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Exoamylase Activity in Vacuoles Isolated from Pea and Wheat Leaf Protoplasts[1]

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ABSTRACT

Vacuoles isolated from pea (*Pisum sativum*), and wheat (*Triticum aestivum*) leaf protoplasts contained considerable activities of electrophoretically highly mobile exoamylases. Vacuoles from spinach (*Spinacia oleracea*) leaf and photoautotrophic *Chenopodium rubrum* suspension culture cell protoplasts were devoid of amylolytic activity. Endoamylase activity was in all cases associated primarily with the chloroplast.

Although amylases are considered to cooperate with phosphorylases in the degradation of the transitory starch accumulated in chloroplasts (3), their overall physiological role in leaves is far from clear. Several reports suggest the presence of considerable activities of these enzymes outside the chloroplast (7, 10, 12, 15). No extrachloroplastic homoglucans which could act as substrates for such amylases are known and no explanations have been tendered to account for the presence of these enzymes outside the chloroplast in which transitory starch turnover is known to take place. The precise intracellular location of extrachloroplastic amylases is not known (15), and a more definite assessment of their compartmentation is required in order to understand their significance for the assimilatory cell. We have analyzed subcellular fractions obtained from the protoplasts of both leaf and photoautotrophically growing suspension culture cells to acquire more insight into the compartmentation of amylases in assimilatory plant cells.

MATERIALS AND METHODS

Buffers for Protoplast Preparation and Fractionation. Buffer A: 5 mM Mes, 1 mM $CaCl_2$, and 0.5 M sorbitol (pH 6.0). Buffer B: 5 mM Mes, 1 mM $CaCl_2$, 0.5 M sucrose, and 20% Percoll (Pharmacia) (pH 6.0). Buffer C: 5 mM Mes, 1 mM $CaCl_2$, 0.4 M sucrose, 0.1 M sorbitol, and 10% Percoll (pH 6.0). Buffer D: 25 mM Hepes, 2 mM EDTA, and 20% Ficoll 400 (Pharmacia) (pH 7.8). Buffer E: 10 mM Hepes, 2 mM EDTA, 0.5 M sorbitol, and 5% Ficoll (pH 7.6). Buffer F: 10 mM Hepes, 2 mM EDTA, and 0.5 M sorbitol (pH 7.6).

Preparation of Protoplasts. All steps of protoplast preparation and fractionation were carried out at room temperature. In separate experiments, 2 g portions of leaves of each of greenhouse-grown pea (*Pisum sativum*, cv Kleine Rheinländerin), wheat (*Triticum aestivum*, cv Kolibri), and spinach (*Spinacia oleracea*, cv vital) were cut into thin strips under buffer A. The strips were vacuum infiltrated and incubated in the dark for 3 h in 30 ml portions of buffer A supplemented with 2% Cellulase TC and 1% Rohament P5 (both Serva) and adjusted to pH 5.5. Protoplasts were released from the leaf tissue by shaking the incubation mixture, decanting the supernatant fluid, and repeatedly washing the leaf strips with buffer A. The crude protoplast suspension (combined supernatants) was filtered through a 100 μm net and centrifuged for 5 min at 165*g*. The sediment was taken up in 50 ml buffer A and centrifuged again. The washed, pelleted protoplasts were then suspended in 7 ml buffer B and overlayered with 2 ml buffer C and 2 ml buffer A. Upon centrifugation at 1000*g* for 10 min intact protoplasts gathered at the buffer C:buffer A interface.

Two g portions of photoautotrophically growing cells of *Chenopodium rubrum* (6) were harvested after 3 weeks of culture and incubated as described above in 30 ml portions of buffer A supplemented with 2% Driselase (Fluka) and 1% Rohament P5 and adjusted to pH 5.5. Protoplasts released from the cells were filtered, sedimented, washed, and purified by flotation as described above.

