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δ-AMINOLAEVULINATE SYNTHETASE IN EXTRACTS OF CULTURED SOYBEAN CELLS

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SUMMARY

1. δ-Aminolaevulinate synthetase has been detected in extracts of soybean callus tissues and the enzyme activity reached its maximum when callus were 11 days old.

2. The presence of a compound which seems to control δ-aminolaevulinate synthetase activity was demonstrated. The enzyme was present in the soluble fraction and was very labile.

3. When crude extracts or 500 × *g* supernatants were stored at 4°, the apparent activity of δ-aminolaevulinate synthetase increased by as much as 3–6 times, while the activities of δ-aminolaevulinate dehydratase and succinyl-CoA synthetase did not significantly change during the storage. Activation was dependent on concentrations of cells suspensions during disruption and aging.

4. Gel filtration with Sephadex G-25 of 2000 × *g* supernatants produced an enzyme fraction 30% more active. An increase in enzyme activity was observed when dark-grown callus were exposed to light.

5. The addition of ATP, gibberellic acid and δ-aminolaevulinate to the culture media diminished activity; iron deficiency also produced an δ-aminolaevulinate synthetase less active.

Introduction

It is generally accepted that the first step leading specifically to the biosynthesis of porphyrins is the formation of δ-aminolaevulinate from succinyl-CoA and glycine by the action of δ-aminolaevulinate synthetase. In spite of its assumed universal occurrence, δ-aminolaevulinate synthetase has only been demonstrated in cell-free extracts of photosynthetic and non-photosynthetic bacteria, of reticulocytes and of liver cells[1], but no activity could be found in proplastids or chloroplasts.

In addition, the enzyme could not be found in plant mitochondrial fractions[2,3], although MILLER AND TENG[4] claimed to have detected δ-aminolaevulinate synthetase in spinach.

In 1958, SHEMIN and associates[5] reported the presence of an inhibitor of δ-ami-

nolaevulinate synthetase in extracts of *Rhodopseudomonas spheroides* and Chromatium but experimental data of these observations were not published and LASCELLES[6] was unable to confirm the occurrence of such an inhibitor. However, more recently MARRIOT *et al.*[7,8] reported the existence of at least two compounds, an activator and an inhibitor which appear to participate in the control of δ-aminolaevulinate synthetase activity in *R. spheroides*, and also the presence of a specific and reversible inhibitor of δ-aminolaevulinate synthetase in *R. spheroides* has been reported previously[9].

This paper presents the results of an investigation of δ-aminolaevulinate synthetase activity in extracts of soybean callus cultures which is a vegetable tissue with an active cell division but which fails to synthetize chlorophyll in amounts equivalent to that found in mature leaves.

MATERIAL AND METHODS

Source material of enzyme

Callus from soybean seeds was obtained and grown according to MILLER[10]. The growth medium and culture conditions have already been reported[11]. Unless otherwise specified, callus cultures which had been grown semi-anaerobically in the dark have been used in this work.

Preparation of cell free extracts

Wound callus tissues (1 g wet wt./ml of 0.05 M Tris–HCl buffer, pH 9.0) were homogenized in a Potter–Elvehjem-type homogenizer or disrupted by ultrasonic treatment in an ultrasonic power unit for 3 min at 4°. The results obtained were the same whether cells were homogenized or disrupted by ultrasonic treatment. In some cases, extracts were centrifuged at 4° for 5 min at 500 × *g* immediately afterwards and the supernatant examined for δ-aminolaevulinate synthetase activity.

Assay of enzymes

δ-Aminolaevulinate synthetase was assayed as follows: 2 ml of reaction mixture containing 100 μmoles of glycine, 100 μmoles of succinate, 10 μmoles of ATP, 3.5 nmoles of CoA, 10 μmoles of $MgCl_2$, 0.25 μmole of pyridoxal phosphate, 10 μmoles of GSH, 50 μmoles of Tris–HCl buffer (pH: 7.2), 1 mg of succinyl-CoA synthetase (specific activity 8–12 μmoles of succinyl-CoA/h per mg) and appropriate amount of extracts, were incubated in a test tube for 30 min at 37°, then the reaction was stopped by addition of 0.5 ml of 25% trichloroacetic acid. δ-Aminolaevulinate formed was determined according to the method of URATA AND GRANICK[12] and identified by thin-layer chromatography[13] of the reaction product itself and of the pyrrole produced from δ-aminolaevulinate with acetyl-acetone.

