Subcellular Localization of the Starch Degradative and Biosynthetic Enzymes of Spinach Leaves

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ABSTRACT

The subcellular localization of the starch biosynthetic and degradative enzymes of spinach leaves was carried out by measuring the distribution of the enzymes in a crude chloroplast pellet and soluble protein fraction, and by the separation on sucrose density gradients of intact organelles, chloroplasts, peroxisomes, and mitochondria of a protoplast lysate. ADP-Glucose pyrophosphorylase, starch synthase, and starch-branching enzymes are quantitatively associated with the chloroplasts. The starch degradative enzymes amylase, R-enzyme (debranching activity), phosphorylase, and D-enzyme (transglycosylase) are observed both in the chloroplast and soluble protein fractions, the bulk of the degradative enzyme activities reside in the latter fraction. Chromatography of a chloroplast extract on diethylaminoethyl-cellulose resolves the R- and D-enzymes from amylase and phosphorylase activities although the two latter enzyme activities coeluted. The digestion pattern of amylase with amylpectin as a substrate indicates an endolytic activity but displays properties unlike the typical a-amylase as isolated from endosperm tissue.

The mode of regulation of starch metabolism in leaves, i.e., accumulation in the light and degradation in the dark, has yet to be firmly established. Starch metabolism may be controlled by 3-PGA/Fr ratio (13, 31), photosynthetic driven pH changes (30, 31), and/or oxidation-reduction (1, 41). Several studies have shown that a relatively high 3-PGA/Fr ratio stimulates starch synthesis presumably by activating ADP-glucose pyrophosphorylase (13, 30, 31) while a low ratio brings about net degradation of starch by simultaneously inactivating ADP-glucose pyrophosphorylase and stimulating phosphorylase.

Much is known about the properties and regulation of the enzymes involved in starch synthesis. In contrast, our knowledge of starch degradation in leaves is scant. Recent studies (13, 19, 28, 36) have shown that leaf starch is degraded both by phosphorylase and amylolysis and it has been suggested that the principal route of carbon is through the former pathway (13). The enzymes which catalyze these steps have yet to be adequately studied. It is clear that our understanding of starch metabolism in higher plants will be limited unless the enzymes responsible for degradation are identified and their regulatory properties elucidated. Here we report the localization of both the starch degradative and biosynthetic enzymes of spinach leaves.

MATERIALS AND METHODS

Plant Material. Protoplasts were isolated from Spinacia oleracea var. Hybrid High Pack (Asgrow Seed Co.) which was grown hydroponically in an 8 h/16 h day/night cycle. Spinach, obtained from the local market, was the source of tissue in which chloroplasts were directly isolated by gentle homogenization and differential centrifugation (40).

Protoplast Preparation. Protoplasts were isolated by treating mature leaves with 0.5% Macerase and 0.5% Cellulysin as described by Ohlrogge et al. (27). The protoplasts were collected by centrifugation at 143g for 5 min and then washed twice with 0.85 M mannitol. These latter steps are particularly important since the commercial enzyme preparations contain about 2.2 units/ml of amylase activity which is easily removed by repeated washing. The protoplasts were disrupted as recommended by Nishimura et al. (26) and then portions of disrupted protoplasts containing 1 to 2 mg of Chl were layered on a 30 to 58% (w/w) linear sucrose gradient containing 20 mM MOPS-KOH (pH 7.0) and centrifuged at maximum speed in a Beckman SW 27 rotor for 4 h at 4 C. About 1-m1 fractions were collected and used for the assay of enzyme activities.

Direct Preparation of Chloroplasts. Chloroplasts were isolated using a modified method of Levi and Preiss (20). Washed leaves (50-500 g) of spinach, purchased from a local market, were homogenized for 2 to 4 s in 50-g batches using 3 volumes of isolation medium containing 0.33 mM mannitol or sorbitol, 5 mM MgCl2, 5 mM Na-pyrophosphate, 20 mM Hepes-NaOH (pH 6.8). The homogenate was filtered through eight layers of cheesecloth and then centrifuged at 2,500g for 1.5 min. The pellets were washed with about 300 ml of the above buffer minus PPI and collected at 2,500g for 1 min. The yield of intact chloroplasts was 15 to 50%, normally about 20%, depending on the experiment, as measured by RuBp carboxylase distribution (16).

