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Amylases in Pea Tissues with Reduced Chloroplast Density and/or Function[1]

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ABSTRACT

Pea (*Pisum sativum* L.) tissues with reduced chloroplast density (*e.g.* petals and stems) or function (*i.e.* senescent leaves and leaves darkened for prolonged periods) were surveyed to determine whether tissues with genetically or environmentally reduced chloroplast density and/or function also have significantly different amylolytic enzyme activities and/or isoform patterns than leaf tissues with totally competent chloroplasts. Native PAGE followed by electrophoretically blotting through a starch or β-limit dextrin containing gel and KI/I_2 staining revealed that the primary amylases in leaves, stems, petals, and roots were the primarily vacuolar β-amylase (EC 3.2.1.2) and the primarily apoplastic α-amylase (EC 3.2.1.1). Among tissues of light grown pea plants, petals contained the highest levels of total amylolytic (primarily β-amylase) activity and considerably higher ratios of β- to α-amylase. In aerial tissues there was an inverse relationship between chlorophyll and starch concentration, and β-amylase activity. In sections of petals and stems there was a pronounced inverse relationship between chlorophyll concentration and the activity of α-amylase. Senescing leaves of pea, as determined by age, and protein and chlorophyll content, contained 3.8-fold (fresh weight basis) and 32-fold (protein basis) higher α-amylase activity than fully mature leaves. Leaves maintained in darkness for 12 days displayed a 14-fold (fresh weight basis) increase in α-amylase activity over those grown under continuous light. In senescence and prolonged darkness studies, the α-amylase that was greatly increased in activity was the primarily apoplastic α-amylase. These studies indicate that there is a pronounced inverse relationship between chloroplast function and levels of apoplastic α-amylase activity and in some cases an inverse relationship between chloroplast density and/or function and vacuolar β-amylase activity.

The physiological roles of amylases in vegetative tissues of higher plants are poorly understood. One reason for this current state of confusion is that at the cellular level most of the amylolytic activity in plant vegetative tissues is extrachloroplastic, away from the site of starch synthesis and degradation. More than 90% of the total amylolytic activity in pea leaves has been reported to be extrachloroplastic (16). Similarily, 70 and 80% of the total amylolytic activity is located in the extrachloroplastic fractions of *Commelina communis* guard cells (23) and spinach mesophyll cells (21), respectively. All or most of the total endoamylolytic activity in pea and barley leaves has usually been found to be extraplastidic (13, 16, 17, 25). However, Ziegler and Beck (28) found endoamylase activity associated primarily with the chloroplasts of pea and wheat leaves. Recently up to 87% of the total α-amylase activity has been localized in the apoplast of pea stems and leaves (1, 2, 24). Studies with *Vicia faba* (6), barley (13), *Arabidopsis* (19), and pea (16) show that most of the leaf exoamylase activity is extrachloroplastic. β-Amylase, the major amylolytic and the major exoamylolytic enzyme in pea leaves (1, 16, 20) appears to be primarily vacuolar (28). Of the extrachloroplastic glucans surveyed, none have been found to act as substrates for either α-amylase (2) or β-amylase (20), and no substantiated explanations have been tendered to account for the presence of these enzymes outside the chloroplast.

In addition to most amylolytic enzymes being primarily extrachloroplastic, the highest abundance of amylolytic enzyme activity in plant vegetative tissues often appears to be in tissues which are photosynthetically less active (*e.g.* stems) than leaf tissues (7). There appears to be an inverse relationship between the production of transitory starch in leaf tissues and levels of amylolytic activity. Starchless *Arabidopsis* mutants have 3.4-fold higher total leaf amylolytic activity than the wild type (18). Most of this amylolytic activity in the mutant is due to increases in β-amylase activity (5). Also, norflurazon-caused photooxidation of leaf chloroplasts and inhibition of chloroplast protein synthesis results in very large increases (up to 86-fold) of α-amylase and a much lower increase in β-amylase (up to 6.7-fold) activity in pea leaves (24). Such findings have led us to conclude that the chloroplast produces a negative signal for the regulation of α-amylase activity in pea (24). In this study we have examined amylolytic enzyme activities in different types of tissues with various degrees of chloroplast density and/or function and environmental treatments which alter chloroplast function. These experiments were conducted to determine whether these environmental and genetically controlled factors act in a manner similar to plastid disruption by chemical means in the regulation of amylolytic enzymes in vegetative tissues.

