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δ-AMINOLEVULINIC ACID SYNTHETASE FROM COLD-STORED POTATOES

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SUMMARY

δ-Aminolevulinic acid synthetase has been obtained in a cell-free state from greening potatoes, which are stored in low temperature (0–4 °C) and dim light (30–40 lux). The maximum enzyme activity is developed within 3 weeks of storage. Higher temperatures or light intensities do not favour the formation of this enzyme. The pH optima for δ-aminolevulinic acid synthetase is 7.0. Supplementation of the reaction mixture with pyridoxal phosphate, Mg^{2+}, ATP, CoA and succinyl-CoA synthetase gives maximum δ-aminolevulinic acid synthesis. Chlorophyllide *a* at 10 μg level in the reaction mixture inhibits the enzyme activity while chlorophyll *a* and pheophytin *a* are ineffective.

INTRODUCTION

After the discovery that δ-aminolevulinic acid is the common intermediate in chlorophyll as well as in heme synthesis[1] a search was begun for an enzyme which could catalyse the formation of δ-aminolevulinic acid in plants. However, until recently δ-aminolevulinic acid synthetase defied all attempts at isolation from green plant tissue. The isolation of this enzyme in a cell-free state from cultured soya bean cells was one of the first successful attempts to demonstrate this activity[2]. In our studies on chlorophyll synthesis in cold stored potatoes[3], we found that green peel tissue slices were able to incorporate ^{14}C-labeled succinate or glycine into the chlorophylls. This observation gave us an incentive to look for δ-aminolevulinic acid synthetase in the peel tissue. The present study describes the conditions for isolation of δ-aminolevulinic acid synthetase in a cell-free state and some of its properties.

MATERIALS AND METHODS

Up-to-date variety of potatoes were stored, 3–4 weeks after harvest, at 0–4 °C in light (30–40 lux) for greening[3]. After 3 to 4 weeks storage these green potatoes were used for the experiment. The green skin peelings were used to isolate the enzyme.

Preparation of enzyme

10 g of green potato peelings were ground in a pre-cooled mortar and pestle, with 10 ml of cold 0.1 M sodium phosphate buffer, pH 7.0, containing 0.35 M NaCl at 0–4 °C and light intensity 30–40 lux. The homogenate was filtered through a double layer of cheese cloth. 3 vol. of cold acetone (−30 °C) were added to the filtrate and the precipitated proteins were immediately centrifuged at 16 000 × *g* for 5 min. The precipitate was dissolved in 10 ml of the grinding medium and again centrifuged at 10 000 × *g* for 10 min at 0–4 °C to remove the insoluble proteins. The supernatant was used as the enzyme preparation. Care was taken to have dim light throughout these operations. The enzyme was prepared freshly every day.

Assay of δ-aminolevulinic acid synthetase activity

The complete reaction mixture for the assay of δ-aminolevulinic acid synthetase consisted of 100 μmoles glycine, 100 μmoles sodium succinate, 0.25 μmole pyridoxal phosphate, 5 μmoles $MgCl_2$, 5 μmoles ATP, 0.25 μmole of CoA, 0.05 μmole EDTA, 0.2 mg succinyl-CoA synthetase protein isolated from spinach by the method described by Kaufman *et al*[4], 1.8 to 2 mg of enzyme protein, 100 μmoles sodium phosphate buffer, pH 7.0, and 700 μmoles NaCl in a final volume of 2 ml. The reaction mixture was incubated at 15 °C in the light (30 lux) for 1 h and the reaction was stopped by the addition of 0.5 ml of 12.5% trichloroacetic acid. δ-Aminolevulinic acid formed was estimated by the method described by Mauzerall and Granick[5] and Narisawa and Kikuchi[6]. 1 ml of deproteinized reaction mixture was adjusted to pH 4.6 with 2.5 M NaOH and 1 M sodium acetate buffer, pH 4.6, and then treated with 0.2 ml of acetyl acetone for 10 min in a boiling water bath to yield the pyrrole derivative. The mixture was cooled to room temperature. To 2.0 ml of this solution 2.0 ml of the modified Ehrlich reagent[5] containing 2 M perchloric acid was added and after 15 min absorbance was measured at 553 nm against a reagent blank. Suitable controls were included in all experiments. Although supplementation with all cofactors gave maximum activation, for routine assays CoA, ATP, pyridoxal phosphate and succinyl-CoA synthetase were omitted from the reaction mixture.