Fractionation of Protoplasts. Purified protoplasts in buffer A were lysed osmotically by gently mixing 4.5 ml buffer D with 3 ml protoplast suspension. The mixture was overlayered with 2 ml buffer E and 2 ml buffer F and centrifuged for 10 min at 1000*g*. Vacuoles banded at the buffer E:buffer D interface and chloroplasts sedimented at the bottom of the centrifuge tube. Suspensions of protoplasts, chloroplasts, and vacuoles were sonicated and centrifuged at 50,000*g* and the supernatant protein extracts were stored frozen until analysis.

Preparation of Zymograms of Amylase Activity. Extracts of protoplasts, chloroplasts, and vacuoles were subjected to PAGE in slab gels of 1.5 mm thickness using the buffers of Davis (8). The separation gel contained 7.5% acrylamide, 0.2% bisacrylamide and 0.2% amylopectin (Serva). After electrophoresis the gels were equilibrated with 0.1 M citrate buffer (pH 6.0) and incubated for 5 h in the same buffer containing 10 mM 2-mercaptoethanol. The gels were then stained with a solution of 14 mM KI and 10 mM I_2 and fixed with a mixture of 30% methanol and 5% acetic acid. The amylopectin in the gel thus stains dark violet and reveals areas of enzyme activity as unstained or differently stained regions according to the type of hydrolytic starch degrading enzyme present (12). Unstained areas indicate complete amylopectin breakdown due to the action of endoamylases, and pink regions reveal the degradation of amylopectin to β-limit dextrin by exoamylases. Debranching enzyme debranches amylopectin to form amylose, which stains blue with iodine.

Enzyme Assays. Amylolytic activity was assayed by measuring the release of reducing groups (as maltose units) in a reaction mixture containing 0.1 M citrate buffer (pH 6.0), 1.5 mM NaF, 0.5% soluble starch (Serva) and protein extract at 30°C with

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dinitrosalicylic acid reagent (4). α-Mannosidase and β-*N*-acetylglucosaminidase were assayed according to Boller and Kende (5) at 30°C. NADP-malate dehydrogenase was activated for 10 min at room temperature at pH 9.0 with 0.5 M NaCl and 0.1 M DTT as described by Scheibe *et al.* (16) and then assayed at 25°C. NAD-malate dehydrogenase was assayed as was NADP-malate dehydrogenase, but using NADH instead of NADPH. PEPcarboxylase was assayed according to Hüsemann *et al.* (9). All assays were corrected for unspecific activities by running blanks without substrates.

Other Analytical Procedures. Soluble protein was determined by dye-binding (17). Chl was extracted into 96% methanol and estimated according to Arnon (2). TLC was carried out as described by Nurok and Zlatkis (14).

RESULTS AND DISCUSSION

Amylase Activity and Pattern in Protoplasts. Protoplasts were obtained in comparable yields from each type of plant material (corresponding to 0.28 to 0.40 mg Chl per g leaf or culture cell fresh weight). Pea and *C. rubrum* protoplasts contained considerably higher amylolytic activities (on an average approximately 110 and 100 nmol reducing groups released · min^{-1} · mg^{-1} protein, respectively) than did those of wheat and spinach (about 45 and 25 nmol · min^{-1} · mg^{-1} protein, respectively). Zymograms of amylolytic activity prepared from extracts of the protoplasts (Fig. 1, lanes P) reveal the types of amylolytic enzymes contributing to the respective starch-hydrolyzing potential. All protoplast extracts contained endo- and exoamylase and debranching enzyme activities, but activity banding patterns showed specific differences with respect to the plant material. Whereas endoamylase activity (bands a) in wheat was limited to very slowly migrating bands, indicating a high degree of affinity of the enzymes for the amylopectin in the gel, additional electrophoretically more mobile forms were evident in the cases of pea, spinach and *C. rubrum*. Debranching enzyme activity (bands b) was strongly pronounced in pea and *C. rubrum*, but was only marginally detectable in the cases of wheat and spinach. Differences in exoamylase activity (bands c) were particularly evident. The zymograms of pea and wheat show strong activities of a sharply banded, electrophoretically highly mobile form of this type of amylase (c1), whereas exoamylase activity in spinach and *C. rubrum* migrated more slowly in the gels and did not band sharply (c2).

