CERTAIN ASPECTS OF CONTROL MECHANISM OF STORED FOODS DEGRADATION DURING SEED GERMINATION

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Abstract: The control mechanism of stored foods degradation during seed germination was studied. When one excised cotyledon was incubated with three embryonic axes, it was found that the embryonic axis may stimulate the proteinase activity, RNA synthesis, and reserve protein breakdown in the excised cotyledons. Cytokinins may replace the embryonic axis function and stimulate the proteinase activity and RNA synthesis in the excised cotyledous.

It is evident that the soybean cotyledon contains more than one proteolytic enzyme. The experimental results show that the embryonic axis may affect *de novo* synthesis of the peak b component of the proteolytic enzyme.

A possible explanation for these results is that the synthesis of proteolytic enzyme in the cotyledons of soybean seedlings is controlled by the embryonic axis and the hormone involed appears to be a cytokinin.

INTRODUCTION

A seed contains an embryonic axis (or embryo) which is in an inactive condition, and germination is its resumption of growth. This young plant, protected by various layers of living and dead tissue, has reserves for metabolism and growth. These reserves are starch, proteins, fats and hemicellulose, and they are all insoluble or colloidal substances. These stored foods cannot be transported from cell to cell for utilization in building up protoplasm and cell walls unless they are changed into a soluble and diffusible form. The process of rendering food soluble and diffusible requires enzymes.

A number of workers[1-3] have extensively reviewed the subject of seed germination, and have recognized that the ability of making new protein is certainly an early requirement during germination. The stored foods are hydrolyzed into low molecular compounds and then transferred from the storage organ to the growing part of the germinatimg seeds. Assimilation is the final step in the utilization of stored foods. Swelling of the seed, due to imbibition of water and growth, is followed by the bursting of the seed coat.

Seed germination has been studied extensively, and quite a few have contributed to our knowledge about the regulation of the processes involved in the degradation of stored foods in germinating seeds. Several workers[4-11] have been investigating

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the possible control mechanism of the degradation of stored foods during seed germination. These reports tend to support the idea that the embryo produces a hormone-like substance which activates or synthesizes some hydrolytic enzymes in the storage tissues. The degradation of storage foods in the seedling of monocotyledonous plants has received more attention. In this experiment the soybean has been chosen because its cotyledons have high protein reserves and the proteinse content of cotyledons increases rapidly in the initial phase of germination[12]. The objectives of this investigation are to determine: First, the influence of the embryonic axis on the reserve protein breakdown and the relative amount of proteinase activity development occurring at the various stages of germination. Second, what kind of plant hormones are released from the embryonic axis which can induce the proteinase activity in cotyledons. Third, the purification of proteinase whose development is controlled by the axis.

MATERIAL AND METHODS

The soybeans (*Glycine max.* var. Taita-Kaoshiung No. 5) were used in this study. The soybeans were purchased from the Kaoshiung Area Agriculture Improvement Station in 1968. The seeds were stored at 3°C for extended periods of time with no noticeable change in subsequent growth.

Uniform-sized seeds were sterilized with 1% sodium hypochlorite for 15 min., rinsed twice with distilled water and then germinated on a double-layer of paper towels moistened with distilled water in petri-dishes. For analysing the effect of the excised axis on excised cotyledons, the sterilized seeds were allowed to imbibe water on a double-layer of paper towels moistened with distilled water for 3 hours and then separated. It was considered that during this period of imbibition no metabolic changes occurred. The excised cotyledons were incubated alone or one excised cotyledon was incubated with three embryonic axes, in double-layers of paper towels moistened by various solutions (see results) in petri-dishes. Duplicate tissue samples were used, and the experiment was repeated. All the manipulations were carried out in sterile conditions. The cultures were maintained in a darkened room at 25±1°C.

