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Starch Degradation and Distribution of the Starch-Degrading Enzymes in *Vicia faba* Leaves[1]

Diurnal Oscillation of Amylolytic Activity and Starch Content in Chloroplasts

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Subcellular localization of the starch-degrading enzymes in *Vicia faba* leaves was achieved by an electrophoretic transfer method through a starch-containing gel (SCG) and enzyme activity measurements. Total amylolytic and phosphorolytic activities were found predominantly in the extrachloroplastic fraction, whereas the debranching enzymes showed homogenous distribution between stromal and extrachloroplastic fractions. Staining of end products in the SCG revealed two isoforms of α-amylase (EC 3.2.1.1) and very low β-amylase activity (EC 3.2.1.2) in the chloroplast preparation, whereas α- and β-amylase exhibited higher activities in the crude extract. However, it is unclear whether the low α- and β-amylase activities associated with the chloroplast are contamination or activities that are integrally associated with the chloroplast. Study of the diurnal fluctuation of the starch content and of the amylase activities under a 9-h/15-h photoperiod showed a 2-fold increase of the total amylolytic activity in the chloroplasts concurrent with the starch degradation in the dark. No fluctuation was detectable for the extrachloroplastic enzymes. The possible roles and function of the chloroplastic and extrachloroplastic hydrolytic enzymes are discussed.

In higher plants, transitory starch, the usual principal product of photosynthetic carbon assimilation, is formed inside the chloroplast, where it is deposited in the stroma before its mobilization during the night. The regulatory mechanisms involved in the starch degradation pathway are incompletely understood. In particular, the functions of most of the starch-degrading enzymes localized outside the chloroplast, away from the site of starch metabolism, have not yet been elucidated (Chapman et al., 1972; Okita et al., 1979; Okita and Preiss, 1980; Jacobsen et al., 1986; Kakefuda et al., 1986; Ziegler and Beck, 1986). Different degradative systems have been characterized in higher plant species and extensively studied in spinach and pea.

The amylolytic and phosphorolytic pathways are thought to be involved in starch degradation in chloroplasts (Okita et al., 1979; Steup and Latzko, 1979). The initial hydrolysis of native starch granules in spinach chloroplasts has been considered to be due to α-amylases (endoamylase 3.2.1.1) (Steup et al., 1983), but recently Sun and Henson (1990) reported that α-glucosidase is capable of initiating attack on native starch granules. Furthermore, these authors report that a dramatic synergism occurs between α-glucosidase and α-amylase activities in the hydrolysis of granular starch. Phosphorylase activity would degrade soluble glucans, which are formed by the preceding hydrolytic attack on starch. Even though spinach chloroplasts contain both α- and β-amylase activities, which could account for the hydrolytic degradation of starch, more than 80% of the total amylase activity present in spinach leaves is extrachloroplastic in origin (Okita et al., 1979). Less than 5% of the amylase and debranching enzyme activities are localized in the plastids. The chloroplastic and extrachloroplastic amylases differ partly in their substrate specificity (Okita and Preiss, 1980). The extrachloroplastic amylase in spinach leaves has been identified as an α-amylase (Okita and Preiss, 1980), whereas in *Vicia faba* (Chapman et al., 1972), pea (Ziegler, 1988), barley (Jacobsen et al., 1986), wheat (Ziegler and Beck, 1986), and *Arabidopsis thaliana* (Lin et al., 1988) most if not all of the β-amylase activity was associated with the extrachloroplastic fraction and may be localized in the vacuole (Ziegler and Beck, 1986). Spinach leaves, like pea leaves, contained one phosphorylase form, which appeared also to be located outside of the chloroplast (Steup and Latzko, 1979). This nonchloroplastic phosphorylase represented a considerable amount of the total phosphorolytic activity in the leaves. As a matter of fact, the specific activity in the leaf extract was more than 2-fold higher than in the chloroplast extract.

