration of the enzyme of the lysine pathway, DAP decarboxylase, as an internal chloroplast component. However, such purified organelles were devoid of spermidine synthase, before or after disruption of the organelle.

In its turn, the 'soluble' spermidine synthase of a crude protoplast extract, comprising 85 to 90% of the total initial activity, was found not to be readily sedimentable, suggesting a size in protoplast extracts not greater than that of the partially purified enzyme estimated by Sephadex fractionation. In additional fractionation steps, although a significant fraction of the nonsedimentable enzyme was associated with the vacuoles isolated from protoplasts, this appears to reflect a contamination by soluble cytoplasmic proteins.

MATERIALS AND METHODS

Plant Material. Seeds of Chinese cabbage (*Brassica pekinensis*, var. Pak Choy) were obtained from Nichols Garden Nursery, Albany, OR. Seedlings and larger plants were grown in a controlled environment chamber set for 18-h d at 28°C and 20,000 lux using incandescent and fluorescent lighting, and 6-h dark periods at 22°C. When the plants were about 20 d old, the rosette of each plant was removed, leaving two leaves which were inoculated by abrading the upper surface and painting with a solution of TYMV (0.1 mg virus/ml in 50 mM acetate, pH 4.8). Control plants were similarly pruned and newly emerging leaves of both healthy and infected plants were used 10 to 14 d later. TYMV was purified as described earlier (6).

Protoplast Isolation. Young, rapidly growing leaves from 20- to 35-d-old plants were used for the isolation of protoplasts. For infected plants and controls, newly emerging leaves from 31- to 35-d-old plants (10–14 d postinfection) were used. Leaves were deribbed, weighed, and gently rubbed with carborundum using a moist Q-tip until the lower surface turned shiny green. The leaves were then washed thoroughly with H_2O, blotted dry and floated on a sterile solution (10 ml/g leaf tissue) of 0.4 M mannitol in 5 mM Mes-KOH (pH 5.6) containing 0.1% BSA and 1 mM $CaCl_2$ and 0.5% each of cellulase and macerozyme in sterile 150-mm Petri dishes. The leaves were incubated at 18°C in the dark.

After 18 h, the enzyme solution was removed gently by aspiration and fresh 0.4 M mannitol in 5 mM Mes-KOH (pH 5.6), containing 0.1% BSA and 1 mM $CaCl_2$ was added. The protoplasts were released by a careful shaking of the dish. The suspension was diluted to about 40 ml per gram leaf tissue. This was filtered through eight layers of cheese cloth and centrifuged at 20*g* for 10 min. The pellets were resuspended in the mannitol solution and washed six times by centrifuging at 20*g*. The protoplasts were then resuspended in G-R medium (0.33 M sorbitol, 2 mM Na_2 EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $Na_4P_2O_7$, 5 mM isoascorbic acid, 50 mM Hepes/NaOH, pH 6.8), counted in a hemocytometer and adjusted to approximately 1 to 2 × 10^6 protoplasts/ml. The preparation and composition of G-R medium has been described (20).

After disrupting the protoplast preparations in passage through a 20-μm nylon mesh fitted to a 5-ml syringe, crude chloroplasts were collected by centrifugation at 210*g* for 10 min. Pure chloroplasts were obtained by centrifugation of the crude chloroplasts on linear gradients of silica sol, Ludox AM as described below.

Preparation of Gradients. Gradients for the purification of chloroplasts were prepared essentially as described by Price *et al.* (20). PEG (1.05 g), BSA (0.35 g), and Ficoll (0.35 g) were dissolved in 35 ml of purified Ludox AM. Ludox was purified as described by Price and Dowling (21). Starting and limiting solutions for the gradients contained 3.5 and 28 ml, respectively, of the Ludox/PEG/BSA/Ficoll mix in a final volume of 35 ml. Both the starting and final solutions also contained the same solutes as the G-R medium plus 6 mg of reduced glutathione/35 ml. Linear gradients of 32 ml derived from 16 ml each of the starting and limiting solutions were generated in 25 × 89 mm centrifuge tubes, respectively. The concentrations of the ingredients of the G-R medium are, therefore, constant throughout the centrifuge tube, but there is a gradient in Ludox/PEG/BSA/Ficoll.

