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Protein Breakdown and Formation of Protease in Attached and Detached Cotyledons of *Phaseolus vulgaris* L.[1, 2]

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HARUGORO YOMO AND KOMALA SRINIVASAN
Kitchawan Research Laboratory of the Brooklyn Botanic Garden, Ossining, New York 10562

ABSTRACT

In contrast to earlier reported results of similar experiments in peas, in which almost no increase in protease activity occurred in incubated detached cotyledons, we report here an increase in protease activity in both attached and detached bean cotyledons. Detached bean cotyledons showed continually increasing protease activity up to the 12th day, while that in attached cotyledons declined after 6 days. The free amino acid level in detached cotyledons reached a maximum at the 11th day; protease formation leveled off after 50% of the original seed protein was digested. These data suggest that high free amino acid levels may inhibit protease formation.

The activity of partially purified protease in aqueous extracts was enhanced by 10 mM 2-mercaptoethanol or cysteine, indicating a sulfhydryl requirement for activation. Protease formation in detached cotyledons was inhibited 30% by 10 μg/ml cycloheximide and 50% by 100 μM abscisic acid. In contrast, α-amylase formation was inhibited 90% by 10 μg/ml cycloheximide and 95% by 20 μM abscisic acid. The cycloheximide data suggest that only a part of the protease, but all of the α-amylase, is synthesized *de novo*; the similar pattern of inhibition by abscisic acid emphasizes the concept that protease may exist in two forms.

Rapid liberation of low molecular weight nitrogenous compounds at the expense of stored proteins at the early stage of bean germination was reported by Pusztai and Duncan (7). They also reported in that paper a time course of protease activity which reached maximum on the 8th day of germination. Seeschaaf and Pirson (8) reported that in half cotyledons of blue lupine, protein breakdown proceeded, and the amounts of protease and several other enzymes, including 6-phosphogluconic acid dehydrogenase, increased during incubation of detached cotyledons more than those in attached cotyledons did during germination. They found that the increase occurred only in the absence of the buds; removal of roots or hypocotyl had no effect. Addition of IAA to incubated cotyledons inhibited the increase of 6-phosphogluconic acid dehydrogenase activity. They concluded that auxin from the bud controls enzyme levels in the cotyledons. However, there are no data to show whether auxin has a similar effect on digestive enzymes. α-Amylase activity was also reported to increase during both germination and incubation of axis-free half cotyledons of beans by Dale (1) and by Gepstain and Ilan (2). Dale suggested that gibberellin is involved in α-amylase formation, while Gepstain and Ilan observed an enhancing effect of kinetin. Yomo has reported that formation of α-amylase in detached bean cotyledons was inhibited by ABA during incubation (10). Yomo and Varner (11) found that in detached cotyledons (*a*) α-amylase activity was also inhibited by ABA, and (*b*) protein breakdown and protease formation were very slight compared with attached cotyledons. We report here on the protein breakdown and the formation of protease in comparison with that of α-amylase, during incubation of excised bean cotyledons, in relation to effects of cycloheximide and ABA.

MATERIALS AND METHODS

General Procedure. Bean (*Phaseolus vulgaris* L. cv. Kentucky Wonder) seeds were sterilized in 1% aqueous NaOCl (Allied Chemical) solution for 10 min and imbibed at 22 C for 16 hr. Imbibed seeds were germinated on moist vermiculite in the dark. Cotyledons from germinated seedlings are referred to as attached cotyledons. For incubation experiments, the imbibed seeds were cut transversely in half and the axis-free half cotyledons (detached cotyledons) were sterilized for 1 min in 0.2% NaOCl solution which had been neutralized with 2 N HCl just before use. They were then put in a 125-ml Erlenmeyer flask containing 10 ml of 2% agar solution which contained water or test chemicals, and incubated at 22 C in the dark.

Preparation of Enzyme Solution. Cotyledons of two germinated seeds (attached cotyledons) or eight excised half cotyledons (detached cotyledons; their volume is the same as that of two seeds) were homogenized by mortar and pestle with 6 ml of water or 2 mM $CaCl_2$ solution (for α-amylase assay), and centrifuged at about 1200*g* for 15 min. The supernatant was used as an enzyme solution (enzyme solution A). For testing the effects of sulfhydryl compounds on protease activity, 5 ml of this supernatant were passed through a column (25 cm × 10.5 mm i.d.) containing 15 ml of coarse Sephadex G-50, which was equilibrated and eluted with 10 mM citric acid buffer, pH 5.5. After the first 5 ml of the elutant were discarded, the following 7.5 ml were used as an enzyme source (enzyme solution B).

