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Purification of a β-Amylase that Accumulates in *Arabidopsis thaliana* Mutants Defective in Starch Metabolism[1]

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ABSTRACT

Amylase activity is elevated 5- to 10-fold in leaves of several different *Arabidopsis thaliana* mutants defective in starch metabolism when they are grown under a 12-hour photoperiod. Activity is also increased when plants are grown under higher light intensity. It was previously determined that the elevated activity was an extrachloroplastic β-(*exo*)amylase. Due to the location of this enzyme outside the chloroplast, its function is not known. The enzyme was purified to homogeneity from leaves of both a starchless mutant deficient in plastid phosphoglucomutase and from the wild type using polyethylene glycol fractionation and cyclohexaamylose affinity chromatography. The molecular mass of the β-amylase from both sources was 55,000 daltons as determined by denaturing gel electrophoresis. Gel filtration studies indicated that the enzyme was a monomer. The specific activities of the purified protein from mutant and wild-type sources, their substrate specificities, and K_m for amylopectin were identical. Based on these results it was concluded that the mutant contained an increased level of β-amylase protein. Enzyme neutralization studies using a polyclonal antiserum raised to purified β-amylase showed that in each of two starchless mutants, one starch deficient mutant and one starch overproducing mutant, the elevated amylase activity was due to elevated β-amylase protein.

Arabidopsis thaliana leaves contain a β-(*exo*)amylase (EC 3.2.1.2) that constitutes a major portion of the crude amylolytic activity (12). Its role in transitory starch degradation is unclear, however, since it is located outside the chloroplast (12) and starch degradation is thought to occur within the chloroplast (18). Leaf β-amylases occur in a wide variety of plants but little is known about their physiological function. Chapman *et al.* (4) purified and characterized a β-amylase from *Vicia faba* vegetative tissue but did not determine its precise localization. Zeigler and Beck (24) found that the dominant β-amylases from pea, wheat, spinach, and *Chenopodium rubrum* leaves were extrachloroplastic, and that those from pea and wheat were confined to the vacuole. The major pea leaf β-amylase has been purified (13). How these extrachloroplastic amylases function in starch degradation or whether they have other functions is presently unknown.

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Recently, elevated activities of the *Arabidopsis* leaf β-amylase were observed in several mutants defective in starch metabolism (3). The activity, up to 40-fold higher than wild type, was only found when plants were grown under a 12 h photoperiod, and not when grown under 24 h light. It was of interest that both starch overproducing and starchless mutants contained the elevated activity. In several of the mutants the phenotype could be attributed to a specific enzyme deficiency. In pgmP-1 the plastid form of phosphoglucomutase is very low (2; L Li, J Preiss, unpublished results), and in adg2-1 and adg1-1 one or both subunits of ADPglucose pyrophosphorylase are missing, respectively (10, 11). It is therefore unlikely that any of the starchless or starch deficient phenotypes are a direct result of the elevated amylase activity. Rather, it is more likely that the inability to store and/or utilize starch effectively in the mutants causes a metabolic change that affects β-amylase activity.

In the present study, an attempt was made to determine whether the level of β-amylase protein in leaves of *Arabidopsis* mutants defective in starch metabolism is elevated or whether the enzyme is posttranslationally activated. The enzyme was purified and characterized from both pgmP-1 and wild-type leaves. Additionally, a polyclonal antibody was raised to the enzyme purified from pgmP-1 to aid in protein comparisons.

MATERIALS AND METHODS

Plant Material

For protein purification, the Columbia wild type and pgmP-1 mutant *Arabidopsis thaliana* plants were grown in a greenhouse in 5-inch pots (12). For comparisons of the mutants, plants were grown in a chamber at a constant temperature of 24°C under a 12/12 h light/dark cycle with a light intensity of 300 $\mu E/m^2$ s. Leaf tissue was harvested at early flowering and frozen at −80°C until used.

Enzyme Purification

All steps of this procedure were carried out at 4°C. One hundred to 150 g of tissue were ground in a mortar and pestle with liquid N_2. The powder was extracted twice in a total of two volumes of buffer A (50 mM Tris HCl [pH 7.5], 2 mM EDTA) per g fresh weight of tissue with sand. After the removal of cell debris by centrifugation at 16,000*g* for 20 min, 50% (w/v) PEG 8000 in buffer B (50 mM Mes [pH 6.0], 150 mM NaCl, 2 mM DTT) was added to the crude extract to

give a final PEG concentration of 12%. After stirring for 20 min the precipitate was collected by centrifugation at 10,000*g* for 15 min and discarded. More PEG was added to the supernatant to give a final PEG concentration of 30%. This 12 to 30% PEG precipitate was collected by centrifugation at 10,000*g* for 15 min, dissolved in buffer B, dialyzed twice against 1000 mL of buffer B overnight, and then clarified by centrifugation at 10,000*g* for 10 min. This fraction was passed through a CHA[2] affinity column (15 × 80 mm) prepared according to Vretblad (22). The sample was applied to the column at a flow rate of 0.4 mL/min. After washing the column with two bed volumes of buffer B, bound protein was eluted with 10 mg/mL CHA in buffer B. Active fractions were pooled and glycerol was added to a final concentration of 10% (v/v). The purified β-amylase was concentrated in a Centricon 10 μconcentrator (Amicon).

