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Metabolism of Inositol Phosphates

I. PHYTASE SYNTHESIS DURING GERMINATION IN COTYLEDONS OF MUNG BEANS, *PHASEOLUS AUREUS*[1]

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ABSTRACT

The degradation of phytin in germinating mung bean seeds has been found to be associated with the increased activity of phytase in the cotyledon. In the differentiated embryo the increase of this activity is very low all throughout the growth periods studied. Phytase appears in the cotyledon during germination. No activity has been detected in the cotyledons of unsoaked seeds. Cycloheximide (10^{-6} M) inhibits the appearance of phytase by 61% during 24 and 48 hours after the start of germination. This phytase increase is dependent on the synthesis of new RNA in the cotyledon. Synthesis of DNA is not detected in the cotyledon during germination.

Phytase or myoinositol hexaphosphate phosphohydrolase (EC 3.1.3.8) which can hydrolyze myoinositol hexaphosphate to myoinositol and inorganic phosphate has long been known (23). Pears (18) showed that in ungerminated wheat the aleurone portion contained 39%, endosperm 34%, and scutellum 15% of the total phytase activity of the whole seeds. It has also been shown by different groups of workers that phytase activity of different grains increases during germination (1, 12, 15). Many reports are now available on the characteristics of the enzyme phytase. Nagai and Funahashi (17) reported a phytase, purified 1500-fold from wheat bran, which shows broad substrate specificity at pH 5.0. Gibbins and Norris (6) purified phytase and an acid phosphatase from the cotyledons of germinated *Phaseolus vulgaris*, the pH optimum of which has been recorded at 5.0. In the present study the activity of phytase in the cotyledons of mung bean seeds (*Phaseolus aureus*) has been found to be increased after soaking for 24 hr. The pH optimum for this system has been recorded at 7.5 instead of 5.0, the level reported with various preparations from different seeds. Very little phytase activity has been detected in the cotyledons of mung bean seeds after soaking for 6 hr. Regarding the appearance of phytase activity there may be two possibilities: (*a*) *de novo* synthesis or (*b*) activation of enzyme protein already present in the seeds. From the present study it appears that phytase is synthesized *de novo* in the cotyledon of mung beans after soaking.

MATERIALS AND METHODS

Mung beans (*Phaseolus aureus*, variety N.P. 23) were purchased from the Botanical Substation, Indian Agricultural Research Institute, Pussa, Bihar. ^{14}C-Amino acid mixture, 2-^{14}C-uridine, and 2-^{14}C-thymidine were obtained from Radiochemical Centre, Amersham, Buckinghamshire, England. Cycloheximide was procured from Nutritional Biochemical Corporation, Cleveland, Ohio. Chromomycin A_3 was obtained as a gift from Takeda Chemical Industries, Ltd., Osaka, Japan.

Isolation of Phytate. Phytate was isolated from mung bean by extraction with 1 M $HClO_4$ (4) and separated by gradient elution chromatography on a Dowex 1-Cl column (19).

Germination Procedure. The dry seeds of mung beans (*Phaseolus aureus*) were washed with water for 5 min and then covered with water (3 ml/g of seed) in a large Petri dish with seeds dispersed uniformly over the dish. The Petri dish was then covered, allowing for a little air passage, and kept at 35° in an incubator. After the seeds were fully soaked (about 12 hr), the water level in the Petri dish was adjusted such that the soaked seeds were less than half immersed in water. Water was added at intervals when the germination was prolonged up to 72 hr. Alternatively, the seeds were sterilized by dipping in 1% NaOCl for 2 min, rinsed with sterilized water, and germinated under aseptic condition. In the former case bacteria were not detected in cotyledons when these were washed thoroughly with distilled water after soaking for a definite period. However, associated with the seed coats and washings the bacterial counts were recorded at 10^7/g of dry seeds.

