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Subcellular Localization and Characterization of Amylases in *Arabidopsis* Leaf[1]

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ABSTRACT

Amylolytic enzymes of *Arabidopsis* leaf tissue were partially purified and characterized. Endoamylase, starch phosphorylase, D-enzyme (transglycosylase), and possibly exoamylase were found in the chloroplasts. Endoamylase, fraction A2, found only in the chloroplast, was resolved from the exoamylases by chromatography on a Mono Q column and migrated with an R_F of 0.44 on 7% polyacrylamide gel electrophoresis. Exoamylase fraction, A1, has an R_F of 0.23 on the polyacrylamide gel. Viscometric analysis showed that A1 has a slope of 0.013, which is same as that of A3, the extrachloroplastic amylase. A1, however, can be distinguished from A3 by having much higher amylolytic activity in succinate buffer than acetate buffer, and having much less reactivity with amylose. A1 probably is also localized in the chloroplast, and contributes to the 30 to 40% higher amylolytic activity of the chloroplast preparation in succinate than acetate buffer at pH 6.0. The high activity of D-enzyme compared to the amylolytic activity in the chloroplast suggests that transglycosylation probably has an important role during starch degradation in *Arabidopsis* leaf. Extrachloroplastic amylase, A3, has an R_F of 0.55 on 7% electrophoretic gel and constitutes 80% of the total leaf amylolytic activity. The results of substrate specificity studies, action pattern and viscometric analyses indicate that the extrachloroplastic amylases are exolytic.

Starch degradation in leaf tissues of plants is usually accomplished by both phosphorolysis and amylolysis (8, 17, 22). For example, spinach leaf chloroplasts contain endoamylase (EC 3.2.1.1.), debranching enzyme (EC 3.2.1.10), phosphorylase (EC 2.4.1.1.), and D-enzyme (EC 2.4.1.25) (14, 15, 19). Amylase and phosphorylase activities constitute the major degradative activity in the chloroplast. In spinach, α-amylase has been the only enzyme demonstrated to attach starch granules isolated from chloroplasts (23).

A different degradative system seems to exist in pea chloroplasts. Starch degradation in pea shoot chloroplasts probably occurs principally by phosphorolysis (9, 25). Pea chloroplasts have been reported to contain two β-amylases (EC 3.2.1.2), R-enzyme (EC 3.2.1.10), phosphorylase and D-enzyme (5). α-Amylase has not been detected in pea chloroplasts (9, 24) and β-amylase activity was very low (9). A recent report has demonstrated that α-amylase is only extrachloroplastic in pea (5). In contrast, Ziegler and Beck (29) have reported that α-amylase is a major amylolytic activity in pea and wheat chloroplasts. However, their conclusions were based solely on the color of bands after iodine staining of starch impregnated native gels.

β-Amylase has also been reported to be an extrachloroplastic enzyme. As yet, no function for this enzyme has been demonstrated. β-Amylase is the major amylolytic activity in pea leaves (24) and may be located in the vacuole (29). β-Amylase has also been found in *Vicia faba* (2), barley (4), and wheat (29) and in all cases was associated with the extrachloroplastic fraction. A similar finding in *Arabidopsis thaliana* is reported herein.

A. thaliana is considered one of the best vascular plants for the study of the molecular biology of plants because of its relatively small genome, suitability for mutation studies, and short generation time (11). The starch degradative systems of leaves in general, and of *A. thaliana* in particular, have not been well characterized. Before the importance of mutants of starch metabolism isolated in this laboratory could be assessed, it was found necessary to characterize the starch degradation system found in the leaves of *A. thaliana*, and characterization of amylases is the subject of this report.

MATERIALS AND METHODS

Reagents. Hexokinase, glucose -6-P dehydrogenase, pancreatic α-amylase, maltotriose, pullulan, PEG 8000 and polyacrylamide were obtained from Sigma Chemical Co. Macerase and cellulysin were from Calbiochem. Amylose (DP 300) and amylopectin (mol wt > 10^6) were products from Nutritional Biochemical Co. and Pierce Chemical Co., respectively. Protein assay reagents were from Pierce Chemical Co. (20). Maltodextrins were purchased from Aldrich Chemical Co. Sweet potato β-amylase was from Boehringer Mannheim. β-Limit amylopectin was prepared as described by Whelan (27). Soluble starch was from Merck & Co., Inc.

Plant Material and Growth Conditions. The Columbia wild-type of *Arabidopsis thaliana* (L.) Heynh. was used. The plants were grown at approximately 22°C, with a 12 h photoperiod under cool-white fluorescent illumination (about 200 $\mu E \cdot m^{-2} \cdot s^{-1}$) on a perlite:vermiculite:sphagnum (1:1:1) mixture irrigated with a mineral nutrient solution.

Enzyme Purification. *Preparation of Crude Extract.* All steps of this procedure were carried out at 4°C. One hundred g of leaf tissue was homogenized in a mortar and pestle in 90 ml of 50 mM Tris-HCl buffer (pH 7.5), containing 2 mM EDTA and centrifuged at 16,000*g* for 20 min. The extraction was repeated one time with 50 ml of the extract buffer.

Polyethylene Glycol Fractionation. The supernatants from above extraction were combined and PEG 8,000 (50% w/v dissolved in 20 mM Bis-Tris-propane buffer [pH 7.0], containing 2 mM DTT [buffer A]) was added to give 3, 10, 15, and 35% final PEG concentration. At each PEG concentration, the precipitate was collected after stirring for 15 min at 4°C. The precipitate from 3 to 10% and 15 to 35% PEG fractions was