MATERIALS AND METHODS

Plant Material

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

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water for 4 h. Seeds were sown about 3 cm deep in flats of vermiculite and grown in a greenhouse at 17 to 22°C until flowering. Nutrient solution (9), supplemented with 10 mM KNO_3, was supplied once a week to saturate the vermiculite. Plants were watered with tap water as needed.

Plants were collected and separated into leaves, stems, roots, and flowers. Fully expanded standards (the largest petal on each flower) and wings (the two lateral petals) were excised from flowers and used for comparisons of amylases in differing tissues and fully expanded standards were only used in studies on the distribution of amylases within petals. Roots were rinsed with distilled water and blotted dry before weighing whereas other plant tissues were first weighed and then rinsed with distilled water and blotted dry before use.

In leaf senescence studies, plants were grown for 3 weeks under greenhouse conditions with supplemental flourescent light, and then transferred to a growth chamber with a 12L:12D light-dark regime with photoperiod fluence rate of 700 μmol m^{-2} s^{-1} and constant 21°C. After 9 d in the growth chamber leaves were harvested at the growth stages of bud, partially expanded leaves, fully expanded mature leaves, and senescing leaves (lower leaves with reduced Chl content).

In the prolonged darkness experiments plants were grown for 2 weeks in the greenhouse with supplemental flourescent lamps. Plants were then transferred to a growth chamber with continuous light (350 μmol m^{-2} s^{-1}) and 21°C for 7 d followed by 12 d of either continuous darkness or continuous light. Fully expanded leaves, that were mature and not senesecent before the beginning of the 12 d treatments, were harvested for enzyme, Chl, and protein determinations.

Enzyme and Starch Extraction

Plant tissues were homogenized in an ice chilled mortar in chilled (about 3°C) grinding buffer (50 mM Hepes-KOH [pH 6.9], 20% [v/v] glycerol, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 1% [v/v] Triton X-100, 20 μM leupeptin, 1 mM DTT), using a plant tissue to grinding buffer ratio of 1:3. Grinding buffer for the enzyme preparations used for the detection of amylases by native PAGE and the electrophoretic transfer technique did not contain glycerol and Triton X-100, and the DTT concentration was increased to 6 mM. Leupeptin and DTT were added to all grinding buffers immediately before use. Homogenates were centifuged at 21,000*g* for 15 min. Supernatants were decanted and immediately frozen at −20°C until analysis of enzyme activities and protein content.

For starch extraction, plant tissues were homogenized in the same grinding buffer as described above. Crude homogenates, without centrifugation, were boiled for 10 min and immediately frozen at −20°C until starch content determinations.

Enzyme Assays

Total amylolytic enzyme and starch debranching enzyme activities were measured by assaying the production of reducing sugars from Lintner soluble starch and pullulan, respectively, by the method of Rick and Stegbauer (22). Total endoamylolytic activity was determined by the β-amylase saturation starch-azure technique as described by Doehlert and Duke (7). β-Amylase activity was approximated by subtracting total endoamylolytic activity from total amylolytic activity. α-Glucosidase activity was determined as described by Beers and Duke (3) at pH 4.5 with PNPG[2] as substrate. D-Enzyme was assayed with maltotriose as substrate by the hexokinase/glucose-6-phosphate dehydrogenase assay as by Kakefuda and Duke (15). Starch phosphorylase was assayed as previously (16).

Starch Measurements

Starch content was determined by the method described by Henson *et al.* (12). Crude boiled homogenates containing starch were digested for 4 h at 40°C with 25 units *Aspergillus niger* amyloglucosidase (EC 3.2.1.3, Sigma Chem Co.) in Na-acetate buffer (0.2 M, pH 4.5). Blanks were comprised of sample aliquots and heat inactivated amyloglucosidase. Glucose produced from starch by amyloglucosidase was determined by using the hexokinase and glucose-6-P dehydrogenase coupled assay.

Protein and Chl Determinations

Total soluble protein content of the 21,000*g* supernatants were determined by the method of Bradford (4), using BSA as a standard. Levels of Chl were measured by the method of Harborne (11), immediately following homogenization of tissues.

[2] Abbreviation: PNPG, *p*-nitrophenyl α-D-glucopyranoside.