Native Mol Wt Determination

The native mol wt of β-amylase purified from wild-type *Arabidopsis* was determined by gel filtration on a Sephadex G75 column (1.4 × 45 cm) previously equilibrated with buffer B. Mol wt standards were bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A. Blue dextran was used to measure the void volume.

Enzyme and Protein Assay

Amylase activity was determined in 1 mL of 40 mM succinate (pH 6.0) with 5 mg/mL amylopectin as substrate unless otherwise noted. After 30 min at 37°C the reaction mix was immersed in a boiling water bath for 1 min. Reducing sugar was measured by the Somogyi assay (14). Units of activity are μmol/min. The activity was linear for at least 2 h. Protein was measured according to Smith *et al.* (16) using BCA reagent (Pierce Chemical) with BSA as the standard. Absorbance at 280 nm was used to estimate the protein concentration in column eluates.

Antiserum Preparation

Polyclonal antibodies were prepared in New Zealand white rabbits by subcutaneous injection of 300 μg of purified β-amylase in Freund's complete adjuvant, followed 4 weeks later by a second injection of 100 μg of protein in Freund's incomplete adjuvant. Ten days later blood was collected and serum was obtained by incubating the blood for 60 min at 37°C, and then overnight at 4°C. The resulting clot was removed by centrifugation at 5000*g* for 15 min.

Neutralization of Enzyme Activity with Antiserum

Enzyme activity neutralization assays were done according to Holmes *et al.* (6) except that the reaction mix contained 0.05 units of β-amylase in 50 mM Hepes (pH 7.0), 150 mM NaCl, and varying amounts of antiserum diluted with preimmune serum. After incubation at 22°C for 30 min the reaction mix was centrifuged at 15,000*g* for 10 min. Amylase activity in the supernatant was determined as above.

[2] Abbreviation: CHA, cyclohexaamylose.

Electrophoresis and Western Blotting

Proteins were separated by SDS-PAGE in a minigel apparatus (Bio-Rad) according to Laemmli (9). The stacking and separating gels contained 4 and 12% acrylamide, respectively. Native proteins were separated by PAGE in a Protean II apparatus (Bio-Rad) as described by Kakefuda and Duke (7). Zymograms of amylase activity after native PAGE were done according to Kakefuda and Duke (7) using soluble starch incorporated in the transfer gel. Proteins were located after both native and SDS-PAGE using silver stain (23). Western blots were performed as described by Towbin *et al.* (21). Nitrocellulose paper was blocked after transfer of proteins with 3% (v/v) liquid fish gelatin (Sigma) in PBS. The primary antibody, anti-β-amylase serum, was diluted 1:10,000, and the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (Boerhinger), was diluted 1:3000.

RESULTS

β-Amylase Purification

Arabidopsis leaves contain at least three major amylases (12). Several other minor amylases are observed when leaf proteins are separated by native PAGE and located by transfer through starch containing gels followed by iodine staining (JD Monroe, G Kakefuda, unpublished observations). The elevated amylase activity observed in mutants of *Arabidopsis*

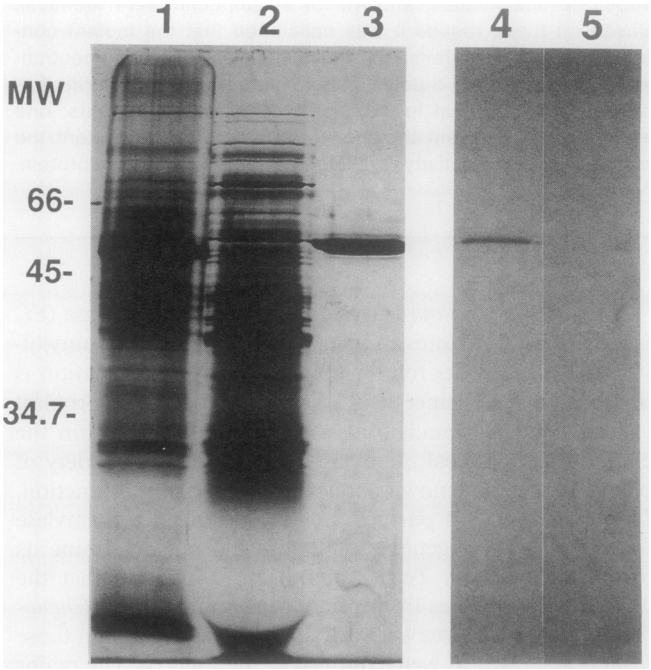

Figure 1. SDS-PAGE of each step of the purification of β-amylase from pgmP-1 plants, and a Western blot of crude extract. Proteins in lanes 1 to 3 were visualized with silver stain; lane 1, crude extract (2 μg protein); lane 2, PEG 12 to 30% precipitate (2 μg protein); lane 3, CHA column eluate (0.5 μg protein). Proteins in lanes 4 and 5 were transferred to nitrocellulose and probed with: lane 4, anti-β-amylase serum; lane 5, preimmune serum. Lanes 4 and 5 contained 35 μg of protein from crude extracts of pgmP-1 plants.