Protease Assay. The method reported previously (11) was used to measure protease activity. Protease activity was expressed as μg released amino acid nitrogen/seed·2.5 hr. Protease activity measured by our method showed an optimum at pH 5.5 and decreased as pH was increased. In experiments using sulfhydryl compounds, the increase in A_{280} was measured because sulfhydryl compounds caused strong color formation in the ninhydrin reaction. Protease activity was expressed in

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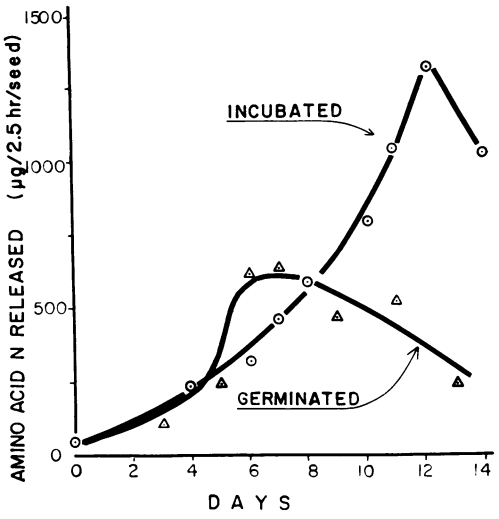

FIG. 1. Change in activity of bean protease in extracts of cotyledons from germinated seeds or of excised half cotyledons incubated on agar containing water during 14 day period.

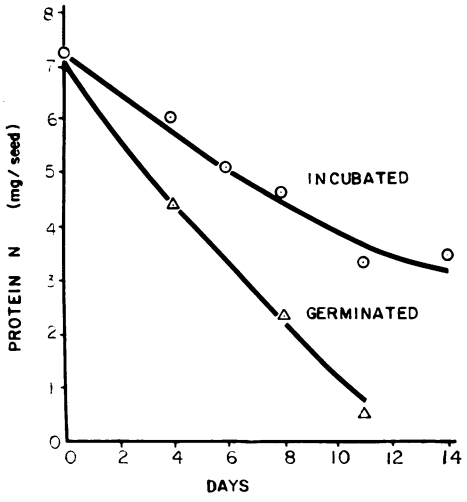

FIG. 2. Changes of protein nitrogen level in cotyledons from germinated seeds or in incubated half cotyledons.

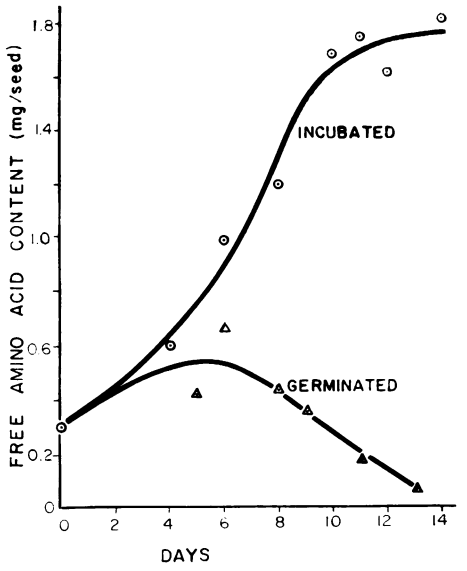

FIG. 3. Changes of amino acid level in cotyledons from germinated seeds or in incubated half cotyledons.

this case as μg released tyrosine equivalent N/seed·2.5 hr. Protease activity as measured by the increase in A_{280} (PA_{280}) was closely related to the activity as measured by the ninhydrin method (9) (PA_N), within the range of 1200 μg released amino acid N/seed·2.5 hr for PA_N and 500 μg released tyrosine equivalent N/seed·2.5 hr for PA_{280}.

α-Amylase Assay. The activity was measured according to the procedure of Jones and Varner (4), except that soluble starch (Mallinckrodt Chemical Works, Detroit) was used in a concentration of 0.1% as a substrate, and the iodine concentration was increased to 0.036%. The activity was expressed as decrease in A_{620} times the dilution factor.

Protein Nitrogen. Cotyledons after germination or incubation were dried at 80 C for 24 hr, and the 80% alcohol insoluble nitrogen was determined by the Kjeldahl method (5).

Free amino acid nitrogen of the cotyledons was measured by the ninhydrin method (9) on a supernatant obtained after centrifuge of a mixture of 1 ml of an enzyme solution A and 0.5 ml of 15% trichloroacetic acid.

RESULTS

Protease activity, which was observed in slight amount in dry seeds, increased slowly at the beginning of germination of intact seeds, turned to rapid increase and reached a maximum on the 6th or the 7th day, while the activity of detached cotyledons steadily increased until the 12th day of incubation, to an amount about twice as great as that of the maximum activity of attached cotyledons and then leveled off (Fig. 1).

α-Amylase activity of cotyledons of germinated seedlings gave the same kind of time course. During germination, the activity started to increase on the 4th day and reached the maximum value of 16 on the 8th day, while during incubation the activity steadily increased until the 10th day, reaching a value of 60, more than three times that of the maximum activity of the germinated seedlings.[3]

To confirm protein breakdown in the cotyledons during germination and incubation, changes in protein nitrogen and free amino acid nitrogen were measured (Figs. 2 and 3). Protein nitrogen in the cotyledons decreased rapidly during germination but more slowly during incubation. The protein nitrogen content during incubation remains constant (at about 50% that of dry seeds) after the 11th day, which coincides with the fact that free amino acid nitrogen in these cotyledons increased until the 11th day and leveled off.