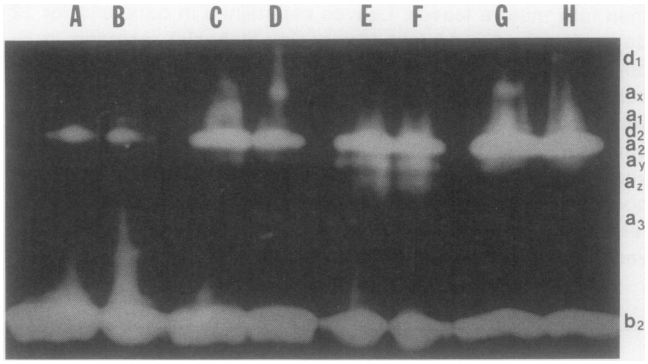

Figure 1. Detection and identification of hydrolytic-amylolytic enzymes in crude homogenates of pea petals (lanes A and B), stems (lanes C and D), roots (lanes E and F), and leaves (lanes G and H). Enzymes were separated by native PAGE and detected by blot transfer through a second native polyacrylamide gel containing Lintner soluble starch, followed by staining of the starch containing gel with KI/I_2 solution. The characteristic staining of limit dextrins from the activities of α-amylase (a_1-a_3 and a_x-a_z), β-amylase (b_1, b_2), and starch debranching enzyme (d_1, d_2) (*cf.* refs. 1, 14, 16, 24) allowed for tentative identification of isoforms of each enzyme. Verification of the identity of isoforms was by the results in Figures 2 and 3. Plants were grown under greenhouse conditions until flowering. Tissue was homogenized as described in "Materials and Methods" except that the grinding buffer to tissue ratio was 1:1 and the DTT concentration was increased to 30 mM. Lanes were loaded with 100 μL (lanes A, C, E, G) or 70 μL (lanes B, D, F, H) of crude enzyme preparation.

Electrophoresis

Native PAGE and electrophoretic transfer through starch containing gels were performed as described by Kakefuda and Duke (14) and as modified by Beers and Duke (1). A 7 to 15% (w/v) linear gradient gel was used for enzyme separation. Imidazole buffer (50 mM, pH 6.5), containing 0.5 mM $CaCl_2$ and 1 mM DTT was used during electrophoretic transfer.

RESULTS

Identification of Hydrolytic Starch Degrading Enzymes in Leaves, Petals, Stems, and Roots

To identify hydrolytic starch degrading enzymes in crude preparations of pea leaves, petals, stems, and roots, enzymes separated by PAGE were blot-transfered through a starch-containing gel and blot gels were stained with KI/I_2 (Fig. 1). Because β-amylase cannot degrade β-limit dextrins while α-amylase can, α-amylase activity was distinguished from the β-amylase activity on the basis of the disappearance of β-amylase activity bands when β-limit dextrin was substituted for soluble starch in blot gels (Fig. 2). Also, as a second proof of amylase identity, crude homogenates were heated to 70°C for 5 to 20 min before PAGE and blot transfer through starch containing gels (Fig. 3). Pea β-amylase is inactivated by 70°C, whereas the major pea α-amylase is not (2, 20, 24, 27). The R_F values (Figs. 1–3), substrate specificity (Fig. 2), and heat stability (Fig. 3) revealed that the primary amylases in all pea tissues tested were the primarily apoplastic α-amylase (a_2) (*cf.* refs. 1, 2, 24) and the primarily vacuolar β-amylase (b_2) (*cf.* refs. 20, 28). However, there were differences in the relative amounts of these two major amylases and in the ability to detect minor isoforms of both α- and β-amylase and starch debranching enzyme. α-Amylase isoforms a_1, a_2, and a_3 had been previously identified (1, 2, 24), whereas isoforms a_x, a_y, and a_z are first reported here. Petals had the same pattern of major amylases as those in leaves. However, four minor forms

