ACTA SOCIETATIS
BOTANICORUM POLONIAE
vol. XXXIX — nr 3
1970

The influence of amino acid analogues on the development of isocitritase activity in peanut cotyledons*

ANNA GIENTKA-RYCHTER** and JOE H. CHERRY

Isocitritase, an enzyme of the glyoxylate cycle, was discovered by Kornberg (Kornberg & Madsen, 1957) in bacteria and has been shown to be present only in tissues where rapid conversion of fat to carbohydrate occurs, namely fat containing seeds upon germination (Carpenter & Beevers, 1959). In peanut cotyledons 50% of the storage material consist of fat. During the first four days of germination of peanut seed a linear increase in isocitritase activity was observed in the cotyledons (Cherry, 1963). Activity of this enzyme increased dramatically during germination and was paralleled by a rapid fat disappearance; after five days isocitritase activity subsequently declined when fat reserves were exhausted (Beevers, 1961). It was shown (Gientka-Rychter & Cherry, 1968) that isocitritase is *de novo* synthesized during seed germination.

Induction of an enzyme involving *de novo* synthesis may be controlled at the transcription and/or translation levels (Spiegelman & Hayashi, 1963).

Inhibition of any step in the production of RNA or translation of RNA suppresses *de novo* synthesis of protein.

Several naturally occuring amino acid analogues have been employed to block protein synthesis which also results in retarded plant growth (Richmond, 1962). p-fluorophenylalanine (Young, 1957; Young *et al.*, 1960) and azetidine-2-carboxylic acid (Fowden, 1963; Fowden (Richmond, 1962). p-Fluorophenylalanine (Young, 1957; Young gues in the inhibition of protein synthesis. From previous work (Presley & Fowden, 1965; Young & Varner, 1959) it is not clear if the analogues result in the production of completely inactive protein or not. Proteins not having the amino acids analogous in the active center may not be affected to the inhibitor.

In this paper we will show the effect of p-fluorophenylalanine and

---

* This work was supported in part by a grant (GB-4593) from the National Science Foundation. Journal paper No 3712 of the Purdue Agriculture Experiment Station.

** Present address: Institute of Botany, Warsaw University, Warsaw, Poland.

azetidine-2-carboxylic acid on the development of isocitritase in cotyledons of germinating peanut seed. The effect of the inhibitors on the incorporation of labelled amino acids into protein is compared and the incorporation of ^{14}C-p-fluorophenylalanine into protein demonstrated.

EXPERIMENTAL

Cotyledons of germinating peanuts (*Arachis hypogaea* L., variety 56-R) were used throughout this study. Seeds were dusted with Spergon (a fungicide) and layered in small petri dishes lined with a moist layer of absorbent paper. Seeds were germinated in the dark at 29° for various times ranging from 18 hours to 5 days.

Enzyme preparation. Seeds were washed several times in distilled water and four cotyledons were blended for 1 min in a high speed homogenizer (Omni-Mixer) with 8 ml of 0.1 M phosphate buffer, pH 7.6. The homogenate was filtered through cheesecloth and the final volume made up to 10 ml. Cellular debris was removed by centrifugation at 1500 x g for 15 min. The temperature of the homogenate was kept between 0° and 4° during the entire procedure. The resulting crude homogenate was used directly for enzyme assay.

Amino acid analogues and inhibitor studies. Azetidine-2-carboxylic acid and p-fluorophenylalanine were used at the ratios of 2 mg per seed and 3 mg per seed, respectively. Since dry seeds imbibe about 0.4 ml of water per seed during 18 hours, the above amounts of the amino acid analogues were dissolved in 0.4 ml of water. Seeds kept for 18 hours in inhibitor solutions were transferred to water and germinated further without inhibitor for up to 5 days.

Enzyme assay. The dinitrophenylhydrazine method described by Rao & Ramakrishnan (1962) was used for enzyme activity determination. The reaction mixture of 1.9 ml contained the following: 200 μ-moles of phosphate buffer, pH 7.6, 20 μ-moles of D,L-isocitrate, 5 u-moles of cysteine hydrochloride, 5 μ-moles of magnesium sulphate and 0.2 ml of enzyme preparation. The reaction mixture was incubated at 30° for 15 min and the reaction stopped by addition of 0.1 ml of 100% (w/v) trichloroacetic acid. Enzyme activity was expressed as millimicromoles of glyoxylate formed per minute per mg of protein.

Protein estimation. Protein was estimated according to the method of Lowry *et al.* (1951).

