SYNTHESIS OF A PROTEASE IN GERMINATING COTTON COTYLEDONS CATALYZED BY mRNA SYNTHESIZED DURING EMBRYOGENESIS

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Received June 23, 1969

It has previously been suggested that a great deal of the protein synthesis that occurs in cotton seed cotyledons during the initial stages of germination is catalyzed by mRNA that is synthesized during the maturation of the seed on the maternal plant (Waters and Dure, 1966). In order to further establish the existence of such a long-lived body of mRNA, the activity of a specific protease that appears to arise de novo during germination in cotton seed cotyledons has been studied.

The protease has been purified 1000 fold and appears homogeneous by disc electrophoresis and column elution. The purification procedures and characteristics of the enzyme will be published elsewhere.

The advent of activity of the protease appears to be the result of de novo protein synthesis in that it is totally sensitive to cycloheximide and has a specific activity (cpm/mgP) five times that of crude supernatant protein when the cotyledons are germinated in the presence of ^{14}C amino acids and the enzyme isolated. However, the advent and extent of its activity has been found to be insensitive to concentrations of actinomycin D that stop all detectable RNA synthesis and that are ultimately lethal to the plantlet. Furthermore, since cotton embryos can be induced to germinate precociously before their maturation is complete by simply dissecting them onto agar gel, it has been possible to follow this actinomycin D insensitive synthesis of the protease back into embryogenesis until both precocious germination and the synthesis of the protease become sensitive to the inhibitor.

Methods

Cotton seeds (Gossypium hirsutum, variety Coker 100) were used throughout.

To study the normal development of enzyme activity during germination, cotton seeds were imbibed in water for thirty minutes, placed on 0.8% agar gel in culture dishes and germinated in the dark at 30°C. To examine the effect of inhibitors supplied at the beginning of germination on the subsequent development of enzyme activity, the dry seeds were imbibed with solutions containing either actinomycin D (20μg/ml) or cycloheximide (1mg/ml) and transferred to agar gels that contained the same concentrations of inhibitors or to agar gels without inhibitors. In order to examine the effects of inhibitors when applied at later stages of germination, the cotyledons were separated from the seed coat and growing axis and placed in petri dishes between two layers of miracloth saturated with the same concentrations of inhibitors as used above. Without inhibitors the maturation of the cotyledons continues under these conditions and greening occurs upon illumination. To follow the development of enzyme activity during precocious germination, immature embryos were removed from the ovules and germinated on 0.8% agar gel as above. The effects of actinomycin and cycloheximide on the advent and extent of enzyme activity during precocious germination were determined by incorporating the above concentration of inhibitors into 0.8% agar gel. Enzyme activity during germination was followed by homogenizing the cotyledons with a Duall motor-driven homogenizer in cold 0.1M sodium phosphate buffer, pH 6.6, containing 0.001M mercaptoethanol. The homogenate was centrifuged at 27,000 x g and the supernatant assayed for enzyme activity by following the hydrolysis of benzoyl-arginine-ethyl-ester (BAEE) at pH 6.6 (Schwert and Takenaka, 1955) with a Cary Model 15 spectrophotometer. An enzyme unit is defined as an increase in A_{253} of 0.01/minute.

Results

Figure 1, line a, shows the time course of the development of enzyme activity in the cotyledons during the first 6 days of germination. No

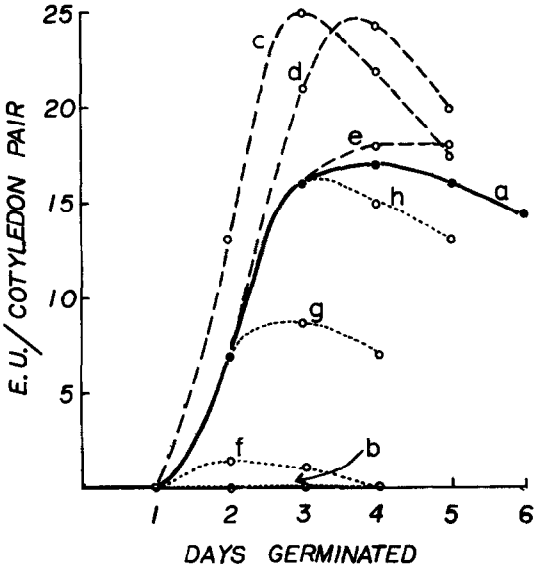

Figure 1. Effect of cycloheximide on enzyme appearance: a, control; c, d and e, excised cotyledon controls; b, f, g and h, cycloheximide treated. See text for details.

activity is detectable until after 24 hours of germination. It does not appear that the enzyme is present but inhibited before that time, since addition of supernatants obtained from cotyledons of imbibed seeds or from 24 hour germinated cotyledons, to active supernatants does not inhibit enzyme activity. Further, subjecting preparations having no activity to the purification scheme for the enzyme fails to show activity in fractions that otherwise would be highly active.