Between 1 and 1.5 ml each of the crude chloroplast suspension was layered onto the gradients. Centrifugation was carried out in SW27 swinging bucket rotors (Beckman No. 2904) at 8100 rpm for 20 min at 4°C. After the centrifugation, three major bands were obtained. The upper band contained thylakoid membranes and the middle band contained stripped chloroplasts. A lower band was comprised of intact refractile chloroplasts. The intact chloroplasts were collected with a Pasteur pipette, diluted 6-fold with G-R medium and centrifuged at 400*g* for 10 min. In two washes, the pellets were resuspended with 0.33 M sorbitol (pH 8.4) and recentrifuged.

Preparation of Vacuoles. Vacuoles were prepared by shearing the protoplasts in a Ficoll gradient. The conditions for preparing the protoplasts were essentially the same as described earlier in "Materials and Methods," except that BSA was omitted from the washing solution. Four ml of the protoplast suspension (20 × 10^6 protoplasts) was loaded onto a 3-step discontinuous Ficoll-400 gradient. The gradient was prepared by layering 10 ml each of 5, 10, and 15% (w/v) Ficoll-400. Each Ficoll gradient contained 0.7 M mannitol and 1 mM EDTA in 10 mM Hepes-NaOH (pH 8.0) at room temperature. The gradient was immediately spun at 100,000*g* for 2 h in a SW27 (No. 2904) rotor in the Beckman ultracentrifuge. Vacuoles were collected from 5, 10, and 15% Ficoll, respectively, diluted 20 times with 0.7 M mannitol containing 1 mM EDTA in 10 mM Hepes-NaOH (pH 8.0), and collected by centrifugation at 300*g* for 15 min. The vacuoles were washed twice with 50 ml of 0.7 M mannitol containing 1 mM EDTA in 10 mM Hepes-NaOH (pH 8.0) and counted in a hemocytometer.

Enzyme Assays. Triton X-100 was added to all the fractions to a final concentration of 0.25% to effect the lysis of protoplasts and chloroplasts before estimating the enzyme activities of the fraction. Spermidine synthase activity was estimated by using [1,4-^{14}C]putrescine as the substrate. The reaction mixture contained 150 mM glycine-NaOH buffer (pH 8.8); 37 μM-[^{14}C] putrescine (107 mCi/mmol); 25 μM decarboxylated SAM; 0.01 to 4.0 units of enzyme and H_2O in a total volume of 0.325 ml. Control mixtures were set up lacking either decarboxylated SAM or the enzyme extract. The tubes were incubated at 37°C for 1 h and the reaction was stopped by adding 1 ml of cold 5% PCA. The analysis of the product, labeled spermidine, and the correction of the enzyme activity due to dilution of the specific radioactivity of [^{14}C]putrescine by putrescine of the extract have been published (25). One unit of spermidine synthase is the amount of enzyme required to form 1 nmol of spermidine/h under the stated assay conditions.

DAP decarboxylase activity was determined by following the release of $^{14}CO_2$ from 1,7-^{14}C-DAP (18). The reaction mixture contained 48 mM K-phosphate buffer (pH 7.0); 24 μM PLP; 1.93 mM [1,7-^{14}C]-DL-DAP (0.1 μCi), an appropriate amount of enzyme and H_2O in a total volume of 0.5 ml. The reaction was carried out routinely in 12 × 75 mm screw-capped tubes. A 21-guage hypodermic needle was inserted through the cap of the tube. The needle carried a 63.6 mm^2 disc of Whatman No. 3MM filter paper wet with 15 μl of 1 M KOH to trap the $^{14}CO_2$ formed during the reaction. The other end of the needle was closed tightly with a cork. After incubation at 37°C for 1 h, the reaction was stopped by injecting 1 ml of 10% cold PCA with a 1-ml syringe. The outer end of the needle was closed immediately after injecting PCA. The tubes were shaken at 37°C for an additional 1 h to ensure the complete release of $^{14}CO_2$. The filter paper disc carrying $^{14}CO_2$ was then transferred to a scintillation vial and counted with 1 ml of H_2O and 10 ml of Aquasol II (New England Nuclear) in a Liquid Scintillation Spectrometer (Packard). The amount of $^{14}CO_2$ formed was proportional to the amount of extract added. One unit of DAP decarboxylase is the amount of enzyme required to form 1 nmol of $^{14}CO_2$/h under the stated assay conditions.