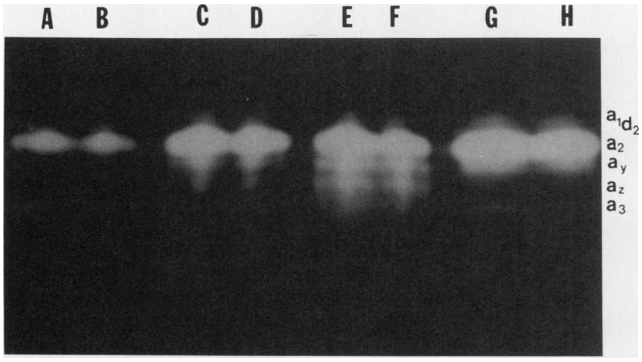

Figure 2. Identification of hydrolytic-amylolytic enzymes in pea petals (lanes A and B), stems (lanes C and D), roots (lanes E and F), and leaves (lanes G and H) by blot transfer of enzymes separated by PAGE through a β-limit dextrin containing gel followed by KI/I_2 staining. All procedures were as for Figure 1 except that β-limit dextrin was substituted for starch. Enzymes are identified as in Figure 1. Lanes were loaded with 100 μL (lanes A, C, E, G) or 70 μL (lanes B, D, F, H) of crude enzyme preparation.

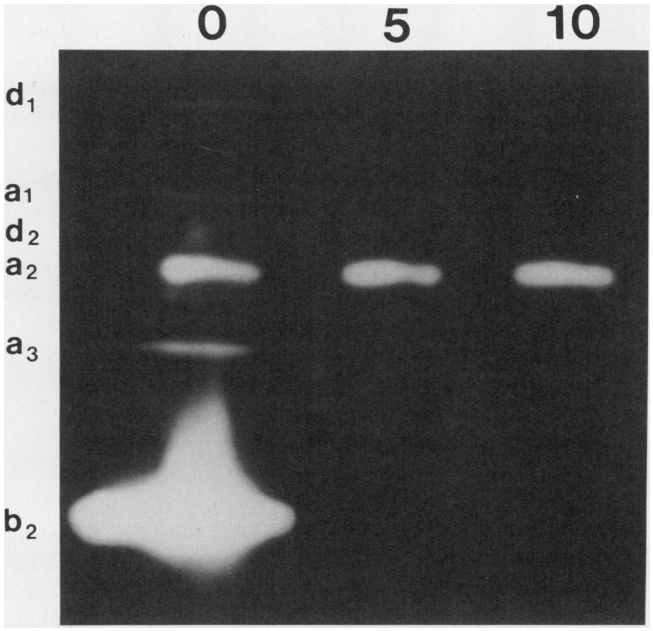

Figure 3. Detection of hydrolytic-amylolytic enzymes in the crude pea petal homogenates after heating at 70°C for different periods (0, 5, and 10 min). All procedures were as for Figure 1 except for the heat treatments. Enzymes are identified as in Figure 1. Homogenates were prepared from the fully expanded petals. Lanes were loaded with 100 μL of homogenate.

of α-amylase (a_x, a_1, a_y, a_z) that were detectable in the crude leaf homogenates were not detectable in petals (Fig. 1). This similarity in hydrolytic starch degrading enzyme isoforms between leaves and petals is not surprising in that petals are considered to be modified leaves (10). However, in petals, activity associated with the major α-amylase (a_2) band was much less pronounced while the activity associated with fast moving β-amylase (b_2) band was much more pronounced than in leaves, indicating a possible difference in gene expression of these isoforms. Stem homogenates contained all of the activity bands present in the crude leaf extracts. The two isoforms a_y and a_z in crude leaf and stem preparations may either be isozymes of α-amylase, the proteolytic degradation products of the a_2, or a_2 that has lost Ca^{2+} before or during PAGE (*cf.* ref. 2).

Root homogenates had the same major amylase activity bands as leaf preparations (Figs. 1 and 2). However, roots had no detectable isoforms of starch debranching enzyme (d_1 and d_2) and two α-amylases (a_x and a_3). The lack of starch debranching enzyme in roots may be due to crude root preparations having activities of these enzymes that are too low to be detected with the blot-transfer technique or to the absence of these enzymes in roots.

Quantitation of Hydrolytic Starch Degrading Enzymes in Leaves, Petals, Stems, and Roots

In pea petals, tissues with relatively low total endoamylolytic activity (Table I), starch debranching enzyme may have contributed significantly to the total endoamylolytic activity.

Table I. *Distribution of Amylolytic Activity and Starch and Chl Concentrations Among Different Tissues of Pea*

Values are means ± SE of three separate tissue preparations. Fully expanded leaves, petals (standard and wing petals only, mature roots, and stems were used for enzyme preparations.