1. Preparation and assay of crude proteolytic enzyme:

The proteinase activity was measured according to the method of Penner and Ashton (9, 10). A crude enzyme extract was prepared by homogenizing the cotyledons in cold 0.05 M Tris-maleic acid buffer (pH 7.0) with mortar and pestle and then homogenized with a glass homogenizer. One cotyledon pair was homogenated with 4 ml. of Tris-maleic acid buffer. The homogenate was filtrated through 4 layers of cheesecloth and the filtrate then centrifuged at 20,000 xg for 30 min. The sediment and upper fat layers were discarded and the supernatant fraction

was used for the assay of proteinase activity. During the preparation of the crude enzyme, the temperature was maintained at 2–3°C.

After an equilibration period of 10 min. in a 35°C waterbath shaker, 2 ml. of crude enzyme extracts were added to a 25 ml. Erlenmeyer flask which contained 4 ml. of 2% casein solution previously adjusted to pH 7.0 and 6 ml. of 0.05 M Tris-maleic acid buffer (pH 7.0). The reaction mixture was incubated in a waterbath shaker for 3 hours. At the beginning and at the end of the incubation period, 3 ml. of the aliquot was used by immediately adding 3 ml. of 15% Trichloroacetic acid. The solutions were mixed, allowed to stand at room temperature for 30 min., and then centrifuged to precipitate the protein. The proteolytic enzyme activity was measured by the determination of the release of ninhydrin positive material using the methods described by Moore and Stein[13]. One milliliter of the supernatant solution was mixed with 1 ml. ninhydrin reagent, heated in boiling water bath for 15 min., cooled and then 15 ml. of 50% ethanol was added. After shaking for 30 seconds, 1 ml of mixture was diluted with 9 ml. of distilled water. The optical density at 570 mμ was measured by Spectronic 20 (Shimadzu Co.). The proteolytic activity was also measured by the release of UV (280 mμ) absorbing materials in a Beckman Du Spectrophotometer. One milliliter of the supernatant was diluted with 19 ml. of distilled water. The results obtained from this measurement agreed with the data obtained by measuring the optical density at 570 mμ. The increase in optical density of the supernatant fraction from the beginning to the end of the 3-hour incubation was indicative of the proteolytic activity.

2. Extraction and quantitative analysis of protein:

Ten pairs of cotyledons were homogenated with 50 ml. of cold Tris-HCI buffer (0.05 M; pH 7.4) in a mortar and pestle, and then homogenized in a glass homogenizer. The homogenate was centrifuged at 1,100 xg for 10 min. to precipitate the cell debris. The supernatant was precipitated with an equal volume of 10% Trichloroacetic acid and the pellet was centrifuged at 2,000 xg for 10 min. The pellet was dissolved in 0.1 N NaOH and an analysis was carried out using the Folin-phenol method as described by Lowry et al.[14] A standard curve was established using bovine serum albumin.

3. Extraction and quantitaive analysis of ribonucleic acids:

RNA analysis was done by the Key and Shannon procedure[15]. The aliquots were made to 0.2 N with respect to Perchloric acid (PCA), thoroughly mixed, and centrifuged at 1,100 xg for 10 min.. The pellets were then suspended and twice washed in 0.2 N PCA. All washed pellets were washed twice at 37°C for 30 min. in 2:2:1 mixture of ethanol-ether-chloroform to remove lipids. RNA was hydrolyzed in 0.3 M KOH for 18 hours at 37°C. After chilling, PCA was added to a final concentration of about 0.3 N, then followed by centrifugation, to remove the PCA precipitate, protein and DNA. 3 ml. of the RNA hydrolysate was removed and added to 6 ml.

of the orcinol acid reagent and 0.4 ml. of 6% alcohol orcinol in each tube, and heated in a boiling water bath for 20 min, the tube was then cooled and the optical density was determined at 660 mμ.

4. Purification of the crude proteinase and chromatography on DEAE-cellulose column:

Salt fractionation of the crude proteinase preparation was accomplished with a saturated solution of $(NH_4)_2SO_4$. The saturated solution was added drop-wise to the enzymatic preparation, which was constantly stirred at 0°C for 10 min.. The protein fraction was precipitated by 30–40% saturated ammonium sulfate. The mixture was then centrifuged for 10 min. at 34,800 xg at 0°C. The pellet was resuspended in 0.05 M Tris-maleic acid buffer (pH 7.0) and assayed for proteolytic activity. Following salt fractionation, the low molecular weight materials were dialysed in Tris-maleic acid buffer (0.05 M, PH 7.0) for overnight at 0°C.