Preparation and Fractionation of Chloroplast Extracts by Ion Exchange Chromatography. Washed pellets were resuspended in 20 mM Hepes-NaOH (pH 7.0) containing 10% glycerol, 1 mM Na2EDTA, 1 mM CaCl2, and 1 mM DTE and passed through a French press at 5,000 to 10,000 p.s.i. The extract was centrifuged at 70,000g for 30 min and the supernatant fluid was passed directly through a DEAE-cellulose column (1.5 × 12 cm). The column was washed with 1 column volume of the above buffer and the enzymes eluted with the same buffer and a 250-ml linear KCl gradient (0-0.5 M).

Reagents. 30PPI was purchased from New England Nuclear. ADP-Glucose, hexokinase, glucose-6-P dehydrogenase, malate, maltooliase, pullulan, and P-glucosidase were obtained from Sigma Chemical Co. Macerase and Cellulysin were from Calbiochell. Pullulanase was purchased from Boehringer Mannheim. Amylose (DP ~ 300) and amylopectin (mol wt > 106 daltons)
were products from Nutritional Biochemical Co. and Pierce Chemical Co., respectively.

ENZYME ASSAYS

All assays were conducted at 37°C and under conditions where linear catalytic rates were obtained with respect to time and enzyme concentration. A unit of enzyme activity is equal to 1 μmol of substrates utilized or products formed/min/mg Chl.

Organelle Marker Enzymes. RuBPCase was assayed according to Bahr and Jensen (3) except that the enzyme was incubated at 37°C for 15 min before RuBP was added. The reaction was allowed to proceed for 5 to 10 min at 25°C and terminated by the addition of 0.5 M HCl. Unreacted 14CO2 was removed by purging the mixture with warm air and the amount of radioactivity determined by liquid scintillation spectrometry. Isocitrate dehydrogenase, a marker enzyme for mitochondria, was assayed according to Cox (7) while the peroxisome enzyme, catalase, was measured by the method of Luck (23).

Assay of Starch Biosynthetic Enzymes. ADP-Glucose pyrophosphorylase was assayed according to Ghosh and Preiss in the pyrophosphorylation direction in the presence of 1 mM 3-PGA (9) while starch synthase and starch branching enzyme were measured by the method of Hawker et al. (11).

Assay of Starch Degradative Enzymes. Amylase was measured in a reaction mixture (1 ml) containing 40 μmol imidazole-HCl (pH 6.0), 5 μmol amylpectin, and 50 to 200 μl enzyme. R-enzyme was measured by substituting pullulan in place of amylopectin. The reaction was allowed to proceed for 30 to 60 min and then terminated by immersing the reaction tubes in a boiling water bath. Total reducing power was determined by the method of Nelson (25). D-enzyme (transglycosylase) was measured in a 250-μl reaction containing 10 μmol imidazole-HCl (pH 6.0), 2.5 μmol maltotriose, and 50 μl enzyme while maltase was assayed in a 1-ml reaction mixture containing 40 μmol acetate (pH 6.0), 10 μmol maltose, and 200 μl enzyme. The reaction mixtures were incubated for 60 min and then terminated by immersing the reaction tubes in boiling water. Released glucose was measured by following the reduction of NADP in the presence of hexokinase and glucose-6-P dehydrogenase (20).

Phosphorylase was assayed in a reaction mixture (1 ml) containing 100 μmol Hepes-NaOH (pH 7.0), 20 μmol Pi, 1 mg debranched amylopectin (17), and 20 to 100 μl enzyme. After incubating for 30 min, the reaction was terminated and glucose-1-P produced was measured by following the reduction of NADP using P-glucosumtase and glucose-6-P dehydrogenase (20).

Analytical Methods. Chl was estimated by the method of Arnon (2). Paper chromatography was done on Whatman No. 1 paper for 36 h in butanol-pyridine-water (6:4:3, v/v). Sugars were detected by AgNO3-NaOH dip (39).

RESULTS

Distribution of Starch-metabolizing Enzymes of a Spinach Leaf Homogenate. As a first approach for the elucidation of the pathways of starch degradation in spinach leaves a study of the subcellular localization of the starch biosynthetic and degradative enzymes was conducted. This study was performed using two different approaches; these enzymes were estimated from a direct preparation of chloroplasts (40) and via sucrose density gradient centrifugation of a protoplast lysate (26).