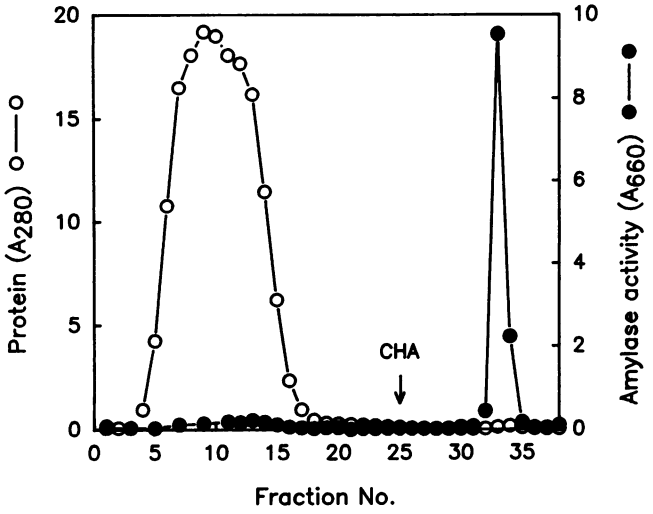

Figure 2. Affinity chromatography of β-amylase from pgmP-1 plants on a CHA column. The flow rate was 0.4 mL/min. After applying the sample, the column was washed with two bed volumes of buffer B (see "Materials and Methods"). At the arrow, 10 mg/mL CHA in buffer B was added to elute the activity. Relative enzyme activity was determined using the Somogyi assay (14). Three microliters of each fraction were assayed for 10 min and the results are expressed as the absorbance at 660 nm.

defective in starch metabolism was identified by Casper *et al.* (3) as a β-amylase based on the result that it comigrated with β-amylase A3 previously characterized by Lin *et al.* (12). In this study, activity staining of proteins after native PAGE was used to confirm that the amylase being purified was β-amylase A3.

Mutant material was used in the initial purification because of its high activity of β-amylase. Purification of the β-amylase activity to homogeneity was accomplished in two steps: PEG fractionation and CHA affinity chromatography (Fig. 1). A typical elution profile from the CHA affinity column is shown in Figure 2. CHA is a competitive inhibitor of β-amylase with a K_i of 40 μM (data not shown). The presence of soluble CHA in the purified enzyme fraction did not significantly affect subsequent measurement of enzyme activity because of the highly concentrated nature of the enzyme. After running the purified enzyme on native PAGE, a single band of activity comigrated with a single band of protein (Fig. 3). The molecular mass of the β-amylase determined from SDS-PAGE was 55,000 D. The native molecular mass of the protein as determined by gel filtration on a Sephadex G75 column corresponded to that of a spherical protein of 45,000 D (Fig. 4). Although the exact molecular mass can not be determined from these data, the native enzyme appears to be monomeric.

Following the same purification scheme, β-amylase A3 was also purified from wild type leaves. The yield of pure β-amylase activity from both pgmP-1 and wild type was approximately 45% of the crude amylase activity (Table I). The specific activity of amylase from pgmP-1 was about fourfold higher than that from wild type in the crude extracts and PEG precipitates, but after the affinity chromatography step the specific activity of the enzyme from both sources was about 1000 μmol/min·mg protein (Table I).

Comparison of β-Amylase from Wild-Type and pgmP-1 Plants

The substrate specificities of the pure enzyme from pgmP-1 and wild-type leaves were similar (Fig. 5). Activity was highest when the substrate was amylopectin. Rabbit liver glycogen, shellfish glycogen, and amylose were poorer substrates, and there was no activity with either β-limit dextrin or pullulan, as would be expected with a β-amylase. This same pattern of substrate specificity was previously observed

