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Partial Purification and Characterization of the Major Endoamylase of Mature Pea Leaves[1]

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ABSTRACT

An endoamylase from leaves of pea (*Pisum sativum*) was purified to near homogeneity by affinity chromatography and ultrafiltration with a yield of about 20%. The purified protein had a specific activity of 686 to 1300 units per milligram protein. Molecular weights of 45 and 41 kilodalton were determined by SDS-PAGE and molecular sieve chromatography, respectively. The purified protein exhibited an action pattern commensurate with that of an endoamylase and exhibited properties indicating it to be very similar to cereal grain α-amylases (calcium requirement, stability to heat, lability to low pH-values, insensitivity to sulfhydryl reagents). Leaf frationation studies indicated that the enzyme was not primarily located in assimilatory mesophyll cells. Chloroplasts isolated from the leaves were found to contain endoamylases, but their activities represented only a small proportion of the total amylolytic potential of the leaf and reflected for the most part properties quite different from those exhibited by the purified enzyme.

Endoamylases appear to be ubiquitous in higher plant leaf tissue (8). In contrast to the well-characterized α-amylases found in germinating cereal grains (26), however, present knowledge concerning the nature and physiological role of the leaf amylases is fragmentary and often even contradictory.

Uncertainty exists as to the properties of endoamylases occurring in leaf tissue. The only endoamylase that has been purified to any significant extent from mature leaf tissue is a nonchloroplastic form from spinach (18). This enzyme, as well as a chloroplastic form from the same source (17), did not possess several of the properties traditionally ascribed to endoamylases as derived from studies on cereal grain α-amylases, and just these properties (calcium requirement, stability to heat, insensitivity to sulfhydryl reagents, lability at low pH-values) are often regarded as diagnostic criteria to establish the presence of endoamylases (20). These findings cast doubt upon the validity of employing such assumed properties as a basis for the determination of endoamylase activity in leaves (21, 25). On the other hand, endoamylases have been identified on the basis of such comparisons in chloroplasts (4), cotyledons (12, 16), and mature leaves (10).

Although there is evidence to suggest that endoamylases initiate the breakdown of assimilatory starch granules, it is not certain that these enzymes are necessarily directly associated with the chloroplasts (24). Ziegler and Beck (29) found endoamylases of protoplasts isolated from mature leaves of pea, wheat, and spinach to be located primarily in the chloroplast and other studies have indicated endoamylases to be present in chloroplasts (2, 4, 18). Endoamylase activity in leaves has, however, also been reported as being exclusively extrachloroplastic in nature (10, 11).

The purification and description of the compartmentation of endoamylases from the mature leaves of species other than spinach should be undertaken to help resolve the uncertainties as to the nature and functional role of these enzymes in assimilatory leaf tissue. This would provide insight into the diversity or similarity shown by leaf endoamylases and allow a more extensive comparison of these enzymes and the cereal grain α-amylases which have hitherto shaped our conception of what constitutes a typical plant endoamylase. Distribution studies would enable an assessment of whether the hydrolytic potential of the enzyme is relevant to the degradation of assimilatory starch.

The present study reports the partial purification and characterization of an endoamylase from shoots of pea plants, along with the results of an investigation into the localization of the enzyme within the leaves.

MATERIALS AND METHODS

Plant material. *Pisum sativum* L. (var Kleine Rheinländerin) was grown in vermiculite in a greenhouse. Fully expanded leaves either alone or attached to stalks (shoots) were harvested 3 to 4 weeks after sowing, before the plants began to flower.

Purification of Endoamylase. Shoots were homogenized in a Waring Blendor with 2 ml of 20 mM calcium acetate containing 20 mM 2-mercaptoethanol (pH 6.0) per g plant material. The homogenate was filtered through cheesecloth and centrifuged at 20,000*g*. The supernatant solution was frozen at −20°C and then thawed and cleared by centrifugation as above. This supernatant solution was slowly filtered through a 3 × 4 cm column of amylose (practical grade from potato, Sigma: bed volume 1 ml per 50 ml extract) equilibrated with extraction buffer in the cold. The column was washed in the cold with 10 bed volumes of 10 mM calcium acetate containing 10 mM 2-mercaptoethanol (pH 6.0), followed by 3 bed volumes of the same buffer without 2-mercaptoethanol. The column was then eluted at room temperature with 10 mM calcium acetate (pH 6.0) containing the 75% ethanol-soluble component of a commercial dextrin preparation (Dextrin 10, Serva) at a concentration corresponding to 2% (w/v) of the original dextrin preparation. Eluate fractions containing high amylolytic activity were pooled and passed to an extent of approximately 99% through the PM 30 membrane of a Centricon ultrafiltration unit (Amicon). The filtrate was then concentrated approximately 50-fold on a Centricon PM 10 ultrafiltration membrane. This final concentrate, along with other fractions from the purification procedure, was stored with 30 to 50% glycerol (w/v) at −20°C until analysis.

Preparation of Protoplasts and Chloroplasts. Protoplasts were

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prepared from leaves as described by Ziegler and Beck (29) and sedimented at 165*g* prior to extraction.

