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Starch Degradation in Spinach Leaves

ISOLATION AND CHARACTERIZATION OF THE AMYLASES AND R-ENZYME OF SPINACH LEAVES[1]

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ABSTRACT

The properties of two amylase activities which differ in their substrate specificity and subcellular location as well as a chloroplast-associated R-enzyme (debranching activity) are reported. An extrachloroplastic amylase is resolved by gel filtration chromatography into two activities of 80,000 and 40,000 daltons. Both extrachloroplastic activities hydrolyze amylopectin and shellfish glycogen and only slowly hydrolyze rabbit liver glycogen, β-limit amylopectin, and amylose. In contrast, the major chloroplastic amylase attacks all of these glucans at comparable rates. Glucan hydrolysis by both the extrachloroplastic and chloroplastic amylase generates not only maltose but appreciable amounts of other oligosaccharides, whereas maltotetraose hydrolysis produces glucose, maltose, and maltotriose. The action patterns displayed by the amylase activities indicate that both are endoamylases, although they lack the typical Ca^{2+} requirement or heat stability of seed endosperm α-amylases. Dithiothreitol, glutathione (oxidized or reduced), ascorbate, dehydroascorbate, and dithiothreitol plus thioredoxin have no effect on either the chloroplastic or extrachloroplastic amylase activities.

The chloroplastic R-enzyme debranches amylopectin, β-limit amylopectin, pullulan, and α-limit dextrins, but not rabbit liver glycogen. An increase in extinction coefficient and λ_{max} is detected when the debranched amylopectin and β-limit amylopectin form a complex with I_2-KI. Based on these properties, the chloroplastic R-enzyme is similar in enzymic activity to the R-enzyme observed in endosperm tissue.

Metabolic studies using intact spinach chloroplasts have demonstrated that starch is degraded by both a phosphorolytic and amylolytic pathway (9, 13, 18). A previous study from this laboratory has shown that spinach leaf chloroplasts contain the necessary complement of degradative enzymes, *i.e.* phosphorylase, amylase, R-enzyme (debranching activity), and D-enzyme (transglycosylase), which are present at more than sufficient levels to account for the *in vivo* disappearance of starch (17). The bulk of the degradative enzymes, however, occur in the soluble protein fraction, presumably residing in the cytoplasm. This is not an unusual observation, at least for amylase activity, since β-amylase is the major amylolytic activity in peas (21) and *Vicia faba* (6) and is extrachloroplastic in origin.

Here, the studies of the starch degradative enzymes present in spinach leaves are extended. The major amylase activity was isolated and partially purified from a whole leaf homogenate and its properties were compared to the chloroplastic amylase. Although there are distinct differences between these amylases in their ability to attack glucans and their resulting action patterns, both activities are apparently endoamylases. The significance of these results will be discussed in relation to the possible regulation of the chloroplastic amylase and to the use of the Briggs' assay (3) in defining α- and β-amylases in leaves. In addition, some properties of the chloroplastic R-enzyme, an activity which has not previously been isolated from leaf tissue, will be presented.

MATERIALS AND METHODS

Buffer Solutions. The following buffer solutions were used: A, 50 mM imidazole-HCl (pH 7.0), 2 mM Na_2EDTA, 50 mM KCl, 2 mM calcium acetate, 10% ethylene glycol, 5 mM 2-mercaptoethanol; B, 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 75 mM KCl, 1 mM calcium acetate, 10% ethylene glycol, 5 mM 2-mercaptoethanol; C, 5 mM K-phosphate (pH 7.5), 10% ethylene glycol, 1 mM DTE[2]; D, 20 mM Hepes-KOH (pH 7.6), 250 mM KCl, 10% ethylene glycol, 1 mM DTE; E, 20 mM Hepes-KOH (pH 7.2), 1 mM calcium acetate, 10% ethylene glycol, 1 mM DTE.

Purification of Extrachloroplastic Amylase. Washed spinach leaves (1,300 g), obtained from a local market, were homogenized for 60 s in 1,500 ml buffer A. The homogenate was filtered under vacuum through two layers of Miracloth and then centrifuged at 16,000*g* for 20 min. The supernatant fluid was collected and made 60% saturated with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, resuspended in 250 ml buffer B, and dialyzed overnight against 4 liters of the same buffer. The enzyme fraction was clarified by centrifugation at 60,000*g* for 30 min and then layered on a DEAE-cellulose column (5 × 21 cm) which had been equilibrated with buffer B. Amylase activity was eluted using a 2.2-liter linear gradient (75–400 mM KCl) in buffer B. Pooled fractions containing amylase activity were concentrated 3-fold using an Amicon Ultrafiltration PM-30 membrane. The amylase fraction then was dialyzed overnight against buffer C and then chromatographed on a hydroxylapatite column (2.6 × 14 cm) equilibrated with buffer C. A 5 to 250 mM K-phosphate linear gradient containing 10% ethylene glycol and 1 mM DTE was used to elute the enzyme. Pooled fractions containing amylase activity were dialyzed overnight against buffer D, made 40% with respect to ethanol by dropwise addition, and then placed on ice for 10 min. The fraction was clarified by centrifugation and the amylase activity was precipitated by the addition of 2% (w/v) shellfish glycogen to a final concentration of 0.18% (14). The glycogen precipitate was collected by centrifugation, resuspended in buffer E, and placed on ice for 18 h to hydrolyze the glycogen. Oligosaccharides were removed by passing the enzyme through a DEAE-cellulose column (1 × 2.5 cm) and eluting with 0.4 M KCl in buffer E. Fractions containing amylase activity were collected, made 25% with respect to ethylene glycol, and dialyzed for at least 4 h against buffer E containing 25% ethylene glycol. The fraction then was