A major difficulty in ascribing any enzyme activity to a specific cellular level is determining the amount of enzyme activity recovered in an organelle preparation relative to the percentage of intact organelles. This problem holds true particularly for those enzyme activities which are localized at more than a single cellular site. Two useful methods for estimating the degree of integrity of a chloroplast preparation have been the light reduction of ferri-cyanide (12) and the recovery of a specific enzyme activity which is restricted only to the chloroplasts (16). The latter method is more useful in subcellular localization studies of enzymes since the ferricyanide method may overestimate the degree of chloroplast intactness. Chloroplasts, which lyse and thus expel their stroma contents but subsequently reseal their envelope membranes, would display the characteristic ferricyanide impermeability (21). In this study we estimated the degree of chloroplast intactness by measurement of RuBPCase in our chloroplast preparations. RuBPCase is an excellent indicator of chloroplast integrity since this activity is restricted to the chloroplast and is the major stroma protein (16).

Table I shows the distribution of the starch biosynthetic enzymes between a crude chloroplast pellet and a soluble protein fraction obtained by differential centrifugation of a spinach leaf homogenate. As estimated by the quantity of RuBPCase recovered in the chloroplast fraction, about 19% yield of intact chloroplasts are isolated from the leaf homogenate. The starch biosynthetic enzymes, ADP-glucose pyrophosphorylase, starch synthase, and starch-branching enzyme are recovered to the same degree as RuBPCase in this crude chloroplast fraction, indicating that these enzymes like RuBPCase are restricted to the chloroplasts.

Table II shows the distribution of the starch degradative enzymes in the same two fractions of a spinach leaf homogenate. In contrast to the biosynthetic enzymes, the degradative enzymes are detected at much lower amounts with respect to the percentage of RuBPCase recovered in the chloroplast fraction. The low activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units in Chloroplast Pellet</th>
<th>Total Units Recovered</th>
<th>% in Chloroplast Pellet</th>
<th>Units/mg Chl</th>
<th>Corrected % in Chloroplast Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>RuBPCase</td>
<td>1.68 ± 0.9</td>
<td>19.4 ± 2.4</td>
<td>100</td>
<td>95.8 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>ADP-Glucose pyrophosphorylase</td>
<td>1.80</td>
<td>12.1</td>
<td>18.3 ± 2.8</td>
<td>0.70</td>
<td>95.8 ± 3.2</td>
</tr>
<tr>
<td>Starch synthase</td>
<td>0.36</td>
<td>2.2</td>
<td>19.4 ± 1.7</td>
<td>0.16</td>
<td>94.7 ± 3.4</td>
</tr>
<tr>
<td>Starch branching enzyme</td>
<td>6.63</td>
<td>49.5</td>
<td>18.5 ± 3.3</td>
<td>2.25</td>
<td>94.6 ± 6.0</td>
</tr>
</tbody>
</table>

1 ± SE.

Table II. Activities of Starch Degradative Enzymes in Chloroplasts Obtained from Spinach Leaf Homogenates

For details see the legend to Table I. Values reported are the average of the number of experiments shown in parentheses.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% of Total Activity Present in Chloroplast Pellet</th>
<th>Units/mg Chl</th>
<th>% RuBPCase of Chloroplast Pellet</th>
<th>Corrected % in Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase (9)</td>
<td>3.0 ± 0.2</td>
<td>0.170</td>
<td>18.9 ± 1.7</td>
<td>16.4 ± 1.2</td>
</tr>
<tr>
<td>R-enzyme (5)</td>
<td>6.9 ± 0.7</td>
<td>0.122</td>
<td>21.2 ± 2.6</td>
<td>34.1 ± 1.0</td>
</tr>
<tr>
<td>Phosphorylase (3)</td>
<td>6.8 ± 0.4</td>
<td>0.123</td>
<td>20.5 ± 1.7</td>
<td>33.7 ± 2.8</td>
</tr>
<tr>
<td>D-enzyme (3)</td>
<td>3.9 ± 0.5</td>
<td>0.020</td>
<td>20.5 ± 1.7</td>
<td>19.5 ± 2.9</td>
</tr>
<tr>
<td>Maltase (3)</td>
<td>&lt;1.0</td>
<td>&lt;0.003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
lysates associated is due both in the enzyme estimation of intactness. The (Table these
in the enzyme
of enzymes. be observed is resolved from other organelles was obtained from other organelles by sucrose
mg plast
and Beck
d) starch-branching enzyme. All activities are expressed as amol substrates utilized or products formed/min-fraction. The peak location of mitochondria (fraction 15) and peroxisomes (fraction 6) are indicated by arrows.