In contrast with spinach, pea chloroplasts appear to contain very low, or no, α-amylase activity (Levi and Preiss, 1978; Stitt et al., 1978; Kakefuda et al., 1986). Although Ziegler (1988) found an endoamylase activity in pea chloroplasts that had properties very similar to those of the cereal α-amylase, Beers and Duke (1988, 1990) reported equivocal evidence about the presence of a chloroplastic α-amylase. Amylopectine in pea chloroplasts has been reported to be degraded mainly via the phosphorylase reaction (Levi and Preiss, 1978). More recently Beers et al. (1990) found α-glucosidase in the chloroplasts of pea. Chloroplastic preparations have been shown to contain pronounced debranching enzyme activity in addition to endoamylases (Ziegler, 1988). The total

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Abbreviations: PEP, phospho*enol*pyruvate; SCG, starch-containing gel.

amylolytic activity localized in pea chloroplasts amounted to only 4 to 5% of that of the leaves.

The lack of information concerning the intracellular distribution and function of the starch-degrading enzymes in *V. faba* leaves prompted us to identify and localize these enzymes and to investigate their regulatory control process.

An electrophoretic transfer method has been used for identification and localization of these enzymes. We also attempted in this study to provide information that will resolve the enigma presented by the extrachloroplastic localization of the starch-degrading enzymes. The diurnal behavior of the intracellular amylase activities was investigated to determine if the extrachloroplastic amylase activities are directly related to starch mobilization and thus regulated by the same degradation-control process as in the chloroplast.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of *Vicia faba* L. cv Weisskernige (Samenhandlung Zwaan Pannevis, Kleve, München, Germany) were grown in a controlled environmental chamber under a 9-h/15-h light/dark cycle at 19°C and 80% humidity. The daily light had an energy flux of 680 μmol min^{-1} m^{-2} (400–700 nm) (HQS-Lamps, Osram, München, Germany). Fully expanded leaves were harvested 2 weeks after sowing and used for chloroplast isolation. Chloroplasts were isolated directly after harvesting.

Chloroplast Isolation

Chloroplasts of *V. faba* leaves were isolated following two procedures.

Procedure I

Leaves were homogenized for 30 s in a blender (Braun, Multimix) using 5 mL/g fresh weight of a chilled isolation medium containing 0.35 M sorbitol, 0.05 M Hepes-KOH (pH 8.3), 4 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 4 mM ascorbate, and 0.5% BSA. The homogenate was filtered through eight layers of cheesecloth and a 20-μm nylon net and centrifuged at 1300*g* for 4 min (Heraeus minifuge, GL). The pellet was gently resuspended in a chilled resuspension medium containing 0.365 M sorbitol, 35 mM Hepes-KOH (pH 8.3), 10 mM $MgCl_2$, 1 mM DTE and sedimented for 5 min at 2300*g*. The resulting pellet was washed three times under the same conditions. All steps of this procedure were carried out at 2 to 4°C. The chloroplast intactness was 60%, as measured by the level of oxygen production (Zimmermann et al., 1989).

Chloroplasts were ruptured after 40 s of sonification in 20 mM Hepes-KOH (pH 6.9) buffer with 3 mM DTE. Aliquots were taken to determine the Chl concentration according to Arnon (1949). The starch determination was carried out with chloroplasts isolated following this procedure.

Procedure II

This isolation procedure was adapted from a modified method of Kakefuda et al. (1986). Leaves of *V. faba* were homogenized as described above in the same isolation buffer containing 2 mM EDTA. After filtration, the resulting filtrate was centrifuged for 3 min at 1300*g*. Pelleted chloroplasts were then loaded on 20 mL of a 25 to 92% Percoll gradient and centrifuged for 7 min at 13,000*g* (L7–55 Ultracentrifuge, Beckman). The Percoll gradient was prepared in the isolation medium containing 0.6 mM GSH, 25 to 92% Percoll, 0.7 to 2.7% PEG 3350, 0.25 to 0.92% Ficoll, and 0.25 to 0.92% BSA. After centrifugation, the upper of the two bands that were generated, containing broken chloroplasts (clumped together), was discarded. The lower band, containing intact chloroplasts, was removed and washed two times by gentle resuspension in a washing medium containing 0.365 M sorbitol, 0.035 M Hepes-KOH (pH 8.3), 10 mM $MgCl_2$, 1 mM DTE and centrifuged for 5 min at 2500*g*. All steps of this procedure were carried out at 4°C. This isolation procedure yielded 85% intact chloroplasts. The Chl concentration was measured in the solution of chloroplasts ruptured as described above.