Identification of the product as δ-aminolevulinic acid

The products which interfere with the assay of δ-aminolevulinic acid are: (1) aminoacetone which is formed by an enzymatic reaction of glycine with acetyl-CoA and (2) porphobilinogen which is the product of δ-aminolevulinic acid dehydratase.

The method described by Urata and Granick[7] to separate these compounds was used to detect whether any interfering compounds were formed during the reaction. The deproteinized reaction mixture was centrifuged and the clear solution was neutralized with 1 M NaOH and pH adjusted to 5–6, with 0.5 M sodium acetate. This solution was first passed through a column of Dowex 1 (acetate) to adsorb porphobilinogen. The column was washed with 6 ml of water. The filtrate and washings were then passed through an Amberlite IRC-50 column adjusted to pH 5.0, to adsorb aminoacetone. The column was washed with 10 ml of water and the filtrate and washings containing δ-aminolevulinic acid were combined and assayed colorimetrically.

For positive identification of the product as δ-aminolevulinic acid, the incorporation of [2,3-^{14}C]succinate and [2-^{14}C]glycine into δ-aminolevulinic acid was deter-

mined. In this case, the reaction mixture was that used for routine assay containing 0.8 mCi/mole of either of the substrates. After stopping the reaction, 100 μg cold δ-aminolevulinic acid was added as carrier and δ-aminolevulinic acid was converted into its pyrrole as described above. The pyrrole was extracted with diethyl ether below pH 4.0. The ether layer was washed with distilled water several times, concentrated, and the solution was quantitatively transferred on to a Whatman No. 3 filter paper along with pyrrole prepared from authentic δ-aminolevulinic acid. The chromatogram was developed using 10 M NH_4OH–*n*-butanol–water (1:49:50, by vol.). The spots were detected by spraying with Ehrlich's reagent. The spot corresponding to δ-aminolevulinic acid pyrrole (R_F 0.23) was cut out and the radioactivity determined.

Chlorophyll *a* and pheophytin *a* were isolated by sucrose column chromatography[8].

Chlorophyllide *a* was prepared by incubating chlorophyll *a* with spinach chlorophyllase according to the method described by Klein and Vishniac[9].

Protein was determined using the biuret procedure[10].

RESULTS

Potatoes when stored at low temperature (4–15 °C) and light intensities between 30 and 40 lux were found to develop chlorophyll within 3 weeks of storage[3]. δ-Aminolevulinic acid synthetase could be isolated from the green peelings of these potatoes. Our studies on the potato enzyme have shown that the conditions for the extraction of the enzyme were critical. The best medium for isolation was found to be 0.1 M sodium phosphate buffer, pH 7.0, containing 0.35 M NaCl. Distilled water or buffer alone could extract only 30–40% of the activity that could be isolated in the presence of NaCl. The presence of NaCl at this concentration even in the reaction mixture was highly essential to obtain maximum activity. Light intensities higher than 100 lux during the extraction also adversely affected the enzyme activity. The enzyme was extremely labile. At room temperature inactivation occurred within 30 min and the activity was completely lost when stored at −30 °C overnight.

Products of the reaction

Under the experimental conditions employed in these studies δ-aminolevulinic acid was the only product. Aminoacetone or porphobilinogen were not found in the reaction mixture when an assay was performed to detect them using Dowex 1 (acetate) and Amberlite IRC-50 resins[7]. The inclusion of EDTA in the reaction mixture facilitated the inhibition of δ-aminolevulinic acid dehydratase activity[11] thus preventing the formation of porphobilinogen. Conformatory evidence for the product as δ-aminolevulinic acid was obtained from the studies on the incorporation of either [2,3-^{14}C]succinate or [2-^{14}C]glycine into the enzyme product and isolating it as δ-aminolevulinic acid pyrrole. All the radioactivity was associated with a single spot (R_F 0.23) and from both precursors almost the same amount of radioactivity (600 cpm) was incorporated into the δ-aminolevulinic acid. When the non-radioactive substrate was omitted from the reaction mixture, no incorporation was observed. The equality in the incorporation of radioactivity demonstrated a strict one to one mole stoichiometry for substrates of δ-aminolevulinic acid synthetase reaction.

