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SYNTHESIS OF PHYTASE DURING GERMINATION IN COTYLEDONS OF PUMPKIN SEEDS (*CUCURBITA MAXIMA*)

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SUMMARY

Phytase activity was totally absent in dry seeds of pumpkin. Its activity was detected only after 48 hr of soaking in water. Increase in the level of this enzyme was inhibited by cycloheximide (1 mM) and was dependent on synthesis of RNA in the cotyledons.

INTRODUCTION

Phytin is generally considered as a store of inorganic phosphate in seeds which which is mobilized during seed germination to meet the needs of phosphorus in growing plant during the early period of germination and development (Tomlinson and Ballou 1962; Lim and Tate, 1971; Maiti and Biswas, 1979). Dephosphorylation of phytin is a stepwise process catalyzed by myoinositol hexakis (dihydrogen phosphate) phosphohydrolase (EC 3.1.3 26) (phytase). The ultimate products are myoinositol and inorganic phosphate (Mayer 1958; Mihailovic *et al.*, 1662; Sartirana and Bianchetti 1967). Further hydrolysis presumably by phosphatases in the plant yields free inositol which becomes available for the metabolic requirement. (Maiti and Biswas, 1979). Germinating seeds contain high activity of phytase (Albaum and Umbreit 1943; Desjobert and Petek 1956; Ergle and Guinn, 1959). In ripe seeds it is either totally absent or present at low levels and during early stages of seed germination, the enzyme activity increases. As a result of phytin breakdown, the levels of Pi in seeds increases progressively leading to the repression of phytase synthesis. In the earlier study, multiple forms of phytase were isolated, purified and characterized from the cotyledons of germinating seeds of pumpkin (Goel and Sharma, 1979).

In the present study developmental pattern of phytase activity has been studied during different stages of germination. The effect of some inhibitors like cycloheximide and chromomycin A_3 on phytase activity and RNA synthesis during germina-

tion in cotyledons were also studied in order to know the mechanism of *de novo* synthesis of phytase during germination.

MATERIALS AND METHODS

Unless otherwise stated all the chemicals used were of analytical grades. ^{14}C-amino acid mixture and ^{3}H-uridine were obtained from B.A.R.C., Bombay. Cycloheximide, chromomycin A_3 and phytic acid were obtained from Sigma Chemicals Co., USA and pumpkin seeds were obtained from N.S.I., Pusa, New Delhi.

Seeds were sterilized and germinated in dark at 30°C under aseptic conditions as described by Mandal and Biswas (1970a). All the subequent operation were carried out at 0-4°C unless otherwise stated. Cotyledons (100 g) were defatted 3 times by homogenizing in a waring blender with chilled (-20°C) acetone. The homogenate was filtered under suction and dried *in vaccuo*. One hundred gm of defatted meal (acetone powder) was extracted in IL of 0.1 M sodium acetate buffer (pH 5) for 19 hr. The homogenate was centrifuged for 1 hr at 10,000 g and the supernatant obtained was referred to as acetone powder extract (Goel and Sharma 1979). The proteins precipitating between 0.3 to 0.8 ammonium sulphate saturation contained all the phytase activity. The precipitate obtained after centrifugation for 1 hr at 20,000 × g was dissolved in a minimum volume of 0.5 M sodium acetate buffer (pH 5.0) and dialyzed for 18 hr against the same buffer. The dialyzed preparation was centrifuged as described above to remove the precipitate and the supernatant was concentrated by ultrafiltration using PM-10 membrane. The procedures for ammonium sulphate fractionation and assay of phytase were the same described by Goel and Sharma (1979). Protein was measured by the method of Lowry *et al.*, (1951) using BAS as a standard. One unit of enzyme activity corresponds to liberation of one μ mole of Pi per min under the assay conditions.

To determine the effect of inhibitors of protein and RNA synthesis on level of phytase in the cotyledons, seeds were germinated under aseptic condition. The seeds were first soaked in water for 24 hr and then allowed to germinate for another 24 hr. Four batches of 30 well germinated seeds were selected. Cotyledons from one batch were taken as control and remaining three were placed in 10 ml water containing either 1000 μM, 10 μM and 1 μM of cycloheximide. These were then incubated at 30°C and after 6 days phytase activity was assayed as described above. To examine the effect of chromomycin A_3, four batches of 40 seeds each were taken in four petri dishes. Two batches were allowed to germinate in water and other two in presence of chromomycin A_3 (15 μg seed^{-1}). These were incubated at 30°C and after days 1 and 6, phytase activity was determined as described above.

To study the effect of cycloheximide and chromomycin in A_3 on protein and RNA synthesis in cotyledons, twenty seeds after soaking for 24 hr were divided into two batches, one of the batch was incubated with 10 ml of sterilized water containing a ^{14}C—amino acid mixture (10 μ Ci/ml) and the other in addition contained cycloheximide (1 mM final concentration). After 5 days of incubation at 30°C, the cotyledons were washed repeatedly with water until the washings were free of radioactivity. The cotyledons were then washed with chase medium ($5x10^{-2}$M cold amino acid mixture). The seeds were homogenized with 2 ml of 2% SDS and an equal volume of 20% TCA was added for deproteinization. The samples were kept overnight in a refrigerator. The precipitate was collected with millipore filtration assembly on Max-flow membrane filters (pore size 0.45 micrometers). The precipitate was dried for 1 hr at 60°C and transferred to the scientillation vials containing 8 ml scintillation fluid. For studying the effect of chromomycin A_3 on RNA synthesis in cotyledons, two batches of ten seeds each were soaked for 24 hr and incubated for 5 days with 10 ml sterilized water containing ^3H-uridine (8 μ Ci/ml) at 30°C. Chromomycin A_3 (15 μg/seed) was added to one batch. After 5 days, cotyledons were separated and washed thoroughly with cold uridine solution (50 mM) Homogenization and precipitation was done in the same way as for protein systhesis. Total RNA was determined by the method of Schneider (1957).