Enzyme Extraction

The chloroplastic starch-degrading enzymes were recovered in the supernatant after centrifugation at 20,000*g* for 20 min (4°C, L7–55 Ultracentrifuge, Beckman) of the suspension of ruptured chloroplasts. Soluble proteins in the supernatant were measured according to the method of Bradford (1976), using BSA as a standard.

The extrachloroplastic starch-degrading enzymes were prepared from crude homogenates obtained by homogenization of leaves from *V. faba* in a 20 mM Hepes-KOH (pH 6.9), 3 mM DTE buffer (Kakefuda et al., 1986). After filtration, the Chl concentration was measured and the filtrate was centrifuged for 20 min at 20,000*g* to remove insoluble membranes and other cellular debris. The protein concentration of the supernatant containing the extrachloroplastic enzymes was determined.

Enzyme Assays

Enzyme activities were assayed the day of extraction. Production of reducing sugar from starch (soluble potato starch, Sigma) was measured using the dinitrosalicylic acid reagent (Bernfeld, 1955) representing the total amylolytic activity. The reaction mixture contained, in a final volume of 1.2 mL, 41.6 mM succinate (pH 6.0) and 0.2% soluble starch. The reaction was initiated by adding 0.2 mL of the enzyme preparation and terminated after 30 min at 37°C by adding 0.2 mL of dinitrosalicylic solution prepared as described by Bernfeld (1955). Color was developed after 5 min of incubation in a boiling water bath and absorbance was read at 540 nm after cooling. Blank values (distilled water replacing starch) were subtracted from the experimental values. Maltose standards were used to construct a calibration curve.

Debranching enzyme activity was assayed with pullulan as substrate, as described above. Ziegler (1990) confirmed that debranching enzyme activity was specifically assayable with pullulan as substrate. One unit of activity is defined as the amount of enzyme required to produce 1 μmol of maltose

per min, and specific activity is expressed as units per mg of protein.

Starch phosphorylase was measured by coupling the production of glucose-1-P from soluble starch to phosphoglucomutase and glucose-6-P dehydrogenase (Steup and Latzko, 1979). The reaction mixture contained, in a final volume of 1 mL, 25 μmol of imidazole-HCl (pH 7.0), 15 μmol $MgCl_2$, 1 μmol of EDTA, 0.3 μmol of NADP, 4 nmol of glucose-1,6-bisP, 6 units of phosphoglucomutase, 2 units of glucose-6-P dehydrogenase, 45 μmol of Pi, 10 mg of soluble starch, and 0.2 mL of enzyme extract.

PEP carboxylase (EC 4.1.1.31) and NAD-malate dehydrogenase (EC 1.1.1.37) were assayed using methods similar to those described by Li et al. (1992). Final NAD-malate dehydrogenase assay concentrations were 50 mM Tris-HCl (pH 7.5), 0.2 mM NADH, and 0.5 mM oxaloacetic acid. Oxidation of NADH was monitored by measuring changes at A_{340}. PEP carboxylase was assayed in a solution containing 0.1 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$, 0.14 mM NADH, 2 mM $NaHCO_3$, 4 mM PEP, and 3 units of NAD-malate dehydrogenase in a total volume of 1 mL.

Starch Extraction and Determination

Starch was extracted from chloroplasts isolated as described in procedure I, using a modified method of Ahluwalia and Ellis (1984). The ruptured chloroplasts were centrifuged for 10 min at 3500*g* (Heraeus minifuge, GL). The pellet, containing starch, was resuspended in 50 mM perchloric acid and incubated in closed test tubes at 96°C for 3 min. Perchloric acid has been demonstrated to be the most efficient solvent for starch extraction from plant tissues, and over the proposed extraction time only 0.2% of starch was degraded by acid hydrolysis (Ahluwalia and Ellis, 1984). After cooling, the extract was centrifuged for 5 min at 12,000*g* (Hettich microrapid/K centrifuge) and the resulting pellet was washed again with cold 50 mM perchloric acid (1 mL). Exhaustive extraction of starch by the perchloric acid method was confirmed by negative iodine staining of the supernatant. The starch-containing supernatants were combined to give the extract for starch determination.

