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Communication

Localization of α-Amylase in the Apoplast of Pea (*Pisum sativum* L.) Stems[1]

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ABSTRACT

Most of the activity of an α-amylase present in crude pea (*Pisum sativum* L. cv Laxton's Progress No. 9) leaf preparations cannot be found in isolated pea leaf protoplasts. The same extrachloroplastic α-amylase is present in pea stems, representing approximately 6% of total stem amylolytic activity and virtually all of the α-amylase activity. By a simple infiltration-extraction procedure, the majority (87%) of this α-amylase activity was recovered from the pea stem apoplast without significantly disrupting the symplastic component of the tissue. Only 3% of the β-amylase activity and less than 2% of other cellular marker enzymes were removed during infiltration-extraction.

The roles of α-amylases in higher plant photosynthetic tissues are poorly understood. It is generally assumed that α-amylase initiates or at least plays a key role in starch degradation in chloroplasts (2, 16). This assumption is partially based on evidence indicating that α-amylase and extrachloroplastic starch phosphorylase are the only enzymes occurring within plant vegetative tissues that can attack starch granules isolated from photosynthetic tissues (4, 17, 18). Due to its compartmentation, it is doubtful that extrachloroplastic starch phosphorylase plays a role in transitory starch breakdown. Similarly, the role of α-amylase in starch breakdown in the photosynthetic tissues of several species is also uncertain. α-Amylase has been localized in the chloroplasts of spinach (14, 15), and one would presume that it has a role in starch degradation in this species. However, its presence in the chloroplasts of other higher plant species is less well documented. Several studies with pea and barley indicate that α-amylase is either absent or in very low activity in the chloroplast (9, 12, 13, 19), whereas one study with pea chloroplasts isolated from protoplasts does indicate that α-amylase is in the chloroplast (20). Regardless of whether α-amylase is present or absent in chloroplasts, it appears that the largest amount of the total α-amylase activity in leaf tissues is extrachloroplastic (9, 12, 14, 20), away from the site of starch accumulation. However, the site of this α-amylase is unknown.

The purpose of this study was to determine the extrachloroplastic location of α-amylases in pea tissues.

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MATERIALS AND METHODS

Plant Tissue. Pea (*Pisum sativum* L. cv Laxton's Progress No. 9) seeds (Old's Seed Co., Madison, WI) were surface-sterilized in 0.5% (w/v) NaClO for 10 min and washed in cold, running tap H_2O for 1 h. Seeds were sown 3 cm deep in flats of vermiculite and grown in a growth chamber under a 21°C/17°C, 14/10 h (day/night) regime. Light was supplied by both fluorescent and incandescent lamps at 200 μmol m^{-2} s^{-1} (400–700 nm). Plants were irrigated with nutrient solution (7) as needed. Plants were placed in the dark 36 to 48 h prior to chloroplast and protoplast purification to deplete the chloroplasts of starch.

Protoplast Preparation. Fully expanded leaves from 4-week-old plants were prepared for protoplast isolation by gently abrading the adaxial surface with alumina type 305 (Sigma), followed by rinsing in buffer consisting of 5 mM Mes (pH 6.0), 1 mM $CaCl_2$, and 0.5 M sorbitol. Leaves were placed adaxial surface down in the above buffer, which was supplemented with 2% (w/v) Onozuka R-10 cellulase (Yakult Honsha Co., Ltd., 1-1-19, Higashi-Shinbashi, Minatoku, Tokyo, 105, Japan) and 0.5% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., 4-13, Koami-cho, Nihonbashi, Chuo-ku, Tokyo 103, Japan). Protoplasts were isolated and purified by the method of Ziegler and Beck (20). Protoplasts were ruptured by gentle passage through a 20 μm nylon mesh attached to the end of a 1 ml syringe. After three passes, the chloroplasts were separated from the extraplastidic fraction by centrifugation at 1000*g* for 5 min at 4°C. Protoplast purification and rupture were conducted at 0 to 4°C. Only whole protoplasts and extraplastidic preparations were used for this study.

Chloroplast Isolation. Chloroplasts were isolated from fully expanded leaves of 4-week-old plants by the method of Kakefuda *et al.* (12).