Isolation of Phytase. The separated tissue was washed with water, homogenized with an equal volume of 0.05 M tris buffer, pH 7.0, and centrifuged for 20 min at 10,000*g* in cold. The supernatant was heated to 60° in a water bath and maintained at that temperature for 5 min. This was cooled in ice and centrifuged at 10,000*g* for 20 min. The supernatant was then made up to 90% saturation with ammonium sulphate, and the precipitate was collected by centrifugation, on standing for 30 min dissolved in a minimum volume of 0.05 M tris buffer (pH 7.0), and dialyzed against a large volume of the same buffer overnight in the cold. The protein solution thus obtained contained the phytase.

Assay of Phytase. Assay mixtures contained the following in a total volume of 1 ml: Tris-HCl buffer, pH 7.5, 100 μmoles; phytate, 1.6 μmoles; appropriate enzyme protein. After incubation for 1 hr at 37° the reaction was stopped by adding an equal volume (1 ml) of 0.4 M trichloroacetic acid in cold and kept at room temperature for 15 min. The mixture was then centrifuged. The liberated P_i was estimated by the method of Lowry and Lopez (10) with an aliquot from the clear supernatant, using appropriate standard.

RESULTS

Phytase Activity in Cotyledons and in Embryos at Different Stages of Germination of Mung Beans. Phytase activity was measured in the cotyledons and embryos at the different stages of germination of mung beans as is indicated in Figure 1. At all

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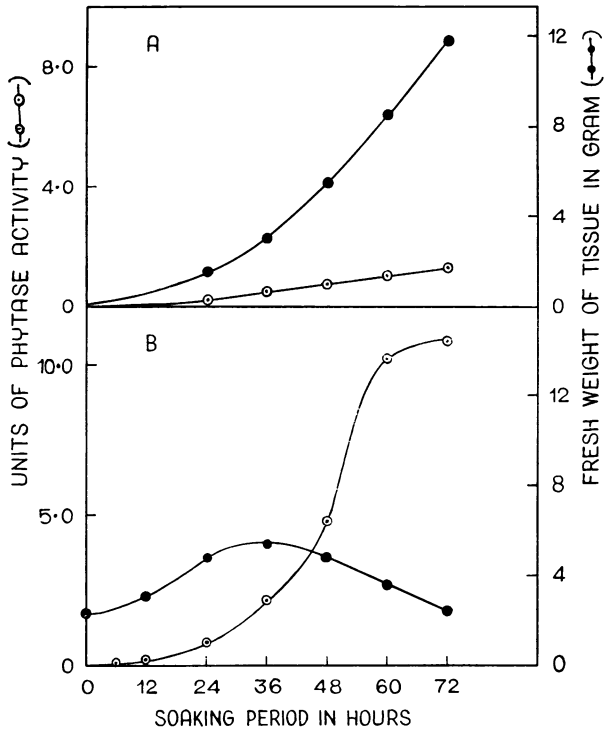

FIG. 1. Phytase activity and fresh weight of tissue as a function of soaking period. Phytase activity and the fresh weight represent the total quantity recovered from cotyledons and embryos obtained from 100 seeds after germination for different periods under the conditions mentioned in the section on method. Phytase was isolated and assayed by the procedure described in that section. One unit of phytase has been defined to be that amount of protein which can liberate 1 μmole of phosphorus from phytate in 1 hr under the assay conditions used. A: Embryo; B: cotyledon.

the stages (from 12 to 72 hr) where phytase is measurable the total phytase activity in the cotyledon is greater than that in the embryo. Enzyme activity is not detectable in the cotyledon of unsoaked seeds. Measurable phytase activity appears in both cotyledon and embryo after 12 hr of soaking. The rate of increase of phytase activity in the cotyledon is initially very low up to 24 hr; after 24 hr it is accelerated and attains a maximum between 48 and 60 hr. When the rate of change in fresh weight of cotyledons is compared with the rate of increase in phytase activity, it is seen that the fresh weight of cotyledon increases up to 36 hr followed by a gradual decrease, and it is at this stage that the phytase activity increases. In the embryo, both fresh weight of tissue and the phytase activity increase with time of soaking, the rate of increase of the latter being very low in comparison with that of its fresh weight as well as with the rate of increase of phytase activity in the cotyledon.