Plant Tissue	Total Amylolytic Activity	Total Endoamylolytic Activity	Starth Concentration	Chl Concentration
	μmol min⁻¹ g⁻¹ fresh wt		*mg g⁻¹ fresh wt*	*μg g⁻¹ fresh wt*
Root	2.40 ± 0.35	0.261 ± 0.067 (11)[a]	3.2 ± 0.5	ND[b]
Leaf	5.02 ± 0.75	1.22 ± 0.21 (24)	42.5 ± 7.3	165 ± 17
Stem	10.8 ± 1.4	1.01 ± 0.08 (9.4)	12.2 ± 2.8	18.1 ± 4.6
Petal	26.5 ± 3.8	0.092 ± 0.024 (0.35)	5.1 ± 1.2	7.72 ± 2.30

[a] Numbers in parentheses = percentage of the total amylolytic activity. [b] Not detectable.

Debranching activity with pullulan as substrate was 21 nmol min^{-1} g^{-1} fresh weight, or about 25% of the total endoamylolytic activity. α-Glucosidase activity with PNPG as substrate was very low in all tissues tested (*e.g.* 2.2 nmol min^{-1} g^{-1} fresh weight for petals), and contributed little to total amylolytic activity. If the activities of these enzymes, with these substrates, are in the range of their activities with starch as substrate, they contributed very little to the total amylolytic and total endoamylolytic activity in pea tissues, with the exception of petal endoamylolytic activity. Hence, total endoamylolytic activity would be almost totally α-amylase activity and total amylolytic activity minus total endoamylolytic activity would be almost entirely β-amylase activity. This conclusion is also supported by the observation that the only two isoforms of starch hydrolyzing enzymes that were detected with very high activity on starch blot gels were the major isoforms of α-amylase (a_2) and β-amylase (b_2) (Fig. 1).

Although we found D-enzyme to be relatively high in activity in pea petals (603 nmol min^{-1} g^{-1} fresh weight) and it is in high activity in leaf tissues (15, 16), it probably would not contribute much to total amylolytic activity, except when PO_4^- was present for starch phosphorylase activity (15). However, even if starch phosphorylase had been included in the total amylolytic activity measurement of pea tissues it would not greatly contribute to the total amylolytic activity. For instance, starch phosphorylase activity in petals was only 101 nmol min^{-1} g^{-1} fresh weight or about 0.4% to the total amylolytic activity without PO_4^-.

Table II. *Distribution of Amylolytic Activity and Chl in Pea Petals*

Values are means of two separate tissue extractions. Fully expanded petals were used for sectioning. Petals (standards only) were cut transversely from base to apex into three sections of equal length.

Petal Section	Total Amylolytic Activity	Total Endoamylolytic Activity	Chl Concentration
	μmol min⁻¹ g⁻¹ fresh wt		*μg g⁻¹ fresh wt*
Basal	29.2	0.045	14.9
Middle	30.1	0.104	9.3
Apical	25.3	0.125	4.9
Petal (whole)	27.9	0.094	8.4

Distribution of Hydrolytic-Amylolytic Enzymes, Chl and Starch in Leaves, Petals, Stems, and Roots

Among the different aerial tissues of pea, petals contained the lowest and leaves contained the highest levels of Chl and starch (Table I). Stems, petals, and roots had 11, 4.7, and 0%, respectively, of the leaf Chl concentration, and 29, 12, and 7.5%, respectively, of the leaf starch content. Petals of cv Laxton's Progress No. 9 are light green at the base, turning progressively closer to white in appearance toward the tips of petals. This is reflected in the Chl concentration in sections of petal tissues from the base to the tip (Table II). The gradient of Chl in petals indicates that either there is a gradient in chloroplast density and/or functional chloroplasts.

There is an inverse relationship between Chl or starch content and total amylolytic activity in aerial tissues (leaves, stems, and petals) of pea (Table I). Petals, which contained low levels of Chl and starch, had β-amylase activity (total amylolytic activity minus total endoamylolytic activity) that was 6.5-fold higher than in leaves. Stem β-amylase was 2.7-fold and higher than in leaves. The higher levels of β-amylase activity in tissues with lower levels of in Chl and starch (Table I) is apparent in Figure 1. Of all of the tissues assayed, petals contained the highest level of total amylolytic, which was primarily β-amylase activity.In pea petals, there was little difference in total amylolytic activity from the base to the tip; however, there was a 3.1-fold increase in total endoamylolytic activity (Table II). There was an inverse relationship between Chl concentration, and presumably chloroplast density and/or function and endoamylase activity. Also, in pea stems there is an inverse relationship between Chl concentration and total endoamylolytic activity. Older, basal stem sections, had 21-fold higher total endoamylolytic activity than apical stem sections, whereas basal stem sections had only 35% of the Chl concentration as compared to apical stem sections (Table III). Total endoamylolytic activity of basal stem sections was 104-fold higher than apical stem sections on a protein basis (data not shown), due to the much lower protein concentration of the older basal stem sections. The difference in age of tissues in the different stem sections may have affected levels of endoamylolytic and Chl content, thus the effects of senescence were further investigated.