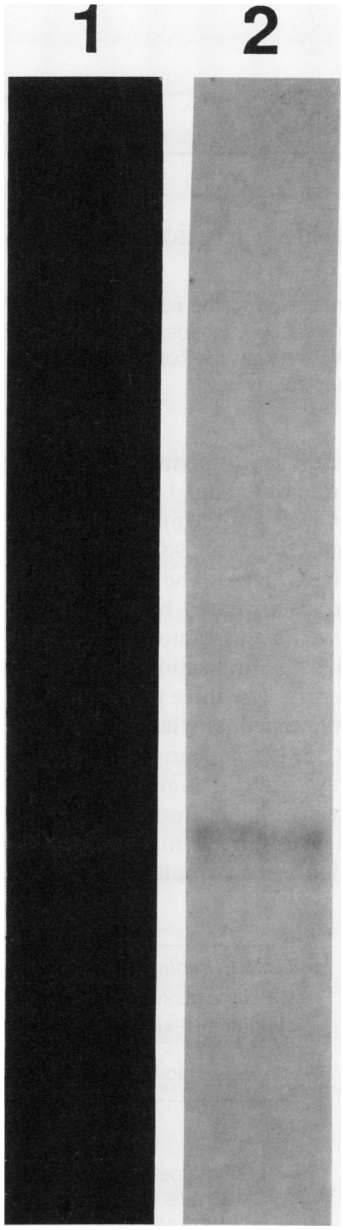

Figure 3. Nondenaturing PAGE of β-amylase purified from pgmP-1 plants. In lane 1, proteins were subjected to a second electrophoresis through a starch-containing gel followed by staining of the second gel with IKI (see "Materials and Methods"). In lane 2, the original gel was silver stained. Lane 1, 0.5 μg protein; lane 2, 2 μg protein.

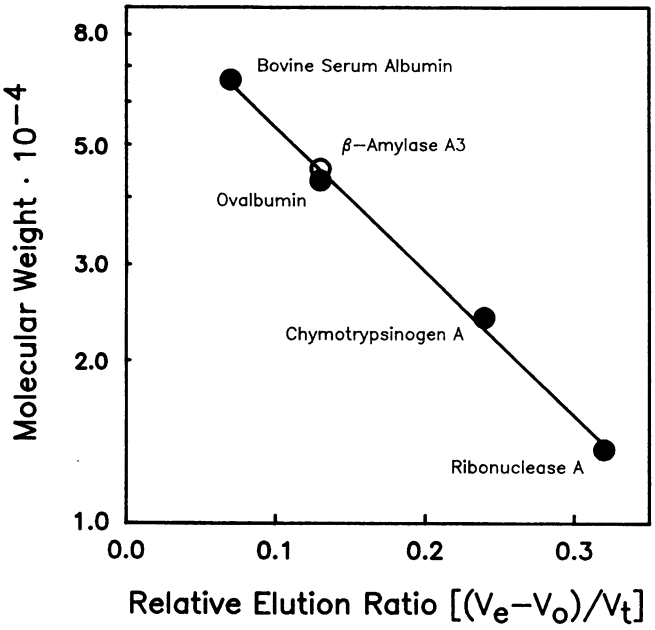

Figure 4. Determination of the molecular mass of β-amylase from wild-type *Arabidopsis* by Sephadex G75 gel filtration. $(V_e - V_o)/V_t$ is the volume to elute minus the column void volume divided by the total volume of the column.

by Lin *et al.* (12). The K_m for amylopectin of the pure enzyme from both sources was about 1 mg/mL (Fig. 6).

Western blot analysis of pgmP-1 crude extract separated by SDS-PAGE showed that anti-β-amylase serum binds specifically to β-amylase (Fig. 1). The binding of antiserum to wild-type crude extract separated by SDS-PAGE was similar to that of pgmP-1 extract (data not shown).

Enzyme activity neutralization was used to test the specificity of the antiserum. The three most abundant amylases from *Arabidopsis* were tested; amylase A1 and A2, isolated previously by Lin *et al.* (12), and amylase A3 purified in this study. Amylase A1 was identified as an extrachloroplastic β-amylase which was distinguishable from β-amylase A3 by substrate specificity and relative mobility on native PAGE. Amylase A2 was identified as a chloroplastic α-amylase (12). Only the activity of β-amylase A3 was inhibited by the antiserum (Fig. 7). Amylase A1 and A2 were not affected by anti-β-amylase serum at the concentrations used in this experiment.

The β-amylase A3 enzymes in crude extracts of wild type and pgmP-1 plants were then compared using enzyme neutralization analysis. Increasing amounts of anti-β-amylase serum inhibited the crude amylase activity from wild type and pgmP-1 plants in the same way (Fig. 8). The crude extracts used in this experiment were the same as those described in Table I. Even though the pgmP-1 material contained about fourfold higher activity than the wild-type material, the indistinguishable pattern of neutralization indicated that the two proteins were immunologically identical. The amount of antiserum required to inhibit 50% of the β-amylase activity was about 4 μL/unit enzyme for both crude extract (Fig. 8) and purified β-amylase (Fig. 7).