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[2] Abbreviation: DTE, dithioerythritol; $Glc_{(n)}$: α(1-4)-linked oligosaccharides.

chromatographed on a Bio-Gel P-150 filtration column (2.6 × 90 cm) equilibrated in buffer E containing 25% ethylene glycol at a flow rate of 10 cm/h.

Isolation of Chloroplastic Amylase and R-Enzyme. These enzymes activities were extracted from crude chloroplast pellets and resolved by DEAE-cellulose chromatography as previously described (17). Fractions containing these enzyme activities were pooled separately and concentrated to about 8 ml.

Enzyme Assays. Amylase activity was usually measured in 1-ml reaction mixtures containing 5 mg amylopectin, 40 μmol sodium acetate (pH 6.0), and 50 to 200 μl enzyme. After incubating for 30 to 120 min at 37 C, the amount of reducing power was measured by the method of Nelson (16) using maltose as a standard. The effect of oxidizing and reducing agents was tested by incubating the amylase in 1 to 10 mM concentrations of the compound in question at 25 C for 1 to 10 min. Samples then were taken and added to standard assay mixture. The effect of thioredoxin was examined by incubating the amylases at 25 C for 10 min in 50 mM Hepes-NaOH (pH 7.0 or 8.0) with 10 mM DTT and 45 to 75 μg spinach cytoplasmic- and chloroplastic-localized thioredoxins that activate spinach chloroplastic NADP-malate dehydrogenase or fructose bisphosphatase (10, 26); amylase activity then was initiated by the addition of amylopectin.

R-enzyme was assayed under the same conditions for the amylase except that 5 mg pullulan was used instead of amylopectin. The products produced by R-enzyme then were examined by reducing power (16) or by the change in spectra formed with I_2-KI. To 1 ml H_2O was added 50 μl reaction mixture, followed immediately by the addition of 2 ml I_2-KI stock solution (11). The iodine reagent was made daily by adding 0.5 ml stock solution (0.26 g I_2 and 2.6 g KI in 10 ml H_2O) to 0.5 ml 1 M HCl in a total volume of 130 ml.

Spinach chloroplastic fructose bisphosphatase and NADP-specific malate dehydrogenase were assayed as described (4, 25).

Mol Wt Determination. Bio-Gel P-150 filtration column equilibrated in buffer E containing 25% ethylene glycol was calibrated by measuring the void volume (V_0) using dextran blue 2000 and the elution volumes (V_e) of lactate dehydrogenase (mol wt, 140,000), BSA (mol wt, 68,000), and myoglobin (mol wt, 17,200). A standard curve was obtained by plotting log mol wt versus V_e/V_0.

Reagents. Maltose, maltotriose, pullulan, pullulanase, amylose, and amylopectin were obtained from commercial sources as described earlier (17). Maltotetraose and maltopentaose were purchased from Koch-Light Laboratories (Colbrook, England). Rabbit liver glycogen type V, shellfish glycogen, and sweet potato β-amylase were from Sigma. Pancreatic α-amylase was a product of Worthington Biochemical Corp. α-Limit dextrins and β-limit amylopectin were prepared as described by Whelan (23). α-Limit dextrins having a degree of polymerization of 6 to 7 were obtained by chromatographing the α-limit dextrin mixture of Bio-Gel P-2 with subsequent analysis by anthrone reagent (2) and reducing power (16). Spinach chloroplastic fructose bisphosphatase, NADP-specific malate dehydrogenase, thioredoxin f, m, and c were generous gifts from B. Buchanan, University of California, Berkeley. Calmodulin and trifluoperazine were kindly provided by P. Cohen, University of Dundee, Scotland.

RESULTS

PURIFICATION OF EXTRACHLOROPLASTIC AMYLASE

Table I summarizes the purification of the major amylase activity present in a spinach leaf homogenate. Large losses of amylase activity appear to occur during the first two steps of the purification. Although a portion of the enzyme loss is due to enzyme instability, these losses may be more apparent than real since the separation of other enzymes and factors, *e.g.* maltase, may significantly contribute to the reducing power measurements. Figure 1 depicts the elution profile from the DEAE-cellulose column. The predominant amylase activity which is initially retained by DEAE-cellulose is eluted as a biphasic peak which indicates that more than one activity, probably the chloroplastic amylase, is present in these fractions. The major amylase activities in fractions 37 to 50 are well resolved from R-enzyme and D-enzyme. A third amylase activity is observed in the spinach leaf homogenate but is not retained by DEAE-cellulose. This activity was not further characterized since it comprises less than 10% of the total amylase activity observed after DEAE-cellulose chromatography.