Some properties of protease were examined by assaying after mixing the enzyme solutions (A or B) with solutions of several chemicals. Protease was not inhibited by 1 mM EDTA but was significantly inhibited by 1 mM p-chloromercuribenzoate. Ten mM 2-mercaptoethanol and L-cysteine each enhanced its activity (Table I). Thus bean protease was found to belong to the so-called sulfhydryl-dependent proteases which is common in plant tissue (as reported, for example, by Pike and Briggs in *Avena* [6]).

To study the nature of protease formation in the cotyledons, excised cotyledons were incubated on agar containing cycloheximide or ABA, respectively for 96 hr or for 8 days, and the effects on formation of protease and α-amylase were compared (Table II and Fig. 4). Ten μg/ml cycloheximide inhibited α-amylase formation by 90% but protease formation by only 30%. Fifty μM ABA inhibited α-amylase formation by

[3] Similar results were obtained by H. Yomo with Dr. J. E. Varner in Michigan State University using *Phaseolus vulgaris* L. cv. resistant Asgrow Valentine and have been briefly reported (10).

95%,[3] but protease formation by only 50%; even 100 μM ABA inhibited the latter only to the same extent.

In contrast, neither gibberellic acid (1–10 μM) nor kinetin (10–100 μM), when added to the incubation medium, affected protease formation during incubation of detached cotyledons.

DISCUSSION

In detached bean cotyledons, the loss of protein nitrogen coincided with an increase in protease activity for 11 days of incubation, after which both leveled off. Only half the protein was broken down.

In earlier studies (3, 11), detached pea cotyledons were found not to develop protease activity upon incubation; protease formation in attached cotyledons was inhibited by the presence of free amino acids; therefore it was suggested that the free amino acid level regulates the protease formation in peas (11). One possible explanation of the present results, therefore, is that in beans also the free amino acid level eventually inhibits protease formation; beans appear less sensitive than peas, however. A similar increase in protease activity and in protein breakdown in detached cotyledons compared with attached cotyledons was found in blue lupine by Seeschaaf and Pirson (8).

α-Amylase activity in excised bean cotyledons showed a similar time course as did that in excised pea cotyledons (11), in that the activity during germination reached a maximum at the 10th (pea) or 8th (bean) day, while that during incubation steadily increased until the 12th (pea) or 10th (bean) day, reaching more than three times higher activity than the maximum obtained during germination.

Table I. *Effects of Several Substances on Bean Protease Activity*

Test substances were added to an enzyme solution (A or B) prepared from excised cotyledons incubated for 12 days, 10 min before the protease assay started. Descriptions of solutions A and B and of methods are in the text.

	Final Concn	Protease Activity	Enzyme Solution	Method
	mM	*% control*		
None		100	A	Ninhydrin
$MnSO_4$	10	98	A	Ninhydrin
EDTA	1	96	A	Ninhydrin
PCMB	1	27	A	Ninhydrin
None		100	B	A_{280}
2-Mercaptoethanol	10	272	B	A_{280}
L-Cysteine	10	252	B	A_{280}

Table II. *Effect of Cycloheximide on Enzyme Formation in Bean Cotyledons*

After 3 days of incubation on plain agar, excised half cotyledons were transferred to an agar solution containing water or cycloheximide and incubated for an additional 96 hr. Enzyme activity was measured before and after the 96-hr incubation and the increase expressed as per cent of control.

Concn of Cycloheximide	Increase of Enzyme Activity	
	α-Amylase	Protease
μg/ml	*% control*	
0	100	100
5	50	93
10	8	71

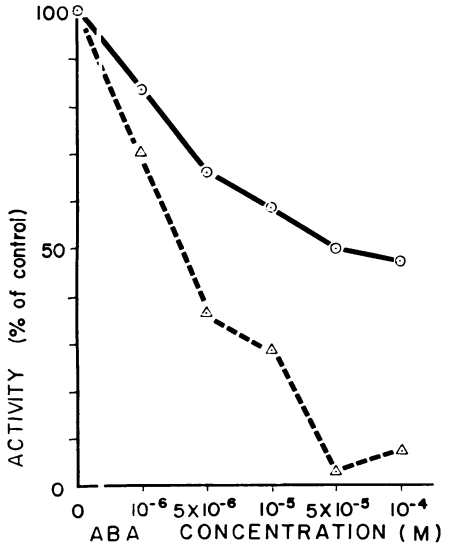

FIG. 4. Inhibition of accumulation of bean cotyledon enzymes by ABA. ○: protease; △: α-amylase. ABA was applied at the beginning of incubation of 8 days.

The facts that (*a*) α-amylase formation in detached bean cotyledons is almost completely inhibited, by a low concentration of cycloheximide, while (*b*) protease formation is inhibited only about 30% (Table II), indicate that cycloheximide works not as a general toxicant, but as a specific inhibitor against enzyme formation under the conditions used in our experiments. Therefore, we conclude that all the α-amylase activity and a part of the protease activity result from enzyme synthesis after imbibition.

In the same way, we can explain the effects of ABA on formation of these enzymes (Fig. 4) and conclude that ABA acid is not a general poison to the plant tissue under our experimental conditions and that α-amylase formation and a part of protease formation can be specifically inhibited by ABA.

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