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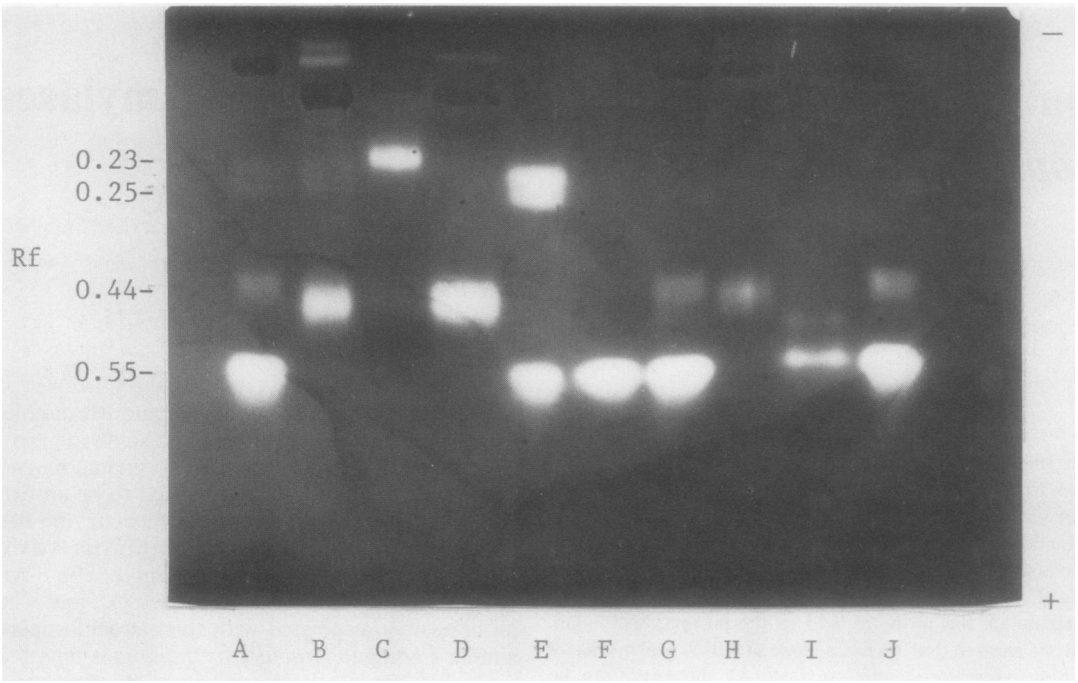

FIG. 1. Detection of amylolytic enzymes in crude extract, PEG fractions, partially purified amylase fractions, chloroplast and cytosolic fractions of *Arabidopsis* leaf by polyacrylamide gel electrophoresis. The staining procedure is described in "Materials and Methods." A, G, and J, crude extract; B, 3 to 10% PEG fraction; C, amylase A1; D, amylase A2; E, 15 to 35% PEG fraction; F, amylase A3; H, chloroplast fraction; I, extrachloroplastic fraction.

Table I. *Purification of Amylases, A1, A2, and A3*

Fraction	Volume	Protein	Total Activity	Specific Activity	Yield
	ml	*mg/ml*	*μmol/min*	*μmol/min·mg protein*	*%*
Homogenate	145	5.9	120	0.14	100
PEG 3–10%	11.2	23.9	42.1	0.16	35
1st DE-52	11.2	4.82	39.7	0.74	33
2nd DE-52	7.1	4.21	20.3	0.68	17
FPLC-Mono Q					
A1	7.1	0.43	8.0	2.61	6.6
A2	1.67	1.28	1.20	0.56	1.0
PEG 15–35%	12.4	7.0	66.9	0.77	56
DE-52					
A3	7.0	0.57	17.9	4.42	15

resuspended in 11.5 and 12.5 ml of buffer A, respectively. The solution was clarified by centrifugation at 16,000*g* for 10 min.

DEAE-Cellulose Chromatography. Protein, 200 mg in 11 ml, was charged onto a DEAE-cellulose column (DE-52) (1.5 cm × 16 cm, 10 ml resin bed volume) which had been equilibrated with buffer A. About 50% of the protein was washed through the column by buffer A. After the absorbance at 280 nm of the eluate decreased to 0.1, the enzyme was eluted with a salt gradient containing 50 ml of buffer A in the mixing chamber and 50 ml of buffer A containing 0.4 M NaCl in the reservoir. The fractions containing amylase activity were pooled and concentrated to about 12 ml using an Amicon PM30 membrane. The solution was dialyzed twice against 500 ml of buffer A for 5 h. The precipitate formed was removed by centrifugation. The clear solution containing 54 mg protein in 11.2 ml was rechromatographed on a second DE-52 column (1 cm × 10 cm; 6.5 ml resin bed volume) which was preequilibrated with buffer A. Amylase activity was eluted as described above in a total gradient volume of 70 ml. The salt concentration in the eluate was determined by measuring the conductivity (Conductivity Bridge, model 31, Yellow Spring Instrument Co., Inc.). Fractions containing enzyme activity were pooled and concentrated 3-fold using an Amicon ultrafiltration PM 30 membrane. The amylase fraction then was dialyzed overnight twice against 500 ml of 20 mM Bis-Tris-Propane buffer (pH 6.5), containing 2 mM DTT and 10% glycerol (buffer B).

FPLC[4]-Mono Q Column Chromatography. The dialyzed solution was centrifuged at 16,000*g* for 10 min and the clear solution was divided into aliquots each containing 10 mg protein. The aliquots were chromatographed on a FPLC Mono Q prepacked HR 5/5 anion exchange column (5 mm × 5 cm, Pharmacia, Uppsala, Sweden) equilibrated with buffer B. After the sample was loaded onto the column, the column was washed with 3 ml of above buffer and then eluted with a 30 ml linear KCl gradient (0.10–0.35 M) in buffer B at a speed of 0.5 ml/min.

[4] Abbreviation: FPLC, fast protein liquid chromatography; BCA, bicinchoninic acid.

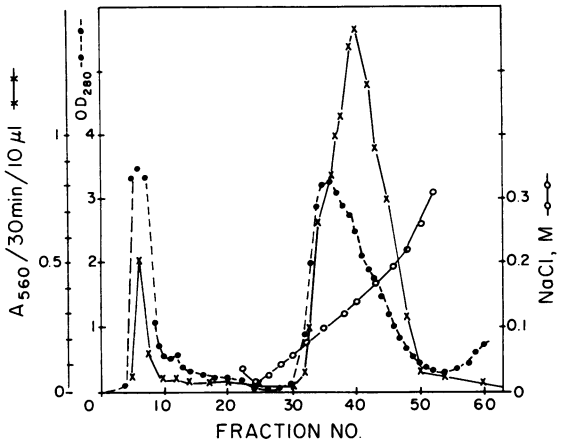

FIG. 2. Second DEAE-cellulose column chromatography of the 3 to 10% PEG fraction. The fractionation procedure is described in "Materials and Methods."