DEAE-cellulose with 0.8 meg exchange groups per grams was prepared according to the usual procedures[16] for the separation of the partially purified proteins on a DEAE-cellulose column. The proteins were eluted with a linear gradient of NaCl from 0 to 1.2 M in a 0.05 M Tris-maleic acid buffer, pH 7.0. The eluates from the DEAE-cellulose column were collected in 3.5 ml. by automatic fractionational collector as a fraction. The protein concentrations of the eluates were determined spectrophotometrically by reading directly at 280 mμ.

RESULTS

1. The effect of the embryonic axis on proteinase activity in cotyledons during germination:

The time-course of the proteinase activity is shown in Fig. 1. There was an increase in the proteinase activity of intact cotyledons during the first 4 days of germination, and followed by a decrease on the fifth day. On the seventh day, the proteinase activity of cotyledon decreased to a level approximately equal to the initial value. The proteinase activity of the excised cotyledens, as in the intact cotyledon, increased through the fourth day and then declined. But the proteinase activity of excised cotyledons only had 79% of that measured from the intact cotyledons on the fourth day of germination. The embryonic axis of 4–day seedlings contained only 13–19% of the proteinase activity of the whole seedling, whereas the cotyledons contained 81–89%.

2. The effect of auxins, gibberellic acid and protein synthesis inhibitors on the proteinase activity of excised cotyledons:

When excised cotyledons were placed in a culture solution containing 1×10^{-6} M IAA or 1×10^{-5} M gibberellic acid, alone or in combination, these had no obvious effect on the level of proteinase activity. Cycloheximide and 2, 4–dinitrophenol somewhat reduced the proteinase activity of excised cotyledons (Table 1).

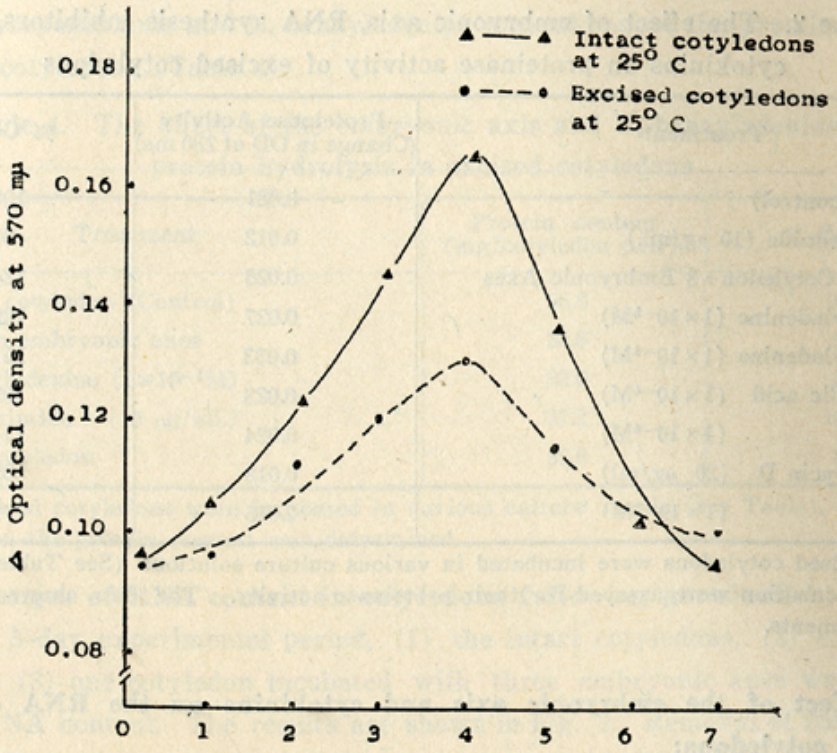

Fig. 1. The change of proteolytic activity in soybean cotyledons following germinaton.

Table 1. The effect of various plant hormones and protein synthesis inhibitors on the proteinase activity of excised cotyledons.

