Protein Mobilization and Proteolytic Enzyme Activities during Seed Germination of Broad Bean (*Vicia faba* L.)

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The protein mobilization from attached and detached seeds of *Vicia faba* L. cv. Eresen 87 (Fabaceae) was investigated. While the total soluble protein content decreased, the free amino acid content increased during the 7 days germination period. Among the three proteolytic enzymes, only endopeptidase activity was found to be affected by the removal of the embryonic axis. Leucine aminopeptidase activity was high at the beginning, then it decreased; carboxypeptidase activity reached the highest value at day 5. In order to examine the effects of plant growth regulators on detached cotyledons incubated with plant growth regulators [10^{-4} M benzyladenine (BA), gibberellic acid (GA_3), indole acetic acid (IAA) and 10^{-5} M abscisic acid (ABA)], only benzyladenine was found promotive on protein mobilization. Our results suggest that the removal of the embryonic axis in seeds of *Vicia faba* L. cv. Eresen 87 decreases protein mobilization and endopeptidase activity.

Key words: Vicia faba L., Protein Mobilization, Proteolytic Activity

Introduction

Research on the role of the embryonic axis on protein mobilization still continues and the modes of enzymatic breakdown of storage proteins during legume seed germination are not yet clearly understood. Two hypotheses have been proposed concerning the axial control of this process. First, the growing axis may act as a sink, which draws off the products of reserve mobilization, and its excision leads to an accumulation of proteolytic end products (Chin *et al.*, 1972; Kern and Chrispeels, 1978; Davies and Chapman, 1979; Mitsuhashi *et al.*, 1984). Second, the growing axis may produce plant growth substances, which stimulate the synthesis of hydrolytic enzymes for reserve mobilization in the cotyledons. The effects of plant growth regulators on protein mobilization could be specific to species and also to cultural variety. Gibberellins or cytokinins are thought to regulate this process in dicots (Allen *et al.,* 1984; Munoz *et al.,* 1990; Nandi *et al.,* 1995; Yoshida and Hirasawa, 1997). In a similar manner gibberellins, arising from the embryo, influence reserve mobilization especially in the endosperm of cereals (Jacobsen and Varner, 1967; Yomo and Varner, 1973).

The regulation of protein hydrolysis in *Vicia faba* cotyledons has been studied in some detail. Lichtenfeld *et al.* (1979) investigated the protein degradation in *Vicia faba* L. var. Minor cotyledons biochemically and by electron microscopy and found that total nitrogen apparently did not change until the 4th day, whereas protein content decreased slightly and the total soluble nitrogen reached the first peak point on the 4th day. Briarty *et al.* (1970) investigated the changes in protein bodies both by electron microscopy and biochemically and defined the germination process to proceed in four stages. Hussin and Briarty (1987) investigated the protease activity of attached and detached cotyledons of *Vicia faba* L. var. Aquadulce and showed changes in the activity depending on the pH value. Yu and Greenwood (1994), purified and characterized a cysteine proteinase from *Vicia faba* L. var. Major cv. 30A Toto cotyledons.

We have examined the protein mobilization during germination of *Vicia faba* L. cv. Eresen 87 seeds in detail. The effect of axis on protein mobilization should be determined. We measured the activities of three proteolytic enzymes, endopeptidase (EP), carboxypeptidase (CP), and leucine aminopeptidase (LAP), and total soluble protein and free amino acid contents of attached and detached cotyledons. To examine the effect of growth regulators on protein mobilization gibberellic acid (GA_3), indole acetic acid (IAA), benzyladenine (BA) and abscisic acid (ABA) were used.

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Material and Methods

Plant material

Vicia faba L. cv. Eresen 87 seeds were used. Seeds were purchased from Turkish Agricultural Ministry Aegean Agricultural Research Institute, Menemen, Izmir, Turkey. Seeds were incubated as attached and detached cotyledons. Some cotyledons were detached from the embryonic axis before incubation. Attached and detached cotyledons were incubated with sterile distilled water for 0 – 1 – 3 – 5 – and 7 days. To determine whether growth regulators can replace the embryonic axis, detached cotyledons were incubated with 10^{-4} M BA, GA_3, IAA and 10^{-5} M ABA for 3 and 5 days. Seeds were surface-sterilized by NaOCI (2%) to prevent contamination. Three seeds or six cotyledons were placed on two sheets of sterilized filter paper in petri dishes. The intact seeds and the detached cotyledons were incubated at 23 °C under sterile conditions in the darkness. At the end of the incubation period, seeds were washed with distilled water and kept at −70 °C until use.