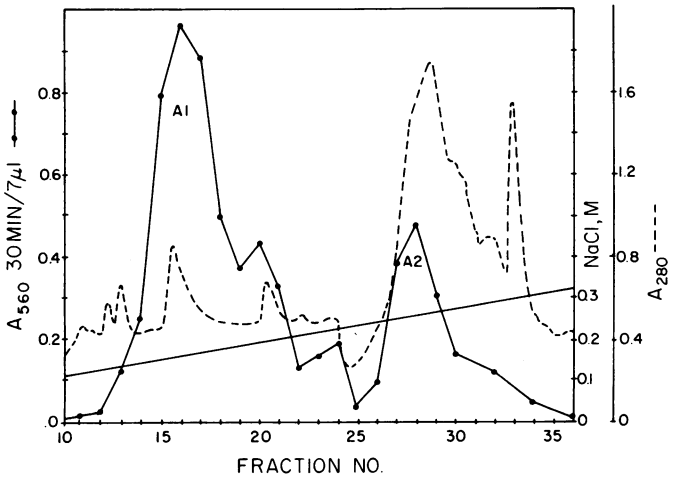

FIG. 3. Elution profile of *Arabidopsis* leaf amylase on FPLC Mono Q. The sample was prepared from the 3 to 10% PEG fraction and chromatographed on DE-52 twice as indicated in the text and in Figure 2. Mono Q fractions were assayed for amylase activity. Fractions 15 to 18, and 27 to 29 were pooled separately and designated as A1 and A2, respectively.

Active fractions were collected and concentrated as mentioned above.

The solution containing the 15 to 35% PEG precipitate was chromatographed on a DE-52 column, 1 × 10 (6.5 ml resin bed volume). About 80 mg of protein was adsorbed onto the column which was equilibrated with 20 mM Bis-Tris-propane (pH 8.0). The column was washed with 20 ml of the equilibration buffer and then the amylase was eluted with a linear gradient (0–0.3 M NaCl) of 66 ml in the above buffer. The active fractions were pooled and concentrated as indicated above. The purified amylase activity was used for the experiments reported in this paper.

Enzyme Assays. Amylase activity was measured in 1 ml reaction mixtures containing 5 mg of amylopectin, 40 µmol sodium acetate or succinate buffer (pH 6.0) and enzyme (14). The reaction mixture was incubated for 30 min at 37°C and the amount of reducing power was measured by the method of Nelson (13) using maltose as a standard. R-enzyme was assayed under the same conditions except that 5 mg of pullulan was used instead of amylopectin. The products generated by R-enzyme were quantified by measuring reducing power (13). D-enzyme was measured in 250 µl reaction mixtures containing 10 µmol sodium acetate or succinate buffer (pH 6.0), and 2.48 µmol

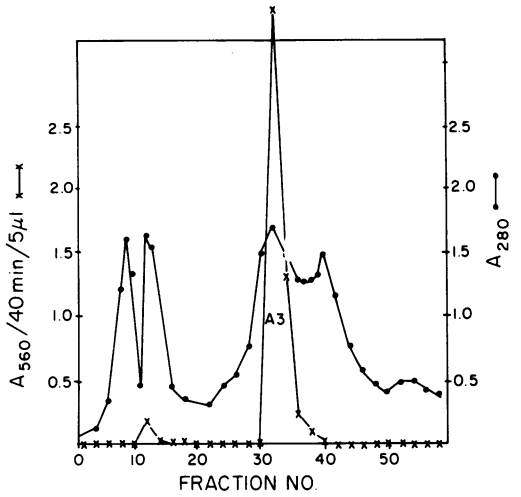

FIG. 4. Fractionation of amylase activities in the 15 to 35% PEG fraction by DEAE-cellulose chromatography. The purification procedure is described in "Materials and Methods." Fractions 30 to 33 were pooled and designated as A3.

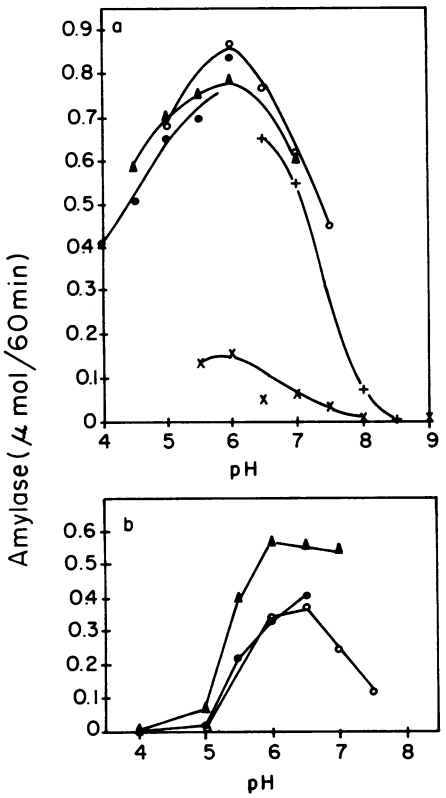

FIG. 5. pH optimum of amylase A3 (a), and amylase A1 (b). Buffers used were Na^+-acetate (●), succinate (▲), Mes (○), Hepes (+), and Bis-Tris-Propane (×).

maltotriose (16). The reaction mixtures were incubated at 37°C for 30 min and terminated by immersing the reaction tubes in boiling water for 30 s. Released glucose was measured by following the reduction of NADP in the presence of hexokinase and glucose 6-P dehydrogenase (9). Phosphorylase was assayed according to Okita *et al.* (14). Glucose 1-P produced was measured by following the reduction of NADP using P-glucomutase and glucose 6-P dehydrogenase (9).

End-Product Analysis. Twenty to 40 µl aliquots of the amylase or D-enzyme assay reaction mixture (enzyme activity was about 0.12 unit per ml) were applied to Whatman No. 1 paper. Sepa-

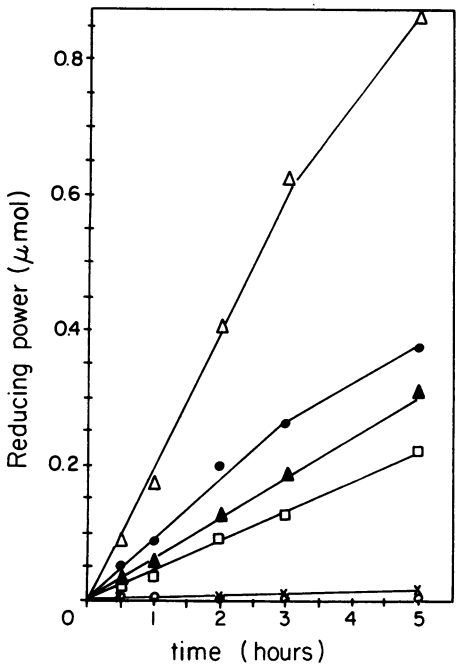

FIG. 6. Substrate specificity of amylase A3. Amylase activities were incubated with 5 mg/ml glucan. At indicated intervals, samples were withdrawn and reducing power measured using maltose as a standard. (●), Amylose; (△), amylopectin; (×), β-limit dextrins; (○), pullulan; (▲), shellfish glycogen; (□), rabbit liver glycogen.

ration was conducted in a descending direction in a butanol:pyridine:water (6:4:3) for about 30 h. Reducing sugars were identified by the method of Trevelyan *et al.* (26).