Infiltration-Extraction. Stems were harvested from 6-week-old plants. Five 1.8 g replicates were cut into 1 cm segments, washed in double distilled H_2O, and blotted dry. Two replicates (controls) were stored at 0 to 4°C. The remaining three replicates were subjected to seven cycles of IE[2] by the procedure of Frehner and Conn (8). Segments were vacuum-infiltrated for 3 min in buffer consisting of 20 mM Hepes (pH 6.9), 3 mM $CaCl_2$, and 3 mM DTT followed by 3 min at atmospheric pressure in the same buffer. Segments were then blotted dry, stacked upright on a 105 μm nylon mesh in the bottom of a disposable syringe to which a microfuge tube had been attached. This apparatus was placed in a centrifuge tube and centrifuged at 1000*g* for 10 min at 4°C to extract the infiltrated buffer.

Tissue Preparation for Enzyme Assays. Following either stor-

[2] Abbreviations: IE, infiltration extraction; NAD-MDH, NAD-malate dehydrogenase; PEP, phosphoenolpyruvate; NAD-GAPDH, NAD-glyceraldehyde-3-phosphate dehydrogenase.

age at 0 to 4°C or seven cycles of IE, stems were homogenized in a chilled mortar with 3.6 ml of extraction buffer (same as for vacuum infiltration) to which 36 μl of 2 mM leupeptin had been added. Homogenized stems were centrifuged at 20,000*g* for 20 min. The supernatant was decanted and stored at −20°C until use. Extraction buffer (2 ml) was added to the pellet. The resuspended pellet was centrifuged as above, and the resultant supernatant was decanted and stored at −20°C.

Chl Measurement. Chl was measured according to Arnon (1) immediately following homogenization of stem tissue.

Enzyme Assays. NAD-MDH (EC 1.1.1.37) and PEP carboxylase (EC 4.1.1.3.1) were assayed using methods similar to those previously described (6). Final NAD-MDH assay concentrations were 50 mM Hepes (pH 7.5), 20 mM oxaloacetate (pH 6.2), 150 μM NADH. Final PEP carboxylase assay concentrations were 50 mM Tricine (pH 8.4), 10 mM $MgCl_2$, 2.5 mM DTT, 150 μM NADH, 10 mM $NaHCO_3$, and 2 mM PEP. NAD-GAPDH (EC 1.2.1.12) was assayed with final assay concentrations of 50 mM Tricine (pH 8.4), 10 mM $MgCl_2$, 2.5 mM DTT, 5 mM ATP, 150 μM NADH, 5 IU PGA kinase, and 5 mM PGA. Production of reducing sugar from Lintner soluble starch (Sigma) (a measure of total amylolytic activity) or pullulan (debranching enzyme activity) was measured using dinitrosalicylic acid reagent (5). Assays were conducted at pH 6.0 (50 mM succinate, 0.5 mM $CaCl_2$) for 15 min (total amylolytic) or 2 h (debranching enzyme) at 30°C. Total endoamylolytic activity was determined by the β-amylase saturation, starch azure technique (5); except that, rather than *Bacillus subtillis* α-amylase, pea leaf α-amylase was used to produce the standard curve.

Pea α-amylase that was used to produce the standard curve for the starch azure assay was extracted from fully expanded leaves of 4-week-old plants. Tissues (20 g) were homogenized in a chilled mortar in 40 ml of the same buffer used for IE extraction. The resultant slurry was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 20,000*g* for 20 min. The supernatant was dialyzed overnight against buffer A (10 mM Mes [pH 5.0], 0.5 mM $CaCl_2$, and 3 mM DTT). This preparation was applied to a DEAE cellulose column (25 × 70 mm) equilibrated with buffer A, and protein was eluted with a 0 to 0.5 M NaCl gradient (300 ml) in buffer A. Fractions (5.5 ml) were collected and assayed for amylolytic activity using the starch azure technique (5). Pooled fractions which could release dye from starch azure were determined to have no β-amylase or starch-debranching enzyme activity by the electrophoretic transfer procedure for identifying amylolytic enzymes (11).

Electrophoresis. Native gel electrophoresis was performed as described by Kakefuda and Duke (11), except that a 7 to 15% (w/v) linear gradient polyacrylamide gel was used for enzyme separation. Electrophoretic transfer was performed as described before (11), except that a 50 mM imidazole (pH 6.5), 0.5 mM $CaCl_2$ buffer was used.

RESULTS AND DISCUSSION

Contamination of Apoplastic Preparations during the Infiltration-Extraction Procedure. Intracellular marker enzymes were used to assess the degree of symplastic contamination of IE solutions extracted from the apoplast of pea stems. Recovery of the intracellular marker NAD-MDH and the cytosolic marker NAD-GAPDH in seven IE cycles did not exceed 1.2% of total activity recovered from pea stem homogenates and stem IE preparations (Table I). Activity of the cytosolic marker PEP carboxylase was not detected in pea stem IE preparations. Approximately 3% of vacuolar β-amylase (20) was recovered in the IE preparations (Table II). Chloroplastic starch-debranching enzyme activity could not be detected in IE preparations (data not shown). These results indicate that very little rupture of or leakage from cells occurred during the IE procedure.