Treatment		Proteinase Activity (Change in optical density)	
		280 mμ	570 mμ
Water (Control)		100	100
Gibberellic acid	(1×10^{-5}M)	110	106
IAA	(1×10^{-6}M)	107	103
GA (1×10^{-5} M)+IAA	(1×10^{-6}M)	102	106
Cycloheximide	(5 μg/ml.)	77	81
2, 4-dinitrophenol	(5×10^{-4}M)	82	88
Intact cotyledons		—	131

The excised cotyledons were incubated in various culture solutions (see table). After 4 days of incubation, they were assayed for their proteinase activity. The results are presented as the percentage of proteinase activity of the excised cotyledons cultured in water. All culture solutions contained 30 μg/ml. streptomycin sulfate.

3. The effect of the embryonic axis, RNA or protein synthesis inhibitors, and cytokinins on proteinase activity of excised cotyledons:

The embryonic axis may stimulate the proteinase activity of excised cotyledons. When one excised cotyledon was incubated together with three embryonic axes, the proteinase activity of excised cotyledons increased about 23.8% (Table 2).

Table 2. The effect of embryonic axis, RNA synthesis inhibitors, and cytokinins on proteinase activity of excised cotyledons

Treatment	Proteinase Activity (Change in OD at 280 mμ)	% Control
Water (control)	0.021	100
Cycloheximide (10 μg/ml.)	0.012	57
Excised Cotyledon+3 Embryonic Axes	0.026	124
N_6-Benzyladenine (1×10^{-5}M)	0.027	129
N^6-Benzyladenine (1×10^{-4}M)	0.033	157
Gibberellic acid (1×10^{-4}M)	0.023	109
Kinetin (1×10^{-4}M)	0.024	114
Actinomycin D (20 μg/ml)	0.018	85.7
5-FU (1×10^{-3}M)	0.015	71 4

The excised cotyledons were incubated in various culture solutions (See Table), and after 4 days of incubation were assayed for their proteinase activity. The data shows the mean of three expreiments.

4. The effect of the embryonic axis and cytokinins on the RNA contents of excised cotyledons:

To evaluate the effect of the embryonic axis and N_6-benzyladenine on ribonucleic acid metabolism in excised cotyledons, excised cotyledons were incubated with various hormones or three embryonis axes and then the RNA content was determined. The results from these experiments are summerized in Table 3. When one excised cotyledon was incubated with three embryonic axes, there was a slight enhancement in the RNA synthesis in the excised cotyledons. The presence of kinetin, and N_6-benzyladenine enhanced the RNA synthesis in excised cotyledons.

Table 3. The effect of cytokinins on the RNA content of excised cotyledons

Treatment	RNA content (mg/cotyledon pair)	% Control
Water (control)	0.988	100
Gibberellic acid (1×10^{-6}M)	0.997	101
Kinetin (1×10^{-4}M)	1.177	119
N^6-Benzyladenine (1×10^{-5}M)	1.195	121
N_6-Benzyladenine (1×10^{-4}M)	1.299	131
+ Three embryonic axes	1.029	104

The excised cotyledons were incubated in various culture media (see Table), after 4 days of incubation the RNA content was determined. All culture media contained 30 μg/ml. streptomycin sulfate.

5. The effect of the embryonic axis and cytokinins on protein hydrolysis in excised cotyledons:

The embryonic axis and N_6-benzyladenine may stimulate the protein hydrolysis in excised cotyledone (Table 4).

Table 4. The effect of the embryonic axis and N_6-benzyladenine on protein hydrolysis in excised cotyledons

Treatment	Protein content (mg/cotyledon pair)	% Control
Excised cotyledon (Control)	36.5	100
+ Three embryonic axes	34.6	94.5
N_6-benzyladenine (1×10^{-4}M)	32.6	89.4
Cycloheximide (10 μg/ml.)	37.2	101.7
Intact cotyledon	35.8	98.0

The excised cotyledons were incubated in various culture media (see Table), after 4 days of incubation the protein content was determined.