Conditions for the development of δ-aminolevulinic acid synthetase in greening potatoes

δ-Aminolevulinic acid synthetase activity was developed only when potatoes were stored in the cold either in dark or light; although the presence of light activated the formation of the enzyme. Freshly harvested potatoes were devoid of this enzyme activity and when they were stored at room temperature (23–25 °C) in light (40 lux) or in dark, this activity did not develop. Maximum activation took place within 3 weeks of storage (Table I) by this time the potatoes contained a detectable amount of chlorophyll[3].

TABLE I

DEVELOPMENT OF δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY IN POTATOES

δ-Aminolevulinic acid synthetase was isolated at different time intervals from peelings of potatoes stored at 0–4 °C and in light at 30–40 lux. The enzyme activity was determined as described under Materials and Methods. The reaction mixture was the one used for routine assay.

Storage time (weeks)	*Specific activity (nmoles of δ-aminolevulinic acid formed per mg protein)*	
	Light	*Dark*
0	0.0	0.0
1	2.6	0.7
2	4.5	1.5
3	5.1	2.3
4	5.6	2.9
5	5.7	3.4

The factors influencing δ-aminolevulinic acid synthetase activity in vitro

The enzyme activity was highly sensitive to the environmental conditions, namely, the temperature and light intensity at which the reaction was conducted. The optimum light and temperature conditions were found to be 30 lux and 15 °C, respectively (Table II). No activity was observed at room temperature or above, or

TABLE II

EFFECT OF TEMPERATURE AND LIGHT ON δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY

δ-Aminolevulinic acid synthetase activity was determined at various temperatures at 30 lux light intensity and various light intensities at 15 °C. The reaction mixture for routine assay was used for this experiment.

Condition for incubation		*Specific activity (nmoles of δ-aminolevulinic acid formed per mg protein)*	
Temp. (°C)	*Light (lux)*	*Routine assay*	*Complete system*
5		2.1	6.06
15		6.0	16.95
20		0.3	0.76
25		0.0	0.0
	0 (dark)	4.2	12.17
	30	6.2	17.42
	60	2.7	7.58
	100	0.0	0.0

TABLE III

INACTIVATION OF δ-AMINOLEVULINIC ACID SYNTHETASE BY LIGHT

δ-Aminolevulinic acid synthetase was irradiated at 0 °C with light of different intensities, *viz.* 0, 30, 60, 100, 200, 500, 1000, 2000 and 4000 lux, for 10 min. The activity of these preparations was compared with the freshly prepared enzyme. The protein concentration was 4.0 mg/ml. The pH of the medium was 7.0 and the irradiation was carried out aerobically.

Light intensity (lux)	*Specific activity (nmoles of δ-aminolevulinic acid formed per mg protein)*	
	Routine assay	*Complete system*
Unirradiated (fresh enzyme)	4.4	17.62
0 (dark)	4.2	16.66
30	4.35	17.04
60	2.36	9.28
100	0.00	0.00

at light intensities above 100 lux. Inhibition of enzyme activity at higher light intensities than 100 lux suggested that the enzyme protein might have been inactivated on irradiation by visible light. An experiment was designed to test this hypothesis. The enzyme was irradiated with light of different intensities, *viz.* 0, 60, 100, 200, 500, 1000, 2000 and 4000 lux at 0 °C for 10 min, and the enzyme activity was determined. The assay was also conducted in freshly isolated unirradiated enzyme side by side. It was found that irradiation with light intensities above 100 lux caused complete inhibition of enzyme activity (Table III); however, no inactivation was observed under the same conditions in the dark. 60 lux caused 45% inactivation.

The pH of the reaction mixture was also very critical. A sharp peak of activity

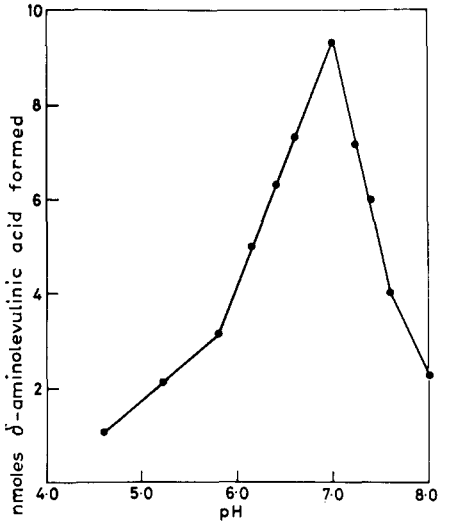

Fig. 1. Experimental conditions are the same as those given for routine assay under Materials and Methods except the pH of the reaction was varied from 4.6 to 8. For pH values 4.0 and 6.0, Na_2HPO_4–citric acid buffer was used and for pH values above 6.0 to 8.0, Na_2HPO_4–NaH_2PO_4 buffer was employed.

with a maximum at pH 7.0 was observed. At alkaline pH the inactivation was more pronounced (Fig. 1).