Chloroplasts were isolated from leaves according to three separate procedures:

(a) Finely sliced leaves (15 g) were homogenized with 150 ml 50 mM Hepes buffer containing 0.33 M sorbitol (pH 7.6) for 5 s using an Ultraturrax (Janke and Kunkel) at 10,000 rpm. The homogenate was filtered through cheesecloth (8 layers) and Miracloth and the filtrate centrifuged at 500*g* for 5 min. The sediment (crude chloroplasts) was suspended in 100 ml of the homogenization buffer and centrifuged again for 5 min at 500*g*. The sediment consisted of washed chloroplasts. All procedures were carried out at 0 to 4°C.

(b) Crude chloroplasts prepared as described above were suspended in 50 ml of the homogenization buffer, layered over AP 100 silicone oil (Wacker), and centrifuged for 15 min at 8,000*g* in a swing-out rotor at 4°C. Intact chloroplasts sedimented to the bottom of the centrifuge tube.

(c) Chloroplasts were isolated from purified protoplasts as described by Ziegler and Beck (29).

Sedimented protoplasts and chloroplasts were ruptured with 50 mM Hepes containing 10 mM 2-mercaptoethanol (pH 7.6) in a Potter homogeniser. Crude extracts prepared by centrifuging the homogenates at 50,000*g* were stored with 50% glycerol (w/v) at −20°C until analysis. Crude enzyme extracts from the leaves used to isolate the chloroplasts were also prepared with the same buffer, using a mortar and pestle for homogenization.

Assay of Amylolytic Activity. Amylolytic activity was assayed according to the following standard procedure, unless otherwise indicated. Protein extract was added to an assay mixture containing 50 mM sodium acetate buffer (pH 6.0), 1.5 mM NaF, and 1% soluble starch (according to Zulkowsky, Serva). Aliquots of the reaction mixture were withdrawn after 0, 10, 20, and 30 min of incubation at 30°C and the release of reducing groups was determined with dinitrosalicylic acid reagent (3). One unit of activity was defined as the release of 1 μmol of reducing power (as maltose equivalents) per min. All activities were linear with respect to the amount of enzyme tested and over a period of 30 min unless otherwise noted. The only reagents used in the present study at the concentrations quoted to affect the intensity of the color developed by maltose with the dinitrosalicylic acid reagent were sulfhydryl-reducing agents. The nonspecific enhancement of the color development due to the presence of these substances was determined in control experiments and was taken into account for calculation of activities.

In some cases amylolytic activity was determined by iodine staining of residual starch, using the assay mixture and incubation and sampling conditions described above. Aliquots of the reaction mixture (0.1 ml) withdrawn subsequent to addition of the protein extract were added to 50 μl of I_2/KI (10/14 mM) dissolved in 0.1 N HCl. The mixture was diluted with 2 ml water and the A read at 555 nm. Amylolytic activity was calculated on the basis of the initial linear decrease in the absorbtion value.

PAGE. PAGE of native enzymes was carried out as described by Ziegler and Beck (29), omitting amylopectin from the separation gel in some cases. Zymograms of amylolytic activity were prepared as described by Ziegler and Beck (29), except that citrate buffer was replaced by 0.1 M sodium acetate containing 1 mM calcium acetate (pH 6.0) for equilibration and incubation of the gels. Zymograms of phosphorylase activity were prepared in a similar manner, but amylopectin was omitted from the separation gel and 0.1 M sodium citrate buffer (pH 6.0) supplemented with 20 mM glucose-1-P, 0.2% soluble starch, and 2 mM 2-mercaptoethanol was used to incubate the gel (23).

SDS-PAGE was carried out according to the same basic system as described above, but with the following modifications. The separation gel contained 12.5% acrylamide, 0.17% bisacrylamide, and no amylopectin. SDS was added to the spacer and separation gels and to the running buffer at 0.1%. Sample protein was precipitated with 80% acetone and boiled for 2 min with 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol dissolved in spacer gel buffer prior to application. Gels were stained with 0.5% Serva Blue R 250 in 40% ethanol and 10% acetic acid, and destained with 30% methanol and 5% acetic acid.

Molecular Sieve Chromatography. A 15 × 295 mm column of Superose 12 (Pharmacia) was used for mol wt determination in conjunction with an HPLC unit from LKB. Purified sample material and calibration proteins were applied to the column in 50 mM sodium acetate (pH 6.0) and eluted with the same buffer. Proteins were detected in the eluate via their absorbance at 280 nm and/or with respect to their specific catalytic activity.

Other Analytical Procedures. Assays of NADP-malate dehydrogenase, PEP-carboxylase, and α-mannosidase activities, determination of soluble protein, estimation of Chl (extracted into 80% acetone) and TLC were carried out as described by Ziegler and Beck (29).

RESULTS

Purification of Endoamylase from Pea Shoots. Amylolytic activity of crude extracts from pea shoots was resolved into two forms of slowly migrating endoamylase and a single rapidly migrating exoamylase upon electrophoresis in amylopectin-containing gels (Fig. 1A, lane 1). Low activities of debranching enzyme detectable in the extracts were not evident on such zymograms. Phosphorylase activity was also present (Fig. 1B, lane 1). Since leaves alone contained essentially the same amylolytic complement as did whole shoots (compare Fig. 6B), the latter were used to purify endoamylase typical of the leaves on a preparative scale.