6. The changes of RNA content in cotyledons following germination:

Over a 5-day experimental period, (1) the intact cotyledons, (2) excised cotyledons, and (3) one cotyledon incubated with three embryonic axes were analyzed for their RNA content. The results are shown in Fig. 2. Removal of the embryonic axis from the cotyledon before germination reduces the RNA content of the coty-

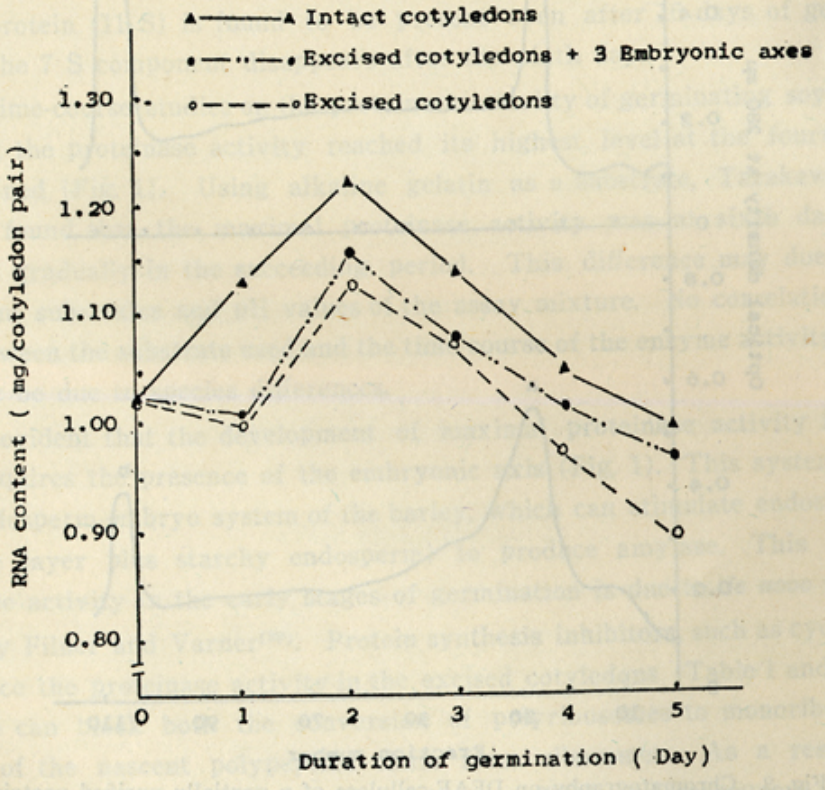

Fig. 2. The change of RNA content in cotyledons following gremination.

ledons. In the presence of three embryonic axes, there was only partial recovery of the RNA content in excised cotyledons. This shows that the hormone-like stimulus secreted by the embryonic axis may diffuse into the cotyledons where it may cause stimuation.

7. Purification of the crude Proteinase and chromatography on DEAE-cellulose:

The eluates from the DEAE-cellulose column chromatography were collected and the absorption at 280 mμ was determined using Beckman Du Spectrophotometer. There are two absorption peaks in tubes 22 to 26 and 104 to 106 which were isolated from intact cotyledons. The excised cotyledon showed two absorption peaks the same as the intact cotyledons, but there was a smaller peak at peak b. The experimental results show that proteinase in the cotyledons may contain more than one component and the embryonic axis may stimulate the synthesis of the b component of proteinase (Fig. 3).

