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Effects of Gibberellic Acid on Enzyme Formation in Cotyledons of Germinating Mung Beans

Kunlayanee Sriboonchuay and Amnuay Jondeung

ABSTRACT

The influences of exogenously supplied gibberellic acid on amylase, catalase and carboxypeptidase B activities in cotyledons of germinating mung beans were studied.

Gibberellic acid caused qualitative and quantitative changes in α-amylase production. Cotyledons of seed germinated in the presence of GA_3 developed more amylase activity than those germinated on water did. The present results indicated that GA_3 induced two different forms [1and 2] of α-amylase. The requirement of GA_3 for the synthesis of α-amylase was also discussed.

In contrast to amylase, catalase and carboxypeptidase B decreased during the time course of germination. No significant development of catalase and carboxypeptidase B activity was found in GA_3- treated cotyledons.

Key words : Gibberellic acid, α-amylase, Catalase, Carboxypeptidase B, Germination

INTRODUCTION

The effects of gibberellic acid on enzyme production have been studied extensively in barley. Gibberellic acid enhanced the synthesis of four α-amylases, ribonuclease [Chrispeels and Varner, 1967 a,b; Jacobsen *et al.*, 1970], carboxypeptidase [Schroeder and Burger, 1987] and of phosphatase in barley aleurone layers [Jones and Carbonell,1984]. Gibberellic acid is continuously required during the period of enzyme formation.

In legume seeds, the influence of gibberellic acid on enzyme development is confusing. Locker and Ilan [1975] found that gibberellic acid has influence on amylase activity in detached cotyledons of pea, but could not bring about full replacement of embryonic axis, which could be accomplished by zeatin or combination of zeatin riboside and gibberellic acid. In contrast, Hirasawa [1989] demonstrated that GA_3 had a little effect, whereas auxin had a significant promotive effect on the formation of α-amylase activity in detached pea cotyledons. The effects of gibberellic acid on other enzyme activities have also been reported. The gibberellic acid [GA_3 showed no significant enhancement of protease formation in pea cotyledons excised from day 3 to 4 seedlings [Yomo and Varner, 1973]. Furthermore, Mitsuhashi *et al.* [1984] also reported that GA_3 exogenously supplied to detached mung bean cotyledons showed no significant effect on developmental patterns of endopeptidase and carboxypeptidase activities. In watermelon seeds, GA_3 promoted germination and accelerated the development of catalase activity in whole embryos and invertase in embryonic axes.

In the present study the effects of exogenously supplied gibberellic acid on the development of amylase, catalase and carboxypeptidase B activities in cotyledons of mung bean during germination were re-examined. It was found that gibberellic acid enhanced the development of α-amylase activity but not that of catalase and carboxypeptidase B activities.

1. Dept. of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

MATERIAL AND METHODS

PLANT MATERIAL AND ENZYME PREPARATION

Mung bean [*Vigna radiata* [L] Wilczek cv. Kampangsaen II] seeds were sterilized for 15 min with 1% [W/V] sodium hypochlorite. After washing with distilled water, the seeds were imbibed in sterile distilled water for 2 h. After imbibition, the seeds were germinated in Petri dishes on moist sterile sand, containing 55 ml of 1000 ppm gibberellic acid or sterile distilled H_20 for control, and incubated at 25°C under illumination providing 16 h daylength for 7 days. The seedlings were watered with 10 ml of appropriate solutions at 2 days interval. The cotyledons were harvested at beginning of germination and every 24 h and stored at 0°C in refrigerator until use.

Cotyledons [1 gm] were homogenized in 10 ml extract buffer [30 mM ethylene-diamine dihydrochloride, pH 4.2, 3 mM $CaCl_2$, 3mM β-mercaptoethanol, 20% v/v glycerol]. The homogenates were centrifuged at 20,000 rpm for 30 min at 4°C. The supernatants were stored at 0°C until use for enzyme and protein assays and for analysis by electrophoresis.

α-AMYLASE ASSAY : The activity was measured according to Rick and Steghauer [1974]. The 0.05 ml extract of cotyledons was incubated with 1 ml of 1% soluble starch dissolved in 20 mM phosphate buffer, pH 6.9, for 10 min at 25°C. The reducing sugar was determined by addition of 3,5 dinitrosalicylic acid. The α-amylase activity was expressed in terms of milligrams of maltose liberated in 1 min by 1 mg of protein in solution.