Succinyl-CoA synthetase was purified 16-fold from soybean callus and assayed by the procedures described by WIDER AND TIGIER[14]. δ-Aminolaevulinate dehydratase activity was assayed according to TIGIER *et al.*[11].

1 unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 nmole of product in 60 min under the standard incubation conditions.

Protein concentration was measured by the method of LOWRY *et al.*[15].

RESULTS

Activity dependence on the age of cultures and its spontaneous activation

It has been found that δ-aminolaevulinate synthetase activity was dependent upon the age of the cultures and reached its maximum on the 11th day of growth (Fig. 1).

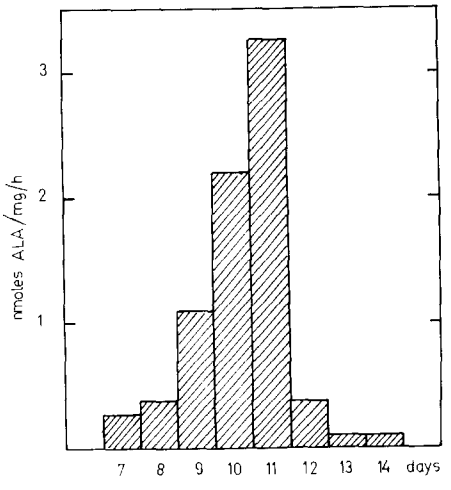

Fig. 1. Variation of δ-aminolaevulinate (ALA) synthetase activity in soybean callus at different days of growth. Wound callus was disrupted and enzyme activity was determined in crude extracts at 40 min after disruption.

As shown in Fig. 2, the activity of δ-aminolaevulinate synthetase in crude extracts or supernatants was found to change when either of them was aged at 4° for various periods of time. Enzymic activity of crude extracts showed a rapid increase

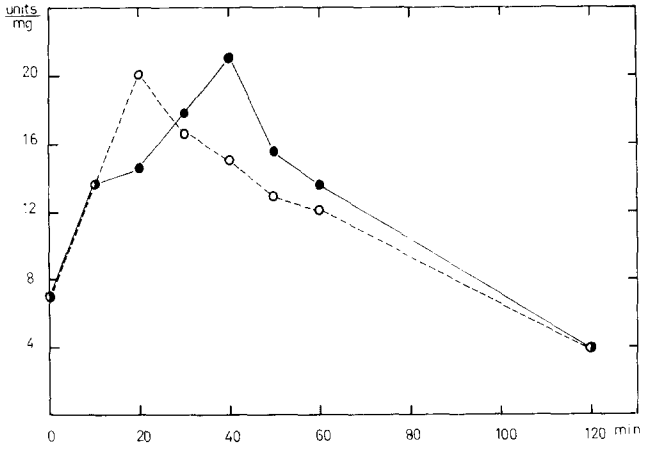

Fig. 2. Effect of aging on activities of δ-aminolaevulinate synthetase in crude extracts (●—●) and supernatants (○ - - -○). Wound callus was disrupted and both crude extracts and the supernatants after centrifugation at 500 × *g* were stored at 4–6°. At different times, portions were removed and δ-aminolaevulinate synthetase assayed as described in MATERIAL AND METHODS.

in the first 40 min; at this time the apparent δ-aminolaevulinate synthetase activity was 3–6 times higher than that in fresh extracts, and after this initial rise the activity began to decrease. This increase could also be observed with the supernatants, and the same maximum activity was reached in 20 min. It is noted that the initial activity is that measured immediately after disruption. No detectable amount of δ-aminolaevulinate was formed at 4° during aging. The rate of δ-aminolaevulinate formation

TABLE I

EFFECT OF AGING ON ACTIVITIES OF SUCCINYL-CoA SYNTHETASE, δ-AMINOLAEVULINATE SYNTHETASE AND δ-AMINOLAEVULINATE DEHYDRATASE IN CRUDE EXTRACTS

Wound callus, 11 days old were disrupted and the assay of enzymes were as described in MATERIAL AND METHODS.