Table I shows the absolute and specific amylolytic activities registered during the course of the purification procedure using freshly harvested shoots as the starting material. Characteristics of the purified preparation as revealed by various PAGE techniques are shown in Figure 1.

The overall purification procedure resulted in a final preparation exhibiting a high specific amylolytic activity (686 units/mg protein: Table I) indicative of amylopectin-degrading activity of the endoamylase type (Fig. 1A, lane 2). This activity corresponded to an essentially single protein species upon SDS-PAGE (Fig. 1C, lane 2), although two bands of amylopectin degradation were observed in zymograms of amylolytic activity. Nondenaturing PAGE of the final preparation in the absence of starch substrate also revealed only one clearly discernible protein-staining band (Fig. 1D, lane 1). This protein co-migrated with amylolytic activity, although a second, less evident area of such starch breakdown activity was also present (Fig. 1D, lane 2), presumably coinciding with protein not sufficient to visibly stain with Serva Blue. It is thus apparent that the single protein species evident upon SDS-PAGE corresponds to two forms of endoamylolytic activity displaying different electrophoretic mobilities. A small amount of phosphorylase activity was also present in the final preparation (Fig. 1B, lane 2; Fig. 1D, lane 3), but this could be removed by heating (see below) and did not interfere with analyses of amylolytic activity, all of which were carried out under phosphate-free conditions.

Preparations of comparable purity were also obtained at similar yields using frozen instead of freshly harvested shoots as the starting material. In these cases a somewhat lower purification factor than that indicated in Table I resulted, since less protein (but similar amylolytic activity) was extracted from the frozen tissues. Specific amylolytic activities of up to 1300 units/mg protein were obtained, presumably due to the presence of less inactive protein in the purified preparations.

All analyses described in the following section were carried

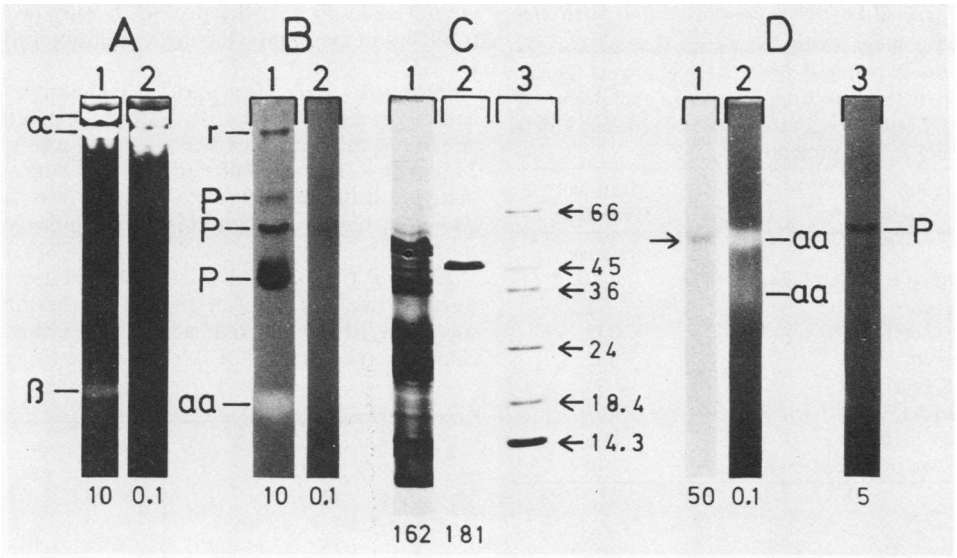

FIG. 1. PAGE of pea shoot crude extract and of the final preparation yielded by the purification procedure. A, Zymograms of amylolytic activity of the crude extract (lane 1) and purified preparation (lane 2). Areas of endoamylase activity show up white (α, no staining) and of exoamylase activity pink (β, β-limit dextrin) against the violet background of the amylopectin-containing gel. B, Zymograms of phosphorylytic activity (lanes 1 and 2 as in A). Starch synthesized by phosphorylases stains darkly (P) against the lighter background of the soluble starch-containing gel. Areas of amylolytic activity are unstained (aa), and Rubisco (r) appears dark due to unspecific protein staining, C, Protein stained with Serva Blue after SDS-PAGE (lanes 1 and 2 as in A). Calibration proteins and their mol wt in kD (see Fig. 3A for details) are shown in lane 3. D, The purified preparation was subjected to nondenaturing PAGE in the absence of amylopectin. Sections of the gel were stained for protein as in C (lane 1: arrow indicates the only visible band), incubated with 0.3% soluble starch dissolved in 0.1 M sodium acetate/ 1 mM calcium acetate (pH 6.0) and stained with iodine (lane 2: areas of amylolytic activity show up light against the darker background: aa), and used to prepare a zymogram of phosphorylytic activity (lane 3). Beginning of the separation gel marked by open frames at the top of the zymograms; number of μl of extract applied to the gel are indicated beneath.

Table I. *Purification of Endoamylase from Pea Shoots*

The starting crude extract was prepared from 330 g freshly harvested shoots. The final yield figure quoted does not take into account the contribution of exoamylase to the activity of the crude extract. When this is considered, the actual yield of the purified endoamylase is about 20%. Values for discarded fractions are included to indicate losses of major components of the starting amylolytic activity.