Table I. *Activities of Cytosolic Marker Enzymes in Crude Stem and Infiltration-Extracted Stem Fractions*

Values are means ± SE of 3 (infiltration-extracted) and 2 (noninfiltration-extracted) separate preparations. 1, 3, 5, and 7 = number of extractions. Data for extraction cycles are cumulative, for the number of indicated cycles.

Enzyme Preparation	Enzyme Activity		
	NAD-malate dehydrogenase	NAD-glyceraldehyde-3-phosphate dehydrogenase	PEP carboxylase
	μmol mg⁻¹ Chl min⁻¹		
Infiltration extraction (IE)			
Extraction cycles			
1	2.82 ± 0.28 (0.7)[a]	0.12 ± 0.06 (0.3)	ND[b]
3	3.99 ± 0.32 (0.9)	0.42 ± 0.24 (1.2)	ND
5	4.10 ± 0.31 (0.9)	0.42 ± 0.24 (1.2)	ND
7	5.08 ± 0.50 (1.2)	0.40 ± 0.09 (1.2)	ND
Infiltration extracted stems	433.88 ± 21.56 (98.8)	33.90 ± 0.96 (98.8)	3.69 ± 0.18 (100)
Noninfiltration extracted stems	413.65 ± 6.22	35.80 ± 4.04	3.34 ± 0.16

[a] Recovery as percentage of total activity. [b] Not detected.

Amylolytic Activity in Stems and Leaves. Total amylolytic activity recovered from IE and non-IE treated pea stems was approximately the same (Table II). α-Amylase activity accounted for only 6 and 5.4% of total amylolytic activity recovered from IE and non-IE treated tissues, respectively. Almost all of the endoamylolytic activity was due to α-amylase. Debranching enzyme activity represented only 0.6% of the total endoamylolytic activity (data not shown). By far, the largest amount of amylolytic activity in pea stems was due to exoamylolytic (β-amylase) activity (94–95%).

Following blot-transfer of crude pea stem preparations through a starch-containing gel, KI-I_2 staining revealed two major and two minor bands of amylolytic activity (Fig. 1). Band b_1, a presumed β-amylase, originates in the plastid (lane D, Fig. 2) (12). It is not found in other parts of the cell (lane C, Fig. 2). Bands a_2 and a_3 are unstained and are due to α-amylase transfer through a starch-containing gel (11). The major band of α-amylase from stem preparations (Fig. 1) has the same R_F value as that found for the major α-amylase in crude pea leaf preparations (Fig. 2). Protoplasts prepared from pea leaves, however, contain very little of this α-amylase (lane B, Fig. 2), suggesting that a large portion of α-amylase activity attributable to this enzyme is located somewhere other than in mesophyll protoplasts. One α-amylase (band a_1, Fig. 2) only appeared in crude leaf tissue homogenates and has not been previously reported.

The fast migrating lower band (b_2) that stains lavender is the result of β-amylase activity (Fig. 1). This band was not apparent when β-limit dextrin was substituted for starch in the substrate containing blot gel (data not shown) and appears to be the same fast-migrating β-amylase that Ziegler and Beck (20) have identified as being located in the vacuole. When blotted through a gel

Table II. *Activities of Pea Stem Amylolytic Enzymes in Preparations from Tissues Homogenized and Centrifuged at 20,000g (A) and from Tissues Infiltration-Extracted 7 Times and then Homogenized and Centrifuged at 20,000g (B)*

Values are mean ± SE of 3 (infiltration-extracted) and 2 (noninfiltration extracted) separate preparations.

Enzyme Preparation	Enzyme Activity		
	Total amylolytic activity	Endoamylolytic activity (α-amylase)	Exoamylolytic activity (β-amylase)
	μmol mg⁻¹ Chl min⁻¹		
(A) Noninfiltration extracted stems			
20,000*g* supernatant	23.24 ± 0.62 (78)[a]	1.44 ± 0.24 (89)	21.80 ± 0.37 (78)
20,000*g* pellet	6.50 ± 0.24 (22)	0.18 ± 0.06 (11)	6.32 ± 0.18 (22)
Total	29.74 ± 0.85 (100)	1.62 ± 0.30 (100)	28.12 ± 0.55 (100)
(B) Infiltration extracted stems			
20,000*g* supernatant	24.78 ± 0.54 (73)	0.16 ± 0.06 (8)	24.64 ± 0.50 (77)
20,000*g* pellet	6.34 ± 0.52 (19)	0.10 ± 0.03 (5)	6.24 ± 0.49 (20)
Infiltration extraction (IE)[b]	2.78 ± 0.86 (8)	1.80 ± 0.27 (87)	0.98 ± 0.58 (3)
Total (20,000*g* extraction + IE)	33.92 ± 1.12 (100)	2.06 ± 0.48 (100)	31.84 ± 0.94 (100)

[a] Recovery as percentage of total amylolytic, endoamylolytic, or exoamylolytic activity. [b] Values are totals for 7 IEs.