Enzymes examined	*Specific activities*		
	Time of aging (min)		
	0	*20*	*40*
Succinyl-CoA synthetase	472	328	320
δ-Aminolaevulinate synthetase	6.9	14.6	20.8
δ-Aminolaevulinate dehydratase	7.9	8.1	8.0

was linear during the time of incubation for crude extract samples taken both immediately after disruption and 40 min later.

On the other hand, the activities of succinyl-CoA synthetase and δ-aminolaevulinate dehydratase did not significantly change during the whole period of aging (Table I) confirming that succinyl-CoA synthetase was not involved in the activation process, which seems to be specific to δ-aminolaevulinate synthetase.

TABLE II

CONCENTRATION DEPENDENCE OF THE ACTIVITY OF SOYBEAN CALLUS δ-AMINOLAEVULINATE SYNTHETASE

Wound callus, 11 days old, were disrupted at the concentrations shown and δ-aminolaevulinate synthetase was determined at the times stated.

Initial concentration (g wet wt./ml)	*Specific activities*		
	Time of aging (min)		
	0	*20*	*40*
1.5	2.0	18.4	4.0
1.5*	5.0	10.1	18.4
1.0	5.0	25.6	29.2
1.0**	5.0	15.0	24.0
0.7	4.0		4.0
0.5	3.5		2.8
0.2	0.2		0.2

* The extract (1.5 g/ml) was immediately diluted to 1 g/ml.
** The extract (1 g/ml) was immediately diluted to 0.5 g/ml.

Optimum conditions for the activation

The best medium for extracting the enzyme was found to be 0.05 M Tris–HCl buffer (pH 9.0) and the simultaneous addition of some thiol compounds such as thioglycollate, CySH or GSH improved neither extraction nor activation. It was observed that activation was dependent upon the concentration of the cell suspensions; large activations were obtained when initial concentrations were approx. 1 g wet wt./ml of buffer (Table II). Concentrated suspensions (1.5 g wet wt./ml) showed lower activity, if they were immediately diluted after disruption to 1 g/ml, maximum activity occurred at 40 min, but was the same as that obtained with the 1.5 g/ml extract at 20 min. They did not reach the highest values determined for the 1 g/ml suspensions.

In addition, if the 1 g/ml extracts were immediately diluted to 0.5 g wet wt./ml, activation was only partially prevented.

TABLE III

EFFECT OF CENTRIFUGATION AND TREATMENT WITH SEPHADEX G-25 ON ACTIVITY OF SOYBEAN δ-AMINOLAEVULINATE SYNTHETASE

Enzyme fraction	*Specific activity*
1. Homogenate	23
2. 1000 × *g* supernatant	20
3. 1000 × *g* precipitate	2.5
4. 2000 × *g* supernatant	20
5. Sephadex G-25 eluate	26
6. 24 000 × *g* supernatant	11
7. 24 000 × *g* precipitate	0

Wound callus, 11 days old, were used .Assay conditions were as described. In Step 1, 5, 6 and 7 activities were determined at 40 min after disruption and in Step 2, 3 and 4 at 20 min. All centrifugations were carried out for 5 min and the precipitates washed once with buffer. The 2000 × *g* supernatant was passed through a Sephadex G-25 column (2 cm × 30 cm) and activity determined in the protein fraction eluated with 0.05 M Tris buffer (pH 7.2).

Effect of centrifugation and gel filtration (Table III)

The intracellular distribution of the enzyme in these cultures was investigated using conventional fractionation procedures and it was found that most of the activity remained in the supernatants. It was also observed that if the supernatant of 2000 × *g* was passed through a Sephadex G-25 column, the activity of δ-aminolaevulinate synthetase in the eluates was increased by 30% as if some inhibitor were eliminated. After spinning at 24 000 × *g*, enzyme activity was rather low, but it must be noted that both the Sephadex G-25 eluate and the 24 000 × *g* supernatant were assayed at 40 min after disruption and it has been found that with the supernatants, maximum activity is reached in 20 min and then began to decrease.