Prior to measurement, the starch extract was incubated with a protease solution (protease type XIV from *Streptomyces griseus*, Sigma) as described by Ahluwalia and Ellis (1984). Protein associated with starch granules can limit enzymic attack; preincubation of the extract for 30 min at 35°C with protease was found to increase the recovery of starch (Ahluwalia and Ellis, 1984). Following this incubation period, the digest was boiled for 50 s to inactivate the protease.

Aliquots (100 μL) from the protease digest were made to 1 mL with 0.2 M sodium acetate buffer, pH 4.8. The use of sodium acetate buffer enables direct and rapid measurement of starch from the acid extract without any further need to neutralize the perchloric acid. Amyloglucosidase (2.8 units) was added and the mixture was incubated at 55°C for 55 min. Appropriate blanks were included to correct for any free glucose not emanating from starch. Glucose released was estimated using hexokinase and glucose-6-P dehydrogenase.

Native and Transfer Electrophoresis

Detection and Identification of α-Amylase and Debranching Enzymes

Native electrophoresis was performed according to Davis (1964), using a 7% polyacrylamide gel. Reservoir and separation buffer consisted of 0.05 M Tris, pH 8.4, 0.38 M glycine, and 0.1% EDTA (w/v). Electrophoresis was conducted at 25 mA/gel for 1 h at about 4°C. Each sample was replicated on two gels so that after electrophoresis one gel could be used for protein staining and one for the electrophoretic transfer and activity staining. Transfer-electrophoresis was performed as described by Kakefuda and Duke (1984).

Detection and Identification of β-Amylase

β-Amylase was separated by acidic electrophoresis on a 15% gel as described by Maurer (1971). The running buffer consisted of 0.35 M β-alanine and 0.08% (v/v) acetic acid. The transfer was performed as described by Kakefuda and Duke (1984).

Amylolytic enzymes standards (α-amylase from *Bacillus* species, β-amylase from barley, pullulanase from *Enterobacter aerogenes*) were purchased from Sigma.

RESULTS

Enzyme Localization and Identification

To discern the localization of the starch-degrading enzymes in *V. faba* leaves, we compared chloroplast stromal preparations with crude extracts. We have used an electrophoretic transfer technique to detect and identify amylolytic activities in both preparations. Enzymes separated by PAGE were blotted through an SCG and stained with a KI/I_2 solution (Kakefuda et al., 1986). Staining of end products in the starch gel with iodine solution indicated the type of amylolytic enzyme activity associated with the bands. Debranching enzyme degraded starch to amylose, which is stained blue, β-amylase produced β-limit dextrins, which stained red, and α-amylase produced α-limit dextrins, which remained unstained. The chloroplast preparation produced two unstained bands (Fig. 1, lane B), whereas the crude extract revealed only one clear area (Fig. 1, lane A). These results indicated the presence of two isoforms of α-amylase in the chloroplasts and a more intense endoamylase activity in the crude preparation. This endoamylolytic activity showed the same electrophoretic migration mobility in both the crude and chloroplast preparations.

Intracellular marker enzymes were used to assess the degree of cytosolic contamination of purified chloroplasts. Activity of the cytosolic marker PEP carboxylase was not detected in the chloroplast preparation, whereas in the crude preparation this activity was 550 milliunits/mg of Chl. Recovery of the intracellular marker NAD-malate dehydrogenase represented approximately 10% of total activity recovered from the crude extract. Thus, the very low amount of α-amylase detected in the chloroplast preparation could be due to extrachloroplastic α-amylase contamination. How-