Compartmentation of Amylases. Gentle osmotic disruption of the protoplasts yielded preparations of chloroplasts and vacuoles found in each case to be largely free from contamination with protoplasts or with vacuoles and chloroplasts, respectively, upon microscopic examination. Chloroplasts were all determined to be highly intact, as judged by ratios of Chl content: NADP-malate dehydrogenase activity (this enzyme is considered to be exclusively chloroplastic: see *e.g.* Ref. 11) calculated for the protoplasts and chloroplasts (better than 80% intactness in all cases). Vacuoles all readily took up neutral red, and the similarities of the ratios of α-mannosidase:β-*N*-acetylglucosaminidase activity in both protoplasts and vacuoles indicated that these organelles, too, were highly intact.

The results of enzymic analysis of the chloroplast and vacuole extracts are summarized in Table I. Whereas the chloroplast preparations contained considerable activities of the primarily mitochondrial NAD-malate dehydrogenase, they demonstrated very little contamination by cytosolic or vacuolar marker enzymes. All vacuole preparations contained only trace activities of enzymes of other subcellular fractions. The amylase activities determined for each of the chloroplast and vacuole fractions can thus be considered to truly be representative of the particular organelle in question.

As indicated in the Table, up to one-fourth of the amylolytic activity of wheat, spinach, and *C. rubrum* protoplasts is attributable to the chloroplasts, whereas this figure can be somewhat higher in the case of pea. Vacuoles of spinach and *C. rubrum* contain practically no amylolytic activity. The considerable proportion of the amylolytic activity of spinach and *C. rubrum* protoplasts thus not accounted for may be ascribed to the cytosolic compartment of these cells. Pea vacuoles contained at least one-half of the amylolytic activity of the protoplasts, and wheat vacuoles almost as much as did the protoplasts. The fact that more than 100% of the protoplastic activity was recovered in the subcellular fractions of wheat could be due to the absence of inhibitory substances present in the protoplast extracts in the chloroplast and/or vacuole extracts.

Zymograms of amylolytic activity of the chloroplast and vacuole extracts are shown in Figure 1 (lanes C and V). Endoamylases present in the zymograms of protoplast extracts represent the major amylolytic activities of the respective chloroplasts. All chloroplasts also contained debranching enzyme activity. The endoamylases in the pea and wheat chloroplast zymograms stand in contrast to results recently published by Kakefuda *et al.* (13) and Jacobsen *et al.* (10), who, working with another pea variety and barley, respectively, and other methods of detection, could not find any endoamylase to be present in the chloroplast. Vacuoles of spinach and *C. rubrum* evidenced no amylolytic activity other than slowly migrating traces. In contrast, vacuoles of pea and wheat contained pronounced activities of the exoamylases characteristic of the protoplasts of these species. The more slowly migrating species of the endoamylase doublet clearly represented in the wheat protoplast zymogram and only weakly