CATALASE ASSAY : The catalase activity of extract of cotyledons was assayed by UV spectrophotometric method [Aebi, 1974]. The extract and H_20_2 in 50 mM phosphate buffer [pH 7.0] were incubated at 25°C for 1 min. The rate of decrease in abso-rbancy at 240 nm of the mixture solution was measured. The enzyme activity was expressed in terms of units per mg protein. One unit of activity was defined as the amount of enzyme which decomposes 1μ mole H_20_2 per minute at 25°C.

CARBOXYPEPTIDASE B ASSAY : The extract and hippuryl-L-arginine in tris buffer [pH 7.6] were incubated at 37°C for 1-2 min. The release of hippuric acid resulted in an increase of absorbancy at 254 nm. The volume activity was calculated as described by Appel [1974] and converted in terms of activities per mg of protein.

PROTEIN ASSAY : Protein concentration of the extract was determined by the method of Lowry *et al.*[1951] with BSA as a standard protein.

ELECTROPHORESIS : Agar gel electrophoresis was carried out according to the procedures described by Jacobsen *et al.* [1970] except that the agar gel [1%] also contained 0.5% starch. Amylase solutions were adjusted to about 100μg per ml for electrophoresis. The electrophoresis was run with stabilized current of 10 ma for 90 min at 4°C.

α-AMYLASE : For visualization of the amylase, the starch-iodine procedure described by Vallejos [1983] was used. Briefly, the gel was incubated in 50 mM Na-acetate buffer [pH 5.6] containing $CaCl_2$ at 37°C for 1 h and then tne gel was flooded with I_2-KI solution for about 1 min. The zone of enzyme activity appeared translucent in a solid blue background.

CATALASE : Starch-iodine system according to Vallejos [1983] was also used for visualizing catalase. The gel was incubated in solution containing thiosulfate and H_20_2 at room temperature for 30 sec and then the gel was immersed in acidified KI-solution. The zones of catalase activity was marked by achromatic areas in the gel.

CHEMICALS : Gibberellic acid [GA_3] and hippuryl-L-arginine were purchased from Sigma Chemical Co..

RESULTS

Mung bean seeds were germinated in sterilized sand containing H_20 or 1000 ppm gibberellic acid solution for 7 days. The activities of α-amylase, catalase, and carboxypeptidase B in cotyledons of germinated seedlings were measured daily [Table 1].

α-AMYLASE : α-amylase activity in cotyledons of control germinated seedling increased slowly during the first 2 days, after that turned to rapidly increase and reached a maximum on the 5th day and then decreased slightly on the 7th day. In contrast, the activity in gibberellic acid-treated cotyledons reached the maximum value on the 1st day, about five times as great as that of the activity at the beginning of germi-

nation, leveled off rapidly. The activity started to increase on the 3rd day and reached a maxinum value on the 6th day and turned to the near level as found in the control seedling [Figure. 1].

CATALASE : Catalase activity in cotyledons of both control and gibberellic acid-treated seedling decreased rapidly about 5 fold of initial activity after 24 h of imbibition. The activity in the control cotyledons showed 2 peaks on the 2nd and 5th day of germination and decreased to low level less than 27 folds of that at the beginning of the experiment. Gibberellic acid exerted a little effect on the catalase activity because the activity reduced about 10 fold of initial activity on 2nd day of treatment and increased slightly after that and reaced a small peak on the 5th day and then declined to the same level as that of the activity of control cotyledons [Figure.2].