Immunological Studies of β-Amylase Expression

Several different *Arabidopsis* mutants known to have elevated β-amylase activity were compared with the wild type. Plants used in this experiment were grown in a growth chamber to reduce the variability in environmental conditions. In all of the mutants tested; pgmP1, adg1-1, adg2-1, and sop1-1, there was a 5- to 10-fold increase in amylase activity in crude extracts compared to wild-type leaves (Fig. 9A). The pattern of neutralization of the activity in all of the mutants by anti-β-amylase serum, however, was identical. One-half of the activity was neutralized by about 4 μL antiserum/unit enzyme (Fig. 9B). The highest level of neutralization observed in this particular wild-type extract was only 40% of the total activity. This was most likely due to the presence of other amylases in the crude extract that were not inhibited by the antiserum. Unlike the wild-type plants used for enzyme purification which were grown in a greenhouse under sunlight, the plants used in this experiment were grown in a growth chamber under a much lower light intensity. The specific activity of amylase in the crude extract of the chamber-grown wild-type plants was only 0.12 unit/mg protein compared to 2.63 units/mg protein in the greenhouse-grown plants. The fact that almost all of the activity in the greenhouse-grown wild-type plants was neutralized by the antiserum (Fig. 8) indicates that the difference between the total crude extract activity of the

Table I. *Purification Steps for β-Amylase from Wild Type and pgmP-1 Mutant Arabidopsis thaliana*

For the purification of wild-type β-amylase, 130 g fresh weight of leaves were used. For the purification of pgmP-1 β-amylase, 105 g fresh weight of leaves were used.

Fraction	Volume	Protein	Total Activity	Specific Activity	Yield	-Fold
	mL	*mg/mL*	*μmol/min*	*μmol/min/mg protein*	%	
Wild type						
Crude	275	3.13	2261	2.63	100	1
PEG 12–32%	25.5	3.59	1568	17.1	69	6.5
CHA	0.895	1.16	1008	969	45	368
pgmP-1						
Crude	173	2.51	4400	10.1	100	1
PEG 12–32%	13.3	3.48	2618	56.6	60	5.6
CHA	0.930	1.88	1901	1086	43	107

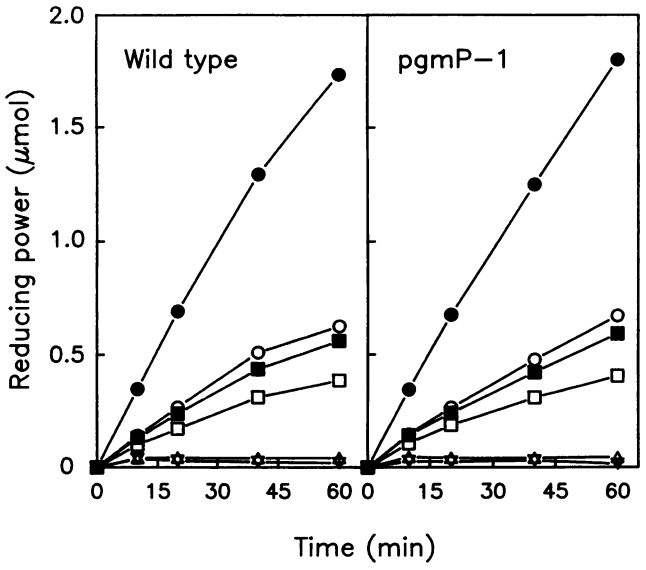

Figure 5. Substrate specificity of purified β-amylase from wild-type and pgmP-1 plants. Substrates were: (●), amylopectin; (○), rabbit liver glycogen; (■), shellfish glycogen; (□), amylose; (△), β-limit dextrin; (▽), pullulan, each at 5 mg/mL.

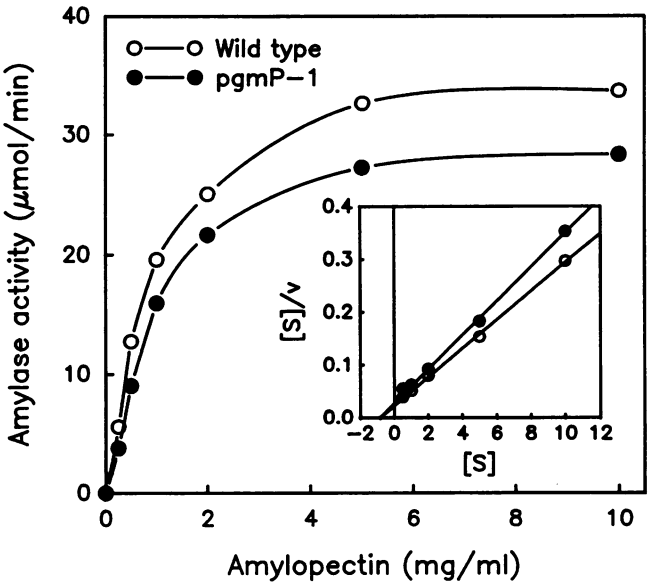

Figure 6. Saturation and Hanes-Woolf plots of purified β-amylase from wild-type and pgmP-1 plants with amylopectin as substrate.

two sets of plants can be attributed to higher β-amylase A3 in the greenhouse-grown plants.