The predominant amylase activity is further purified by hydroxyapatite chromatography where a single symmetrical activity peak is observed (not shown). The resulting amylase then is precipitated by glycogen which has been demonstrated by others to be a useful technique in purifying α-amylase (14, 22). Gel filtration chromatography resolves the amylase into two activity peaks with apparent mol wt of 80,000 (amylase I) and 40,000 (amylase II) daltons (not shown). Since both of these activities possess identical properties (see below), the larger amylase is probably a dimer form of the smaller entity. Although an overall yield of about 3% is obtained with a 115- to 350-fold purification, these final preparations are substantially free of maltase, R-enzyme, and D-enzyme.

These purified amylases are probably of extrachloroplastic origin since they display different properties than the chloroplastic amylase fraction (see below). The chloroplastic activity has an apparent high mol wt which probably forms a complex with glucan (17). This infers that the chloroplastic amylase is removed at the glycogen precipitation step due to its insolubility in 40% ethanol.

CHARACTERIZATION OF EXTRACHLOROPLASTIC AND CHLOROPLASTIC AMYLASE ACTIVITIES

pH Optima. The effect of pH on the enzymic activity of the extrachloroplastic and chloroplastic amylases was determined over the pH 5.0 to 8.0 range. The extrachloroplastic amylases have a pH optimum between pH 6.0 to 6.5 (Fig. 2a). Maximum hydrolysis occurs in imidazole-HCl or K-phosphate buffers, and only 67% and 18% of the activity are observed at pH 7.0 and 8.0, respectively. The chloroplastic amylase exhibits a similar pH response between 6.0 to 7.5 (Fig. 2b) and prefers acetate buffer. Unlike the extrachloroplastic amylases, higher amylolysis is observed in Hepes-NaOH than in imidazole-HCl.

Substrate Specificity and Action Pattern of Extrachloroplastic Amylase. This amylase activity hydrolyzes amylopectin faster than shellfish glycogen (Fig. 3a). It hydrolyzes, albeit at low rates, amylose, β-limit amylopectin, and rabbit liver glycogen. This unusual response toward glucans by the extrachloroplastic amylases is not an artifact produced during the preparation of these substrates since, under identical experimental conditions, the preparations of β-limit amylopectin from this laboratory are attacked by pancreatic α-amylase and pullulanase but not by sweet potato β-amylase, whereas the remaining glucans are hydrolyzed by these commercial enzyme preparations. The hydrolysis of β-limit amylopectin suggests that the extrachloroplastic amylase is an α-amylase. Chromatographic analysis of the products generated by amylopectin hydrolysis reveals not only maltose but significant levels of maltotriose, maltotetraose, and lesser amounts of glucose and larger oligosaccharides (Fig. 4). Glucose and other oligosaccharides other than maltose are detected only after prolonged incubations (12 h or more) of extrachloroplastic amylase with amylopectin. The liberation of glucose, maltose, and a mixture of oligosaccharides from amylopectin is indicative of α-amylase action since β-amylase can release only maltose from amylopectin (22).

Table I. *Purification of Extrachloroplastic Amylase Activities*

Fraction	Volume	Protein	Specific Activity	Total Activity	Yield
	ml	*mg/ml*	*μmol/min/mg protein*	*μmol/min*	*%*
16,000*g* Supernatant	2,005	5.7	0.02	227	100
60% Ammonium sulfate	240	29.0	0.013	92.6	40.7
DEAE-cellulose	86	8.4	0.05	36.1	15.9
Hydroxylapatite	85	3.4	0.12	35.1	15.4
Glycogen precipitate	8.0	1.0	1.57	13.0	5.7
P-150 Gel filtration					
Amylase I	7.0	0.2	2.33	3.3	2.9
Amylase II	7.0	0.07	7.05	3.4	