Enzyme assays

Homogenization was carried out at 0 to +4 °C. Seeds were homogenized in a chilled mortar and Ultra Turrax homogenizer with cold 50 mM Tris-HCl [tris-hydroxymethyl)aminomethane-hydrochloride], pH 7.4, containing 10 mM 2ME (2-mercapto ethanol) buffer. The homogenate was passed through four layers of cheesecloth and centrifuged at +4 °C at $10000 \times g$ for 20 min. The supernatant was collected and a part was dialysed against 50 mM sodium acetate buffer, pH 5.4, containing 10 mM 2ME for 24 h. Prepared supernatants were placed in Eppendorf tubes and kept in −70 °C until subsequent analysis. Freezed supernatants were melted in a 40 °C water bath when used for analysis.

LAP activity was measured according to Chrispeels and Boulter (1975). L-Leucine 4-nitroanilide was used as substrate for the LAP activity assay. 2 ml of 2 mM substrate were incubated with 0.1 ml enzyme at 37 °C for 20 min. The reaction was stopped by addition of 1 ml 1 N $HClO_4$ at the end of the incubation. The precipitate was removed and optical density measured at 410 nm. Controls for each sample were similarly treated but without incubation. Enzyme activity was defined as released μmoles *p*-nitroanilide into reaction medium per cotyledon under the experimental conditions.

EP activity was assayed using 1% (w/v) azocasein in 75 mM sodium acetate buffer, pH 5.4, containing 5 mM 2ME as substrate (Mitsuhashi *et al.,* 1984). The reaction mixture containing 0.15 ml of substrate and 0.15 ml of enzyme was incubated for 2.5 h at 37 °C in a water bath. The reaction was stopped by addition of 0.7 ml of 5% (w/v) TCA. Controls for each sample were similarly treated but without incubation. After standing 30 min in ice, precipitates were removed by centrifuging at $10000 \times g$ for 25 min and the TCA-soluble reaction products were measured at 366 nm. Enzyme activity was defined as the amount of enzyme required to cause an increase of 1.0 in absorbancy under the assay conditions and expressed as units/h.

CP activity was measured according to Chrispeels and Boulter (1975). N-CBZ-Phe-Ala at 2 mM concentration in 25 mM citrate phosphate buffer, pH 5.0, containing 0.5 mM EDTA was used as substrate. The reaction mixture contained 1 ml of substrate and 0.05 ml of dialyzed enzyme solution incubated at 37 °C in a water bath for 1 h. The reaction was stopped by additon of 0.5 ml of 20% (w/v) TCA. After standing in ice for 30 min, the reaction mixture was centrifuged at $300 \times g$ for 15 min. Free amino acids in supernatant were determined by the ninhydrin method (Yemm and Cocking, 1955). Leucine was used as standard for the ninhydrin reaction. Enzyme activity was expressed as μmoles of amino acid released/cotyledon under the assay conditions.

Total soluble protein and free amino acid contents

Total soluble protein content of homogenates was determined according to Bradford (1976) with Coomasie Brilliant Blue using bovine serum albumin as standard. Results were expressed as mg/cotyledon.

Statistical analyses

The differences among the days regarding the investigated parameters (total soluble protein, free amino acid contents, endopeptidase, carboxypeptidase, leucine aminopeptidase activities) of attached and detached cotyledons were tested by one-way ANOVA. Tukey's HSD test was used to determine the differences among the days. All statistical analyses were based on a significance level of 0.05 (Zar, 1984). Also the relationship among the investigated parameters was tested.

Results

Changes in total soluble protein and free amino acid contents

Total soluble protein content decreased during the germination in both attached and detached cotyledons. Total soluble protein content in attached cotyledons was (104.8 ± 14.2) mg/cotyledon at the beginning of the germination, then it decreased to (31.8 ± 14.2) mg/cotyledon at the end of the 7th day (Fig. 1A). The protein content reached a peak point at the 3rd day, and then decreased at the 7th day in attached cotyledons. In detached ones, the protein content increased at the days 1 and 3, then decreased at the days 5 and 7. The highest total soluble protein content was at the 3rd day with BA incubated detached cotyledons. Similar results were found for the 5th day of BA incubated detached cotyledons.

Total free amino acid content increased during the germination in attached cotyledons, whereas it increased at the 3rd day, then decreased in detached ones (Fig. 1B). Incubation with plant hormones did not significantly affect the free amino acid content on the 3rd day (Table I). The free amino acid content of GA_3 and BA incubated cotyledons was significantly higher than controls.