Comparing β-amylase from pgmP-1 plants grown in the greenhouse with those from the growth chamber, greenhouse grown plants had more than 10-fold higher amylase activity in the crude extracts than growth chamber grown plants; 10.1 *versus* 0.9 units/mg protein, respectively (Table I; Fig. 9). The amount of anti-β-amylase serum required to inhibit 50% of the crude amylase activity, however, was the same; 4 μL antiserum/unit enzyme (Figs. 8, 9).

A Western blot of crude extracts from barley, pea, potato, spinach, and tobacco leaves separated by SDS-PAGE failed to show any cross-reaction with the antiserum to *Arabidopsis* β-amylase (data not shown).

DISCUSSION

Affinity chromatography using CHA bound to epoxy-activated Sepharose 6B, as described by Vretblad (22), proved to be an extremely useful step for purifying β-amylase from *Arabidopsis* leaves. Previous workers noted that β-amylase from leaves does not bind to CHA columns (19). We found that the addition of 150 mM NaCl to the buffer overcomes this problem. The same column was used repeatedly with consistent results.

The molecular masses of β-amylase from different sources are highly variable. *Vicia faba* leaf β-amylase is a tetramer of 26,000 D subunits (4). The sweet potato enzyme is also tetrameric but it has 64,700 D subunits (20). Other reported nonseed β-amylases are monomeric with molecular masses of 65,700 and 41,700 D in alfalfa roots (5), and 55,000 D in pea epicotyls (13). *Arabidopsis* leaf β-amylase is similar to alfalfa and pea being a monomer with a molecular mass of 45,000 to 55,000 D. However, the fact that polyclonal antiserum raised to *Arabidopsis* β-amylase does not appear to cross-react with leaf proteins extracted from barley, pea, potato, spinach, or tobacco indicates that β-amylases are a diverse group of enzymes.

The purified β-amylases from wild-type and pgmP-1 *Arabidopsis* were identical in each aspect studied including specific activity, mobility on SDS and native PAGE, substrate specificity, and K_m for amylopectin. For these reasons we suspected that the elevated activity observed in the mutants was due to an elevated level of the β-amylase protein and not due to a posttranslational activation of the enzyme. Neutral-

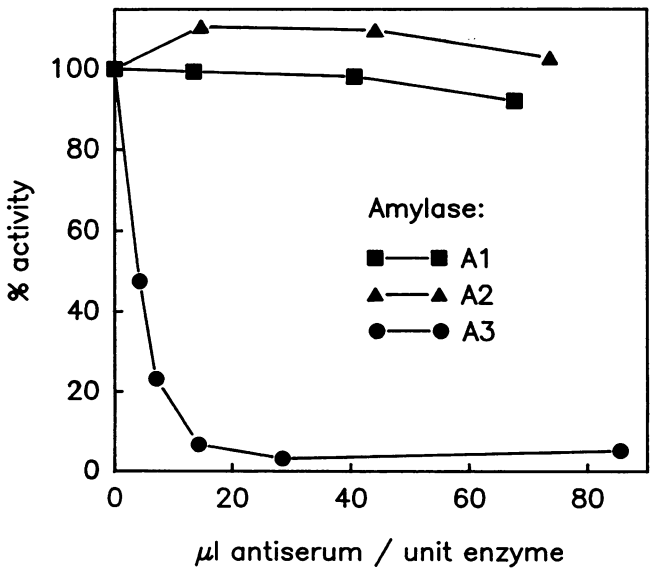

Figure 7. Neutralization of amylases A1, A2, and A3 (purified β-amylase) from wild-type leaves by anti-β-amylase serum. Neutralizations were performed as described in the text.

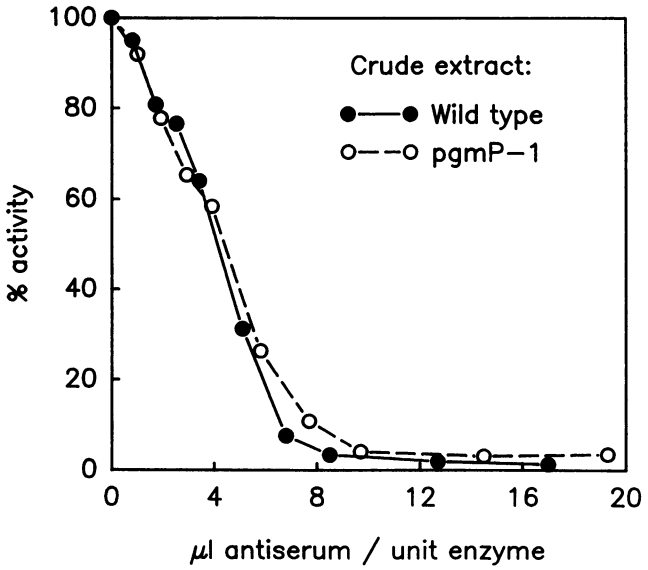

Figure 8. Neutralization of amylase activity in crude extracts from wild-type and pgmP-1 leaves by anti-β-amylase serum. The extracts used were the same as those reported in Table I and were obtained from plants grown in the greenhouse.

ization of β-amylase activity with antibodies raised to purified β-amylase supported this hypothesis. Even though 5- to 10-fold more amylase activity was observed in the mutants than in wild-type leaves, the amount of anti-β-amylase serum required to neutralize 50% of the β-amylase activity was identical in each of the mutants and wild type. If the enzyme had been posttranslationally activated in the mutants, less antibody would have been required to inhibit 50% of the activity. It is unlikely that the enzyme was modified during purification since the same pattern of activity neutralization was obtained from crude extracts and purified β-amylase.