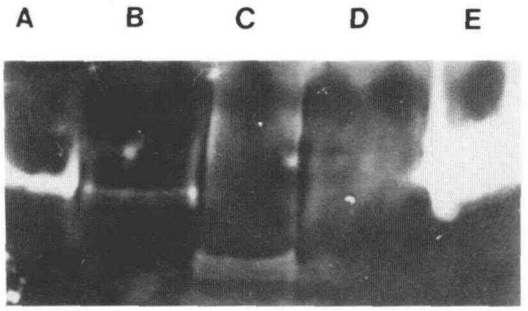

Figure 1. Detection of amylolytic enzymes in chloroplast and crude preparations from *V. faba* leaves by native electrophoresis (7% polyacrylamide gel) followed by electrophoretic transfer through an SCG. Chloroplasts were isolated following procedure I (see "Materials and Methods"). Lanes in the SCG are crude extract (A), chloroplast extract (B), *Enterobacter aerogenes* pullulanase (C), barley β-amylase (D), and *Bacillus* α-amylase. Lanes for transfer were loaded with 85 μg (A), 85 μg (B), 1.28 mg (28.2 milliunits) (C), 11.19 μg (795 milliunits) (D), and 3.36 μg (8.4 milliunits) (E). The identification of β-amylase was not possible in this system.

ever, it is unclear whether this activity is a contamination or α-amylase that is associated with the chloroplast. The blue-stained band produced by the bacterial pullulanase (Fig. 1, lane C) was not detectable in either extract.

The identification of β-amylase activity was not possible in this system, as shown by the absence of activity of the barley β-amylase used as standard (Fig. 1, lane D). This enzyme required an acidic separation on a 15% polyacrylamide gel prior to transfer. The iodine-stained SCG (Fig. 2) revealed a distinct red-pink band in the crude extract (lanes A and C), indicating a β-amylase activity. A very low amount of this β-amylase appears to be associated with chloroplasts (lane B); however, because 10% of the cytosolic marker NAD-malate dehydrogenase was measured in the chloroplast preparation, this activity could come from an extrachloroplastic β-amylase contamination. This localization is in agreement with the extrachloroplastic compartmentation of β-amylase found in *V. faba* leaves by Chapman et al. (1972). In both preparations, the migration of the extrachloroplastic activity was lower than that of the standard (barley β-amylase). The mol wt of the β-amylase purified from *V. faba* leaves (Chapman et al., 1972) has been estimated to be 107,000, whereas the mol wt of the barley enzyme is 90,000.

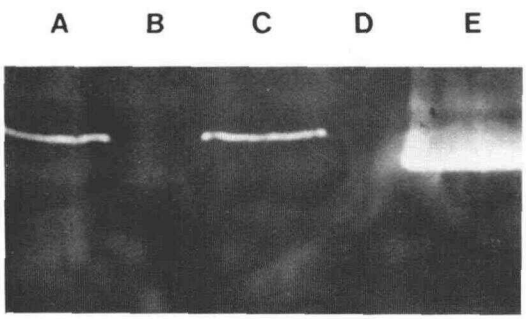

Figure 2. Detection of β-amylase in chloroplast and crude extracts from *V. faba* leaves by acidic PAGE (15% polyacrylamide gel) followed by electrophoretic transfer through an SCG. Chloroplasts were isolated as described in procedure I (see "Materials and Methods"). Lanes in the SCG are crude extract (lanes A and C), chloroplast extract (lanes B and D), and barley β-amylase (lane E). Lanes for transfer were loaded with 90 μg (A), 90 μg (B), 90 μg (C), 80 μg (D), and 104 μg (7.42 units) (E).

Distribution and Activities of the Starch-Degrading Enzymes in *V. faba* Leaves

The chloroplast isolation procedure was carried out at the beginning of the light period to avoid heavy starch accumulation, which would have prevented the isolation of intact plastids. Comparing the two isolation procedures (Table I), a 2-fold higher specific amylase activity was determinated in the stroma from Percoll-isolated chloroplasts, whereas this activity was 6-fold higher when expressed per mg of Chl. During the isolation according to procedure I, some chloroplasts are ruptured, releasing part of their amylase activity. Following procedure II, 85% of the chloroplasts were intact. Consequently, the chloroplastic starch-degrading enzyme activities were determinated in chloroplasts isolated following procedure II (see "Materials and Methods").