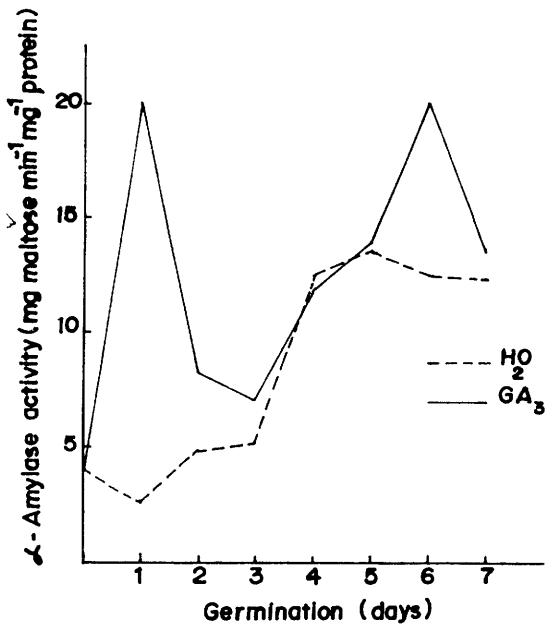


Figure 1 α-amylase activities in mung bean cotyledons treated with H_20 and GA_3 during germination.

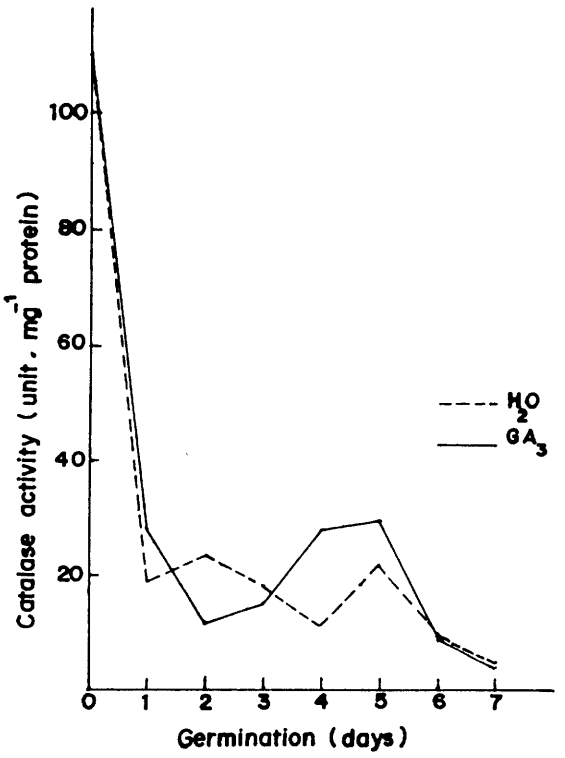


Figure 2 Changes in catalase activity in germinating mung bean cotyledons, with H_20 and GA_3.

Table 1 Changes in protein contents and activities of α-amylase, catalase, and carboxypeptidase B in extracts from cotyledons of germinated mung bean seeds.

Germination Time (day)	α-amylase (mg maltose min^{-1} mg^{-1} Protein)		Catalase unit.mg^{-1} Protein		Carboxypeptidase B unit.mg^{-1} Protein		Protein content (mg/ml)	
	H_2O	GA_3	H_2O	GA_3	H_2O	GA_3	H_2O	GA_3
0	4.000	4.000	109.940	109.940	43.30	43.30	0.010	0.010
1	2.667	20.000	18.600	27.705	31.10	43.30	0.015	0.010
2	4.750	8.333	22.904	11.571	19.40	18.75	0.024	0.024
3	5.161	7.027	17.815	14.875	16.10	15.31	0.031	0.037
4	12.400	11.795	11.173	27.746	10.00	15.81	0.050	0.039
5	13.460	13.928	21.388	29.653	11.80	12.795	0.052	0.056
6	12.410	20.000	9.653	9.311	11.78	13.33	0.058	0.060
7	12.330	13.538	4.513	4.214	9.72	9.23	0.060	0.065

CARBOXYPEPTIDASE B : Carboxypeptidase B activity was assayed by using hippuryl-L-arginine as substrate. Fig.3 shows the changes in the levels of carboxypeptidase activity in cotyledons of control and gibberellic-acid treated seedlings. Carboxypeptidase activities in both cases decreased to one half of initial activity after two days of germination. In control cotyledons, the carboxypeptidase activity continued to decrease until day 4 and remained steady until day 7 whereas the activity in gibberellic - acid treated cotyledons leveled off until day 5 and then decreased to the low level less than 3 times of that at the beginning on day 7.