of amylase and phosphorylase in the chloroplasts are in general agreement with results of Pongratz and Beck (29). Amylase, R-enzyme, phosphorylase, and D-enzyme are present in the chloroplasts at about 16 to 34% of the total activity observed in the leaf homogenate if a correction is made for the percentage of broken chloroplasts. The majority of these enzyme activities are recovered in the soluble protein fraction. Only trace activity of maltase is observed in our chloroplast preparation although significant activity is present in the soluble protein fraction at about 0.063 units/mg Chl. Our results indicate that maltase activity is extremely low and is not present at the quantities as suggested by others (29).

Distribution of Starch-metabolizing Enzymes on Sucrose Density Gradients. The isolation of protoplasts and subsequent separation of the organelles on sucrose density gradients have proven to be a sensitive method for defining the subcellular localization of enzymes. Using a modification of the procedure of Nishimura et al. (26), a high percentage of intact chloroplasts (greater than 75%) was obtained on sucrose density gradients which were well resolved from other organelles (Figs. 1a and 2a).

Figure 1 shows the distribution of the starch biosynthetic enzymes obtained by sucrose density gradient centrifugation. Chl and RuBPCase were used as markers for chloroplasts and for the estimation of intactness. The percentages of Chl and RuBPCase in the intact chloroplasts are in excellent agreement which indicates these parameters are valid indicators of chloroplast integrity (Table III). Quantitatively, all of the ADP-glucose pyrophosphorylase and starch synthase and more than 90% of the starch-branching enzyme are associated with the chloroplasts (Table III).

The small amount of the branching enzyme not chloroplast-associated is due to the relative difficulty in obtaining enzyme linearity in the phosphorylase-coupled assay (11). The over-all results from both the sucrose density gradient centrifugation of protoplast lysates and distribution of enzymes from leaf homogenates clearly indicate that the starch biosynthetic enzymes are restricted to the chloroplasts.

Distribution of Starch Degradative Enzymes. The activities of amylase, R-enzyme, and phosphorylase in fractions from sucrose density gradients are shown in Figure 2. These enzyme activities are restricted to the intact chloroplasts and the soluble protein fraction. As shown previously, the bulk of the degradative enzymes with the exception of R-enzyme are localized in the soluble protein fractions. R-enzyme is equally distributed between the chloroplast and soluble protein fractions.

Table III. Activities of Starch Biosynthetic and Degradative Enzymes in Chloroplasts Isolated by Sucrose Density Gradient Centrifugation of Protoplast Lysates

Twice-washed protoplasts of hydroponically grown spinach were lysed and the organelles resolved on a 30 to 56% (w/w) sucrose density gradient as described in the text. Representative examples of the distribution of enzyme activities, Chl, and organelles on sucrose density gradients are illustrated in Figures 1 and 2. The total activity of each enzyme associated with intact chloroplasts was obtained by summing the activity of each fraction in the area where intact chloroplasts sedimented. Each value reported is the average of the number of experiments shown in parentheses.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units in In-tact Chloroplasts</th>
<th>Units/mg Chl</th>
<th>% of Total Activity Observed in Sucrose Density Gradient</th>
<th>Corrected % in Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl (4)</td>
<td>3.1</td>
<td>0.68</td>
<td>78.5</td>
<td>100</td>
</tr>
<tr>
<td>RuBPCase (4)</td>
<td>0.82</td>
<td>0.48</td>
<td>77.6</td>
<td>98.9</td>
</tr>
<tr>
<td>ADP-Glu pyrophosphorylase (2)</td>
<td>0.68</td>
<td>0.48</td>
<td>77.5</td>
<td>98.9</td>
</tr>
<tr>
<td>Starch synthase (2)</td>
<td>0.04</td>
<td>0.03</td>
<td>78.1</td>
<td>99.7</td>
</tr>
<tr>
<td>Starch branching enzyme (2)</td>
<td>1.61</td>
<td>0.82</td>
<td>70.8</td>
<td>90.3</td>
</tr>
<tr>
<td>Amylase (3)</td>
<td>0.33</td>
<td>0.24</td>
<td>15.9</td>
<td>20.6</td>
</tr>
<tr>
<td>R-enzyme (3)</td>
<td>0.31</td>
<td>0.22</td>
<td>40.0</td>
<td>52.0</td>
</tr>
<tr>
<td>Phosphorylase (2)</td>
<td>0.08</td>
<td>0.07</td>
<td>30.7</td>
<td>38.4</td>
</tr>
<tr>
<td>D-enzyme (2)</td>
<td>0.02</td>
<td>0.02</td>
<td>29.8</td>
<td>37.2</td>
</tr>
</tbody>
</table>