Table III. *Distribution of Amylolytic Activity and Chl in Pea Stems*

Plants were grown for 4 weeks in a greenhouse as described in "Materials and Methods." Apical sections consisted of the internode below the stem apex, middle sections were the fifth and sixth internodes, and basal sections were the second and third internodes. Values are means ± SE of three separate tissue extractions.

Stem Section	Total Amylolytic Activity	Total Endoamylolytic Activity	Chl Concentration	Protein Concentration
	$\mu mol\ min^{-1}\ g^{-1}$ fresh wt		$\mu g\ g^{-1}$ fresh wt	$mg\ g^{-1}$ fresh wt
Apical	38.0 ± 7.5	0.927 ± 0.241	16.9 ± 2.5	22.4 ± 3.82
Middle	16.7 ± 4.7	1.99 ± 0.33	15.7 ± 3.1	11.7 ± 2.65
Basal	24.1 ± 3.7	19.4 ± 3.2	5.87 ± 2.20	4.51 ± 1.33
Whole stem	27.5 ± 4.9	7.72 ± 1.71	13.2 ± 2.45	12.3 ± 2.67

Effects of Senescence and Darkness on Hydrolytic-Amylolytic Activity in Leaves

As leaves progressed from fully expanded mature leaves to senescent leaves, protein and Chl concentrations decreased 88 and 51%, respectively (Table IV). Senescent leaves appeared yellowish. Over this time, total amylolytic activity increased 6.3-fold on a protein basis but decreased 25% on a fresh weight basis, indicating that amylolytic activity was less affected than other proteins during senescence. The increase in total amylolytic activity in senescing leaves on a protein basis was primarily due to a 32-fold increase in endoamylase activity over fully expanded leaves. On a fresh weight basis α-amylase increased 3.8-fold with senescence, indicating that the enzyme was either induced or activated during senescence. There was a net loss of β-amylase activity in senescent leaves, accounting for the slight decrease in total amylolytic activity over fully expanded mature leaves. The increase in α-amylase activity was readily apparent in native PAGE gels of crude preparations of fully mature and senescent leaves blotted through starch containing gels and then treated with KI/I_2 and the α-amylase that increased in activity was the primarily apoplastic a_2 enzyme (data not shown). Also, the increase in α-amylase activity appeared to be continuous from the leaf bud stage through senescence, whereas β-amylase activity decreased on a fresh weight basis over this period (Table IV).

Continuous light had little or no effect on total amylolytic or total endoamylolytic activities, and protein and Chl concentrations over a 12 d period (Table V). In contrast, in continuous darkness, total endoamylolytic activity increased 14-fold and Chl and protein concentrations decreased 38 and 73%, respectively. Chl usually decreases more slowly than protein when plant tissues are placed in darkness (26). There was no starch in plants grown under continuous darkness (data not shown). The endoamylase that was increased in darkness was identified by PAGE followed by blotting through starch containing gels as is the a_2 α-amylase (data not shown). In darkness, as is the case with leaf senescence, β-amylase (fresh weight basis) activity decreased (Table V).

DISCUSSION

It has been shown that aerial tissues with low chloroplast densities and thus low Chl concentrations, such as stems, often have higher amylolytic activity, and in particular β-amylase activity, than leaves (7), the primary site of transitory starch degradation. In tissues with reduced chloroplast density and/or function and thus reduced Chl and starch concentrations, such as pea petals and stems, β-amylase is elevated in activity over leaf tissues (Table I; Fig. 1). This appears to be similar to the situation that exists in starchless mutants of *Arabidopsis* (5, 18) and for photobleached leaf tissues of pea that have been treated with inhibitors of pea chloroplast protein synthesis such as norflurazon (24). In contrast, subterranean tissues with high levels of storage starch and no Chl and photosynthesis, such as alfalfa roots and sweet potato

Table IV. *Effect of Natural Senescene on Amylolytic Activity in Pea Leaves*

Values are means ± SE of three separate tissue preparations. Leaves were harvested from the apex (apical leaves), from one node below the apex (partially expanded young leaves), the middle of the primary stem (fully expanded mature leaves), and from the first or second node of the plant (senescing leaves) of plants that were 4 weeks and 2 d old.