Electrophoresis and Amylase Activity Stain. Electrophoresis was performed according to Davis (3) using a 3% stacking gel and 7% running gel. The electrophoresis was conducted at 20 mA until the tracking dye had passed the stacking gel and 25 to 30 mA thereafter for a total 5 to 6 h. The gel was washed in distilled H_2O and incubated in 2% soluble starch, 0.1 M sodium acetate buffer (pH 5.0) at 37°C for 2 h. The gel was then washed with distilled H_2O to remove the excess starch solution and the gel left in a capped container for 2 h at 37°C. The gel was stained in 0.2 M HCl containing 5.7 mM I_2 and 43.3 mM KI. The activity bands were visible within 1 min of staining.

Native gel electrophoresis was routinely used to assess the identities of the amylases. Originally a starch solution of pH 6.0 was used for incubating the gel but the banding pattern was poor. A preliminary study was undertaken to see the effect of pH on the activity band. The results consistently showed that pH 5 or even lower gave the best result. At higher pHs, pH 6.0 or above, an optimum pH for hydrolysis, not only are the bands less intense but sometimes some of the bands are missing. This is probably because the pH optimum for the reaction is obtained only after incubating the gel in low pH. The gels have been made of 367 mM Tris-HCl at pH 8.9 and also have been saturated with running buffer, pH 8.3 for several hours. For this reason the gels were always incubated in a starch solution at pH 5.0.

Viscometric Analysis. Viscosity measurements were made at 37°C with 3 ml incubation mixtures containing 2% amylopectin, 40 mM Na^+ acetate (pH 6.0) and enzyme of 0.01 to 0.03 unit (μmol/min) per ml using an Ostwald viscometer to monitor specific viscosity (η_{sp}).

To estimate the relative contribution of endolytic or exolytic hydrolysis by the partially purified enzymes, the increase in specific fluidity, ϕ_{sp}, of the reactions was plotted against the concomitant increase in reducing power and the slope calculated (10). Porcine pancreatic α-amylase and sweet potato β-amylase were used as standards for comparison and exhibited slopes of 0.71 and 0.009, respectively.

Protoplast and Chloroplast Preparation. Chloroplast isolation procedures were adapted from the method of Somerville *et al.* (21). Protoplasts were prepared by chopping 5 to 8 g leaves with a razor blade. The leaves were then floated on 50 ml of buffer containing 0.5 M sorbitol, 10 mM Mes (pH 5.5), 1 mM $CaCl_2$, 1.6% macerase, and 1.6% cellulysin. The mixture was incubated for 1 h, at which time the buffer was removed and replaced with 50 ml fresh buffer. The digestion was continued for 2 h. The protoplasts were collected by filtration through a layer of Miracloth, chilled at 4°C, and pelleted by centrifugation for 4 min at 100 to 200*g* using an HS4 rotor. Protoplasts were resuspended in 5 ml of 0.5 M sorbitol, 40% Percoll, 10 mM Mes (pH 6.0), and 1 mM $CaCl_2$. Percoll gradients of 5 ml each of 20, 10, and 0% were layered on top of 40% Percoll and centrifuged at 400*g* for 10 min in HS4 rotor. The protoplast layer (interface of 10 and 20% Percoll) was removed with a Pasteur pipette and diluted into 2 volumes of 0.5 M sorbitol, 10 mM Mes (pH 6.0), 1 mM $CaCl_2$. Protoplasts were pelleted again by centrifugation for 5 min at 100*g* and resuspended in 50 ml of disruption buffer containing 0.3 M sorbitol, 20 mM Tricine (pH 8.4), 12 mM EDTA, 0.1% BSA. The suspension was transferred with a pipette into a 5 ml syringe from which the end had been removed and replaced with 15 μm nylon net, and was gently expelled through the net. Chloroplasts were collected by centrifugation for 120 s at 270*g*. The supernatant was used as the cytoplastic fraction.

Resuspended chloroplasts, in 0.5 to 1.0 ml of the disruption buffer mentioned above were layered on 24 ml of Percoll gradient, 10 to 80% (v/v) containing 1% Ficoll 400, 3% PEG 8,000, 0.33 M sorbitol, 50 mM Hepes (pH 8.3), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ascorbic acid, and 2 mM DTT (12). The gradients were centrifuged at 10,000*g* for 8 min. Intact chloroplasts were collected and diluted five times with the buffer containing 375 mM sorbitol, 35 mM Hepes (pH 8.3), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM DTT. The chloroplasts were collected by centrifugation to 4,000*g* in a SS-34 rotor and then decelerated to a stop. The chloroplasts were suspended and disrupted with 1 ml of 10 mM Tris-HCl (pH 7.5) and microfuged in an Eppendorf centrifuge for 5 min. Chl was measured by the method of Arnon (1), PEP carboxylase activity assay was according to Wong and Davis (28), and RuBPase was assayed according to Pierce *et al.* (18).

Protein Assay. Protein concentration was determined by the method of Smith *et al.* (20) using the Pierce Chemical Co. prepared BCA reagent and BSA as the standard. Absorbance at 280 nm also was used to estimate the protein concentration for the eluates eluted from the column.

RESULTS

Purification of Amylases. Native gel electrophoresis of crude homogenates of *Arabidopsis* leaf shows that there are three groups of amylases present and these groups are defined according to their R_F values as being group A (R_F 0.55), group B (R_F 0.44), and group C (R_F 0.25) (Fig. 1, lane A). Group C amylases have very low activity with amylopectin, and will not be discussed further. The purification of group A and B amylases are summarized in Table I.

Polyethylene glycol fractionation of the crude homogenate separated the amylases into two fractions. The PEG 3 to 10% fraction contained predominantly the group B amylases (Fig. 1, lane B) whereas the 15 to 35% fraction contained the group A and C amylases (Fig. 1, lane E).