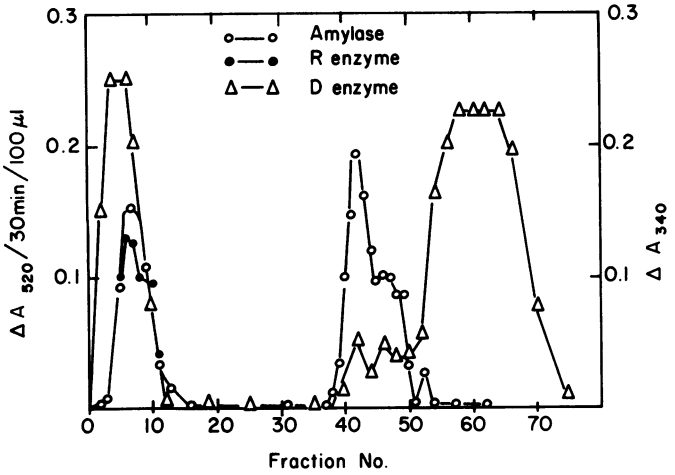

FIG. 1. Elution profile of the major spinach leaf amylases on DEAE-cellulose. A spinach leaf homogenate was prepared, subjected to $(NH_4)_2SO_4$ precipitation, and chromatographed on DEAE-cellulose as described in the text. Fractions were assayed for amylase, R-enzyme, and D-enzyme using amylopectin, pullulan, and maltotriose, respectively. Products generated by amylase and R-enzyme were measured by the Nelson's alkaline copper reagent (19), whereas glucose produced by D-enzyme was measured by glucose-6-P dehydrogenase reduction of NADP at 340 nm (18). The predominate amylase activities are eluted between fractions 37 to 50 and include the major activity found in spinach chloroplasts. A third amylase activity comprising less than 10% of total enzymic activity was detected in the void fractions.

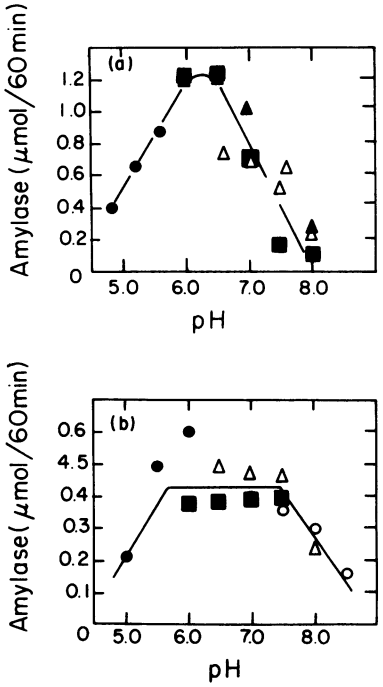

FIG. 2. pH optima of the extrachloroplastic amylase I (a) and chloroplastic amylase (b). The pH response of the extrachloroplastic amylase II was identical to that of amylase I. Buffers used were Na^+-acetate (●), imidazole-HCl (■), K^+-phosphate (▲), Hepes-NaOH (△), and Tris-HCl (○). All buffers were at a final assay concentration of 40 mM.

The extrachloroplastic amylase then was tested for its ability to hydrolyze the oligosaccharides, maltotriose, maltotetraose, and maltopentaose. Maltotriose is slowly hydrolyzed to maltose and glucose. Maltotetraose is cleaved mainly to maltose but also to maltotriose and glucose, whereas maltopentaose is rapidly hydrolyzed to maltotriose and maltose with a trace amount of glucose. The results are not shown but are identical to those obtained for the chloroplastic amylase fraction shown in Figure 5. The release of maltotriose and glucose from maltotetraose is a strict property of α-amylase since β-amylase can produce only maltose from substrates having an even number of glucose residues (22).

Substrate Specificity and Action Pattern of Chloroplastic Amylase. Our earlier study (17) suggested that the predominant amylase activity as resolved by DEAE-cellulose chromatography of a chloroplast extract was an endoamylase, *i.e.* α-amylase. Further support for this notion is shown in Figure 3b. Like the extrachloroplastic amylase, the chloroplastic amylase prefers amylopectin, but it also attacks at comparable rates both rabbit liver and shellfish glycogen, β-limit amylopectin, and amylose. It has negligible activity on pullulan, indicating little contamination with R-enzyme.

The action pattern of glucan hydrolysis as revealed by paper chromatography is shown in Figure 6. Maltose is the principal product of hydrolysis except for pullulan, but significant amounts of larger oligosaccharides with a trace of glucose are easily discernible after 5 h hydrolysis. These action patterns mediated by the chloroplastic amylase are similar to those obtained of α-amylases from endosperm tissue (22).

The products obtained from hydrolysis of the oligosaccharides maltotriose through maltopentaose are shown in Figure 5. The slight conversion of maltotriose to maltose and glucose and the production of maltotriose and glucose from maltotetraose are further evidence for α-amylolysis.

Effect of Oxidizing-Reducing Agents and Thioredoxin on Extrachloroplastic and Chloroplastic Amylases. An earlier study (19) suggested that the chloroplastic amylase may be regulated by light-driven redox transitions. To test whether such a control exists for the spinach leaf amylases, the extrachloroplastic and chloroplastic amylases were initially incubated with different concentrations of DTT (0.1 to 10 mM) and then the hydrolysis of amylopectin was measured. No effect by DTT, however, was ever observed in any of our studies (results not shown). Other reducing and oxidizing agents, GSH, GSSH, ascorbate, and dehydroascorbate, which are metabolically active in the chloroplasts (24) were also without apparent effect on subsequent amylopectin hydrolysis.