Cyt *c* oxidase and NAD-triose phosphate dehydrogenase were estimated according to the methods described in references (26) and (9), respectively. Catalase was estimated by the method of Lück (17) and glucose-6-phosphate dehydrogenase by that of DeMoss (8).

Chl was determined by the method of Arnon (1). DNA was estimated by the method of Giles and Myers (10).

Chemicals. D-Mannitol, BSA, reduced glutathione, Hepes, D-sorbitol, NAD, Cyt *c*, Triton X-100, PLP, DTT, Mes, and DL-glyceraldehyde-3-phosphate, and glucose-6-phosphate were purchased from Sigma Chemical Co. Polyethylene glycol 6000, Na_2 EDTA, and $CaCl_2$ were obtained from J. T. Baker. Ficoll 400 and isoascorbic acid were bought from Pharmacia and Eastman Kodak Co., respectively. Cellulase and macerozyme were obtained from Yakult Biochemicals Co., Ltd., Nishinomiya, Japan and Pectolyase Y-23 from Seishin Pharmaceutical Co., Ltd, Nihonbashi, Tokyo. [1,4-^{14}C]Putrescine·2HCl and 1,7-^{14}C-DAP (29 mCi/mmol) were from New England Nuclear and ICN, Irvine, CA, respectively. Putrescine·2HCl and spermidine·3HCl were purchased from Calbiochem and were recrystallized before use. DL-DAP was from Research Organics, Inc. Ludox AM was a gift of the Industrial Chemicals Division of E. I. du Pont de Nemours. Decarboxylated SAM was a generous gift from Dr. K. Samejima of Tokyo Biochemical Research Institute, Tokyo. All other chemicals were of the highest purity available commercially.

RESULTS

Localization of Diaminopimelate Decarboxylase in Chloroplasts. The pathway of lysine biosynthesis has been shown to proceed in many plants via DAP. This biosynthetic pathway had been described in many procaryotic organisms, and, as a conse-

quence of the postulated homology of chloroplasts and cyanobacteria, it was suggested that DAP decarboxylase was a chloroplast enzyme (4). This suggestion was tested in extracts of *Vicia faba* and the enzyme was found to be localized in this organelle (18, 29). In some plants, such as *Lupinus polyphyllus*, in which cadaverine is a precursor in the synthesis of a quinolizidine alkaloid, *e.g.* lupanine, in chloroplasts, lysine decarboxylase has also been found in the chloroplast (12). For these reasons, DAP decarboxylase was used as a marker in the fractionation and study of the distribution of spermidine synthase.

In various experiments in which the initial protoplasts and number of chloroplasts liberated after disruption were counted, the protoplasts were found to contain 50 to 100 chloroplasts per cell. The value of 63 was obtained in the experiment reported in Table I. It can be seen that the disrupted protoplasts were rich in this enzyme, which was then found almost entirely in the crude chloroplast fraction. The yield of purified intact chloroplasts in several isolations was 28 ± 6% of the chloroplasts liberated by disruption of the protoplasts, as a result of lysis of chloroplasts during the various steps. The activity of the enzyme released per purified intact chloroplast is comparable to the activity found for lysed protoplasts. Thus, in confirmation of the earlier report (18), the enzyme was essentially entirely localized in the chloroplast.

When the chloroplasts were lysed with H_2O and centrifuged at 400*g* for 20 min, more than 80% of the enzyme was found in the supernatant fraction. No significant difference has been found in the activity of this enzyme in healthy or TYMV-infected protoplasts or the derived chloroplasts.