Effect of the presence of various compounds in culture media (Table IV)

The content of porphyrins was very low in callus, dark- or light-grown, or in the presence of gibberelic acid or ATP, but the addition of δ-aminolaevulinate and iron deficiency stimulated porphyrin accumulation; within a few hours after sub-

TABLE IV

EFFECT OF SOME COMPOUNDS ADDED TO THE BASAL MEDIA ON THE SYNTHESIS OF PORPHYRINS AND δ-AMINOLAEVULINATE SYNTHETASE ACTIVITY FROM SOYBEAN CALLUS

Expt.	*Light conditions*	*Additive*	*Specific activity*	*Porphyrins formed (nmoles/g wt.)*
1.	light	None	5.72	0.060
	dark	None	4.28	0.065
	light	ATP, 2 mM	3.84	0.050
	dark	ATP, 2 mM	2.42	0.050
	light	Gibberellic acid 3.3 μg/ml	2.80	0.100
	dark	Gibberellic acid 3.3 μg/ml	2.48	0.030
2.	dark	None	8.1	0.060
	dark	δ-Aminolaevulinate, 0.1 mM	5.6	0.115
	dark	δ-Aminolaevulinate, 0.3 mM	5.0	0.500
	dark	Without Fe	7.0	0.538
	dark	Without Fe + δ-aminolaevulinate, 0.1 mM	1.4	0.523

Extracts were prepared from callus grown in the light or dark as stated and δ-aminolaevulinate synthetase activity determined as described at 40 min after disruption. Concentrations of the additives shown are the final concentrations in the media. Extractions of porphyrins from callus cultures were carried out as described by LLAMBIAS AND BATLLE[16]. In Expt. 1 callus were 12 days old and in Expt. 2 they were 9 days old.

culturing, the callus had the characteristic red fluorescence of porphyrins and a brown-greenish colour. On the other hand, it has been found that when dark-grown callus was illuminated by 60-W bulbs at an appropriate distance, δ-aminolaevulinate synthetase activity was slightly increased. The presence of ATP, gibberelic acid, δ-aminolaevulinate or iron deficiency produced a significant decrease in enzyme activity.

DISCUSSION

The most important fact that has emerged from this work is that it was possible to obtain the conditions to measure δ-aminolaevulinate synthetase activity in a vegetable tissue; the results obtained are reproducible, although initial activities varied with different batches of callus cultures. Some properties of soybean callus δ-aminolaevulinate synthetase resemble those of the *R. spheroides* synthetase[7,9]. As has been suggested by MARRIOT *et al.*[7], the presence of some compound which seems to control δ-aminolaevulinate synthetase activity has also been detected in this tissue and could account for the changes in activity during aging of crude extracts or supernatants. Although we have not yet obtained direct evidence for the existence and chemical nature of this compound, it appears to be unstable. As maximum activity was reached at lower intervals by the supernatants, it is suggested that probably some inhibitor was partially separated in the precipitate.

It was observed that the rate and extent of activation was dependent on concentration during disruption and activation; apparently suspensions more concentrated than 1 g wet wt./ml contained a higher proportion of inhibitor, and those having less than 1 g/ml probably extracted less enzyme and also less inhibitor, since no significant activity changes occurred during aging.

An increase in activity of δ-aminolaevulinate synthetase was found when dark-grown callus were exposed to light; this could be due to the fact that the inhibitor is sensitive to light[9]. However, as this increase in enzyme activity was paralleled by an increase in chlorophyll content[16], light could have induced the new synthesis of enzymes necessary for chlorophyll production[17].

Finally, the addition of ATP to the media diminished activity; the same effect on other enzymes of the pathway has been observed[16], suggesting that ATP could function repressing enzyme formation. The presence of δ-aminolaevulinate or iron deficiency in the media greatly stimulated porphyrin formation, but inhibited δ-aminolaevulinate synthetase activity; possibly the amount of porphyrins or related intermediates accumulated under these conditions, significantly interfere with the activity of δ-aminolaevulinate synthetase, simply operating by a negative feed back control, as proposed by LASCELLES[1]. It has been suggested that iron is involved in the synthesis of δ-aminolaevulinate synthetase[18–20] and, although iron deficiency produced an enzyme 10–20% less active, these results are not conclusive evidence to indicate that iron is involved in the action of δ-aminolaevulinate synthetase in soybean callus. The enzyme is extremely labile and attempts to purify it were as yet unsuccesful.

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