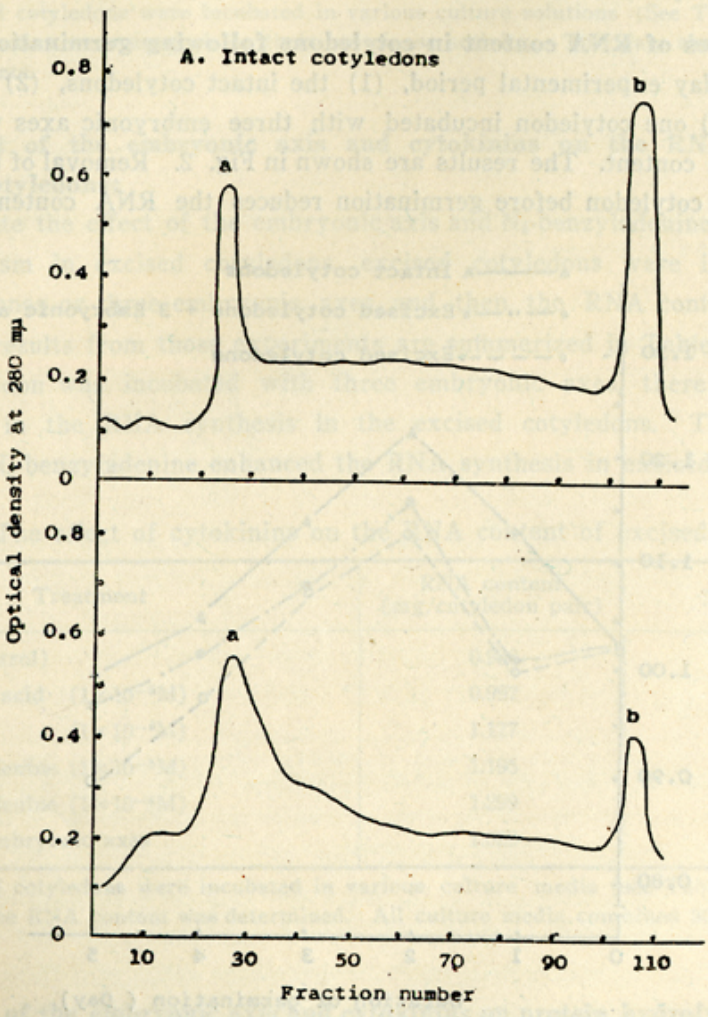

Fig. 3. Chromatography on DEAE-cellulose of a partially purified proteinase preparation from the intact and excised cotyledons germinated in water.

DISCUSSION

Germination is characterized by a rapid uptake of water which activates the metabolic system and faciliates the mobilization and the utilization of reserve food for embryo growth. Although this initial uptake of water is a dominant factor in the induction of germination, its mode of entry, and the sequence of events initiated by its entry, are difficult to define[3,17-20]. Recently the work of Marcus and Feeley[21-23] indicates that the entire apparatus necessary for protein synthesis is functional in the cotyledons of ungerminated peanut seeds, but it may be due to the spatial separation of m-RNA from the ribosomes to form the functional polyribosomes and to synthesize now proteins. They conclude that the formation or activation of polyribosome during imbibition may be a general phenomenon in seed germination.

The reserve proteins in soybean seeds appear to be located in the cotyledon in subcellular entites designated as protein bodies. Tombs found that the only protein, glycinin (11 S component) which is the major soybean protein, could be detected in protein bodies. Morphological changes of protein bodies in soybeans during germination have also been observed by the use of the electronmicroscope[24]. However, Catsimpoolas et al.[25] reported that at least six distinct components were found to be present in the proteins of the isolated soybean protein bodies. These components are metabolized at different rates during germination. The major soybean protein (11 S) is found to be present even after 16 days of germination, whereas the 7 S component disappears after the ninth day.

The time-course studies on the proteinase activiity of germinating soybean seeds show that the proteinase activity reached its highest level at the fourth day and then declined (Fig. 1). Using alkaline gelatin as a substrate, Tazakawa and Hirokawa[12] found that the maximal proteinase activity was at sixth day and then decreased gradually in the succeeding period. This difference may due to the use of different substrates and pH values of the assay mixture. No correlation has been found between the substrate used and the time-course of the enzyme activity[11-12,26-29]. This may be due to species differences.

It is evident that the development of maximal proteinase activity in the cotyledons requires the presence of the embryonic axis (Fig. 1). This system is similar to the endosperm-embryo system of the barley, which can stimulate endosperm tissue (aleurone layer plus starchy endosperm) to produce amylase. This increase in proteinase activity in the early stages of germination is due to *de novo* synthesis as shown by Filner and Varner[30]. Protein synthesis inhibitors, such as cycloheximide, can reduce the proteinase activity in the excised cotyledons (Table 1 and 2). Cycloheximide can block both the conversion of polyribosomes to monoribosomes and the loss of the nascent polypeptide chain from ribosomes. As a result, couples with a failure of attachment of ribosomes to m-RNA and subsequent initiation of

new peptide chains[31]. 2, 4–dinitrophenol at 10^{-3} M may inhibit soybean seed germination[32]. This may be due to the fact that DNP uncouples oxidative phosphorlation[33]. In the imbibition phase of seed germination, ribosome activation and polyribosome formation requires ATP[23].