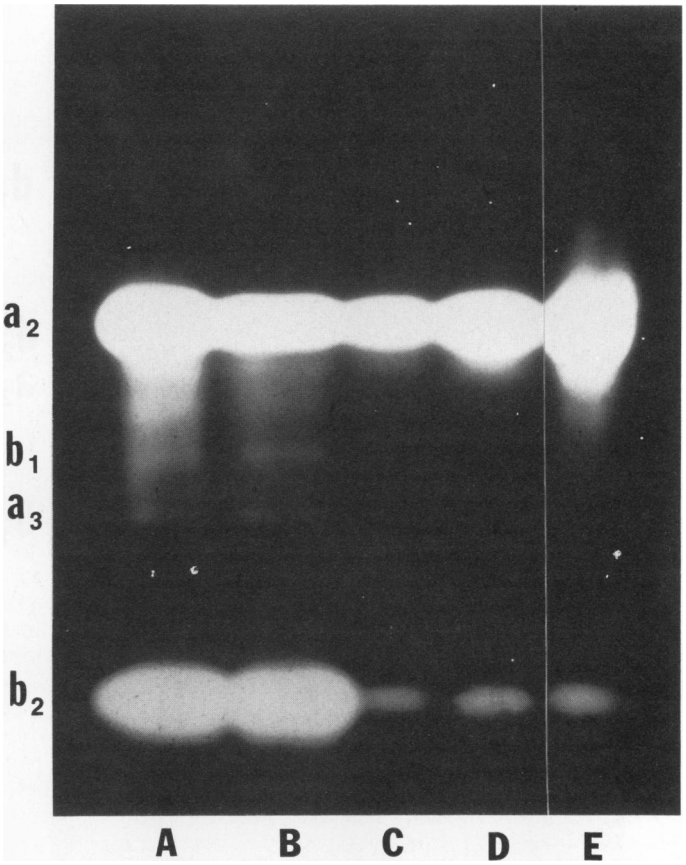

FIG. 1. Detection of amylolytic enzymes in preparations of crude non-IE stems (lane A), crude IE stems (lane B), and cumulative IEs after 1 (lane C), 3 (lane D), and 7 (lane E) infiltration-extraction cycles by native polyacrylamide gel electrophoresis followed by electrophoretic transfer through a polyacrylamide gel containing Lintner soluble starch and staining with KI-I_2 solution. α-Amylase (a_2 and a_3) produces small dextrins which diffuse from the gel, leaving unstained bands. Chloroplast β-amylase (b_1) was reported previously by Kakefuda *et al.* (12). Identification of the rapidly migrating β-amylase (b_2) was based on the disappearance of this band when β-limit dextrin is substituted for soluble starch in the blot gel. Lanes were loaded on a Chl equivalent basis. Chl equivalents loaded (in μg) in lane A were 3.4; B, 3.7; C, 3.8; D, 3.6; E, 3.4.

at pH 8.45, this β-amylase produces a limit dextrin which stains blue with KI-I_2 staining. Due to this phenomenon, this β-amylase was originally misidentified by Kakefuda *et al.* (12) as a debranching enzyme. Amylose, the limit dextrin of debranching enzyme, stains blue with KI-I_2 staining (11). These findings indicate that there is no extrachloroplastic debranching enzyme in pea.

Starch-hydrolyzing enzymes present in pea chloroplasts consisted of two debranching enzymes, one or two β-amylases, and one α-amylase (possibly of extraplastidic origin) (Fig. 2). The slowest migrating debranching enzyme (d_1) was not detected in a previous study (12), possibly because it is very labile and can only be detected in chloroplasts very soon after their isolation. The plastidic β-amylase (b_1) and fastest migrating debranching enzyme (d_2) were identified previously (12). The band containing an apparent plastidic α-amylase (a_2) co-migrates with a previously identified plastidic β-amylase (12). We think that this α-amylase is probably extrachloroplastic contamination because it has the same R_F value as the IE α-amylase (Fig. 1), its presence is accentuated when we find contamination by the vacuolar β-amylase (b_2) (data not shown), and even a tiny amount of α-amylase contamination could be detected because blot gels containing starch are much more sensitive to α-amylases than other amylolytic enzymes (11).

