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 integrity 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 phosphorylactic 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. Amylopectin 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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* Corresponding author; fax 49–228–695168.
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 m−2 s−1 (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 MgCl2, 1 mM MnCl2, 4 mM ascorbate, and 0.5% BSA. The homogenate was filtered through eight layers of cheesecloth and a 20-μm nylon net and centrifuged for 20,000g 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 1300g. Pelleted chloroplasts were then loaded on 20 mL of a 25 to 92% Percoll gradient and centrifuged for 7 min at 13,000g (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 MgCl2, 1 mM DTE and centrifuged for 5 min at 2500g. 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 Assays

Enzyme activities were assayed the day of extraction. Production of reducing sugar from starch (soluble potato starch, Sigma) was measured using the dinitrosoalicylic 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 dinitrosoalicylic 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.
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 stained through an SCG and stained with a KI/L 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-

Starch-Degrading Enzymes in Vicia faba Leaves

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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.

However, it is unclear whether this activity is a contamination or α-amylase that is associated with the chloroplast. The bluestained 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.

Consequently, the chloroplastic starch-degrading enzyme activity was determined 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 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.

### 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").

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Amylase activity (milliunits/mg of protein)</th>
<th>Amylase activity (milliunits/mg of Chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24.84</td>
<td>63.43</td>
</tr>
<tr>
<td>II</td>
<td>53.32</td>
<td>356</td>
</tr>
</tbody>
</table>
Starch-Degrading Enzymes in Vicia faba Leaves

Table II. Distribution of starch-degrading enzyme activities in V. faba leaves

<table>
<thead>
<tr>
<th></th>
<th>Chloroplast Extract</th>
<th>Crude Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>milliunits/mg of protein</strong></td>
<td><strong>milliunits/mg of Chl</strong></td>
<td><strong>milliunits/mg of protein</strong></td>
</tr>
<tr>
<td>Total amylolytic activity</td>
<td>53.32</td>
<td>368</td>
</tr>
<tr>
<td>Pullulanase</td>
<td>25.19</td>
<td>158</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>10.35</td>
<td>62</td>
</tr>
</tbody>
</table>

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-

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**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 determined 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 determined to what extent the endoamylases are involved in the degradation of starch in Vicia chloroplasts, considering their relatively low activities.

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**LITERATURE CITED**


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