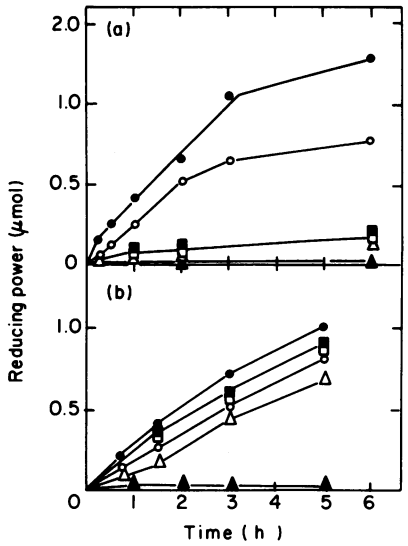

FIG. 3. Substrate specificity of the extrachloroplastic amylase (a) and chloroplastic amylase (b). Amylase activities were incubated with 5 mg/ml glucan. At indicated intervals, samples were withdrawn and reducing power measured (19) using maltose as a standard. (●), amylopectin; (○), shellfish glycogen; (■), rabbit liver glycogen; (□), β-limit amylopectin; (△), amylose; and (▲), pullulan.

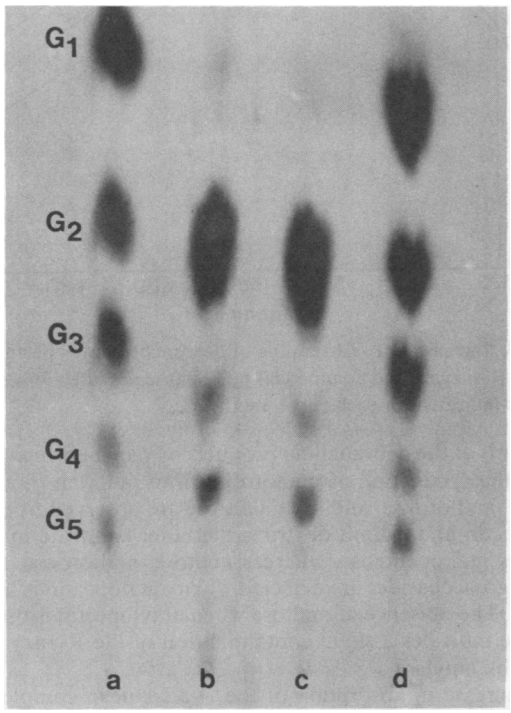

FIG. 4. Action pattern of the extrachloroplastic amylase I and II on amylopectin. Amylases were incubated with 5 mg/ml amylopectin; at periodic intervals, 40-μl samples were withdrawn and spotted on Whatman No. 1 paper and sugars were resolved in butanol-pyridine-H_2O (6:4:3). Products shown are those present at 24 h hydrolysis. a and d, Glc – $Glc_{(5)}$ standards; b, amylase I; c, amylase II.

The combined effect of DTT and thioredoxin then was tested on both amylase preparations at pH 7.0 and 8.0. Amylases were incubated with 10 mM DTT and with either chloroplastic thioredoxin f (specific for enzymes of the reductive pentose phosphate cycle), chloroplastic thioredoxin m (specific for NADP-malate dehydrogenase), or cytoplasmic thioredoxin c. However, no significant increase or decrease in amylopectin hydrolysis was observed when either the extrachloroplastic or chloroplastic amylases were preincubated with DTT-thioredoxin. Under the same conditions, the spinach chloroplastic fructose bisphosphatase and NADP-specific malate dehydrogenase were activated 2- to 4-fold.

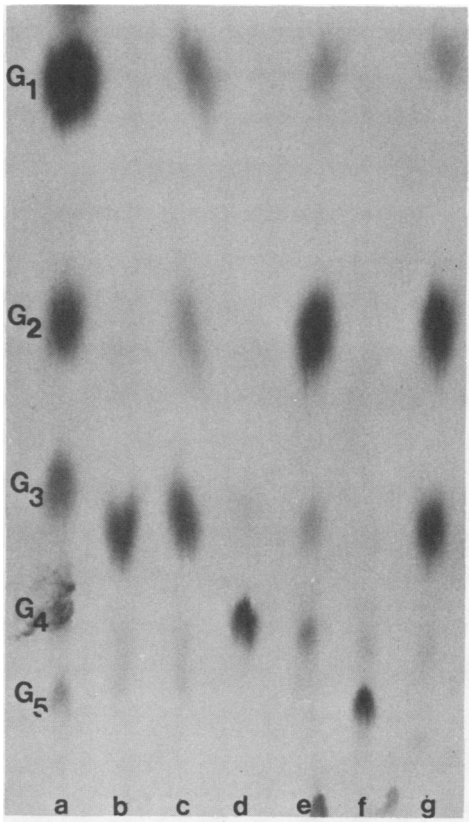

FIG. 5. Action of spinach leaf amylases on maltotriose, maltotetraose, and maltopentaose. The various amylases were incubated at 37 C with 10 μmol/ml oligosaccharide and 40 μmol/ml Na-acetate (pH 6.0). After 4 h incubation, 20-μl samples were withdrawn and spotted on Whatman No. 1 paper and chromatographed as described in the legend to Figure 4. Digestion patterns shown were that obtained from the chloroplastic amylase, although similar patterns were observed for the extrachloroplastic amylase I and II. a, Glc - $Glc_{(5)}$ standards; b and c, maltotriose; d and e, maltotetraose; f and g, maltopentaose. b, d, and f are zero time points, whereas c, e, and g are 4-h samples.