Effect of Cycloheximide on Increase of Phytase Level and on Protein Synthesis in Cotyledon during 24 to 48 hr of Soaking. The effect of different doses of cycloheximide on the increase of phytase activity in the cotyledon is shown in Table I. The normal increase in phytase activity is inhibited by cycloheximide, the degree of inhibition being dependent on the concentration of the inhibitor. Thus at a concentration of 10^{-6} M, the inhibition is 61% while with 2×10^{-5} M it is about 90%. The data also indicate that the normal loss in the weight of the cotyledon is inhibited by cycloheximide.

The data in the Table II show that cycloheximide at 10^{-6} M inhibits protein synthesis in the cotyledon from 24 to 48 hr after soaking, as indicated by the inhibition of ^{14}C-amino acid incorporation into the protein. That the inhibition of incorporation of ^{14}C-amino acid into protein by cycloheximide is not due to inhibitory effect of the antibiotic on the uptake of the precursor by cotyledon is shown by the fact that the total radioactivity recovered in the acid-soluble fraction in both control and the antibiotic-treated sets are approximately same.

Table I. *Effect of Different Concentrations of Cycloheximide on Increase of Phytase Activity in Cotyledons during 24 to 48 hr of Soaking*

Seeds were soaked for 24 hr under the conditions mentioned in the section on methods. From this lot, six batches of 80 seeds each were picked from among well germinated ones. Phytase was isolated and assayed from the cotyledons of one batch. Of the remaining five batches, four were treated with four different concentrations of cycloheximide in 5 ml of water, and the other was kept as a control. All were incubated at 35°, and, after another 24 hr, phytase was isolated and assayed by the procedures described in the section on methods. A unit of phytase activity has been defined in Figure 1.

Experiment	Cycloheximide Concn	Fresh Wt of Cotyledon at 48 hr	Total Unit of Phytase at 24 hr	Total Unit of Phytase at 48 hr	Units of Phytase Synthesised during 24 to 48 hr	Inhibition of Phytase Synthesis during 24 to 48 hr
	M	*g*				*% of control*
1	Control	3.7	0.75	3.33	2.58	...
2	1×10^{-6}	4.3	0.75	1.75	1.00	61
3	5×10^{-6}	4.3	0.75	1.07	0.32	88
4	1×10^{-5}	4.7	0.75	1.07	0.32	88
5	2×10^{-5}	4.8	0.75	1.01	0.26	90

Table II. *Effect of Cycloheximide on Protein Synthesis in Cotyledon during Germination*

Seeds were germinated under aseptic condition. Sixteen seeds after soaking for 24 hr were divided into two batches, each comprised of eight seeds. Both the batches were incubated in 0.5 ml of sterile water containing a ^{14}C-amino acid mixture (equivalent to 2.4×10^5 cpm of specific activity 6.66×10^5 cpm/μ atom carbon) in each and with the addition of cycloheximide (1×10^{-6} M final concentration) to one batch, the other being kept as control. After another 24 hr at 35°, the cotyledons were separated batchwise, washed repeatedly with water until the washings were free of radioactivity, and then homogenized with 4 ml of 1 M perchloric acid in the cold and centrifuged at 10,000 *g* for 10 min. The pellet was washed 4 times with 5% perchloric acid, and the supernatant and the respective washings were pooled and neutralized with 10 M KOH. This neutral solution was used to determine acid-soluble radioactivity. The pellet was dissolved in a known volume of 0.01 N NaOH, an aliquot from this solution was used to determine the acid-insoluble radioactivities, and the rest, after being kept for 30 min at room temperature, was reprecipitated with perchloric acid and washed 6 times again with perchloric acid. Finally the pellet was dissolved in 0.1 N NH_4OH. Radioactivity of this protein solution was determined and protein was estimated by the method of Lowry *et al.* (11).