Stage/Fraction	Volume	Protein	Amylolytic Activity		
			Absolute	Specific	Yield
	ml	*mg*	*U*	*U/mg protein*	*% initial*
Crude extract					
1. Freshly prepared	730	2251	2336	1.04	100
2. Freeze-thawed	700	851	2240	2.63	95.2
Chromatography on amylose					
3. Filtrate (discarded)	670	787	1568	1.99	67.1
4. Dextrin eluate	103	0.74	244	330	10.5
PM 30 membrane filtration					
5. Concentrate (discarded)	1.0	0.193	67.5	350	2.9
6. Filtrate	99.5	0.26	174	670	7.4
PM 10 membrane filtration					
7. Concentrate	2.2	0.243	167	686	7.2

out with the purified preparation documented in Table I and Figure 1.

Characterization of the Purified Endoamylase. *Substrate Specificity and Action Pattern.* The relative rates of hydrolysis of various homoglucans by the purified enzyme are shown in Table II. Amylose was degraded most readily, and branched starch preparations from higher plant material were also effectively hydrolyzed. The enzyme was able to degrade β-limit dextrins prepared from amylopectin and soluble starch, and its complete lack of activity with pullulan as a substrate showed that it was free of debranching enzyme activity. This substrate specificity pattern is typical of an endoamylase.

Exhaustive hydrolysis of soluble starch by the purified enzyme yielded a mixture of glucose, maltose, and maltooligosaccharides with a degree of polymerization of less than 8 (Fig. 2), products typical of endoamylolytic hydrolysis. Incubation of maltooligosaccharides having a degree of polymerization of 4 to 7 revealed that only maltoheptaose was hydrolyzed to a significant extent, although some release of glucose, maltose and smaller maltooligosaccharides from all tested substances was observed. These observations suggest that the binding site of the enzyme is fully effective only over a span of 8 glucose units. An identical action pattern was obtained with a crude preparation of α-amylase from germinated wheat obtained by heating a crude extract at 70°C

Table II. *Activity of the Purified Endoamylase with Various Substrates*

The β-limit dextrins were prepared by exhaustive treatment of the branched substrates with sweet potato β-amylase (Boehringer), precipitation with 75% ethanol (twice) and lyophilization of the alcohol-insoluble residue. The relative rate 100 refers to 620 μmol reducing groups (as maltose) produced · min^{-1} · mg $protein^{-1}$.

Starch substrate at 5 mg/ml	Rate of Hydrolysis
	relative
Soluble starch (according to Zulkowsky, Serva)	100
β-Limit dextrin thereof	71
Amylopectin (potato, Serva)	135
β-Limit dextrin thereof	132
Amylopectin (corn, Sigma)	74
Amylose (potato, Merck)	157
Glycogen (rabbit liver, Sigma)	6
Pullulan (*Aureobasidium pullulans*, Sigma)	0

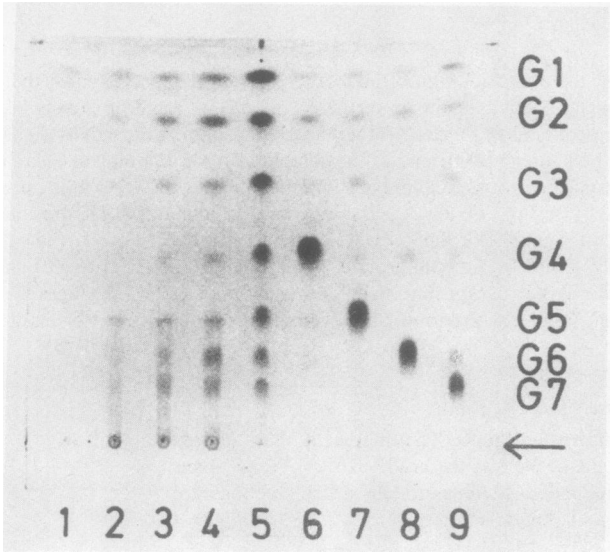

FIG. 2. Action pattern of the purified endoamylase with soluble starch and maltooligosaccharides as revealed by TLC. Purified endoamylase (0.75 units) was incubated with 1% (w/v) of soluble starch or maltooligosaccharide (degree of polymerization 4 to 7, Boehringer) buffered with 10 mM sodium acetate (pH 6.0) for 4 h at 30°C. Samples (2 μl) of the reaction mixture were spotted onto silica gel 60 HPTLC plates (10 × 10 cm: Merck) and developed by 6 replicate runs in acetone:water 4:1 (v:v). Lane 1, enzyme incubated without substrate for 4 h; lanes 2, 3, and 4, incubation with soluble starch for 1, 2, and 4 h; lane 5, standards (glucose G1, maltose G2, and maltooligosaccharides from maltotriose G3 to maltoheptaose G7); lanes 6 to 9, incubation for 4 h with G4 to G7, respectively.

as described by Tkachuk (27).

The activity of the purified enzyme with soluble starch, the substrate used for all other kinetic analyses, showed a maximum velocity of 686 units/mg protein at a substrate concentration of 7.5 mg/ml and higher, and an apparent K_m of 1.0 mg/ml respective of this substrate was calculated.