If these seeds are exposed to cycloheximide during their initial water imbibition which commences germination, no enzyme activity develops, regardless of whether or not they are maintained on cycloheximide or transferred to agar gel without the inhibitor (Figure 1, line b).

When cotyledons which have begun germination are removed from the seed coat, separated from their axis and transferred to wet miracloth (so as to insure a more rapid and uniform uptake of inhibitors at these later stages) a stimulation of the rate of accumulation of enzyme activity occurs if inhibitors

are left out. This stimulation is shown in Figure 1, lines c, d and e which correspond to cotyledons transferred to miracloth at 24, 48 and 72 hours respectively. The effect of inhibitors applied at these later stages of germination must be compared with these miracloth controls.

When cotyledons, dissected at various stages after imbibition, are transferred to and maintained on miracloth containing cycloheximide, enzyme accumulation ceases (compare line f with c, g with d and h with e). This sensitivity to cycloheximide does not in itself demonstrate the de novo synthesis of the protease during germination. However, this likelihood is reinforced by the fact that the purified enzyme prepared from cotyledons germinated in the presence of ^{14}C amino acids has a specific activity five times that of the total soluble protein of the tissue. Details of this experiment will be published elsewhere.

On the other hand, actinomycin D in concentrations that inhibit all RNA synthesis that is detectable by $^{32}PO_4$ incorporation (as demonstrated by Waters and Dure, 1966) has little or no effect on the development of the protease activity as shown in Figure 2. Seeds that imbibe actinomycin D during their initial imbibition and are maintained for three days on actinomycin D (line b)

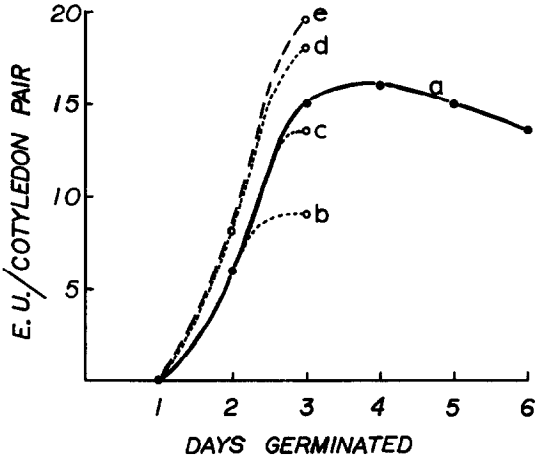

Figure 2. Effect of actinomycin D on enzyme appearance: a, control; e, excised cotyledon controls; b, c and d, actinomycin D treated. See text for details.

show the normal accumulation of the enzyme at 48 hours, but a much reduced value when compared with the normal value (line a) at 72 hours. We believe that this low 72 hour value is due to the pathological condition of the cotyledons after three days in actinomycin D. Large necrotic areas develop by the third day and the cotyledons appear flaccid. Seeds that are imbibed in actinomycin D and subsequently germinated on agar without actinomycin D accumulate the enzyme to approximately the same extent as the controls (line c). Cotyledons dissected after 24 hours and placed on miracloth containing actinomycin D also develop enzyme activity (line d) approaching that of the dissected cotyledon controls (line e). From these data it would appear that the protease enzyme is synthesized de novo in the cotyledons during germination without the requirement for RNA synthesis during this period, i.e. from mRNA synthesized during the embryogenesis of the seed on the maternal plant.

In order to establish the existence of the mRNA for this enzyme in embryos from immature seeds, embryos were dissected from the ovules on the plant and precociously germinated on 0.8% agar gel with and without actinomycin D. The fresh weight of mature embryos before desiccation is 125 mgs. As can be seen from Table I, embryos that weigh more than 100 mgs. develop enzyme activity in the presence of actinomycin D when allowed to germinate precociously. However, embryos that weigh less than 100 mgs. while developing the enzyme activity slowly without the inhibitor, fail to develop it at all (and fail to progress very far into germination) when incubated with actinomycin D.

These data suggest that after embryo development has reached the point corresponding to 100 mgs. of fresh weight, the mRNA for the protease is present in the cells of the cotyledons. Prior to this stage in development, however, the appearance and accumulation of the enzyme requires RNA synthesis.

This apparent example of "stored" mRNA is analogous to the indication that mRNA is stored for subsequent utilization in sea urchin eggs (Gross and Cousineau, 1963). The mechanism by which the translation of the mRNA is proscribed until subsequent events take place is not yet understood in either system.

TABLE I

Development of Enzyme Activity During Precocious Germination

Age	Enzyme Units/Cotyledon Pair -Actinomycin D	+Actinomycin D
115 mg Embryos 2 days germinated	12.48	9.33
100 mg Embryos 4 days germinated	6.23	8.65
95 mg Embryos 6 days germinated	8.58	0

This research was supported in part by an NSF grant and a USAEC contract.

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