Casper *et al.* (3) observed that β-amylase activity in both wild-type and pgmP-1 leaves was elevated when plants were grown under higher light intensity. We observed the same large variation in total leaf amylase activity when comparing plants grown in the greenhouse under sunlight with those grown in a chamber under much lower light intensity (Table I; Fig. 9). The results of enzyme activity neutralization experiments indicated that the enzymes from plants grown in the greenhouse and chamber were identical. Growing plants under high light intensity, like the starch mutants, results in an accumulation of β-amylase protein.

In a previous study it was found that leaves from pgmP-1 plants grown in a 12 h photoperiod had a fourfold higher concentration of soluble sugars than wild-type leaves (2). This difference in soluble sugar concentration did not occur in plants grown under continuous light. Similarly, β-amylase activity was not elevated in plants grown under continuous light (3). The factor(s) causing accumulation of β-amylase in the starchless mutants may therefore be related to sugar metabolism. The same factor(s) could also cause β-amylase accumulation in the high light intensity grown plants since the soluble sugar concentration in leaves is likely to increase with light intensity up to the point of light saturation. Photosynthesis in *Arabidopsis* was not saturated at the levels of irradiance used in the growth chamber (2).

Because the starch biosynthetic enzymes are confined to the chloroplast (15) it has been assumed that degradation of starch must also occur in the chloroplast (1). Being located outside the chloroplast (2), the physiological function of β-amylase in *Arabidopsis* is therefore not known. Washed protoplasts from *Arabidopsis* leaves contained detectable levels of β-amylase activity (JD Monroe, unpublished observations); therefore, the enzyme is probably located in the cytosol or vacuole. Localization studies of β-amylase in *Arabidopsis* tissues are in progress. There is some evidence that extrachloroplastic amylases are not required for transitory starch degradation. Chloroplasts are known to contain α- and β-amylases, and starch phosphorylases capable of degrading

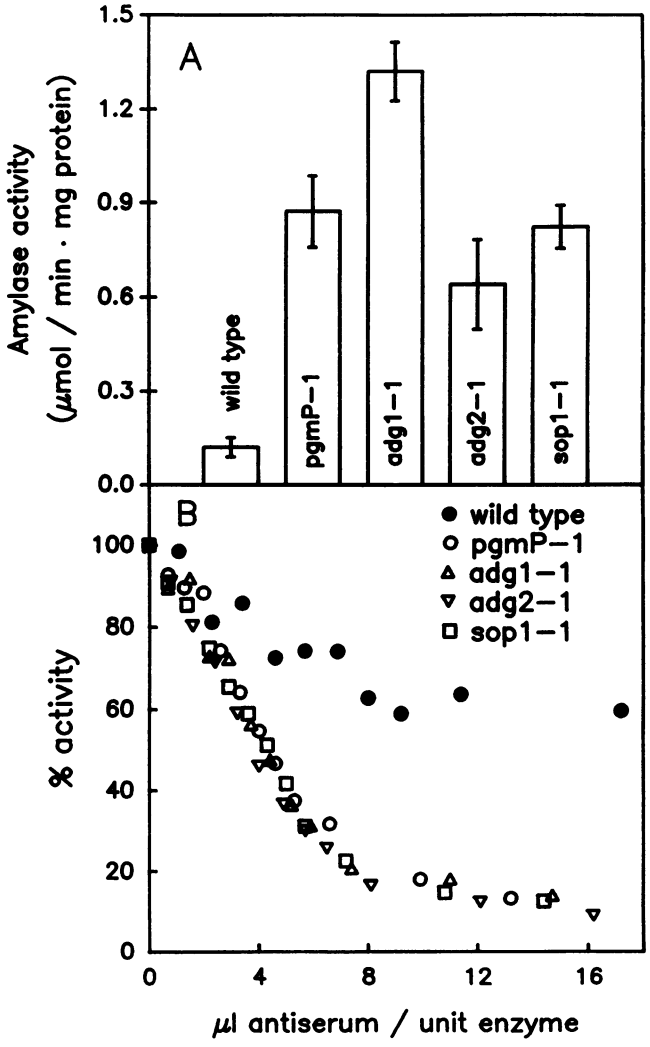

Figure 9. Amylase activity and neutralization of activity in wild type and several starchless or starch accumulating mutants of *Arabidopsis* grown in a growth chamber. A, Total amylase activity in crude extracts prepared from leaves of wild type, pgmP-1, adg1-1, adg2-1, and sop1-1 mutants of *Arabidopsis*; B, neutralization of the activity in each of the extracts from panel A with anti-β-amylase serum.

starch (8, 15). Stitt *et al.* (17) and Stitt and Heldt (18) have observed that isolated chloroplasts from pea (17) and spinach (18) can degrade starch at rates comparable to those observed in whole leaves.