In the δ-aminolevulinic acid synthetase reaction equimolar amounts of substrates were combined to give the product. The effect of both substrates on the reaction was determined by fixing the concentration of one substrate at 100 μmoles. The maximum activity which gave 7.3 nmoles of δ-aminolevulinic acid was observed when both the substrates were present at a level of 100 μmoles in the reaction mixture. None of the substrates at higher concentration effected either an activation or inhibition of the reaction.

Requirements for δ-aminolevulinic acid synthetase

δ-Aminolevulinic acid synthetase isolated from green potatoes showed some activity without supplementing the reaction mixture with any cofactors (Table IV).

TABLE IV

COFACTORS REQUIREMENTS

RM indicates the reaction mixture contained only 0.05 μmole of EDTA, along with the substrates.

Additions	*nmoles of δ-aminolevulinic acid formed*
No cofactors (RM)	2.8
1. RM + pyridoxal phosphate (0.25 μmole)	4.00
2. RM + $MgCl_2$ (5 μmoles)	7.13
3. RM + ATP (5 μmoles)	8.42
4. RM + CoA (0.25 μmole)	6.25
5. RM + pyridoxal phosphate + $MgCl_2$ + ATP + CoA	24.60
6. RM + pyridoxal phosphate + $MgCl_2$ + ATP + CoA + succinyl-CoA synthetase (0.2 mg)	36.25

δ-Aminolevulinic acid synthetase required pyridoxal phosphate as prosthetic group and addition of pyridoxal phosphate caused an activation of the reaction. The addition of Mg^{2+}, ATP, and CoA individually to the reaction mixture produced an enhancement in the δ-aminolevulinic acid formation. About 9-fold activation of the reaction was observed when all these cofactors were added together into the reaction mixture. Gibson *et al.*[12] has demonstrated that succinyl-CoA was one of the substrates for δ-aminolevulinic acid synthetase. In our studies, when purified succinyl-CoA synthetase was added together with other cofactors, there was an additional 13-fold increase in δ-aminolevulinic acid production. The purified succinyl-CoA synthetase was free from δ-aminolevulinic acid synthetase activity. A metal requirement for δ-aminolevulinic acid synthetase was evident from the activation of the reaction by Mg^{2+}.

Product inhibition

It is well known that heme synthesis is controlled by the feed back inhibition of δ-aminolevulinic acid synthetase by hemin[13], or by a repressor mechanism that controls the synthesis of δ-aminolevulinic acid synthetase[14]. δ-Aminolevulinic acid synthetase in cell free extracts of *Rhodopseudomonas spheroides*[15] was shown to be inhi-

TABLE V

EFFECT OF CHLOROPHYLL *a*, PHEOPHYTIN *a*, AND CHLOROPHYLLIDE *a* ON δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY

The reaction mixture was the one used for routine assay and it also contains the following components in the concentrations mentioned below.

Addition	*Concentration (μg)*	*nmoles of δ-aminolevulinic acid formed*	
		Routine assay	*Complete system*
None		8.4	31.74
Chlorophyll *a*	20	7.6	26.79
Pheophytin *a*	20	7.74	29.53
Chlorophyllide *a*	10	4.04	15.39
	20	1.31	5.78
	25	0.00	0.00

bited by hemin. We have investigated the ability of the final products of the chlorophyll synthetic reaction, *viz.* pheophytin *a*, chlorophyll *a* and chlorophyllide *a*, to inhibit the first step (Table V). Of these chlorophyllide *a* alone showed good inhibition of the enzyme activity. 10 μg caused more than 50% inhibition of the reaction whereas pheophytin *a* and chlorophyll *a* were ineffective even at a level of 20 μg in the reaction mixture.