The crude extract exhibited an approximately 10-fold higher total amylase activity than chloroplasts (Table II), which confirmed the predominant extrachloroplastic localization of the hydrolytic enzymes. Only 9% of the total amylolytic activity measured in the leaves was located in the stroma. In contrast, no predominant localization of the debranching enzymes was found. An approximately equal activity was measured in the stroma and in the extrachloroplastic fractions, 25.19 and 22 milliunits/mg of protein, respectively. These low activities could be responsible for the lack of detection of debranching enzymes by transfer electrophoresis. Indeed, these activities indicate that debranching activity of 2.14 and 1.87 milliunits/mg of protein, respectively, were loaded on the gel, which are too low to be detected.

Whereas debranching enzymes showed a homogenous distribution between stroma and extrachloroplastic fractions, starch phosphorylase exhibited a primarily extrachloroplastic compartmentation, with a 14-fold higher activity than in the stroma. The phosphorylase activity measured in *V. faba* leaves (62 milliunits/mg of protein) was 5-fold higher than in spinach leaves (Lin et al., 1988), where both chloroplastic and extrachloroplastic phosphorylases have been found to differ in their substrate specificity (Steup et al., 1983).

Because the phosphorylase activity localized in the stroma accounted for 11% of the starch-degrading enzyme activity

Table I. *Total amylolytic activity in chloroplasts isolated from V. faba leaves*

Comparison of two chloroplast isolation procedures (I and II, see "Materials and Methods").

	Procedure I	Procedure II
Amylase activity (milliunits/mg of protein)	24.84	53.32
Amylase activity (milliunits/mg of Chl)	63.43	356

Table II. *Distribution of starch-degrading enzyme activities in V. faba leaves*
Chloroplasts were isolated following procedure II (see "Materials and Methods").

	Chloroplast	Extract	Crude	Extract
	milliunits/mg of protein	*milliunits/mg of Chl*	*milliunits/mg of protein*	*milliunits/mg of Chl*
Total amylolytic activity	53.32	368	576	4246
Pullulanase	25.19	158	22	180
Phosphorylase	10.35	62	107	883

measured against 60% for the amylolytic activity, we suggest that starch mobilization in *V. faba* chloroplasts is preferentially catalyzed through the amylolytic pathway.

Diurnal Behavior of the Amylase Activity and Starch Content

Illumination of darkened leaves led to an increase in the starch content (Fig. 3). During the first hours of light (from 8 to 13 h), a low starch accumulation was observed. Because photosynthesis-driven pH changes in the stroma during the night-day transition (pH 7.0–8.0) have been demonstrated to account for the regulation of the amylase activity (Pongratz and Beck, 1978; Okita and Preiss, 1980), it seemed that a remaining hydrolytic enzyme activity slowed the rate of starch synthesis until the pH in the stroma reached an inhibitory value for this activity (pH 8.0). This was observed after 5 h of illumination, where synthesis exceeds degradation. The starch content reached a maximal value (2.32 mg/mg of Chl) at the end of the light period.

Transfer to darkness after 16 h of light resulted in a rapid breakdown of the starch. After 4 h in darkness, the starch concentration reached 0.661 mg/mg of Chl. Chloroplasts and crude preparations were used to study the diurnal behavior of the total amylolytic activity that was concurrent with starch breakdown (Fig. 4). The chloroplasts were isolated following procedure I, because under illumination heavy starch accumulation disturbed the isolation of intact plastids through the Percoll gradient. Under the light period, the total amylolytic activity in both preparations showed very low variation, fluctuating between 63.43 and 62.74 milliunits/mg of Chl in the chloroplasts and between 3.69 and 3.33 units/mg of Chl in the crude extract. The same amylase behavior has been observed under a 12-h light period in pea (Saeed and Duke, 1990) and spinach leaves (Pongratz and Beck, 1978). During the first hours of darkness, the total chloroplastic amylolytic activity increased to 129 milliunits/mg of Chl, whereas the extrachloroplastic enzyme showed no fluctua-