The study of extrachloroplastic amylases will be aided by the existence of different genetic mutations and an environmental factor, light intensity, each of which leads to an accumulation of extrachloroplastic β-amylase in *Arabidopsis*. The identification of the factor(s) that regulate the level of β-amylase activity may help to elucidate the physiological function of the enzyme.

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LITERATURE CITED

1. **Beck E, Ziegler P** (1989) Biosynthesis and degradation of starch in higher plants. Annu Rev Plant Physiol Plant Mol Biol **40:** 95–117
2. **Casper T, Huber SC, Somerville C** (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. Plant Physiol **79:** 11–17
3. **Casper T, Lin TP, Monroe J, Bernhard H, Spilatro S, Preiss J, Somerville C** (1989) Altered regulation of β-amylase activity in mutants of *Arabidopsis* with lesions in starch metabolism. Proc Natl Acad Sci USA **86:** 5830–5833
4. **Chapman GW, Pallas JE Jr, Mendicino J** (1972) The hydrolysis of maltodextrins by a β-amylase isolated from leaves of *Vicia faba*. Biochim Biophys Acta **276:** 491–507
5. **Doehlert DC, Duke SH, Anderson L** (1982) Beta-amylases from alfalfa (*Medicago sativa* L.) roots. Plant Physiol **69:** 1096–1102
6. **Holmes E, Boyer C, Preiss J** (1982) Immunological characterization of *Escherichia coli* B glycogen synthase and branching enzyme and comparison with enzymes from other bacteria. J. Bacteriol **151:** 1444–1453
7. **Kakefuda G, Duke SH** (1984) Electrophoretic transfer as a technique for the detection and identification of plant amylolytic enzymes in polyacrylamide gels. Plant Physiol **75:** 278–280
8. **Kakefuda G, Duke SH, Hostak MS** (1986) Chloroplast and extrachloroplastic starch-degrading enzymes in *Pisum stivum* L. Planta **168:** 175–182
9. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277:** 680–685
10. **Lin TP, Casper T, Somerville C, Preiss J** (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADPglucose pyrophosphorylase activity. Plant Physiol **86:** 1131–1135
11. **Lin TP, Casper T, Somerville CR, Preiss J** (1988) A starch deficient mutant of *Arabidopsis thaliana* with low ADPglucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. Plant Physiol **88:** 1175–1181
12. **Lin TP, Spilatro SR, Preiss J** (1988) Subcellular localization and characterization of amylases in *Arabidopsis* leaf. Plant Physiol **86:** 251–259
13. **Lizotte PA, Henson CA, Duke SH** (1990) Purification and characterization of a pea epicotyl β-amylase. Plant Physiol **92:** 615–621
14. **Nelson N** (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem **153:** 375–380
15. **Okita TW, Greenberg E, Kuhn DN, Preiss J** (1979) Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaves. Plant Physiol **64:** 187–192
16. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Garner FH, Provenzano MD, Fugimoto EK, Goehe NM, Olson BJ, Klenck DC** (1985) Measurement of protein using bicinchoninic acid. Anal Biochem **150:** 76–85
17. **Stitt M, Bulpin PV, ap Rees T** (1978) Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. Biochim Biophys Acta **544:** 200–214
18. **Stitt M, Heldt HW** (1981) Physiological rates of starch breakdown in isolated intact spinach chloroplasts. Plant Physiol **68:** 755–761
19. **Subbaramaiah K, Sharma R** (1985) Affinity chromatography of mustard β-amylase on starch columns. J Biochem Biophys Methods **10:** 315–320
20. **Thoma JA, Spradlin JE, Dygert S** (1971) Plant and animal amylases. *In* PD Boyer, ed, The Enzymes, Ed 3 Vol 5. Academic Press, New York, pp 115–189
21. **Towbin H, Staehelin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354
22. **Vretblad P** (1974) Immobilization of ligands for the biospecific affinity chromatography via their hydroxyl groups. The cyclohexaamylose-β-amylase system. FEBS Lett **47:** 86–89
23. **Wray W, Boulikas T, Wray VP, Hancock R** (1981) Silver staining of proteins in polyacrylamide gels. Anal Biochem **118:** 197–203
24. **Ziegler P, Beck E** (1986) Exoamylase activity in vacuoles isolated from pea and wheat leaf protoplasts. Plant Physiol **82:** 1119–1121