Molecular Weight. A mol wt of 45 kD was determined by SDS-PAGE for the essentially homogeneous protein of the purified preparation (Figs. 1C and 3A). Amylolytic activity eluted as a single peak at a position corresponding to a mol wt of 41 kD upon molecular sieve chromatography on Superose 12 (Fig. 3B). The similarity of these values indicate that the purified endoamylase was present exclusively as a monomer.

Although the mol wt of the enzyme was determined to be greater than 40 kD, the protein readily passed through an ultrafiltration membrane having a retention rating of 30 kD (Table I).

Response to pH. The purified endoamylase showed maximal activity at pH 6.0 (Fig. 4), and no appreciable change in the activity at this pH value was observed when sodium phosphate, Mes or Bis-Tris[2] were substituted for sodium acetate as the buffer. Activity in the pH range of 7.0 to 8.0 was also identical to that shown in Figure 4 when Hepes or Tricine were used to buffer the assay.

Effect of Calcium. All enzyme assays described in this section were carried out with an amount of the purified enzyme (dissolved in 10 mM calcium acetate) resulting in an endogenous calcium concentration of 0.6 μM in the assay mixture.

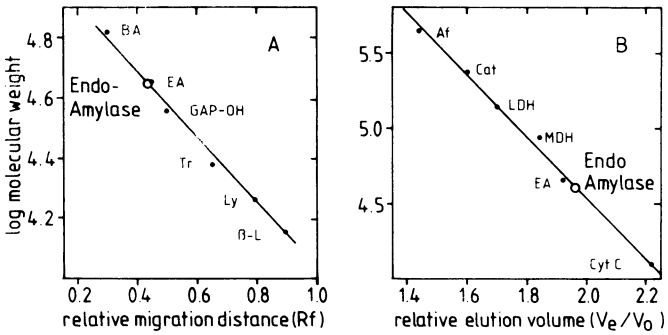

FIG. 3. Determination of the mol wt of the purified endoamylase. A, According to SDS-PAGE; B, according to molecular sieve chromatography on Superose 12. Calibration proteins (all from Sigma) and their mol wt in kD: A: BA, bovine albumin (66); EA, egg albumin (45); GAP-DH, glyceraldehyde 3-P dehydrogenase (36); Tr, trypsinogen (24); Ly, lysozyme (18.4) β-L, β-lactoglobulin (14.3). B: Af, apoferritin (443); Cat, catalase (240); LDH, lactate dehydrogenase (140); MDH, malate dehydrogenase (70); EA, egg albumin (45); Cyt *c*, (12.5). The single protein species in the purified preparation (Fig. 1C, lane 2) and the Superose 12 eluate fraction having the highest amylolytic activity are represented by (○, endoamylase).

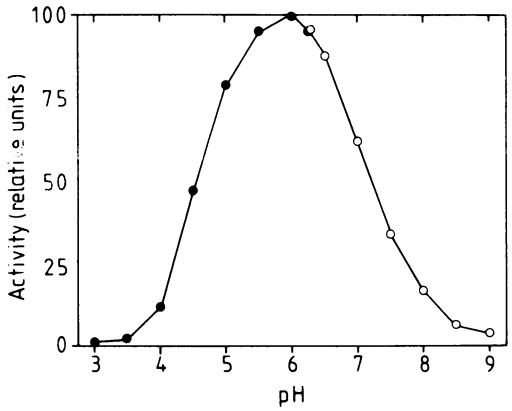

FIG. 4. Effect of pH on the activity of the purified endoamylase. The enzyme was assayed in 50 mM sodium acetate (●) and in 50 mM Bis-tris propane (○). Activity was stable for 30 min at pH 5 to 9. Activity at pH 3 to 4.5 decreased during the first 20 min of the assay to the values indicated.

[2] Abbreviations: Bis-tris, bis(2-bishydroxyethyl)imino-tris-(hydroxymethyl)-methane; Rubisco, ribulose bisphosphate carboxylase; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)-methylaminol-propane; Diamide, azodicarboxylic acid bis(dimethylamide); DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide.

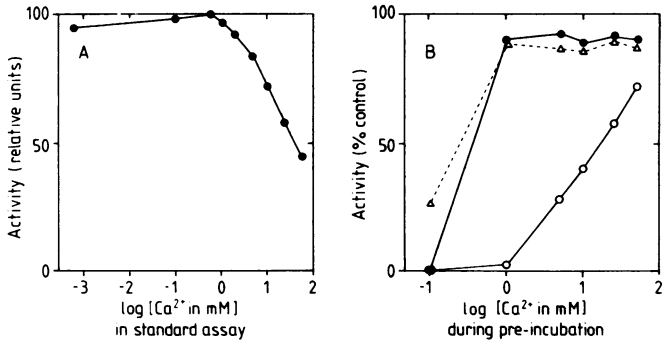

FIG. 5. Effect of calcium on the activity of the purified endoamylase. A, Activity in the standard assay in the presence of 0.6 μM to 50 mM calcium acetate; B, aliquots of the enzyme either without added calcium (endogenous concentration 0.1 mM) or supplemented with calcium acetate to 0.5 to 50 mM were preincubated at 70°C for 10 min at pH 6.0 (——●——), or at pH 4.5 (—△—) and pH 3.6 (——○——) for 30 min at room temperature. Activity was then assayed with 1% soluble starch in an 18-fold excess of 75 mM sodium acetate buffer (pH 6.0). Control values were calculated in each case for a parallel sample preincubated at pH 6.0 at room temperature.