Absence of Spermidine Synthase in Purified Chloroplasts. When protoplasts of uninfected leaves are disrupted as described above, a small but significant fraction of the spermidine synthase was found in the crude chloroplast fraction. This activity was retained in this fraction after several washings. In Table II, the crude chloroplast fraction contained 87% of the DAP decarboxylase and 10% of the spermidine synthase, as assayed by the method in which a correction was applied for the dilution of the radioactive precursor, putrescine, by extract putrescine (25). However, as presented in Table II, after fractionation in the Ludox gradient, the purified chloroplasts were essentially devoid of spermidine synthase activity, although retaining its DAP decarboxylase. Similarly, no spermidine synthase was found in the purified chloroplasts prepared from the protoplasts of TYMV-infected Chinese cabbage. Results similar to that in Table II have been obtained in five separate experiments.

Admixture of purified chloroplasts to crude chloroplasts and assay of enzyme after lysis did not reduce the synthase activity of the crude chloroplasts. Protoplasts exposed to the Ludox gradient were not disrupted, and when reisolated and fractionated had not lost spermidine synthase activity. Crude chloroplasts, sedimented twice through the starting gradient concentration of the Ludox mix (1/10 of the limiting concentration), retained their activity of spermidine synthase.

Although the presence of 50% Ludox in the assay mixture inhibited the activity in the crude chloroplasts or the soluble enzyme by 85%, two washes of the crude chloroplasts incubated in 53% Ludox at room temperature for 30 min completely restored the synthase activity to the sedimented fraction. It has been concluded that the absence of the synthase in the purified organelle is due to a further purification of the organelle.

Subcellular Distribution of the 'Soluble' Spermidine Synthase. Protoplasts prepared from healthy or TYMV-infected cabbage leaves were disrupted by passage through a 20 μm nylon mesh and centrifuged at 400*g* for 20 min. Most of the spermidine synthase activity was found in the supernatant fraction (Table III). This fraction was subjected to differential centrifugation, as indicated in Table III, and the enzyme activity was assayed. Most of the spermidine synthase activity (>90%) was found in the cytosol fraction. By comparison to the distribution of DAP decarboxylase, Cyt oxidase, catalase, and NAD-triose phosphate dehydrogenase, after various centrifugation fractionations, the enzyme appeared in the same fraction as the NAD-triose phosphate dehydrogenase. The presence of Cyt *c* oxidase in the crude chloroplast fraction indicates the presence of mitochondria in this fraction. Catalase is found mainly in peroxisomes, which also sediment in large part in the 400*g* pellet, *i.e.* the crude chloroplast fraction.

Vacuoles and Spermidine Synthase. The disruption of protoplasts by passage through a needle prior to fractionation also disrupts vacuoles. If this organelle contained a soluble spermidine synthase, the enzyme would appear in the cytosolic fraction. In initial experiments, a significant portion (about 20 to 40%) of the spermidine synthase of a protoplast extract was found associated with preparations of purified vacuoles (27), whose yields were quite low. The activities of a mixture of vacuoles and protoplasts were additive, *i.e.* no inhibitory substance was detected in the vacuoles. Spermidine synthase paralleled the recov-

Table 1. *Diaminopimelate Decarboxylase Activity in Various Fractions of Chinese Cabbage Protoplasts*

Fraction Assayed[a]	Enzyme units/h·10^6 Protoplasts or Equivalent Chloroplasts
Disrupted protoplast	98.5
Supernatant fraction	2.2
Crude chloroplasts	109.0
Pure chloroplasts	100.5

[a] All fractions were adjusted to identical concentraions (0.25%) of Triton X-100.

Table II. *Recovery of DAP Decarboxylase and Spermidine Synthase in Fractions of Chinese Cabbage Protoplasts*

Fraction Assayed	DAP Decarboxylase		Spermidine synthase	
	Enzyme units/10^6 protoplasts or equivalent	Recovery	Enzyme units/10^6 protoplasts or equivalent	Recovery
		%		%
Disrupted protoplasts	55.3	100	2.87	100
Supernatant fraction	15.0	27	2.78	97
Crude chloroplasts	48.0	87	0.274	10
Pure chloroplasts	20.0	36[a]	<0.03[a]	<1.0

[a] The recovery of 36% of the DAP decarboxylase is considered to represent the per cent recovery of the purified chloroplasts. In the assay of spermidine synthase, the purified chloroplast fraction was analyzed at 5.5 times the concentration of the disrupted protoplast fraction.