DISCUSSION

Cell-free synthesis of δ-aminolevulinic acid has not hitherto been demonstrated in higher plant tissues. The only other report on the isolation of this enzyme from a plant source is that of Wide De Xifra *et al.*[2] which describes the isolation of a cell-free system from cultured soya bean cells. In these studies they have found an inhibitor, similar to the one reported by Marriott *et al.*[16] in *Rhodopseudomonas spheroides*, for δ-aminolevulinic acid synthetase in crude extracts, which could account for the change in activity during aging of the crude extract. We could not detect the presence of a natural inhibitor if it is present at all in the crude extract, owing to the lability of the enzyme.

The most important observation to emerge from these studies is the involvement of light and temperature in the development of the enzyme activity. Light is not absolutely essential for its formation, although there is a 2-fold enhancement of activity in dim light (Table I). At light intensities above 100 lux, enzyme activity does not develop in potatoes nor does the isolated enzyme show any activity (Table II). Light dependence for the formation of this enzyme has been reported in plants[2] as well as in bacterial systems[17]. Exposure to light caused an enhancement in δ-aminolevulinic acid synthetase activity of dark grown soya bean cell cultures. Formation of this enzyme in *Rhodopseudomonas spheroides* was shown to be dependent on dim light[17]. The organisms grown in dim light contained more enzyme activity than those grown in bright light. So it is quite conceivable that, in the case of potatoes, at higher light intensities the enzyme formed may be inactivated or the formation of the enzyme itself may be blocked. The mechanism of light-induced inactivation of this enzyme is not known at present. This light sensitivity of δ-aminole-

vulinic acid synthetase may be one of the critical factors involved in the isolation of a cell-free system for this reaction.

The difficulty in the isolation of δ-aminolevulinic acid synthetase from plants has impeded progress in understanding the metabolic control of chlorophyll biosynthesis. Several recent investigations indicate that this pathway is also regulated at the first step, *i.e.* the synthesis of δ-aminolevulinic acid. Studies on etiolated seedlings by Granick[18] suggest that enzymes involved in the conversion of δ-aminolevulinic acid to protochlorophyllide are present in non-limiting quantities whereas the rate-limiting step which regulates chlorophyll synthesis is δ-aminolevulinic acid synthetase. There is evidence[19,20] to show that the rate of δ-aminolevulinic acid synthesis is controlled by a negative feed-back offered by protochlorophyllide or protochlorophyllide holochrome, which inhibits δ-aminolevulinic acid synthetase activity. Recent studies on the nature of feed-back inhibition of δ-aminolevulinic acid synthetase by hemin in cell-free extracts of *Rhodopseudomonas spheroides*[21] suggest that hemin inhibits the enzyme by forming a co-ordination complex with the enzyme through its iron atom. Inorganic iron is itself an inhibitor for this enzyme. It is interesting to note that ferric iron is a potent inhibitor for potato δ-aminolevulinic acid synthetase. The concentration for 50% inhibition of the reaction by ferric iron is found to be $1 \cdot 10^{-4}$ M. In plants iron deficiency caused a decrease in the synthesis of chlorophyll and this was suggested to be due to the decreased formation of succinyl-CoA and ferredoxin[22]. This shows that iron is not directly involved in the catalytic activity of δ-aminolevulinic acid synthetase, although it can inhibit the activity in an isolated system.

The observation that δ-aminolevulinic acid synthetase can be inhibited by the final product of chlorophyll synthesis, namely, chlorophyllide *a* (Table V) gives further support to the contention that end products can regulate the chlorophyll synthesis by inhibiting the first enzyme in the sequence. The inefficiency of chlorophyll *a* and pheophytin *a* to inhibit this enzyme may be attributed to their insolubility in aqueous solvents. Another regulatory mechanism for chlorophyll synthesis may be offered by light. Gassman and Bogorad[23] have suggested that light regulates chlorophyll synthesis. Nadler and Granick[24] have presented evidence to show that brief illumination results in enhanced synthesis of at least one protein responsible for δ-aminolevulinic acid synthesis and the control of light is at translational level. Hence, it is possible that the dim light and low temperature at which potatoes are stored may induce the formation of enzymes of the chlorophyll synthetic pathway. Further studies on the nature of inhibition of potato δ-aminolevulinic acid synthetase by light and chlorophyllide *a*, may contribute to the understanding of light and temperature dependence of chlorophyll synthesis in potatoes.

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