The activity of the purified endoamylase was strongly inhibited by the inclusion of EDTA in the standard assay mixture (10 and 1 mM effected a reduction of activity of over 95 and 80%, respectively, within 10 min). The use of 50 mM sodium citrate (pH 6.0) as a buffer in place of sodium acetate also resulted in a loss of activity amounting to about 90% after 20 min under assay conditions. The purified enzyme exhibited considerably reduced activity in zymograms of amylolytic activity prepared with amylopectin-containing gels incubated with sodium citrate buffer, and no activity was evident in amylopectin-free gels incubated with soluble starch dissolved in sodium citrate buffer (Fig. 1D, lane 3). The inhibitory action of these calcium-chelating compounds could be reversibly counteracted by addition of calcium acetate to the assay mixture or gel incubation buffer. This indicated that calcium is required for the activity of the enzyme. Figure 5A shows the activity of the purified endoamylase in the presence of this ion over a wide range of calcium acetate concentrations. Activity was highest at 0.5 mM calcium, but even far lower levels of this ion were sufficient to effect essentially all of this maximal activity. Higher calcium concentrations resulted in a progressive reduction of activity.

Figure 5B shows that excess calcium stabilized the purified endoamylase against the deleterious effects of high temperature and low pH values, as is the case with the calcium-dependent α-amylases of cereal grains (26). The purified enzyme was totally inactivated by heating for a few minutes at 70°C at a calcium concentration of 0.1 mM or less. One mM of calcium was sufficient for effective stabilization at 70°C for 10 min. The progressive inhibition of the activity of the purified endoamylase under assay conditions at pH values of less than 5 (Fig. 4) was mirrored by considerable and total losses of activity upon preincubation at pH 4.5 and 3.6, respectively, at a low calcium concentration (0.1 mM). The denaturing effect of acidic conditions could be counteracted by higher calcium concentrations. Calcium at 1 mM was sufficient for fairly complete stabilization at pH 4.5. The decreased protective effect of even much higher concentrations of this ion at the lower pH value, however, indicates that the purified enzyme was indeed quite labile at pH 3.6. Preincubation at pH 8.0 or 9.0 resulted in no significant loss of amylolytic activity.

Preincubation of highly diluted preparations of the purified endoamylase (100-fold dilutions of the final concentrate described in Table I) without substrate for 10 to 30 min as described in Figure 5B always resulted in the loss of a considerable proportion of the activity determined when the standard assay was started by addition of concentrated enzyme. This indicates that the presence of substrate itself had a stabilizing effect on the enzyme in a diluted form.

Table III. *Inhibition of the Activity of the Purified Endoamylase by Various Substances*

The iodine-staining assay of amylolytic activity was employed to test the effect of maltose and maltotriose. The standard assay was used for the other tests.

Test Substance	Concentration Required to Effect a Reduction of Activity of		
	10%	50%	90%
$HgCl_2$	0.7 μM	3.5 μM	8.5 μM
$CuCl_2$	0.065 mM	0.28 mM	0.52 mM
Zn-acetate	0.12 mM	0.63 mM	4.0 mM
Acarbose	0.035 μg/ml	0.28 μg/ml	2.2 μg/ml
Bay-e4609	0.10 μg/ml	0.73 μg/ml	5.0 μg/ml
Maltose	4.0 mM	67 mM	ND[a]
Maltotriose	2.5 mM	35 mM	ND[a]

[a] Not observed even with 100 mM test substance.

Table IV. *Effect of Sulfhydryl-Oxidizing and -Alkylating Reagents on the Activity of the Purified Endoamylase*

Diluted aliquots of endoamylase were preincubated for 30 min at room temperature either with the indicated test substances at pH 6.0 (diamide, NEM) or pH 8.0 (all others) or without the test substances at the same pH value. The subsequent assay was performed at pH 6.0. GSSG was from Boehringer, all other test substances were from Sigma. Controls were preincubated and assayed without the test substance.

Test Substance	Activity, Assay with 1 mM Test Substance, Preincubation	
	Without Test Substance	With 10 mM Test Substance
	% control	
Sulfhydryl oxidizing		
Diamide	95	93
Oxidized glutathione	97	78
DTNB	104	16
Sulfhydryl alkylating		
N-Ethylmaleimide	98	68
Iodoacetamide	94	78

Effect of Amylase Inhibitors. Cyclodextrins have been found to strongly inhibit some amylolytic enzymes (14, 15). Neither α-, β-, nor γ-cyclodextrin (cyclohexa- to cyclooctaose: all from Sigma) at a concentration of 1 mg/ml had any effect on the activity of the purified endoamylase. No indication of the potent inhibition shown, for example, by β-cyclodextrin on assimilatory tissue debranching enzyme (14) was thus evident with these compounds.