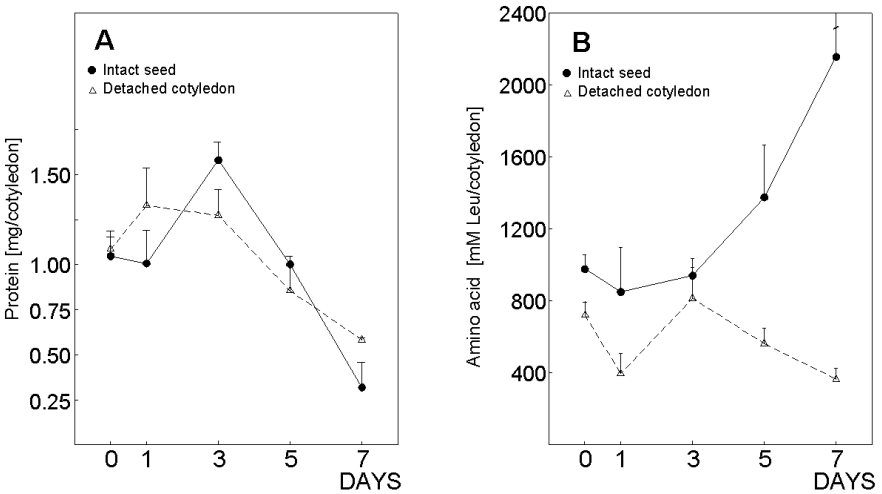

Fig. 1. Changes in protein (A) and amino acid contents (B) of intact seeds and detached cotyledons during a 7 days germination period of *Vicia faba* L. cv. Eresen 87.

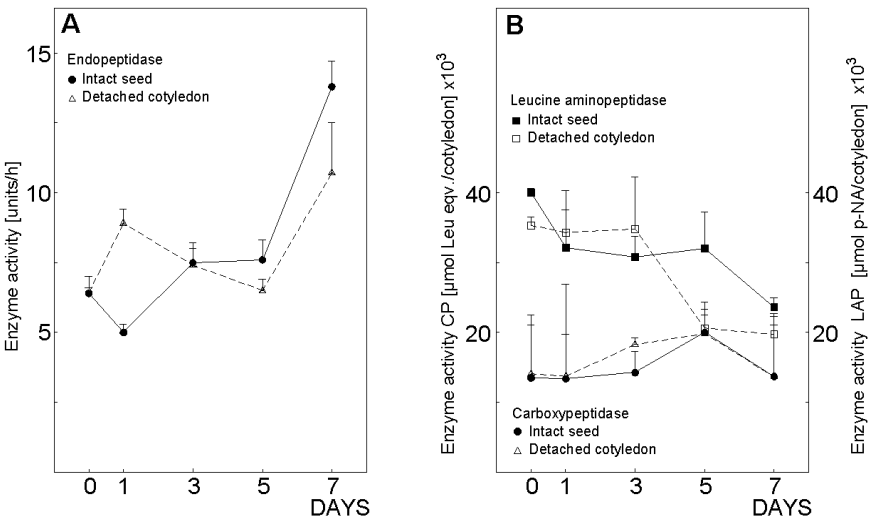

Fig. 2. Changes in endopeptidase (A), leucine aminopeptidase (LAP) and carboxypeptidase (CP) activities (B) of intact seeds and detached cotylledons during a 7 days germination period period of *Vicia faba* L. cv. Eresen 87.

Table I. Changes in protein, amino acid and proteolytic activity of 3 and 5 days with plant growth regulators incubated detached cotyledons (GA_3, BA, IAA 10^{-4} M; ABA 10^{-5}M; distilled water as control) of *Vicia faba* L. cv. Eresen 87 ($P < 0.05$, significant; $P > 0.05$, not significant; ± standard deviation).