It is likely that the embryonic axis may secrete a hormone which stimulates proteinase synthesis in cotyledons. If the axis is absent, the presence of cytokinins in the culture solution enables the cotyledons to increase the proteinase activity to the maximum level (Table 2). A possible explanation for this observation is that the embryonic axis may produce a cytokinin at a level optimal for the development of proteinase activity during the early stages of germination. The cytokinin then is translocated to the cotyledons where it exerts its influence.

Osborne[34] suggested that the primary action of kinetin might be the regulation of synthesis of a particular RNA fraction. Further evidence suggests that kinetin may effect the synthesis of ribosomal and soluble RNA[35–36]. However, Cherry showed that benzyladenine effected all fractions of nucleic acid similarly as judged by the separation on MAK column[37]. He also showed that above the concentration of 2×10^{-3} M benzyladenine inhibited the RNA synthesis. It is suggested that benzyladenine may affect RNA syuthesis at the nucleotide precussor level[37]. At the concentration of 1×10^{-5} M and 1×10^{-4} M benzyladenine may enhance the RNA content in the excised cotyledons (Table 3).

Since one excised cotyledon incubated with three embryonic axes can evoke the stimulation of ribonucleic acid synthesis, and furthermore since the presence of a cytokinin also stimulates the ribonucleic acid synthesis in excised cotyledons (Table 3). These results indicate that the synthesis of proteinase in the cotyledon of the soybean seedling is hormonally controlled via the embryonic axis and that the hormone involved appears to be a cytokinin.

A proteinase from the cotyledons of a 4–day old seedling and excised cotyledons was partially purified and separated on DEAE-cellulose column. From these experimental results it is shown that the embryonic axis may stimulate the *de novo* synthesis of proteinase in the peak b component (Fig. 3) and that this component may be a major enzyme component to hydrolyze the reserve proteins during seed germination.

SUMMARY

The purpose of this experiment was primarily to evaluate the effect of the embryonic axis on the reserve protein breakdown and the relative amount of the proteinase activity occurring at the progressive stages of germination, and secondarily to see what kind of plant hormones are released from the embryonic axis which can induce the proteinase activity in cotyledons.

The time-course studies of the proteinase activity in intact cotyledons showed

the proteinase activity increased rapidly over the first 4 days of germination period, reached the maximum on the 4 th day, and then decreased. Removal of the embryonic axis from the cotyledons before germination reduced the proteinase activity in the cotyledons. The embryonic axis of 4-day old seedling contained only 13–19% of the proteinase activity of the whole seedling, whereas the cotyledons contained 81–87%.

The addition of gibberellic acid, IAA or a mixture of GA and IAA to the culture solution failed to restore the loss in proteinase activity caused by the removal of the embryonic axis. In the presence of protein synthesis inhibitors, there was a reduction of proteinase activity.

When one excised cotyledon was incubated with three embryonic axes there was a stimulation of proteinase activity, RNA synthesis and reserve protein breakdown in the excised cotyledon. Cytokinins may replace the embryonic axis and stimulate the proteinase activity and RNA synthesis. It appears that at least a part of the proteinase enzyme in the cotyledons of germinating soybean seeds is a newly synthesized protein and that the synthesis is controlled by a stimulus originating in the axis part of the embryo.

It is evident that the soybean cotyledon contains more than one proteinase emzyme. The experimental results show that the embryonic axis may affect the *de novo* synthesis of the peak b component in cotyledons.

A possible explanation for these results is that the synthesis of a proteinase in the cotyledons of soybean seedlings is controlled by the embryonic axis and that the hormone involved appears to be a cytokinins. It is suggested that benzyladenine may affect RNA synthesis at the nucleotide precussor level.

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