FIG. 2. Localization of starch degradative enzymes. For details see legend to Figure 1. a) Chl and RuBPCase; b) amylase; c) R-enzyme; and d) phosphorylase. Mitochondria peaked in fraction 12 as indicated by the arrow.
A comparison of the results obtained by these two methods (Tables I–III) clearly indicates that there are distinct differences in the activities of the starch biosynthetic and degradative enzymes based on Chl. Spinach grown in the field (Table I) contains more activity of the starch biosynthetic enzymes than spinach obtained from water culture (Table III). For instance, there is about 5-fold higher starch synthase activity in field-grown spinach. On the other hand, spinach grown hydroponically has more amylase and R-enzyme but less phosphorylase activity, and in addition, a higher percentage of the degradative enzymes, especially R- and D-enzyme, localized in the chloroplast (Table III). Most of these differences in enzyme activity and percentage distribution can probably be accounted for by different growth conditions, although it could also be due to selective enzyme lability. For example, R-enzyme in the soluble protein fraction may be more labile than the chloroplast-associated enzyme. Thus, during sucrose density gradient centrifugation in which there is a considerable period (about 6 h) between protoplast lysis and assay of density gradient fractions, a loss of soluble R-enzyme may have occurred resulting in a higher percentage being assigned to the chloroplasts.

In spite of these differences, the results from both experiments clearly show that the bulk of the degradative enzymes, with the possible exception of R-enzyme, are not localized in the chloroplasts.

Less than 5% of the amylase or R-enzyme activity is observed if the integrity of the chloroplasts is maintained during the assay (data not shown).

DEAE-Cellulose Chromatography of Chloroplast Starch Degradative Enzymes. Inasmuch as the assays for amylase, R-enzyme, and D-enzyme are not specific, e.g. amylase may hydrolyze maltotriose which is the substrate used to measure D-enzyme (for another example see ref. 17 and below), the starch degradative enzymes from isolated chloroplasts were resolved by ion exchange chromatography. Single peaks of activity are observed for R-enzyme, D-enzyme, and phosphorylase while amylase is eluted in two overlapping peaks. The larger amylase activity coelutes with phosphorylase while the smaller activity is coincident with R-enzyme (Fig. 3). This latter amylase activity may be more apparent than real since R-enzyme can debranch amylopectin which is the substrate used in the amylase assay (17). Attempts to separate the phosphorylase and amylase activities by gel filtration failed, suggesting that the two are complexed to glucan. The enzyme activities observed in the first few fractions of ion exchange chromatography are probably due to overloading, since these activities are retained by DEAE-cellulose if rechromatographed.

Some Properties of Amylase and R-Enzyme. The products of R-enzyme and amylase were analyzed by paper chromatography and are shown in Figure 4, A and B, respectively. Maltotriose is the principal product of debranching activity but other oligosaccharides are also observed when pullulan is used as the substrate. A trace amount of maltose is barely detected, indicating a slight contamination by amylase activity. The hexose observed in assays of the crude chloroplast extract is mainly sorbitol which is from the chloroplast isolation buffer. Maltose is the first product detected under amylysis and at later incubations (2 h and on), maltotriose and higher oligosaccharides are readily observed. Trace levels of glucose are also detected after 24 h of hydrolysis. This pattern of hydrolytic products indicates an endolytic activity, i.e. α-amylase. A β-amylase activity has never been observed in any of these preparations.