Other Studies. General properties of α-amylases are their insensitivity toward sulfhydryl-oxidizing agents and their strict dependence on Ca^{2+} for enzymic activity (22). In the presence of excess Ca^{2+}, endosperm α-amylase is stable at 70 C for 20 min. However, results from our earlier study (17) showed that the chloroplastic amylase does not display these characteristic α-amylase properties, although its substrate specificity and action pattern indicate otherwise. The extrachloroplastic amylase displays similar responses to these treatments as the chloroplastic amylase. Incubation of the extrachloroplastic amylase in either 10 mM EDTA or ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid for 10 min at room temperature has no apparent effect on subsequent hydrolysis of amylopectin. Likewise, no enzymic activity is observed when the enzyme preparation is initially incubated at 65 C for 10 to 20 min in 10 mM Ca^{2+}. Incubation with 1 mM *N*-ethylmaleimide or 5,5'-dithiobis(2-nitrobenzoic acid) results in a rapid decline in amylolysis; less than 5% of the original activity remains after 5 min.

PROPERTIES OF CHLOROPLASTIC R-ENZYME

Complete degradation of starch demands the presence of R-enzyme. Since the authors are unacquainted with any study of this

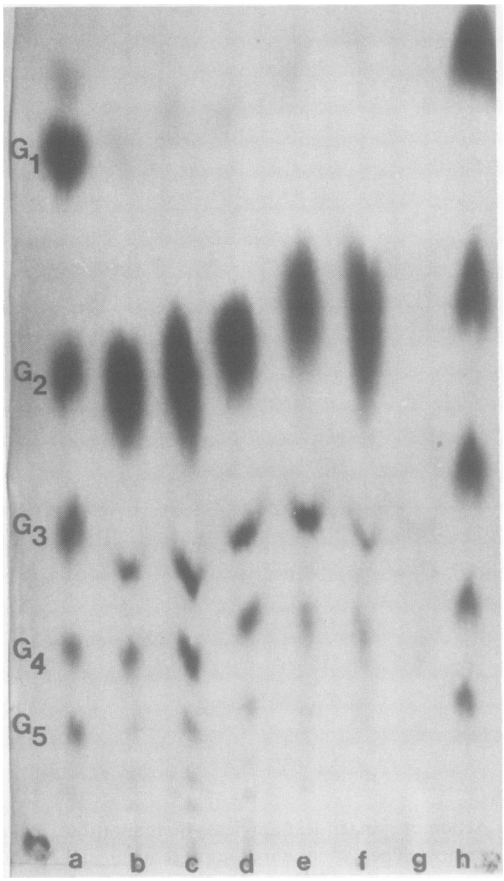

FIG. 6. Action pattern of the chloroplastic amylase on various glucans after 5 h hydrolysis. See the legend to Figure 4 for details. a and h, Glc - $Glc_{(5)}$ standards; b, amylopectin; c, rabbit liver glycogen; d, shellfish glycogen; e, β-limit amylopectin; f, amylose; and g, pullulan.

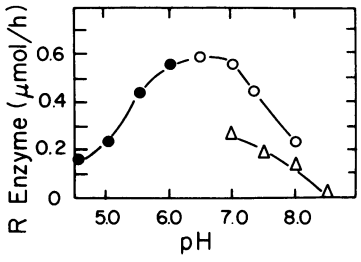

FIG. 7. pH response of R-enzyme. Buffers used were: (●), Na-acetate; (○), Hepes-NaOH; and (△), Tris-HCl.

enzyme from leaf tissue, some of its properties are reported here.

pH Optimum. The dependence of R-enzyme with pH is shown in Figure 7. R-enzyme displays a pH response similar to that obtained for the chloroplastic amylase, having a broad pH optimum of pH 6.0 to 7.0 with about 30 to 40% of the activity remaining at pH 8.0.

Substrate Specificity. The action of R-enzyme on various glucans is shown in Figure 8. α-Limit dextrins (degree of polymerization 6 to 7) are rapidly hydrolyzed, followed by pullulan, β-limit amylopectin, and amylopectin. Only slight activity is observed when R-enzyme is incubated with rabbit liver glycogen for 12 h. In terms of substrate specificity, the chloroplastic R-enzyme is similar to the debranching activity found in endosperm tissue (12). The initial debranching rate of β-limit amylopectin is about the same as pullulan, which differs from endosperm R-enzyme that hydrolyzes β-limit amylopectin at a rate 2.5-fold greater than that of pullulan.