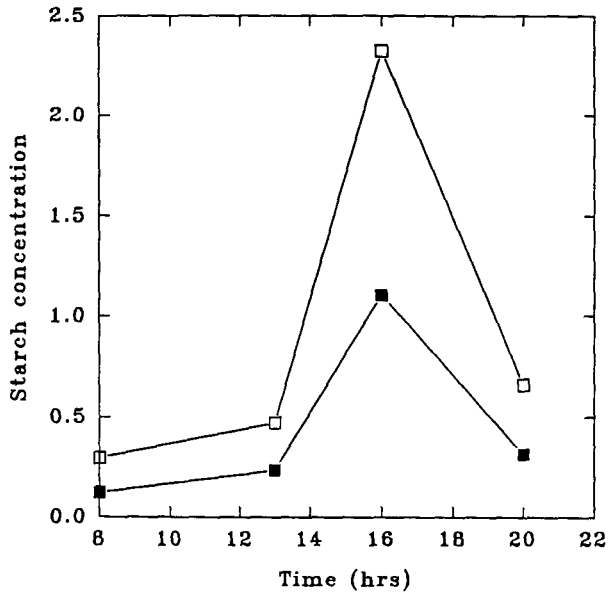

Figure 3. Diurnal fluctuation of the starch content in chloroplasts from *V. faba* plants that were kept under a light/dark regimen of 9/15 h. The light period begins at 8 h and ends at 16 h. Chloroplasts were isolated following procedure I and starch was enzymically measured as described in "Materials and Methods." □, Starch content, mg/mg of Chl. ■, Starch content, mg/mg fresh weight.

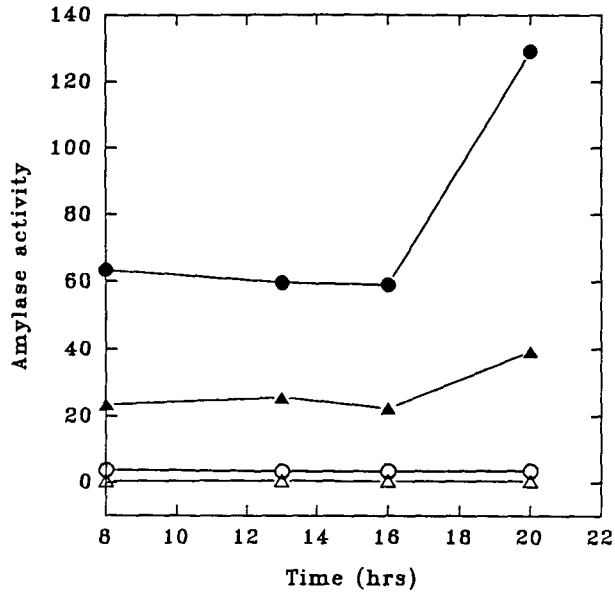

Figure 4. Diurnal behavior of the amylolytic enzyme activities in chloroplast and crude preparations from *V. faba* leaves that were kept under a light/dark regimen of 9/15 h. The light period begins at 8 h and ends at 16 h. Chloroplasts were isolated following procedure I (see "Materials and Methods"). Hydrolytic activity was determinated from the liberation of reducing groups from soluble starch. Total amylolytic activity in chloroplasts: ●, milliunits/mg of Chl; ▲, milliunits/mg of protein. Total amylolytic activity in crude preparations: ○, units/mg of Chl; △, units/mg of protein.

tion. This 2-fold diurnal fluctuation in the stroma was also observed in spinach chloroplasts (Pongratz and Beck, 1978).

A control process in *V. faba* maintains low chloroplastic total amylolytic activity at a constant level during the light period. In the dark, an inverse relationship was observed between the starch content and the stromal enzyme activities. This negative correlation did not exist with the extrachloroplastic amylases, which led us to the conclusion that extrachloroplastic amylases are not involved in the degradation of assimilatory starch.