Recovery of Amylolytic Activity in Infiltration-Extraction Preparations. In contrast to cellular marker enzymes (Table I), almost 90% of the total pea stem α-amylase was removed from tissues during the IE process (Table II), indicating that this enzyme is primarily apoplastic. Activity of the slowest migrating α-amylase (a_2) increased with successive IE cycles (Fig. 1). Infiltration-extracted β-amylase activity, however, did not change significantly throughout the extraction process (b_2, Fig. 1). Activity of α-amylase recovered in the homogenate supernatant of non-IE treated pea stems was 11-fold greater (percent total basis) than that found in the corresponding IE stem preparation (Table II).

We considered it possible that, rather than being located in the apoplast before IE, α-amylase extracted by this procedure is secreted from the cytosol in response to the IE treatment, which requires about 5 h for seven IE cycles. While Ca^{2+}-stimulated secretion of α-amylase from barley aleurone (10) and barley aleurone protoplasts (3) has been demonstrated, Ca^{2+}-stimulated secretion of α-amylase from mature photosynthetic tissue has not. Attempts to alter the amount of α-amylase recovered in protoplasts from pea leaves by desalting fungal cellulose preparations in the presence of EDTA prior to use and preparing protoplasts in the presence or absence of Ca^{2+} plus EDTA had no effect on the amount of α-amylase recovered in protoplasts (data not shown). Thus, although it appears that the majority of α-amylase activity may reside in the apoplast of pea stems, its role as an apoplastic enzyme is unclear. Lack of knowledge concerning the *in situ* substrate for both this α-amylase and the vacuolar β-amylase further hampers any attempts to propose a role for these enzymes.

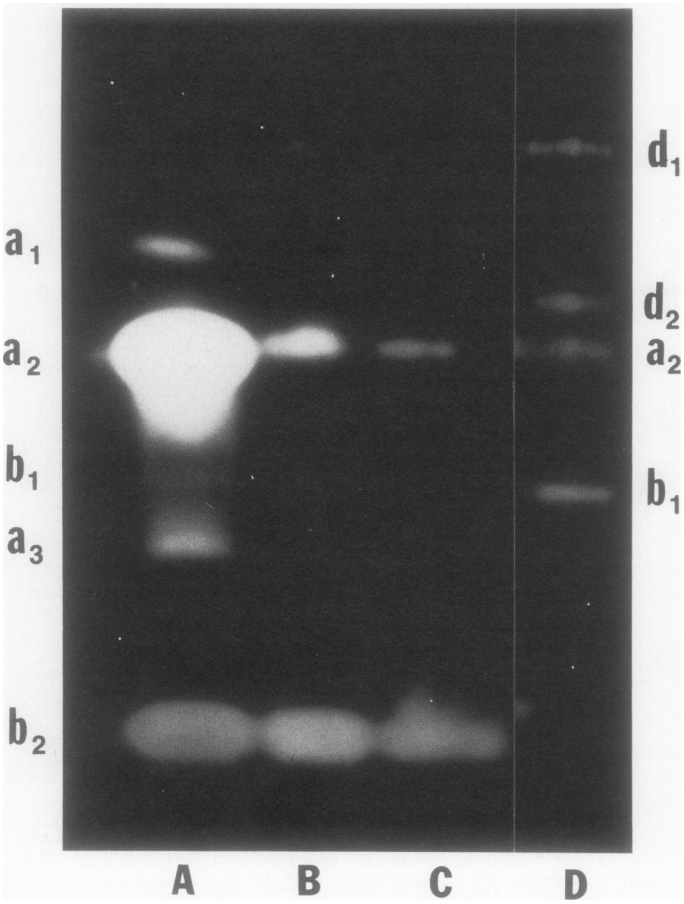

FIG. 2. Detection of amylolytic enzymes (under Fig. 1 conditions) in crude (lane A), protoplast (lane B), extrachloroplast from protoplasts (lane C), and chloroplast (lane D) preparations from pea leaves. Bands are labeled as in Figure 1. In addition, the slowest migrating α-amylase (a_1) is not detected in stem preparations and debranching enzyme (d_1 and d_2) produces amylose which stains blue. Lanes were loaded on a Chl-equivalent basis. Chl equivalents loaded (in μg) in lane A were 27; B, 52; C, 52; D, 110.

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