Leaf Stage of Development	Total Amylolytic Activity	Total Endoamylolytic Activity	Chl Concentration	Protein Concentration
	$\mu mol\ min^{-1}\ g^{-1}$ fresh wt		$\mu g\ g^{-1}$ fresh wt	$mg\ g^{-1}$ fresh wt
Apical leaves	21.2 ± 4.5	0.163 ± 0.032	71 ± 11	51 ± 5.3
Partially expanded young leaves	11.6 ± 2.1	0.965 ± 0.188	156 ± 29	49 ± 6.1
Fully expanded mature leaves	6.68 ± 1.90	1.03 ± 0.21	152 ± 34	26 ± 3.9
Senescing leaves	5.02 ± 1.33	3.91 ± 0.87	74 ± 15	3 ± 0.8

Table V. *Effect of Prolonged Darkness on Amylolytic Activity in Pea Leaves*

Values are means ± SE of three separate tissue preparations. Pea plants were grown in the greenhouse for 2 weeks under natural light conditions supplemented with fluorescent lamps. Thereafter, plants were transferred for 7 d to a growth chamber with continuous light followed by 12 d of either continuous dark or continuous light at a constant temperature of 21 ± 1°C. Fully expanded mature leaves were harvested for enzyme preparations.

Treatment Time	Total Amylolytic Activity	Total Endoamylolytic Activity	Chl Concentration	Protein Concentration
h	*μmol min⁻¹ g⁻¹ fresh wt*		*μg g⁻¹ fresh wt*	*mg g⁻¹ fresh wt*
Continuous light				
0	7.63 ± 2.37 (100)[a]	0.439 ± 0.104 (100)	117 ± 14 (100)	25.3 ± 2.1 (100)
120	5.41 ± 1.89 (71)	0.509 ± 0.095 (116)	128 ± 12 (109)	23.2 ± 2.5 (89)
288	6.15 ± 2.14 (81)	0.465 ± 0.071 (106)	121 ± 21 (103)	24.8 ± 1.9 (98)
Continuous dark				
0	7.32 ± 1.53 (100)	0.373 ± 0.063 (100)	123 ± 13 (100)	26.7 ± 3.4 (100)
120	6.15 ± 1.68 (84)	2.62 ± 0.519 (702)	108 ± 9 (88)	13.9 ± 2.1 (50)
288	6.82 ± 1.62 (93)	5.21 ± 1.35 (1397)	76 ± 10 (62)	7.4 ± 1.5 (27)

[a] Numbers in parentheses = percentage of time zero.

tubers, contain very high levels of β-amylase activity (7, 8). β-Amylase is a vacuolar enzyme in pea and wheat leaves (28) and it has been previously speculated that it may function in hydrolyzing maltodextrins in the vacuole (20). From the data presented here (Table I; Fig. 1) and elsewhere (5, 18, 24) it would appear that tissues with low levels of transitory starch or no starch would be the best candidates for examining vacuoles for potential substrates for β-amylase.

Within the same tissue, α-amylase activity is elevated in tissue sections with reduced Chl concentrations (Tables II and III). At least in stems, this elevation in α-amylase activity is correlated with age and the loss of chloroplast function. Also, α-amylase is greatly increased in tissues that are naturally senescing (Table IV) or have been induced to lose plastid function by physiological manipulation such as placing plants in continuous darkness over an extended period (Table V) or treating plants with inhibitors of chloroplast protein synthesis such as norflurazon, chloramphenicol, or lincomycin (24). The α-amylase that is elevated with loss of plastid function is the primarily apoplastic α-amylase (1, 2, 24). These data indicate that the primarily apoplastic α-amylase is associated with senescence and/or other physiological processes that results in loss of plastid function. Since there are no known substrates in the apoplast for this α-amylase (2), the role of this enzyme in senescence or other physiological processes remains a mystery.

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