The PEG 3 to 10% fraction was chromatographed on DE-52 twice. Some of the contaminating protein and group A amylase can be further removed by the second DE-52 column chromatography. The group A amylase contaminating the 3 to 10%

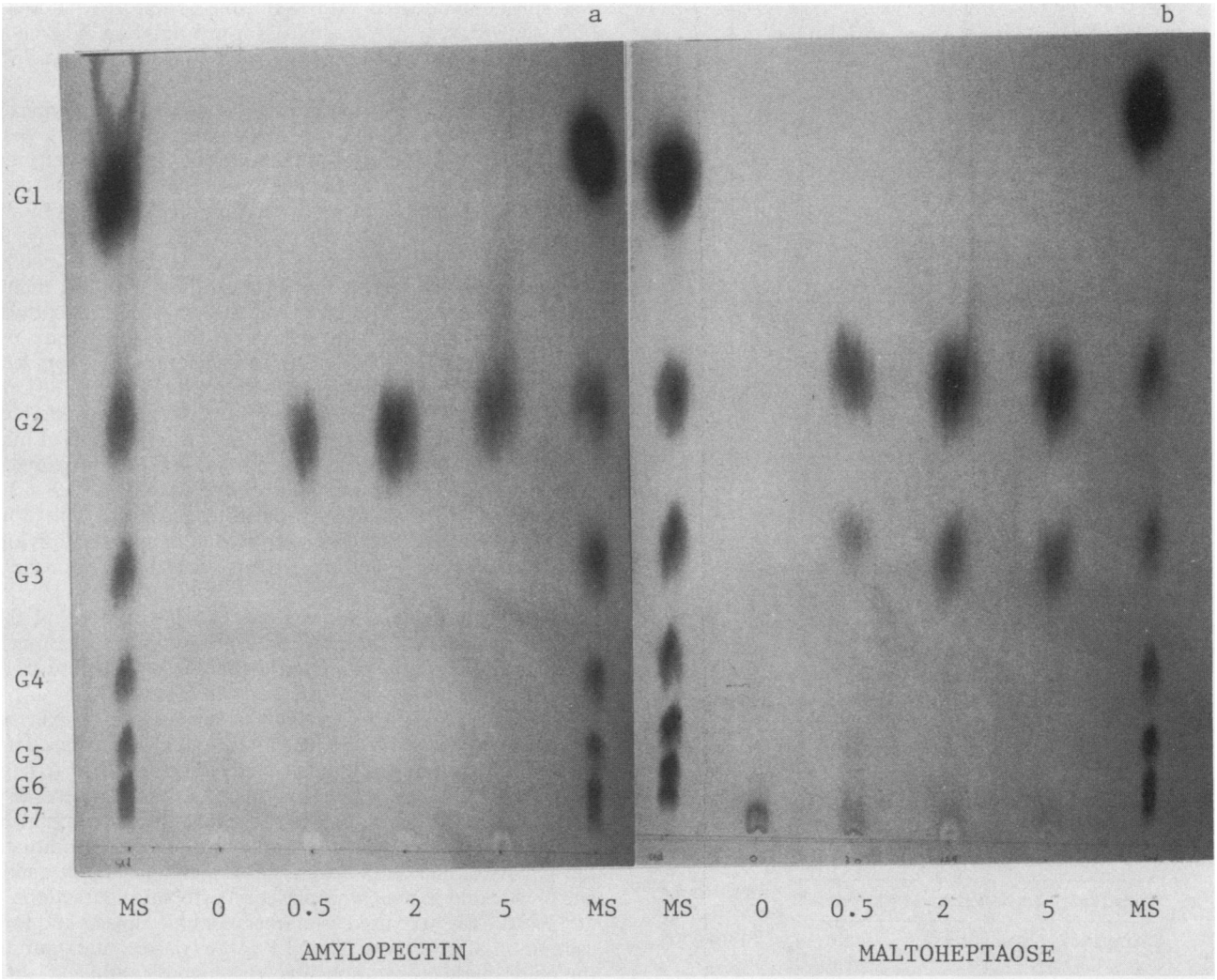

FIG. 7. Pattern of action of amylase A3 on amylopectin and maltoheptaose. The reaction mixture contained 5 mg/ml substrate, 40 mM Na acetate buffer (pH 6.0), and 0.12 activity units (μmol/min) of amylase A3 per ml. Incubation time in hour and substrate are listed under the paper chromatograms. MS, maltodextrin standards.

PEG fraction did not bind to DE-52 at pH 7.0 whereas the group B amylases did bind. One major activity was eluted at about 140 mM NaCl (Fig. 2). This amylase activity largely overlapping with D-enzyme activity, was further chromatographed by ion-exchange on a Mono Q column using FPLC. Two major peaks of activity were found, and these fractions were designated A1 and A2, in order of their elution (Fig. 3). The procedure gives the high resolution necessary to separate the amylases which elute as a single peak from the DE-52 column.

In the PEG 15 to 35% fraction, amylase A3 is the major activity. At pH 8.0, amylase A3 adsorbed onto a DE-52 column, and a single peak of activity was obtained (Fig. 4).

Characterization of Partially Purified Amylase A3 (Derived from 15–35% PEG Fraction), and Amylases A1 and A2 (Derived from 3–10% PEG Fraction). These groups of amylases partially purified from the DE-52 and Mono Q columns were used for study.

pH Optimum. The effect of pH on the enzymic activity of these two groups of amylase was determined from pH 4 to 8.5. A3 has a pH optimum of 6.0 (Fig. 5a) and in acetate, succinate, and Mes buffers there was comparable hydrolysis at pH 6.0. Bis-tris-propane and Tris are poor buffers due to the interaction of the buffer with the Somogyi reagent and thus causing a very low color yield.

The amylase of the PEG 3 to 10% fraction which was eluted from DE-52 and amylase A1 exhibit a pH optimum about 6.0 to 6.5 (Fig. 5b) and has higher activity in succinate buffer than acetate buffer. Acetate buffer gives about 50 to 60% activity compared with succinate buffer at pH 6.0. At pH 4.0, almost no activity can be detected for this preparation while the amylase A3 still has about 50% of the activity seen at pH 6.0. Amylase A2 shows a similar pH response as A1 and with a pH optimum about pH 6.0 to 6.5 (not shown).