^{14}C-labelling of the total protein. Cotyledons from seed germinated for two or four days in a ^{14}C-amino acid solution (0.5 μc/ml of reconstituted ^{14}C-protein hydrolysate or 1 μc/ml of ^{14}C-p-fluorophenylalanine, purchased from Schwarz BioResearch, Inc.) were washed several times with distilled water. Four cotyledons were homogenized for 20 min at high speed in

a Vir-Tis homogenizer with 8 ml 0.1 M phosphate buffer, pH 7.6. The final volume of the homogenate was made up to 10 ml and centrifuged for 15 min at 1500 x g. An aliquot of each sample (0.1 ml) was placed on filter paper disks and total radioactivity of protein determined according to the method of Mans & Novelli (1961). Radioactivity was counted directly in a Packard liquid scintillation spectrometer.

Purification of isocitritase on a Sephadex G-200 column. The enzyme was purified according to the method described by Gientka-Rychter & Cherry (1968). The isocitritase activity in the fractions from the column was assayed by the method of Olson as described by Carpenter & Beevers (1959). In this case, the enzyme unit was defined as the change in optical density of 0.001 at 252 mμ per minute.

RESULTS

Seeds imbibed for 18 hours in azetidine-2-carboxylic acid solution and then transferred to water, were stunted in growth. As shown in Table 1 azetidine-2-carboxylic acid had no effect on the development of isocitritase activity. p-Fluorophenylalanine slightly influenced seedling growth but did not inhibit the development of isocitritase activity. The amounts

Table 1

Effect of amino acid analogues on the development of isocitritase activity in penaut cotyledons

Peanut seed were first germinated for 18 hrs. in water or a solution of one of the inhibitors and then in distilled water for times indicated. Enzyme activity was measured in crude extracts from detached cotyledons as described under "Experimental"

Days after 18 hrs. pretreatment	Isocitritase activity (mμmoles glyoxylate/min/mg protein)		
	Control	Azetidine-2-carboxylic acid (2 mg/seed)	p-Fluorophenylalanine (3 mg/seed)
0	8.3	16.7	21.7
1	16.7	25.0	20.0
2	45.0	36.7	40.0
3	55.0	58.4	51.7
4	61.6	66.7	65.0

of amino acid analogues imbibed during the 18 hour imbibition period appear to be sufficient to inhibit protein synthesis, since growth was inhibited.

In other experiments, seeds imbided in cycloheximide (50 μg/ml) virtually stopped the production of isocitritase. Seeds that were transferred to absorbent paper moistened with water after cycloheximide trea-

Table 2

Effect of cycloheximide on the development of isocitritase activity in peanut cotyledons

Peanut seed were first germinated for 18 hrs. in water or a solution of cycloheximide then in distilled water for times indicated. Enzyme activity was measured in crude extracts from detached cotyledons as described under "Experimental"

Days after 18 hrs. pretreatment	Isocitritase activity (mμmoles glyoxylate/min/mg protein)	
	Control	Cycloheximide (50 μg/ml)
0	16.7	0
1	23.4	6.7
2	38.4	20.0
3	55.0	25.0
4	65.0	—
5	71.6	33.3

tment recovered to a great extent by 3 days (Table 2). In this case the level of isocitritase was about 50% of that of the control.

Imbibition of seeds in actinomycin D (100 μg/ml) for 18 hours followed by transfer to water slightly reduced the rate of isocitritase production for two days (Table 3). Subsequently, isocitritase did not increase. These data suggest that, if actinomycin D prevents the transcription of RNA, the RNA synthesis is not essential for the development of isocitritase activity during the first two to three days of germination. However, RNA synthesis appears to be required thereafter for the synthesis of the enzyme.

In additional studies, seeds were germinated for 2 days in a solution containing ^{14}C-amino acid (0.5 μc/ml) and one of the following inhibitors: azetidine-2-carboxylic acid, p-fluorophenylalanine, cycloheximide, or actinomycin D, and the incorporation of ^{14}C-amino acid into protein was examined. As seen in Table 4, cycloheximide and actinomycin D inhibited

Table 3

Effect of actinomycin D on the development of isocitritase activity in peanut cotyledons

Peanut seed were first germinated for 18 hrs. in water or a solution of actinomycin D and then in distilled water for times indicated. Enzyme activity was measured in crude extracts from detached cotyledons as described under "Experimental"

Days after 18 hrs. pretreatment	Isocitritase activity (mμmoles glyoxylate/min/mg protein)	
	Control	Actinomycin D (100 μg/ml)
1	40.0	30.0
2	78.4	33.3