Assay series	Protein [mg/cot.]	Amino acid [mM Leu/cotyledon]	Endopeptidase [units/h]	Leucine aminopeptidase [μM p-NA/ cotyledon] x10^3	Carboxypeptidase [μM Leu eqv./ cotyledon] x10^3
Day 3					
Control	127.4^{ab} ± 14.3	813.3^{a} ± 220.3	7.4^{a} ± 0.8	34.8^{a} ± 7.3	16.5^{a} ± 0.2
GA_3	66.0^{a} ± 12.2	595.0^{a} ± 37.7	5.3^{ab} ± 1.1	32.3^{a} ± 7.0	11.0^{ab} ± 6.1
ABA	175.0^{a} ± 25.9	695.0^{a} ± 116.5	6.6^{ab} ± 0.7	26.4^{a} ± 2.8	6.7^{b} ± 0.4
BA	439.6^{b} ± 84.7	811.7^{a} ± 188.2	5.5^{ab} ± 0.3	39.3^{a} ± 4.9	10.2^{ab} ± 2.6
IAA	351.3^{bc} ± 28.9	700.0^{a} ± 86.6	4.7^{b} ± 0.8	26.7^{a} ± 1.6	4.2^{b} ± 0.6
α: 0.05	$P < 0.05$	$P > 0.05$	$P < 0.05$	$P > 0.05$	$P < 0.05$
Day 5					
Control	85.5^{b} ± 13.8	561.3^{b} ± 84.5	6.4^{ab} ± 0.4	20.6^{b} ± 2.7	19.6^{a} ± 5.3
GA_3	74.3^{b} ± 6.7	845.0^{a} ± 74.6	6.6^{a} ± 0.4	25.9^{b} ± 1.5	16.8^{a} ± 4.7
ABA	125.3^{b} ± 24.5	683.3^{ab} ± 65.1	7.7^{a} ± 0.5	22.8^{b} ± 1.8	6.3^{b} ± 1.2
BA	379.0^{a} ± 43.9	891.6^{a} ± 101.0	6.1^{ab} ± 1.0	36.3^{a} ± 3.7	6.4^{b} ± 0.6
IAA	377.0^{a} ± 12.2	683.3^{ab} ± 76.4	4.8^{b} ± 0.5	19.8^{b} ± 0.8	7.4^{b} ± 0.6
α: 0.05	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

EP, LAP and CP activities

The differences of EP activities among the days were found significant in both attached and detached cotyledons ($P < 0.05$). EP activity increased during the germination in attached cotyledons (Fig. 2A). None of the hormone treatments could reach to control at the 3rd day in detached cotyledons. At the 5th day of the germination only ABA was found to stimulate the EP activity (Table I).

LAP activity was high at the beginning of germination but it decreased during the germination with both attached and detached cotyledons (Fig. 2B). BA increased the LAP activity in both 3 and 5 days incubated detached cotyledons (Table I). The inhibition effect of ABA was not observed, or the used dose was not effective enough to inhibit the enzyme activity.

CP activity increased significantly at the 5th day, and then decreased in both attached and detached cotyledons (Fig. 2B). None of the growth regulators reached the CP activity of control in both 3 and 5 days incubations (Table I).

Discussion

The free amino acid contents increase during germination because of the combined activities of proteinolytic enzymes (Müntz, 1996). The highest free amino acid content was found on day 7 at which the EP activity reached the highest value in attached cotyledons (Fig. 2B). Negative significant relationship between total soluble protein and free amino acid content was found ($P < 0.05$; $r = -0.691$). But the free amino acid content of detached cotyledons did not increase regularly during the incubation (Fig. 1B). The relationship between free amino acid content and total soluble protein was not significant ($P < 0.05$; $r = 0.350$).

The CP activity increased until the 5th day, and then decreased in both attached and detached cotyledons. CP activity was found to be not affected by the removal of the embryonic axis (Fig. 2B). Despite this, in *Vigna mungo* cotyledons (Mitsuhashi *et al.,* 1984), CP activity was affected by the removal of the embryonic axis. It was reported in *Arachis hypogea* L. cv. Virginia 56-R seeds (Mikola, 1976) that CP has not an important role on protein mobilization.

Among the hormone treatments only BA is found to have a stimulatory effect on LAP activity. BA did increase the LAP activity and total soluble protein content at the days 3 and 5, and also the free amino acid content at the day 5. GA_3 and IAA application did not stimulate the protein mobilization. ABA was not effective with the used

dose (10^{-5}M). It was reported that in *Lupinus luteus* (Nandi *et al.*, 1995), BA was more effective than kinetin (KIN) and stimulated the protein mobilization, in *Cicer arietinum* L. (Munoz *et al.,* 1990) zeatin has a promotive effect on protein mobilization, zeatin was promotive and ABA and KIN were not effective on protein mobilization in *Pisum sativum* L. (Malek, 1987). According to the literature the effects of plant growth regulators could be specific to species and also to cultivar.

In the present study it was determined that when the embryonic axis was removed from the seeds of *Vicia faba* L. cv. Eresen 87, protein mobilization continued but at a slower rate. While total soluble protein decreased, free amino acid contents increased during the germination of intact seeds. Among the three proteolytic enzymes examined, only EP was found to be affected by the removal of the axis. The highest LAP activity was at the beginning of germination, and then it decreased as the germination proceeds. The highest CP activity was at the 5th day, then it decreased. In order to determine whether the growth regulators can replace the embryonic axis the detached cotyledons were incubated with the growth regulators. Only benzyladenine was found to have a promotive effect on protein mobilization.

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