Agar gel electrophoresis exhibited qualitative and quantitative differences in the α-amylase in cotyledons of seedlings treated with H_20 or GA_3. In cotyledons of seedlings treated with H_20, the amylase had at leact 2 bands [amylase 3 and 4] [Figure 4 a]. Amylase 3 showed low activity on the 4th day, increased in intensity on the 5th day. The second band [amylase 4] appeared with high intensity on the last

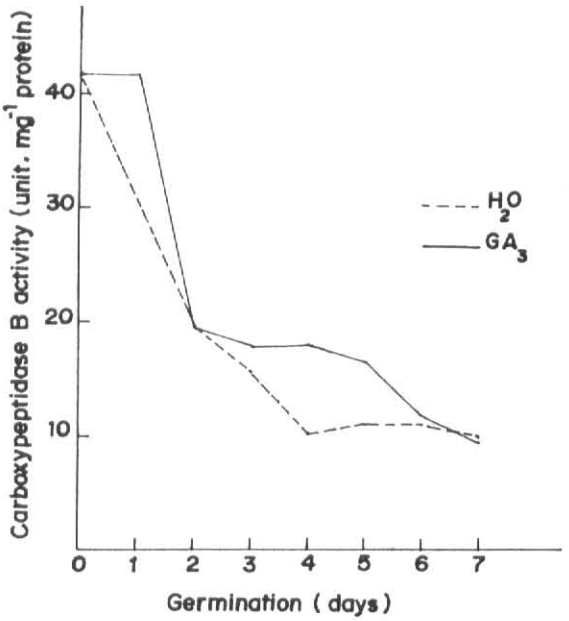
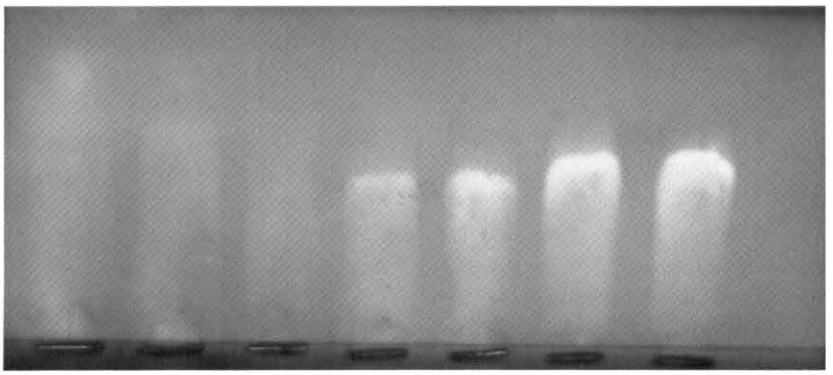
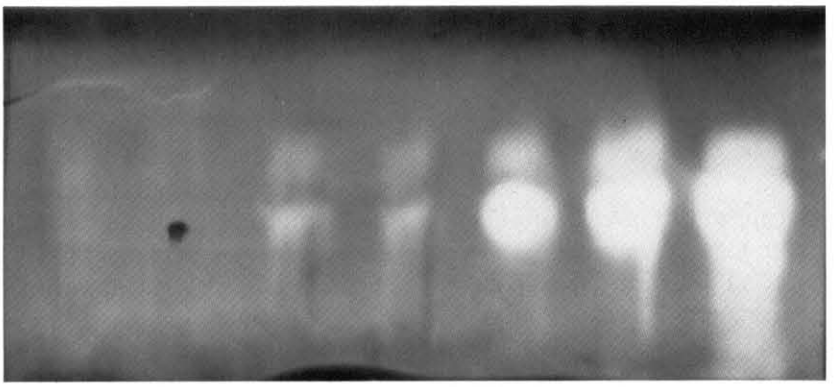


Figure 3 Development of carboxypeptidase B in germinating mung bean cotyledons, with H_20 and GA_3.



Germination (days)



Germination (days)

Figue. 4 Zymograms of α-amylases developed in mung bean cotyledons [a] with H_20 and [b] with GA_3 during germination.

two days. In contrast, the zymogram pattern of α-amylase in GA_3-treated cotyledons showed a maximum of 4 bands. The intensities [activities] of amylase 3 and 4 increased from the 3rd to 7th day of experiment. Amylase isozyme 1 and 2 were found only in cotyledons which have been treated with GA_3. The results showed that GA_3 exerts its effect on both quantitative and qualitative distributions of α-amylase isozymes in mung bean cotyledons.