Substrate Specificity, Action Pattern, and Viscometric Analysis of Amylase A3. Amylase A3, the major activity in the PEG 15 to 35% fraction, comprises about 65 to 80% of the total amylolytic activity in the leaf homogenate. When resolved on a 7% electrophoretic gel, amylase A3 appears as a clear band with R_f values of about 0.55 (Fig. 1, lane F). Amylase A3 has higher activity with amylopectin than with the other glucans tested (Fig. 6). Amylose is a better substrate than shellfish glycogen and rabbit liver glycogen. Almost no hydrolysis can be detected with pullulan and β-limit dextrins.

Paper chromatography analysis of the products generated from amylopectin hydrolysis revealed only maltose (Fig. 7a). Maltose and maltotriose were generated from maltoheptaose (Fig. 7b). Maltopentaose, was also observed. Glucose was not detected after prolonged incubation of maltodextrins and A3. When the

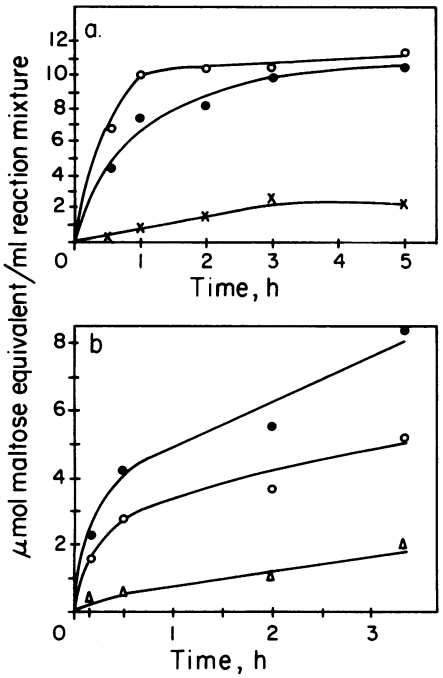

FIG. 8. Action of amylases on maltodextrins. Amylases activities were incubated with 5 mg/ml maltodextrin, 40 mM Na^+-succinate (pH 6.0). At indicated interval, samples were withdrawn and reducing power measured. a, Amylase A3; b, amylase A1; (●), amylopectin; (×), maltotriose; (○), maltopentaose; (△), maltotetraose.

Table II. *Properties of Amylase Fractions A_1, A_2, and A_3 and the Extent of Their Contamination with D-Enzyme Activity*

Fraction	Activity Ratios with Glucans			Slope of ϕ_{sp} *versus* Reducing Equivalents
	D-Enzyme Amylase	*Amylopectin*[a] Amylose	*Amylopectin*[a] β-Limit dextrin	
A1	0.006	13.6	17.4	0.013
A2	1.11	7.7	3.5	0.47
A3	0	2.4	73	0.013
Pancreatic α-amylase				0.71
Sweet potato β-amylase				0.009

[a] The reaction mixtures contained 5 mg/ml substrate, 40 mM Na succinate (pH 6.0), and amylase. The activity ratios of amylases using different substrates were calculated from the μmol reducing equivalents produced.

hydrolysis of substrates was followed by measuring the release of reducing equivalents, maltotriose was shown to be a poor substrate compared to amylopectin (Fig. 8a). When compared with amylopectin as the substrate for A3, oligosaccharides with chain length greater than maltotetraose have comparable or even higher rates of hydrolysis (Fig. 8a). This has not been observed for A1 and A2.

Viscosity changes during a 2 h incubation of amylopectin with amylase A3 were recorded. The slope of specific fluidity *versus* μmol reducing equivalent produced is 0.013 (Table II). This is similar to the slope 0.009 of sweet potato β-amylase and very different from the value of 0.71 obtained with pancreatic α-amylase.

The results of the three different experimental approaches described above suggest that amylase A3 is an exoamylase.

Action Pattern and Viscometric Analysis of Amylases A1 and A2. Amylase A1 showed no detectable D-enzyme activity, however, even after repeated FPLC chromatography, amylase A2 was still contaminated with D-enzyme (Table II). A1 and A2 both showed very low reactivity toward amylose. The activity ratio of amylopectin to amylose were 13.6 for A1 and 7.7 for A2.

Gel electrophoresis showed that A2 yielded an activity band of R_F 0.44 which stained red after iodine staining. A1 yielded bands of R_F 0.23 and most of the time can hardly be detected in the crude and 3 to 10% PEG fractions.

Chromatographic analysis of the products generated by incubating A1 with amylopectin revealed only maltose (Fig. 9A). Maltose and maltotriose were observed as the major products when maltoheptaose was the substrate (Fig. 9b). No maltotetraose was observed. This may indicate that A1 is an exoamylase. A1 was also studied with respect to the reaction rate using different maltodextrins as substrate. The longer the chain length of the maltodextrin, the higher the reaction rate (Fig. 8b). Maltotriose and maltotetraose are poor substrates. Almost no reducing equivalents could be detected after 1 h incubation of maltotriose with 0.12 unit of enzyme per ml reaction mixture. The same patterns of action were obtained from amylase A2. A2 can be distinguished from A1 by the high reactivity with β-limit dextrins (Table II). Analyses using β-limit dextrins or amylopectin as the substrate reveals the production of maltose and glucose (Fig. 9C and 9D).

Viscometric analysis showed that A1 has a slope of 0.013 (Table II). This is consistent with the above result suggesting that A1 is an exoamylase. Viscometric analysis gave a slope of 0.47 for A2, indicating that amylase A2 is an endoamylase.

Chloroplast Isolation and Compartmentation of Amylases. A chloroplast preparation completely without cytoplasmic contamination is critical for the amylase study since the amylase activity largely resides in the cytoplasmic fraction. It is also necessary to have high purity chloroplasts in order to study the compartmentation of the different forms of amylases. Table III summarizes the activity of marker enzymes, starch synthetic and degradative enzymes found in the chloroplast and cytoplasmic fractions. Chl and RuBPCase were used as markers for chloroplasts and for the estimation of intactness. The PEP carboxylase contamination in the chloroplast preparation was less than 1%, and 82% of the intact chloroplasts were recovered. ADPglucose pyrophosphorylase and starch synthase are only localized in the chloroplast and with the same activity distribution as RuBPCase activity. Nineteen percent of the total amylase activity was found in the chloroplasts. Only 4% of the starch phosphorylase was found in the chloroplast. In contrast, most of the D-enzyme is localized in the chloroplast.