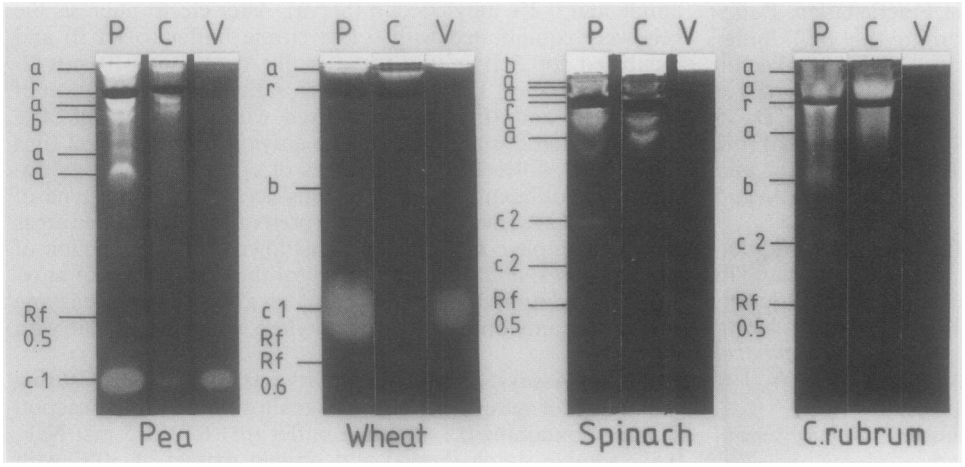

FIG. 1. Zymograms of amylolytic activity in extracts of protoplasts from pea, wheat and spinach leaves and from *C. rubrum* culture cells (P) and of chloroplasts (C) and vacuoles (V) isolated from these protoplasts. Areas of endoamylase activity show up white (a, no staining), those of exoamylase activity pink (c), and those of debranching enzyme activity blue (b) against the violet background of the amylopectin-containing gel. Ribulose bisphosphate carboxylase appears as a dark band (r) due to unspecific iodine staining of the protein. The R_F-value quoted refers to bromophenol blue as the front marker.

Table I. *Recovery of Protoplast Enzyme Activities in Chloroplast and Vacuole Fractions*

Amylolytic activity and the activity of marker enzymes of subcellular compartments in extracts of chloroplasts and vacuoles isolated from protoplasts of pea, wheat, and spinach leaves and *C. rubrum* suspension culture cells. The figures quoted are average values from two experiments: significant variation is indicated.

Enzyme Activity	Enzyme Recovery as Percent of Activity in Protoplasts							
	Chloroplasts				Vacuoles			
	Pea	Wheat	Spinach	*C. rubrum*	Pea	Wheat	Spinach	*C. rubrum*
Marker enzymes								
NADP—malate dehydrogenase (chloroplasts)	83 ± 2	76 ± 3	74 ± 9	42 ± 6	1	1	1	1
NAD—malate dehydrogenase (non-chloroplastic, -vacuolar)	36 ± 4	50 ± 9	36 ± 4	22 ± 4	2	1	2	1
PEP—carboxylase (cytosol)	1	1	1	1	1	1	1	1
α-Mannosidase	4 ± 1	2 ± 1	1	2	17 ± 5	14 ± 4	14 ± 1	7 ± 0.5
β-Acetylglucosaminidase (vacuoles)	4 ± 1	2 ± 1	2 ± 0.5	2	17 ± 4	12 ± 2	15	8
Amylolytic activity								
Actual recovery	28 ± 7	13 ± 3	19 ± 2	10 ± 2	10 ± 4	13 ± 3	1	1
Assuming 100% recovery of respective marker enzyme	33 ± 8	17 ± 4	25	23 ± 1	57 ± 7	95 ± 6	1	1
Type found: (___: major; ( ): minor	endo debr. (exo)	endo (debr.) (exo)	endo (debr.) (exo)	endo debr. exo	(endo) exo	endo exo	(endo)	(endo)

pronounced in the corresponding chloroplast zymogram is also quite evident in the vacuoles from this plant. Amylolytic enzymes present in the protoplasts of pea (the most rapidly migrating endoamylases), spinach (exoamylase), and *C. rubrum* (debranching enzyme and exoamylase) but not or to only a minor extent present in either of the respective chloroplast or vacuole fractions must be considered to be primarily cytosolic in nature.

The pink iodine-staining color resulting from the action of the pea and wheat vacuolar amylases suggests that these enzymes are exoamylases (classically termed β-amylases) which degrade amylopectin to differently staining β-limit dextrins. This conclusion is supported by three additional findings: (a) no amylases at locations comparable to those of c1 along gels were evident in zymograms prepared with β-limit dextrin as a substrate, (b) no reducing groups were released when vacuole extracts were incubated with β-limit dextrin instead of soluble starch, and (c) maltose was the sole degradation product observed upon incubation of vacuole extracts with soluble starch as determined by TLC.

The fact that a large component of the amylolytic activity of the leaf cells of some plants is associated with the vacuole will necessitate a reassessment of the present concepts concerning starch breakdown metabolism in assimilatory organs, particularly as regards the significance of potential starch degradation phenomena in organelles other than the chloroplast. It remains to be clarified if the exoamylases described here are unique to the vacuole and whether they indeed play any role in the metabolism of reserve polysaccharides or are, as has been suggested respective of this type of enzyme (1), irrelevant to starch metabolism.

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