The amylase was further characterized to determine whether it possessed properties similar to α-amylase present in germinating seeds. Incubating the amylase with EDTA or EGTA (10 mM) prior to the assay had no effect on amylolytic activity whereas complete activity was lost when the preparation was incubated for 10 to 20 min at 70 C in the presence of 10 mM CaCl₂. Amylase activity was susceptible to treatment with 1 mM NEM or DTNB, with about 20% of the original activity remaining after a 10-min incubation.

DISCUSSION

The results described in this study support the findings of others (13, 19, 28, 36) that radioactive starch is degraded both by a phosphorolytic and amylolytic pathway in isolated chloroplasts. The localization and isolation of an amylase displaying endolytic activity in chloroplasts suggest that the initial step in starch degradation is the formation of maltose and a complex mixture of oligosaccharides (Fig. 5) since this is the only enzyme capable of directly attacking starch granules (31). This supports the observations from Gibbs' laboratory (19, 28) that maltose is a major
results of Peavey et al. (28) do not support the occurrence of maltose transport. In spite of our lack of knowledge concerning the fate of maltose and glucose, the isolation of the aforementioned enzyme activities clearly indicates that spinach leaf starch can be degraded to sugar phosphates, maltose and glucose.

The endoamylase activity observed in the spinach chloroplast does not display the typical properties of an α-amylase. The spinach activity has no apparent requirement for Ca²⁺, lacks heat stability, and is sensitive to sulfhydryl-oxidizing agents. The first two properties are atypical of plant α-amylases while the latter property is characteristic of β-amylase (38). In the club of the spadix of Arum maculatum, an endoamylase activity has been described (5) having properties similar to the chloroplast activity, except that it is not affected by sulfhydryl-oxidizing agents. Thus, the basis by which α-amylase activity is assigned in higher plants must be redefined.

In previous studies (20, 24), high levels of ADP-glucose pyrophosphorylase and starch synthase were observed in chloroplast preparations. We have verified and extended these observations and show by two different methods—the direct isolation of chloroplasts, and sucrose density gradient centrifugation of a leaf homogenate—that all of the biosynthetic enzymes of starch metabolism are restricted to the chloroplasts which is consistent with the location of starch in the plant cell. The surprising result is that the bulk of the degradative enzymes are detected in the soluble protein fraction. A function for the starch degradative enzymes in the cytosol is presently unknown. A survey of the literature indicates that in pea (37) and Vicia faba (6), the predominant amylase activity is a β-type restricted to the soluble protein fraction while insignificant amylase activity is observed in the chloroplasts. Other studies have shown α- and β-amylase activity in the chloroplasts of sugarcane (4) and Stellaria media (10), respectively.

It is highly unlikely that the association of starch degradative enzymes with the chloroplasts is the result of cytoplasmic contamination, since amylase and R-enzyme activity are detected only upon lysis of the chloroplasts. Also addition of 1H1-acetyl carrier protein to the protoplast lysate reveals no detectable radioactivity with the intact chloroplasts in the sucrose density gradients indicating little nonspecific binding of cytoplasmic proteins (27). The apparent distribution of the degradative enzymes is probably due to multiple forms (isozymes) present in spinach leaves. The possibility of selective leakage from the chloroplasts, however, cannot be discounted. Chloroplast and cytoplasmic phosphorylase activities have been resolved by polyacrylamide gel electrophoresis (35) and they display different affinities toward glycogen (Greenberg and Preiss, unpublished observations). Differences in the substrate specificity of the cytoplasmic and chloroplast amylase activities have also been observed (unpublished observations). These and other results of the amylase and phosphorylase activities present in spinach leaves will be presented in our next communications.

LITERATURE CITED


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**Fig. 5.** Proposed scheme for degradation of leaf starch. Solid lines represent enzyme-catalyzed reactions observed in this report or others (13, 19, 28, 32, 36); broken lines reflect tentative reaction steps which remain to be resolved.
The image contains text from a scientific document, which appears to be a page from a Journal. The text is in a dense, scientific format, typical of academic research. The text includes references to various scientific studies and authors, mentioning topics such as starch synthesis, glycogen, and other carbohydrate-related processes. The text is formatted in a standard academic style, with proper citation of literature.