The effects of three other groups of substances potentially inhibitory to amylolytic activity are compiled in Table III. Heavy metal ions, which have been found in some cases to selectively inhibit plant amylases (9, 16), strongly inhibited the activity of the purified endoamylase at varying concentrations. Only mercury proved to be extremely potent in this respect, and was nearly 100-fold more effective than copper. Zinc was strongly inhibitory only at millimolar concentrations, and 10 mM nickel was required for 50% inhibition. Acarbose and Bay-e4609, both kindly provided by Bayer (Leverkusen), each strongly inhibited the activity of the purified endoamylase at concentrations of less than 1 μg/ml, with acarbose being about three times as effective. Maltose has been reported to inhibit the activity of leaf amylases (2, 28). Both this substance and maltotriose, another end product of

amylolytic starch degradation, significantly inhibited the activity of the purified enzyme at concentrations of 10 mM. Inhibition at even 100 mM of these compounds, however, was still far from complete (46 and 36% of control activity remained with maltose and maltotriose, respectively).

Effect of Sulfhydryl Reagents. The activity of the purified endoamylase was not affected by the presence of the sulfhydryl-reducing agents 2-mercaptoethanol, DTT (both Sigma), or reduced glutathione (Boehringer) at concentrations of up to 10 mM, upon either inclusion in the assay mixture or preincubation with the enzyme in the absence of substrate. Preincubation of the purified endoamylase in the presence of sulfhydryl-oxidizing and -alkylating agents resulted in a moderate loss of activity respective of GSSG, NEM, and iodoacetamide, and in a considerable inactivation in the presence of DTNB (Table IV). None of these compounds, however, had a significant effect on the activity when included in the standard assay at up to 10 mM. These results show that the catalytic activity of the purified endoamylase is insensitive to sulfhydryl reagents in the presence of substrate. Sulfhydryl groups requiring full reduction for maximum activity are nevertheless indicated to be present, but these are only susceptible to chemical modification in the absence of substrate.

DTNB also affected the normally very short migration distance of the purified enzyme upon PAGE in the presence of amylopectin. Preincubation of the enzyme with 10 mM DTNB for 30 min resulted in a 5-fold increase in the R_F values representing endoamylolytic activity in such gels, a phenomenon reversible by the addition of an excess of 2-mercaptoethanol to the application buffer immediately prior to PAGE. This indicates that the reversible loss of catalytic activity due to oxidation of sulfhydryl groups is either coupled to or results alone from an impairment of the ability of the enzyme to bind to its substrate.

Compartmentation of Amylolytic Enzymes in Pea Leaves. Protoplasts prepared from pea leaves were all tightly packed with chloroplasts, and were thus taken to represent primarily the mesophyll cells of the leaves. Chloroplasts prepared either directly from the leaves by mechanical homogenization and washing (chloroplasts 1) or centrifugation through silicon oil (chloroplasts 2) of the sedimented crude organelle preparation or via osmotic rupture of the protoplasts (chloroplasts 3) were all seen to be free of larger cell structures upon microscopic examination. Enzyme activities determined for crude extracts obtained from the leaves and from the protoplast and chloroplast preparations are summarized in Table V, where their basis on a common Chl content show them to represent equivalent amounts of chloroplastic material. Zymograms of amylolytic activity present in the extracts are shown in Figure 6.

Leaves of pea plants contained amylolytic activities very similar to those obtained from whole shoots (*cf.* Fig. 6B, lane L with Fig. 1A, lane 1). The amylolytic activity of protoplasts prepared from the leaves was, however, much lower than that found in the leaves on a Chl basis (Table V), and also showed a different distribution pattern. In Figure 6A a protoplast extract is compared with a whole leaf extract representing 4-fold less leaf Chl. Although the exoamylase activity of the two samples was approximately equal, the protoplast extract contained far less endoamylase activity than did the leaf extract. These data indicate that much of the exoamylase and most of the endoamylase activity present in pea leaves is not located in the assimilatory mesophyll cells. This is especially true of the endoamylase purified in the present study, the two characteristic zymogram bands of which are clearly evident in Figure 6A, lane L. Since the bulk of the leaf endoamylase activity has not otherwise been localized, however, it is conceivable that such activity actually present in the mesophyll cells was unstable under the conditions employed to prepare protoplasts and was thus lost.

The approximately equal activities of NADP-malate dehydrogenase (an enzyme considered to be exclusively chloroplastic, [29]) in all extracts investigated (Table V) indicates that all chloroplast preparations were essentially intact with respect to their integrity for chloroplast enzymes. The chloroplasts contained very low activities of cytosolic (PEP-carboxylase) and vacuolar (α-mannosidase) marker enzymes and were thus largely free of contamination with enzymes of other major subcellular compartments. The amylolytic activities determined for the chloroplast extracts were much lower than those found in protoplast extracts (Table V). This indicates that the amylolytic activity of pea chloroplasts represents only a very small proportion of that present in whole leaves, and constitutes only a minor fraction of that present in assimilatory mesophyll cells.