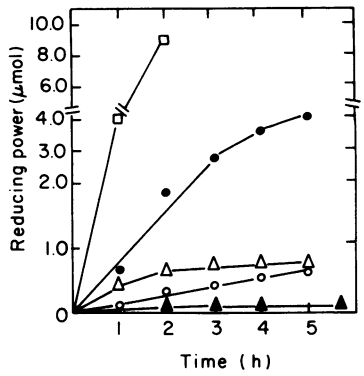

FIG. 8. Action of chloroplastic R-enzyme on various glucans. For details see the legend to Figure 3. (□), α-limit dextrins; (●), pullulan; (△), β-limit amylopectin; (○), amylopectin; (▲), rabbit liver glycogen.

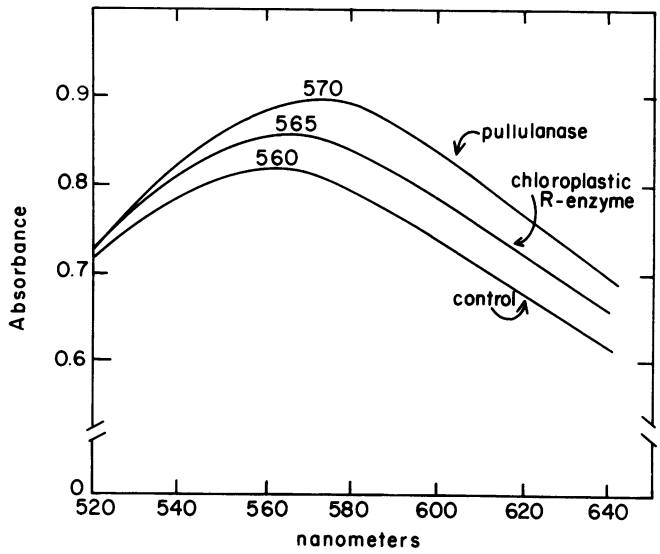

FIG. 9. Increase in I_2-KI staining of debranched amylopectin by chloroplastic R-enzyme and commercial pullulanase. For details see the text. Numbers indicated for each curve are the λ_{max}.

Analysis of the debranching products by paper chromatography reveals the production of maltotriose from pullulan (not shown). Maltose, maltotriose, and maltotetraose are observed from β-limit amylopectin and α-limit dextrins; the latter substrate in addition produces maltopentaose, whereas maltose, maltotriose, and very large oligosaccharides are detected when amylopectin is incubated for 24 h. The presence of maltose when amylopectin is used as the substrate indicates a slight contamination of the R-enzyme preparation by amylase.

An increase in absorption of the I_2-KI-glucan complex is also observed during debranching of amylopectin catalyzed by the chloroplastic R-enzyme (Fig. 9). The extinction coefficient increases about 14% and λ_{max} shifts from 560 to 565 nm. Under the same conditions, a commercial preparation of pullulanase raises the extinction coefficient about 20% and shifts λ_{max} from 560 to 570 nm (8). Similar responses are also observed when β-limit amylopectin is debranched (results not shown). The slight contamination by amylase activity probably prevents maximum increases of extinction coefficient and λ_{max} shifts by the chloroplastic R-enzyme.

DISCUSSION

In an earlier paper (17) it was reported that spinach chloroplasts, the cellular site of starch metabolism, contain a major amylase

activity which can account for the observed hydrolytic degradation of starch (13). A second amylase activity is also observed in spinach chloroplasts and co-elutes with R-enzyme from DEAE-cellulose. About 80% of the total amylase activity present in spinach leaves, however, appears to be extrachloroplastic in origin (17).

To increase the understanding of starch degradation in spinach leaves, the properties of the major chloroplastic amylase activity purified by DEAE-cellulose have been compared to those of the major amylase activity purified from crude leaf homogenates. The chloroplastic amylase has comparable hydrolytic activity on amylopectin, rabbit liver glycogen, shellfish glycogen, amylose, and β-limit amylopectin. Hydrolysis of these glucans produces maltose and also minor amounts of glucose and larger oligosaccharides. In contrast, the amylase activity purified from total leaf homogenates prefers amylopectin and shellfish glycogen and has slight activity with the remaining glucans (<5%). Maltose is the major hydrolytic product while glucose and other oligosaccharides are detected only after a prolonged incubation of enzyme with amylopectin. This striking difference in substrate specificity suggests that the amylase activity purified from total leaf homogenates is extrachloroplastic in origin.

The biphasic elution of amylase activity from DEAE-cellulose chromatography of total leaf homogenate suggests the presence of the chloroplastic amylase in these fractions. Some evidence from this is that the chloroplastic amylase is eluted from DEAE-cellulose at about the same ionic strength (17). In addition, pooled enzyme fractions from DEAE-cellulose hydrolyze amylose and β-limit amylopectin at about 12 to 15% of the amylopectin degradation rate. After subsequent purification steps, this hydrolysis rate for amylose and β-limit amylopectin decreases to less than 5% of amylopectin hydrolysis. This is consistent with the notion that the chloroplastic amylase which can efficiently hydrolyze amylose and β-limit amylopectin is present with the extrachloroplastic amylase and is removed by further purification probably at the glycogen precipitation step.