DISCUSSION

Total amylolytic and phosphorolytic activities were found predominantly in extrachloroplastic fractions, whereas the debranching enzyme showed a homogenous distribution between stroma and extrachloroplastic fractions. Approximately 9% of the total amylolytic activity measured in the leaves was located in the stroma. As shown by electrophoretic transfer, α-amylase accounts mostly for the chloroplastic hydrolytic activity, whereas α- and β-amylase exhibited higher activities in the extrachloroplastic fraction. However, α-amylase activity associated with chloroplasts could originate from extrachloroplastic contamination. Recently, the chloroplastic localization of an α-amylase has been reported in sugar beet by Li et al. (1992).

Because an approximately 5-fold higher amylase than phosphorylase activity has been measured in *V. faba* chloroplasts, we suggest that endoamylases are possibly involved in starch granule degradation. The same degradative system catalyzed by an endoamylase has been found in spinach chloroplasts (Okita and Preiss, 1980; Steup et al., 1983). In pea chloroplasts, the pathway of starch breakdown is subject to controversy. Kakefuda et al. (1986) found only α-amylase in crude preparations, indicating an extrachloroplastic localization. Ziegler (1988) found pronounced debranching enzyme activity and unmistakably evident endoamylase (with properties very similar to the cereal α-amylase) in three different chloroplast preparations from pea leaves. More recently, Beers and Duke (1990) reported equivocal evidence for the presence of α-amylase in pea chloroplasts.

The study of the diurnal behavior of the amylase activity in *V. faba* leaves showed a 2-fold diurnal fluctuation of the chloroplastic total amylolytic enzyme activities, whereas no fluctuation was detectable for the extrachloroplastic enzymes. Thus, we suggested that most of the extrachloroplastic starch-degrading enzyme potential of *V. faba* leaves was not directly related to the degradation of assimilatory starch and, consequently, was not controlled by the same regulatory mechanisms that manage starch degradation in the plastids. The diurnal oscillation of chloroplastic amylolytic activity was probably controlled by an endogenous rhythm in which starch accumulation would be the result of a dynamic process where synthesis exceeds degradation. It has been suggested that photosynthesis-driven pH changes in the chloroplast during the day-night transition could account for the regulation of the amylase activity, because this enzyme exhibits a rather acidic pH optimum (Pongratz and Beck, 1978; Ziegler, 1988). The 2-fold increase in the chloroplastic enzyme activity was observed when the pH was decreased from pH 8.0 to pH 7.0, which is reached in the stroma in the dark. This activation of the amylolytic system might be superimposed by the formation of a complex with an endogenous dithiol (Pongratz and Beck, 1978). Regulation by high maltose concentrations could be envisioned, although in spinach chloroplasts this regulation process seemed to play a minor role (Pongratz and Beck, 1978).

A major regulatory role in starch synthesis may be ascribed to the ratio Pi/PGA, which controls the activity of ADP-glucosepyrophosphorylase. A decrease in the pH of the stroma during a dark period would lead to a release of the starch synthesis substrate PGA from the chloroplast to the cytoplasm.

All these regulatory mechanisms are possible candidates for starch metabolism control in the chloroplast, whereas such mechanisms implicated in the regulation of the extrachloroplastic enzymes are still unknown. Jacobsen et al. (1986), studying the effect of water stress on barley leaves, demonstrated that extrachloroplastic α-amylase levels increased as leaf water potential decreased and that this increase was related to elevated synthesis of α-amylase mRNA and protein. This study may indicate that the bulk of the extrachloroplastic amylolytic activity in higher plants could be related to a response to environmental conditions. More recently, Caspar et al. (1989) found that the amount of β-amylase in leaves of *Arabidopsis thaliana* could be very high in mutants with altered starch metabolism, and they implied that this enzyme could be induced in response to high levels of soluble sugars that accumulate during the photoperiod in the mutants.

The precise physiological role of the extrachloroplastic starch-degrading enzymes remains unknown, and further investigations might help to determine whether or not the high activities of these enzymes in *V. faba* leaves play a significant role in leaf carbon metabolism. It also remains to be determinated to what extent the endoamylases are involved in the degradation of starch in *Vicia* chloroplasts, considering their relatively low activities.

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