RESULTS AND DISCUSSION

It is evident form the fig. 1 that phytase activity was totally absent in the dry seeds, and was detected after 40 hr of soaking in water. Following this initial lag, phytase activity increased rapidly during the next 120 hr of germination and then began to fall. Thus the germination period between 2-7 days represents a period of rapid mobilization of reserve phytate phosphorous It has infact been shown that mobilization of phytate is closely linked to germination (Tomlinson and Ballou 1962; Lim and Tate 1973; Maiti and Biswas 1979). As the germination proceeds, there is an increase in the rate of disappearance of phytate as well as an increase in phytase activity in the whole seedling (Sartirana and Bianchetti 1967; Mandal and Biswas 1970b). The appearance of phytase during germination may be due to any of the following reason : *de novo* synthesis during germination, activation of an inactive form of the enzyme or presence of some inhibitor in ripe seeds. To identify the probable mechanism involved, effect of inhibitors of protein and RNA synthesis were examined.

The effect of cycloheximide on the level of phytase during 2-6 days of germination is shown in Table I. Cycloheximide at 1000 μM and 10 μM and 1 μM inhibited

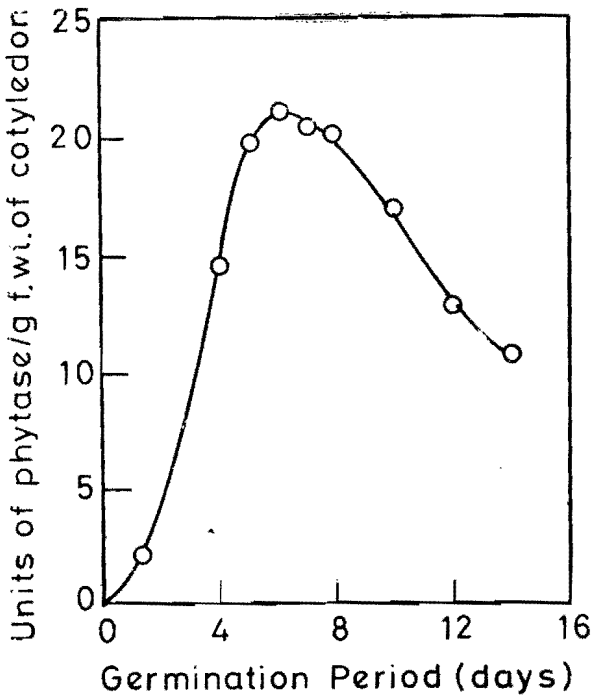

Figure 1. Changes in Phytase activity in cotyledons during germination. At each stage of germination the enzyme was extracted from 100 seeds with 200 ml of 0.05 M acetate buffer pH 4.8 and the total enzyme activity was measured as described in the method.

Table I. Effect of cycloheximide on phytase activity in cotyledons during 2 to 6 days germination

Cycloheximide concentration (μM)	Units of phytase activity/ gr f wt of cotyledon		Increase in activity during 2-6 days germination	Inhibition %
	2 daye germination	2 days germination		
0 (control)	2.0	21.0	19	—
1	2.0	11.5	9.5	50
10	2.0	9.5	7.5	60.5
1000	2.0	5.5	3.3	82.6

increase in phytase activity by 82. 6%, 60.5% and 50% respectively. Cycloheximide did not affect the uptake of labelled amino acids as indicated by the total radioactivity recovered in the control and cycloheximide treated sets (data not show). The results thus suggest that the increase in phytase activity in the cotyledons during germination involves *de novo* synthesis of the enzyme.

Increase in phytase activity was arrested when cotyledons were treated with chromomycin A_3 (15 μg/seed). When it was added at the beginning of soaking, the phytase synthesis was arrested (Table II) and under similar condition, the incorporation of ^{14}C uridine into RNA was inhibited by 78% (Table II).

Table II. Effect of chromomycin A_3 on phytase activity and RNA synthesis in cotyledons during germination

Treatment	Units* of phytase/g fr wt of cotyledon		% Inhibition phytase synthesis 1 to 6 days germination	Total radio activity recovered cpm	^{14}C uridine incorporation cpm/mg protein
	1 days	6 days			
Control	0.5	21.0	—	30,129	295
Chromomycin A_3 from beginning of soaking	0	6.3	69.3	22,500	65
Chromomycin A_3 added after 8 hr soaking	0.15	7.0	66.6	—	—

* Unit—see in Materials and Methods

** Chromomycin A_3—15 μg seed^{-1}

The experiment thus indicate that increase in phytase activity during germination requires both RNA synthesis and protein synthesis and thus involves its *de novo* synthesis during seed germination.

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