Experiment	Total Radioactivity Recovered in		Incorporation of ^{14}C-Amino Acid
	Acid-soluble fraction	Acid-insoluble fraction	
	cpm		*cpm/mg protein*
Control	10,200	27,600	280
Control + cycloheximides	12,500	18,300	170

Effect of Chromomycin A_3 on Phytase and RNA Syntheses in Cotyledon during Germination. Is the increase of phytase activity in cotyledons affected by arresting RNA synthesis during germination? Because actinomycin D was not effective even in high concentration in blocking RNA synthesis in cotyledons, chromomycin A_3 was tried as an alternative. In seeds soaked in chromomycin A_3 phytase synthesis is arrested (Table III). Under the same conditions the incorporation of 2-^{14}C-uridine into RNA is inhibited by about 71% (Table IV). The total radioactivity in the acid-soluble fraction in the control and the treated sets is found to be the same, indicating that the inhibition of incorporation of 2-^{14}C-uridine into RNA is not due to impairment of the entry of uridine into the cell in the presence of the antibiotic.

Table III. *Effect of Chromomycin A_3 on Phytase Synthesis in Cotyledon during Germination*

Four batches of 80 seeds each were well washed with water and taken in four petri dishes. Two batches were allowed to soak in the presence of 5 ml of water and the other two in the presence of chromomycin A_3 (10 μg per seed) in 5 ml of water, at 35°. When the liquid had been soaked in fully, a little more water was added to each. At 24 and 48 hr of soaking, seeds from both control and chromomycin A_3-treated batches were taken out, and the phytase was isolated and assayed from each batch of cotyledon by the procedures mentioned in the section on methods. The unit of phytase has been defined in Figure 1.

Experiment	Units of Phytase/g, Fresh Wt, of Cotyledon		
	6 hr	24 hr	48 hr
1. Control	...	0.25	0.84
2. Chromomycin A_3 from beginning of soaking	...	0.06	0.075
3. Chromomycin added after 6 hr of soaking	0.015	0.11	...

Table IV. *Effect of Chromomycin A_3 on RNA Synthesis in Cotyledon during Germination*

Since the extent of incorporation of amino acids into protein was found to be approximately the same under aseptic and nonaseptic conditions, the seeds were not germinated under sterile condition in the following experiments. Two batches of 10 seeds each were well washed with water. To each of two small beakers was added 2-^{14}C-uridine (2.07×10^5 cpm, specific radioactivity 5.23×10^6 cpm/μmole) followed by the addition of a solution of chromomycin A_3 (10 μg per seed) to one of them, in a final volume of 0.6 ml in each beaker at 35°. After 12 hr, when the liquid had been soaked in, 1 ml of water was added and kept for another 12 hr. After that period, the seeds were washed thoroughly and the cotyledons were separated batchwise. The RNA was then isolated by the method of Scherrer and Darnell (21). The radioactivity and the optical density at 260 mμ of the isolated RNA in solution were determined. Different sets of experiments with 2-^{14}C-uridine were done under identical conditions for assaying total acid-soluble and acid-insoluble radioactivities. The methods were essentially the same as that described in Table II.

Experiment	Total Radioactivity Recovered in		Incorporation of ^{14}C-Uridine
	Acid-soluble fraction	Acid-insoluble fraction	
	cpm		*cpm/OD at 260 mμ*
Control	43,400	10,450	375
Control + chromomycin A_3	57,300	3,150	108

Table V. *Relative Synthesis of DNA in Cotyledon and Embryo at Different Periods of Soaking*

Sixty well washed seeds were put into 4 ml of water containing 2-^{14}C-thymidine (4.8×10^5 cpm of specific radioactivity, 2.88×10^6 cpm/μmole) in a small crystallizing dish and incubated at 35°. Twenty seeds each at 8, 16, and 24 hr were taken out, washed carefully to remove external radioactivity, then decoated by means of a pair of forceps. The cotyledons and the whole embryos were then separated batchwise, and DNA was isolated from each by the method of Schneider (22). Then radioactivity and the optical density at 260 mμ of the isolated DNA in solution were determined.