In case of catalase, it was not found that there are different isozymes in cotyledons. The intensity of a catalase band was very low, therefore, it was not shown here.

DISCUSSION

The results show different developmental patterns of α-amylase in cotyledons of H_2O-and GA_3-treated seedlings. In H_2O-imbibed cotyledons, α-amylase activity during the first 3 days remained low similar to that of pea cotyledons, in which the amylase was low during the first 4 days of germination [Juliano and Varner, 1969]. In contrast, GA_3showed a significant enhancement of α-amylase formation at 1st and 6th day of experiment. The effects of gibberellic acid on α-amylase production were intensively studied in barley aleurone layers [Chrispeels and Varner, 1967 a,b; Jacobsen *et al.* 1970;Jones and Carbonell, 1984] and in pea cotyledons [Locker and Ilan, 1975]. Chrispeels and Varner [1976 a,b] found that α-amylase synthesis is inhibited by inhibitors of protein synthesis and RNA molecules. However, the increase in amylase content and synthesis have to be accompanied by the level of translatable mRNA, which increased markedly in cotyledons of mung bean during the beginning of imbibition [Morohashi *et al.* 1989]. Thus the possible explanation for α-amylase formation is that GA_3 accelerates enzyme synthesis from translatable preexisting mRNA and enhance the synthesis of specific α-amylase mRNA, which would be expressed as α-amylase enzymes at later stages of germination.

Agar gel electrophoresis revealed qualitative and quantitative differences in the α-amylase isozymes in cotyledons of H_2O-and GA_3 treated seedlings. The amylase isozyme 3 and 4 occurred separately at different stages of germination whereas these 2 isoenzymes were produced simultaneously in the presence of GA_3. Moreover, GA_3 caused the production of isozyme 1 and 2. The exogenous GA_3 also increased the amounts of 4 α-amylase bands [Fig. 4b]. By SDS-PAGE analysis of *in vitro* translation products of RNA extracted from *Vigna mungo* cotyledons, Tomura and Koshiba[1985] reported that α-amylase is synthesized as a polypeptide precursor [mol wt of 45 KD] and is later processed to a native form [mol wt 43 KD]. Therefore, we cannot exclude the possibility that amylase 3 might be precursor of amylase 4 and, the maturation processes would be accelerated in the presence of GA_3. Therefore, we found these 2 enzymes on the same days of germiation. Furthermore, the present experiments indicate that GA_3 induced two different forms [1 and 2] of α-amylase separable by agar gel electrophoresis. The GA_3 triggered synthesis of new amylases in cotyledons has not been reported, however, Jacobsen *et al.* [1970] found 4 α-amylases in isolated barley aleurone tissue induced by GA_3

Catalase activities were high at the beginning and decreased more than 5-times during 2 days of germination. After that catalase activity in GA_3-treated cotyledons increased slightly between 3th to 5th days. The GA_3 enhanced development of catalase activities during germination were reported in cotyledons of light-incubated watermelon seeds [Evensen and Loy, 1978] and in rice seedling [Goyal and Baijal, 1980]. Evensen and Loy [1978] mentioned that catalase is a marker enzyme involved in lipid degradation. Since GA_3 had little noticeable effect on catalase activity, it is conceivable that lipid degradation is not a major catabolic processes during germination of mung bean seeds.

As shown in this study, carboxypeptidase B decreased sharply at the beginning and then declined slightly between day 2 to day 7. The results did not agree with previous reports. The increase of carboxypeptidase activities during germination have been reported in cotyledons of mung bean [Chrispeels and Boulter, 1975; Mitsuhashi *et al.* 1984] and in barley half-kernels [Schroeder and Burger, 1978]. From inhibition experiment of enzymatic activity by PMSF, Chrispeels and Boulter [1975] also suggested that there are different carboxypeptidases involved in proteolytic activities during mung bean germination. Thus, the carboxypeptidase B is likely to play an important role in degradation of storage protein only at the beginning of germination.

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