Table III. *Distribution of Chl, DNA, Spermidine Synthase, and Activities of Marker Enzymes following Fractionation of Protoplasts*

Fraction Assayed	Chl	DNA	Enzyme Activities				
			Spermidine synthase	DAP decarboxylase	Catalase[a]	Cyt *c* oxidase[b]	NAD-Triose-P dehydrogenase[c]
	μg/10⁶ protoplasts		*units/10⁶ protoplasts*				
Protoplast	139	5.6	1.30	85.0	75.7	0.011	2.67
400*g* pellet	137	5.2	0.14	61.5	47.2	0.007	0.38
800*g* pellet	<1	0	0.03	0	8.3	0.002	0.02
20,000*g* pellet	0	0	0.09	0	4.5	0.001	0
100,000*g* pellet	<1	0	0.03	0	0.8	0	0.05
100,000*g* Supernatant	<1	0.1	1.19	16.3	6.4	0	2.09

[a] One unit of enzyme is defined as the amount of enzyme required to liberate half the peroxide oxygen in 100 s under the stated assay conditions.

[b] The enzyme units have been described in terms of the rate constant observed/s as described in Ref. 26.

[c] One enzyme unit is defined as the amount of enzyme required to cause an increase of 0.01 A_{340} per s under the stated assay conditions.

Table IV. *Presence of Spermidine Synthase and Glucose 6-Phosphate Dehydrogenase in the Protoplasts and Vacuoles of Chinese Cabbage Leaves*

Fraction Assayed	Spermidine Synthase Activity	Glucose 6-Phosphate Dehydrogenase Activity
	%	
Disrupted protoplast	100	100
Vacuoles isolated from 5% Ficoll	18	19
Vacuoles isolated from 10% Ficoll	33	44
Vacuoles isolated from 15% Ficoll	73	73

ery of the soluble enzyme, glucose 6-phosphate dehydrogenase (3), in the vacuolar fractions, as presented in Table IV. These results indicated, as had been reported earlier, the difficulties of obtaining uncontaminated vacuoles.

DISCUSSION

The working hypothesis that spermidine synthase, an enzyme present in certain procaryotic cells, *e.g. Escherichia coli*, is present in the chloroplasts of plant protoplasts is disproved in this communication. Until chloroplasts were extensively purified, some spermidine synthase was associated with the organelle. After purification on a silica sol gradient, a true chloroplast marker, *i.e.* DAP decarboxylase, was conserved in the intact chloroplast fraction but spermidine synthase was eliminated. It is not known if the spermidine synthase was adsorbed to chloroplasts or associated with fragments of other contaminating organelles. Just how crude such a fraction indeed is, despite isolation after presumably gentle shearing of protoplasts, is revealed by the large amounts of DNA, Cyt oxidase, and some catalase in this fraction (Table III). It is also important to note the work of Robinson (23) in which crude chloroplasts from spinach leaves had considerable glycerate 3-P phosphatase activity. However, a further purification by a Percoll step gradient yielded intact chloroplasts in 95% yield with a phosphatase activity reduced more than 90%. This result is similar to our own experience with spermidine synthase.

The results of this study indicate, as in analyses of the spermidine synthase of other cells, that this plant enzyme is a soluble enzyme and is not localized in a particular organelle. This result calls attention once again to the surprising exclusion of several steps of methionine and SAM biosynthesis and metabolism from some chloroplasts (28). Chloroplasts prepared from barley leaf protoplasts, although able to synthesize essentially all of certain amino acids, *e.g.* threonine, lysine of the plant (28) are unable to make methionine or certain of its metabolites which fulfill many essential roles in chloroplast function. It appears then that in barley, at least, methionine, SAM, and possibly spermidine as well must be taken up by the auxotrophic organelle.

TYMV, which causes chloroplast vesiculation, aggregation, and eventual disruption in the course of virus assembly within the pockets produced in chloroplast aggregates, has not affected the amount of the typically chloroplast enzyme, DAP decarboxylase. It will be of interest to determine if any chloroplast functions are altered in the course of this infection.

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