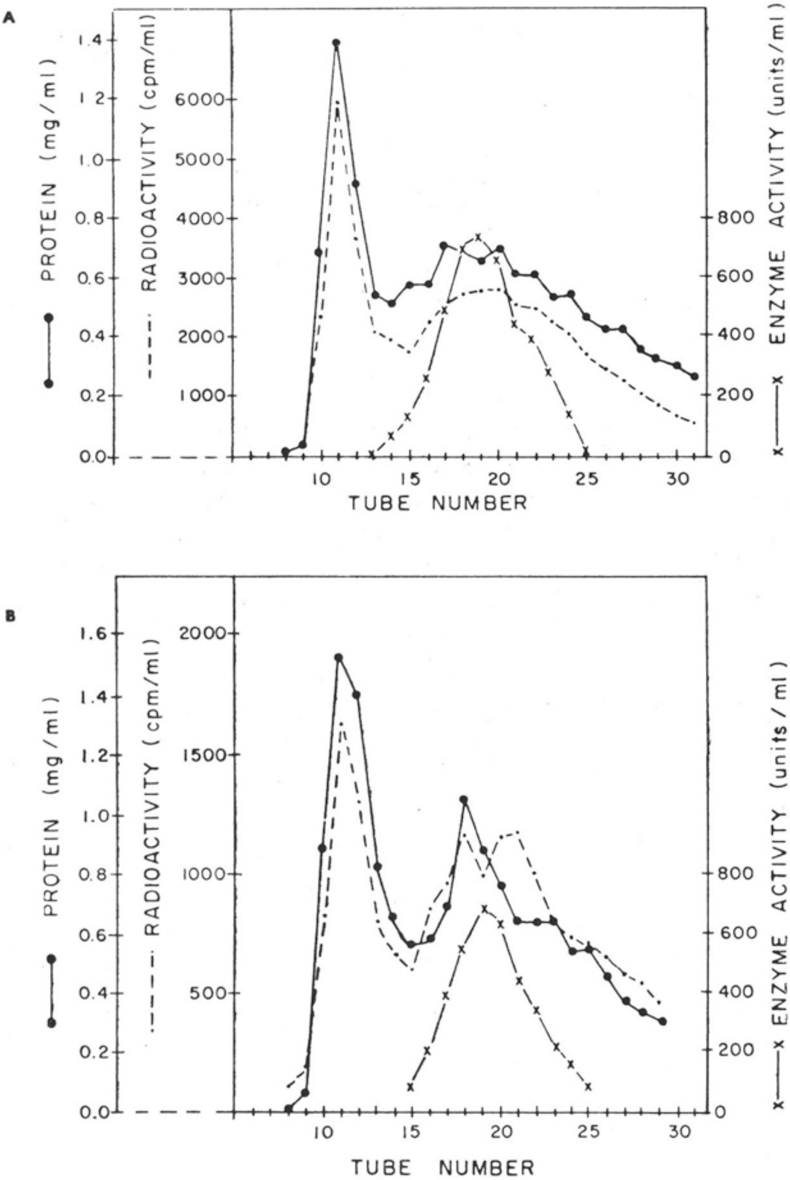

Fig. 1. Fractionation of a purified ^{14}C-labelled isocitritase preparation on a Sephadex G-200 column. Peanut seed were germinated for four days on:

(A), ^{14}C-labelled reconstituted protein hydrolysate (0.5 μ c/ml); (B), ^{14}C-p-fluorophenylalanine (1 μc/ml). Isocitritase was extracted from cotyledons and purified (Gientka-Rychter & Cherry, 1968). Ca. 30 mg protein was applied to a Sephadex G-200 column (200×20 mm) and eluted with 0.1 M phosphate buffer, pH 7.6. Fractions of ca. 2 ml were collected and analyzed as described under "Experimental". Enzyme unit: the change in optical density of 0.001 at 252 m per minute.

Table 4

Effect of various inhibitors on the incorporation of ^{14}C-amino acid into total protein

Peanut seed were germinated for 2 days in a solution of ^{14}C-amino acids (reconstituted protein hydrolysate; 0.5 μc/ml) with or without an inhibitor. The incorporated radioactivity was measured by the method of Mans & Novelli (1961) in crude extracts from detached cotyledons.

Inhibitor	Concentration	cpm/mg protein	% inhibition
none	—	883	0
p-fluorophenyl-alanine	3 mg/seed	1042	−18
azetidine-2-carboxylic acid	2 mg/seed	797	10
actinomycin D	50 μg/ml	711	20
cycloheximide	50 μg/ml	533	40

the incorporation of ^{14}C-amino acid into protein. Azetidine-2-carboxylic acid only slightly inhibited the incorporation of ^{14}C-amino acid, while p-fluorophenylalanine, in contrast, promoted incorporation (18% increase). The total uptake of ^{14}C-amino acids by the p-fluorophenylalanine treated seed, as estimated by total radioactivity in the crude homogenate, was greater than in control. In all experiments where p-fluorophenylalanine promoted ^{14}C-amino acid incorporation into protein, a concomitant increase in ^{14}C-amino acid uptake by the tissue was observed.