When the cytoplasmic fraction was used for viscometric analysis, the slope of ϕ_{sp} *versus* reducing equivalent was 0.013, the same value obtained for amylase A3. When the cytoplasmic fraction was electrophoresed, a clear band of R_F 0.55 was the only detectable amylase activity with the same R_F as amylase A3 (Fig. 1, lane G). When the chloroplast extract was resolved via native gel electrophoresis, a red band with an R_F of 0.44 was the only amylase activity observed (Fig. 1, lane H). These results indicate that the endoamylase A2 is localized solely in the chloroplast. The exoamylase A3 which is the major amylolytic activity in the leaf homogenate, is associated only with cytoplasmic fraction.

Other Properties of Amylases. The heat stability of amylases have been studied. Amylases A1, A2, and A3 lost their activities when the enzyme preparations are initially incubated at 60°C for 10 min either in the presence or absence of 20 mM $CaCl_2$.

Several pseudo-oligosaccharides known as inhibitors for amylase and glucosidases were tested for their effect. Incubating amylase A1, A2, or A3 with acarbose, Bay e 4609 or 1-deoxnojirimycin (200 μg/ml) or α-amylase inhibitor from wheat germ (4 inhibition units/ml, Sigma) had no effect on amylolytic activ-

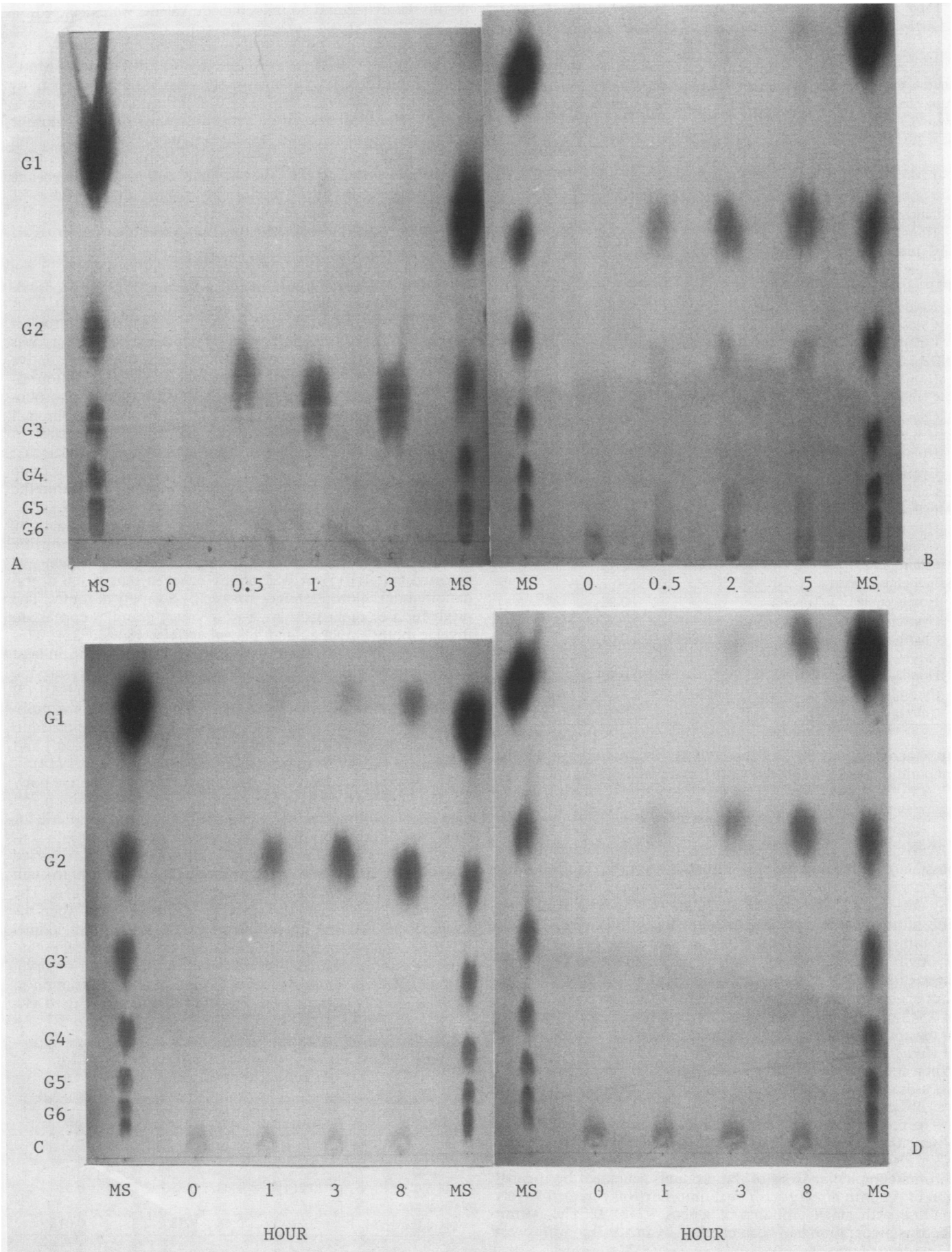

FIG. 9. Pattern of action of amylase A1 (A and B) and amylase A2 (C and D). The reaction conditions are those described for the legend of Figure 7, except that Na^+-acetate was replaced by Na^+-succinate buffer. MS, maltodextrin standards. The substrate used for A and C, amylopectin; B, maltoheptaose; D, β-limit dextrins.

Table III. *Distribution of Starch Synthetic and Degradative Enzyme Activities in the Chloroplast Fraction Isolated by 10 to 80% Percoll Gradient Centrifugation*

The experiment was started with 19 g fresh leaves. The Chl recovered in the cytosolic and chloroplast fractions were 0.045 and 0.40 mg, respectively. The protein recovered in cytosolic and chloroplast fractions were 6.3 and 3.3 mg, respectively.

Enzyme	Activity		Activity in Chloroplast
	μmol/min unit/mg Chl		%
Ribulose bisophosphate carboxylase			
Homogenate		16.5	
Cytosolic	0.58		
Chloroplast	2.62	6.6	82
PEP carboxylase			
Homogenate		0.41	
Cytosolic	0.22		
Chloroplast	0.0012	0.003	0.5
ADPglucose pyrophosphorylase			
Homogenate		0.75	
Cytosolic	0.03		
Chloroplast	0.15	0.39	83
Starch synthase			
Homogenate		0.056	
Cytosolic	0.0044		
Chloroplast	0.018	0.044	80
Amylase			
Homogenate		1.44	
Cytosolic	0.33		
Chloroplast	0.08	0.12	19
Starch phosphorylase			
Homogenate		0.22	
Cytosolic	0.11		
Chloroplast	0.0044	0.011	4
D-enzyme			
Homogenate		0.40	
Cytosolic	0.031		
Chloroplast	0.12	0.31	80

ity, whereas about 90% of the activity was inhibited when the preparations were incubated with 1 mM *N*-ethylmaleimide.