Time of Soaking	Incorporation of ^{14}C-Thymidine	
	Cotyledon	Embryo
hr	*cpm/OD at 260 mμ*	
8	26	114
16	22	276
24	30	374

DNA Synthesis in Cotyledons and in Embryos during Germination. In order to find out the extent of DNA synthesis in cotyledons and embryos during different periods of soaking, the relative incorporation of 2-^{14}C-thymidine into DNA of embryos and cotyledons was measured as is indicated in Table V. It is apparent from the data that incorporation of radioactivity into DNA of the embryo increases almost linearly with time whereas in the cotyledon this is constant and negligible. This suggests that there is no DNA synthesis in the cotyledon during germination.

DISCUSSION

It is now well established that the phytate phosphorus is mobilized in the seeds, and this mobilization closely follows germination (15). The increased rate of disappearance of phytate with germination has been shown to be paralleled by a simultaneous increase in the phytase activity (15, 24, 25) in the whole seedling. In the present report we have studied the increase of phytase activity both in cotyledons and differentiated embryos during germination of mung bean. The increase of phytase activity in the embryo seems to be proportional to the fresh weight, so that the net increase is dependent on the growth of the embryo. But in the case of cotyledons the situation is different. In the unsoaked cotyledons phytase activity is absent but appears after soaking the seeds for 12 hr and reaches its maximum between 48 and 72 hr. The absence of phytase activity in unsoaked cotyledons and its subsequent appearance during germination might be due to (*a*) the absence of the enzyme itself in the unsoaked cotyledon and subsequent synthesis of this enzyme during the period of soaking, (*b*) the presence of some inhibitor, which could interfere with the activity of phytase normally present in the cotyledon at the earlier stages of germination, or (*c*) the activation of phytase by the proteolytic enzymes. Neither inhibition of phytase by addition of extract from ungerminated seeds isolated after heat treatment to enzyme from seeds germinated for 72 hr nor activation of phytase by using trypsin (1 μg/5 mg of protein) in the extract from seeds germinated for 6 hr was obtained in experiments performed separately. These observations seem to have ruled out the latter two possibilities. The experiments with cycloheximide, a potent inhibitor of protein synthesis (2, 7, 16), under different conditions (Tables I and II), suggest that the increase of phytase activity in the cotyledon

with an increased period of soaking is due to the synthesis of the enzyme.

The next question arises whether this synthesis of phytase in the cotyledon is dependent on the synthesis *de novo* of messenger RNA. When chromomycin A_3 (9, 26) (10 μg per seed) is added from the beginning of soaking, at 24 hr the phytase synthesis (Table III) and the RNA synthesis (Table IV) have been inhibited by 76 and 71%, respectively. If the chromomycin A_3 is added after 6 hr of soaking, there is also an inhibition of phytase synthesis (Table IV), the inhibition being about 60% of the increased value from 6 to 24 hr. Thus only 16% of the activity from 6 to 24 hr could be initiated by messenger RNA synthesized within 6 hr of soaking. Although we have not provided direct proof of induction, induction of phytase synthesis in the cotyledon after soaking of mung bean seeds is in agreement with Jacob and Monod's concept involving transcription and translation processes (8). Similar type of induction of other enzymic activities in germinating seeds has been reported in other cases as well (14). That the degradation of phytate in the developing wheat embryo in isolated culture is regulated by the level of inorganic phosphate in the medium has been shown by others (3, 20). This regulation by inorganic phosphate has been shown to be effected by the repression of phytase synthesis at the transcriptional level in this growing tissue. It is interesting that for the mobilization of phytate from the cotyledon the phytase system had to be developed during germination but the presence of phosphoinositol phosphotransferase (5) could be detected in the ungerminated mung bean and declines gradually during germination (unpublished result). Furthermore, it has been observed that phytate is synthesized in the cotyledon of the mung bean during the early period of soaking (13), which seems to be a reflection of the situation at the ripening stage. Thus far what is evident is that rigid control systems are in operation for the metabolism of phytate during germination.

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