Inasmuch as the chloroplastic amylase probably initiates starch degradation (15), regulation of this hydrolytic activity would control the degradative process overall. In this context, Pongratz and Beck (19) observed an amylase activity which underwent a 2-fold diurnal fluctuation, as measured from crude chloroplast extracts. These workers suggested that photochemical reduction superimposed on the effects of photosynthetic-driven pH changes could account for this fluctuation in amylase activity and, thereby, regulate starch degradation, Therefore, the chloroplastic amylase has been examined for any possible regulatory properties, specifically for photoreduction. Under these conditions, one would expect that photoreduction, a light-mediated phenomenon, would deactivate amylase activity since starch degradation is a dark-related event. The chloroplastic amylase is inhibited by the sulfhydryl-oxidizing agents *N*-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid), whereas a 2-fold stimulation by added DTT is observed by others (19). Whether the presence of an essential free sulfhydryl group is indicative of amylase control by photoreduction still remains to be clarified, although prior incubation of the chloroplastic amylase under the conditions reported here with excess DTT, DTT plus thioredoxin f, m or c, ascorbate, dehydroascorbate, GSH, or GSSH has no effect upon subsequent hydrolysis of amylopectin. Since these are conditions which have been shown by others (1, 21, 22) to activate or deactivate several enzymes of chloroplast carbon metabolism, the studies here suggest no direct involvement of photoreduction control.

Unlike α-amylases from other sources, the chloroplastic amylase is not dependent on free Ca^{2+} for maximal stability or enzyme activity (22). Alternatively, Ca^{2+} may regulate indirectly via calmodulin (7). Incubation of the chloroplastic amylase with calmodulin in the presence of Ca^{2+}, however, exhibited no significant change in amylase activity as did trifluoperazine a potent inhibitor of calmodulin (unpublished observations; ref. 20). The above results suggest that the chloroplastic amylase is not regulated by divalent cations. Likewise, the chloroplastic amylase is not influenced by several glycolytic intermediates and adenylates whose levels fluctuate during photosynthesis (unpublished observations).

The apparent lack of regulation by photoreduction, calmodulin, divalent cations, and metabolites and the response of the chloroplastic amylase activity toward pH suggest that photosynthetic-driven pH changes alone may control amylase activity. In agreement with others (19), a 2-fold decrease of amylase activity is observed when the pH is increased from pH 7.0 to 8.0, a condition which may occur in the chloroplast during the night-day transition. Thus, the persistent, albeit lower, level of amylase activity in the light suggests that starch accumulation is the result of a dynamic process where synthesis exceeds degradation. Indeed, the reduced level of amylolysis in the light may be a mechanism to prevent excessive flow of carbon in starch and may facilitate starch synthesis by increasing the number of available nonreducing ends by which glucose can be transferred to by starch synthase.

The procedure devised by Briggs (3) has been used to measure α-amylase activity in plant extracts containing a number of contaminating enzymes, in particular β-amylase. α-Amylase of endosperm tissue is heat-stable in the presence of excess Ca^{2+}, whereas β-amylase is denatured under these conditions. The sensitivity toward heat treatment under these conditions by the extrachloroplastic and chloroplastic amylases suggest that both activities are β-amylases. However, the digestion pattern obtained from glucan hydrolysis and the formation of glucose and maltotriose from maltotetraose by spinach leaf amylases are indicative of α-amylases or endoamylase activity. In addition, the extrachloroplastic amylase binds to glycogen which has been a useful step in purifying α-amylases (14). Since both activities are essentially free of R-enzyme and D-enzyme, we ascribed both the extrachloroplastic and chloroplastic amylases as endoamylases which do not display the classical α-amylase properties. These results and others (5, 17) indicate that a cautionary approach should be taken when defining the intracellular amylases of leaves based on the Briggs assay which was devised to distinguish the extracellular amylases of endosperm tissue.

The isolation of a spinach extrachloroplastic amylase possessing endolytic activity appears to be at variance to the studies in peas and *Vicia faba* (6, 21). In leaf tissue of both plants, the predominant activity is a β-amylase. However, these plants contain at least 25-fold more amylase activity, based on a protein content, than that observed in spinach leaves (17). The extrachloroplastic endoamylase, if present in these plants at quantities equivalent to that found in spinach leaves, could easily be overlooked during any protein purification. Because of the low quantities of chloroplastic endoamylase present in spinach (17), the large amounts of extrachloroplastic β-amylase present in peas and *Vicia faba* would obscure any attempts to assign amylase activity in the chloroplast of these plants.

Finally, the location and function of the extrachloroplastic amylase remain unknown at present. Subcellular localization studies reported earlier (17) do not differentiate between enzymes compartmentalized in specific tissues of leaves nor their distribution between the cytoplasm or vacuole. Such a study might help to determine whether or not the extrachloroplastic amylases plays a significant role in leaf carbon metabolism.

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