DISCUSSION

Amylase A2, the chloroplastic amylase, was thought to be a possible debranching enzyme initially; however, the following observations do not support this conclusion. A2 does not have any detectable activity toward pullulan. A2 can hydrolyze amylose, although at a lower rate than amylopectin (Table II), while the commercial pullullanase (from *Aerobacter aerogenes*) has no reactivity toward amylose. The absorption spectrum of glucans digested by A2 and then stained with iodine did not show the shift of absorption maximum from λ560 to λ570 for amylopectin or λ545 or λ565 for β-limit dextrins (data not shown). The shifts in the absorption maximum are the characteristics of pullulanase of *Aerobacter aerogenes* or R-enzyme of spinach leaves (7, 15). When amylopectin was used as a substrate for viscometric analysis, a dramatic decrease of the viscosity in the early incubation was observed.

The conclusion that A2 is an endoamylase was based on the observation that A2 can hydrolyze β-limit dextrin (Table II) and A2 gives a slope of 0.47 in the viscometric analysis. However, chromatographic analysis of the products generated by incubation of A2 with amylopectin or β-limit dextrins revealed largely maltose with small amounts of glucose (Fig. 9). The β-limit dextrins preparation had been checked by incubating with sweet potato β-amylase and no reducing equivalents were detected.

There is no direct evidence for compartmentation of exoamylase A1. A1 is the major activity in the PEG 3 to 10% fraction and comprises 85% of the amylase activity eluted from the Mono Q column (Table I). A1 shows much higher amylolytic activity in sodium succinate buffer (pH 6.0) than in the sodium acetate buffer (pH 6.0) (Table IV). This is different from the cytosolic amylase A3 which shows similar amylolytic activity in both buffers. The chloroplastic preparation always give 30 to 40% higher hydrolytic activity in succinate buffer than in acetate buffer at pH 6.0. These results may suggest that amylase A1 probably also is present in the chloroplast and contributes to the higher activity of chloroplastic fraction in succinate buffer at pH 6.0.

This study shows that the starch degradative activity in the chloroplast of *Arabidopsis* includes endoamylase (A2), D-enzyme, starch phosphorylase, and possibly exoamylase A1. This system, however, is different from that in the spinach chloroplast (14) in several aspects. Surprisingly high D-enzyme activity was found in the *Arabidopsis* chloroplast whereas other degradative enzymes are mostly localized in extrachloroplastic fraction especially the starch phosphorylase. This distribution of phosphorylase largely reduces the role of starch degradation through phosphorolysis of starch to glucose 1-P in the chloroplast in *Arabidopsis*. No debranching enzyme activity has been detected in the leaf homogenate or in the chloroplastic fraction. It is quite possible that the R-enzyme activity is too low and not within the sensitivity of the assay.

The ratio of D-enzyme activity to the total amylolytic activity in the chloroplasts is 1.5:1 (Table III). This high D-enzyme activity also has been observed in pea chloroplasts, which was reported as 1.1:1 (5). It is generally believed that starch degradation in the chloroplast may proceed by a variety of routes. The production of monosaccharide from intact granules appears to involve both amylases and phosphorylases (8, 17, 22). This scheme should be extended to include transglycosylation, at least in pea and *Arabidopsis*, since the ratio of activity of D-enzyme to phosphorylase in pea chloroplast was reported to be 8.4:1 (5) and 28:1 in *Arabidopsis* (Table III). Transglycosylation is probably a minor pathway in the spinach chloroplast, since the ratio of D-enzyme activity to the total amylolytic activity is 0.06:1 and the ratio of D-enzyme to phosphorylase activity is 0.25:1 (14).

The resulting colors of amylase activity bands on native polyacrylamide gel which have been treated with starch solution and then stained with iodine solution have been used as a criteria for amylase identification in the literature (5, 6, 29). Amylose generated by the action of debranching enzyme has been reported to stain blue and β-limit dextrin formed by β-amylase to stain red, and α-limit dextrins by α-amylase are not stained. The exoamylase, amylase A3, however, yielded a clear ban when the gel was incubated with 2% soluble starch and stained with iodine. A2, the endoamylase however, gave a red band. The sweet potato β-amylase showed a green-brown band on the gel (our observation and Ref. 6). Thus, the color formation and the relation to amylase identity is variable and by itself should not be used as a

Table IV. *Amylolytic Activity Measured in Sodium Succinate Buffer (pH 6.0) and Sodium Acetate Buffer (pH 6.0) for the Different Amylase Preparations*

The assay conditions are described in "Materials and Methods.

Buffer	Homogenate	A1	A2	A3	Chloroplast
	μmol/min·ml				
Na^+ succinate, pH 6.0	0.26	1.03	0.38	2.75	0.020
Na^+ acetate, pH 6.0	0.27	0.46	0.28	2.83	0.013

sole criterion for identifying the nature of the amylase (exo or endo).

Amylase A3, the exoamylase, constitutes about 80% of the total leaf amylolytic activity associated with the cytoplasmic fraction. Also about 80% of the total amylase activity present in spinach leaves appears to be extrachloroplastic in origin (14). Large amounts of extrachloroplastic amylase activity are also present in *Vicia faba* (2), barley (4), pea, wheat, *Chenopodium rubrum* (29). The preferential localization of amylase activity in the extrachloroplastic fraction may therefore be a phenomenon in most vascular plants. This provides, however, little insight into its physiological significance. Recently, Jacobsen *et al.* (4) in studying the effect of water stress on barley leaf demonstrated that extrachloroplastic α-amylase level increases as leaf water potential decreases and that this increase is related to elevated synthesis of α-amylase mRNA and protein. High levels of amylolytic activity have also been observed in *Arabidopsis*, especially in some starchless mutants, when they are grown under various conditions and this increased activity has been ascribed to changes in the level of amylase A3 (TP Lin, T Caspar, C Somerville, J Preiss, unpublished data). These studies may indicate that the bulk of amylolytic activity in the cytosolic fraction of higher plants may be regulated in response to environmental conditions, perhaps in addition to being controlled according to the availability of starch in the chloroplasts.

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