When the proteins were extracted from the seeds germinated for four days either in a ^{14}C-amino acid mixture or in ^{14}C-p-fluorophenylalanine and fractionated on a Sephadex G-200 column, each preparation gave the same profile of both radioactivity and enzymic activity (Fig. 1 A and B). The conclusion from these results is that p-fluorophenylalanine might be incorporated into the enzyme molecule but it does not interfere with its enzymatic function.

DISCUSSION

Recent work by Walton (1966) showed that p-fluorophenylalanine stimulated axis growth. He suggested that the stimulation might involve other metabolic effects in addition to replacement of phenylalanine in proteins. For example, p-fluorophenylalanine might interfere with metabolic pathways from phenylalanine to various compounds such as phenolics, coumarin. and flavonoids. Unexpected results on the stimulation of ^{14}C-amino acid incorporation into protein by p-fluorophenylalanine (Table 4) are difficult to explain. p-Fluorophenylalanine may inhibit protein degradation and thus decrease the endogenous amino acid pool which, in turn, would increase the specific activity of these pools and the protein synthesis from it. Furthermore, p-fluorophenylalanine may change the permeability of the cell membrane. An enhanced ^{14}C-amino acid incorporation was correlated with an increased uptake of labelled amino acids.

From the density labelling experiments (Gientka-Rychter & Cherry, 1968) it was concluded that isocitritase is *de novo* synthesized during peanut seed germination. Another possibility should be considered, namely, that synthesis of enzyme may start after one or two days of germination and that the enzyme appearing during earlier phases of germination is produced by an activation.

Horticulture Department
Purdue University, Lafayette
Indiana, U.S.A.

(Entered: February 20, 1970)

SUMMARY

1. The pattern of the production of isocitritase (isocitrate-glyoxylate lyase; E.C.4.1.3.1.) during early stages of peanut seed germination in the presence of cycloheximide and actinomycin D indicates that continual synthesis of protein is essential for the production of isocitritase but the requirement for RNA synthesis develops only after two days of germination. 2. Examination of the effects of azetidine-2-carboxylic acid and p-fluorophenylalanine on the development of isocitritase activity and the incorporation of radioactive amino acids into protein in germinating peanut seed suggests that proline and phenylalanine probably are not involved in the active portion of isocitritase.

REFERENCES

Beevers H., 1961, Nature 191: 433.

Carpenter W. D. & Beevers H., 1959. Plant Physiol. 34: 403.

Cherry J. H., 1963, Plant Physiol. 38: 440.

Fowden L., 1963, J. Exp. Botany 14: 387.

Fowden L & Richmond M. H., 1963, Biochim. Biophys. Acta 71: 459.

Gientka-Rychter A. & Cherry J. H., 1968, Plant Physiol. 43: 653.

Kornberg H. L. & Madsen N. B., 1957, Biochim. Biophys. Acta 24: 651.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J., 1951, J. Biol. Chem. 193: 265.

Mans R. V. & Novelli G. D., 1961, Arch. Biochem. Biophys. 94: 48.

Presley H. J. & Fowden L., 1965, Phytochem. 4: 169.

Rao A. N. & Ramakrishnan T., 1962, Biochim. Biophys. Acta 58: 262.

Richmond M. H., 1962, Bacteriol. Rev. 26: 398.

Spigelman S. & Hayashi M., 1963, Cold Spring Harbor Symp. Ouant. Biol. 28: 161.

Walton D. C., 1966, Plant Physiol. 41: 1549.

Young J. L., 1957, Feder. Proc. 16: 275.

Young J. L., Huang R. C., Venecko S., Marks J. D. & Varner J. E., 1960, Plant Physiol. 35: 288.

Young J. & Varner J. E., 1959, Arch. Biochem. Biophys. 84: 71.

Wpływ analogów aminokwasów na aktywność liazy izocytrynianowej w liścieniach orzecha arachidowego

Streszczenie

1. Zmiany aktywności liazy izocytrynianowej (glioksylano-liaza treo-D_s-izocytrynianu E.C.4.1.3.1.) podczas wczesnego stadium kiełkowania orzecha arachidowego w obecności cykloheksimidu i aktynomycyny D wskazują, że dla stałego wytwarzania tego enzymu niezbędna jest ciągła synteza białka. Konieczność syntezy RNA ujawnia się natomiast dopiero po dwóch dniach kiełkowania.

2. Zbadanie wpływu kwasu azetydyno-2-karboksylowego i p-fluorofenyloalaniny na aktywność liazy izocytrynianowej oraz na włączanie radioaktywnych aminokwasów do białka w kiełkujących nasionach orzecha arachidowego wskazuje, że prolina i fenyloalanina prawdopodobnie nie są składnikami centrum aktywnego liazy izocytrynianowej.