Figure 6 indicates that exoamylase activity was virtually absent from the chloroplast extracts. All chloroplast preparations contained pronounced debranching enzyme activity, and endoamylases were also unmistakably evident. The distribution patterns of the chloroplastic endoamylases differed only slightly with respect to the method of chloroplast preparation. Chloroplasts contained essentially all of the endoamylolytic complement of the protoplasts from which they were isolated (Fig. 6A). Electrophoretic analysis of amylolytic activity on an equivalent Chl basis under conditions optimal for the development of the purified endoamylase showed that essentially all of this latter activity characteristic of whole leaf extracts was absent from the chloroplast extracts (Fig. 6B). Zymograms prepared with 5-fold more concentrated extracts under conditions less favorable for the activity of the purified endoamylase revealed the presence

Table V. *Enzymic Activities Present in Crude Extracts of Pea Leaves and in Extracts of Protoplasts and Chloroplasts Prepared from the Leaves*

The Chl content corresponding to each extract was determined from the homogenate of the material prior to centrifugation. The modes of chloroplast preparation according to different procedures (1–3) are described as designated in the text and "Materials and Methods."

Extract	Enzyme Activities			
	NADP-malate dehydrogenase	PEP-carboxylase	α-Mannosidase	Amylolytic
	$\mu mol \cdot (mg\ Chl)^{-1} \cdot min^{-1}$			
Leaves	2.38	0.463	2.10	2.07
Protoplasts	2.49	0.251	1.17	0.408
Chloroplasts				
1	2.37	ND[a]	ND[a]	0.083
2	2.48	ND[a]	ND[a]	0.094
3	2.51	ND[a]	ND[a]	0.093

[a] Not detected to a significant extent (present to less than 5% of the value quoted for the protoplast extract).

of numerous additional endoamylolytic activities in the chloroplast extracts, which could now also be recognized in the whole leaf extract (Fig. 6C). The intensity and distribution of endoamylolytic activities in a chloroplast preparation (chloroplasts 1) appeared to be independent of the type of incubation buffer employed, and heating of the extract in the presence of calcium destroyed most of the chloroplastic endoamylase forms (Fig. 6D). These data indicate that chloroplasts (and mesophyll cells) of pea leaves contain several forms of endoamylase which differ from the main endoamylolytic activity of whole leaves in that they apparently do not require calcium and are heat-labile.

Quantitative Distribution of Endoamylolytic Activity in Pea Leaves. Approximately 30% of the total amylolytic activity of the crude extracts of both leaves and shoots could still be observed after heating the extracts for 10 min at 70°C in the presence of 10 mM calcium. PAGE analysis of the heated extracts revealed that essentially all of the original endoamylolytic activity was still evident, whereas all exoamylase and phosphorylase activity had been destroyed. Assuming that 90% of the endoamylolytic activity of the crude extract survived the heat treatment (as described above respective of the purified endoamylase), it follows that endoamylase activity accounted for approximately one-third of the total amylolytic activity measured in shoot or leaf extracts. This indicates that the final recovery of endoamylase activity from pea shoot crude extracts as obtained by the purification procedure was about 3-fold higher than that quoted in Table I and was thus in the order of 20%.

The principal amylolytic activity present in pea leaf or shoot extracts was thus indicated to be due to an exoamylase. Since this enzyme was identified in the present study only on the basis of its degradative action on amylopectin upon PAGE, however, further studies will be required to unequivocably demonstrate its exolytic character.

The total amylolytic activity of pea chloroplasts amounted to only about 4 to 5% of that of the leaves and to less than one-quarter of that of the protoplasts from which the organelles were isolated. Since these chloroplasts contained practically no exoamylase and otherwise only debranching enzyme activity (which degrades soluble starch only very slowly) in addition to endoamylases (Fig. 6), it is evident that at most 15% of the total leaf endoamylolytic activity was associated with mesophyll cell chloroplasts. Since chloroplastic endoamylase activity represented essentially all of the corresponding activity in protoplasts (Fig. 6A), the figures quoted in Table V indicate that at least 75% of the amylolytic activity of the protoplasts could be ascribed to exoamylase (*cf.* Ref. 29). It can also be calculated that only about 25% of the total exoamylolytic activity of the leaf was associated with the mesophyllic cells.

DISCUSSION

The amylolytic enzyme purified by the procedure described above was unequivocably an endoamylase, as shown by its ability to degrade branched starch forms and their β-limit dextrins and its action pattern with soluble starch. The decisive purification step with respect to isolating the endoamylase was adsorption of the enzyme onto amylose, which removed other amylolytic enzymes and most extraneous protein, and desorption with a soluble dextrin preparation. This affinity technique is based on the well-known property of endoamylases to adsorb to starch substrates (22), which is also illustrated by the very limited electrophoretic mobility of the endoamylase in polyacrylamide gels containing amylopectin. A similar technique was recently employed to isolate phosphorylase from crude extracts of pea leaves (6). The use of amylose as described in the present study also resulted in the co-isolation of phosphorylase, which could, however, be mostly removed by selective ultrafiltration. The purity of the final preparation was shown by the essentially single protein species observed upon SDS-PAGE and the lack of contamination with exoamylase or debranching enzyme activity.

The specific activity of the purified endoamylase (686–1300 units/mg protein) was far higher than that determined for the only other endoamylase isolated from mature leaves (7.05 units/mg: 18), and was comparable to that of α-amylase purified from germinating wheat (1300 units/mg: 27) and with those of the best commercially available endoamylases (from hog pancreas, Calbiochem: 750 units/mg, and from *Bacillus subtilis*, Serva: 660 units/mg). The mol wt calculated for the purified enzyme according to either SDS-PAGE (45 kD) or molecular sieve chro-

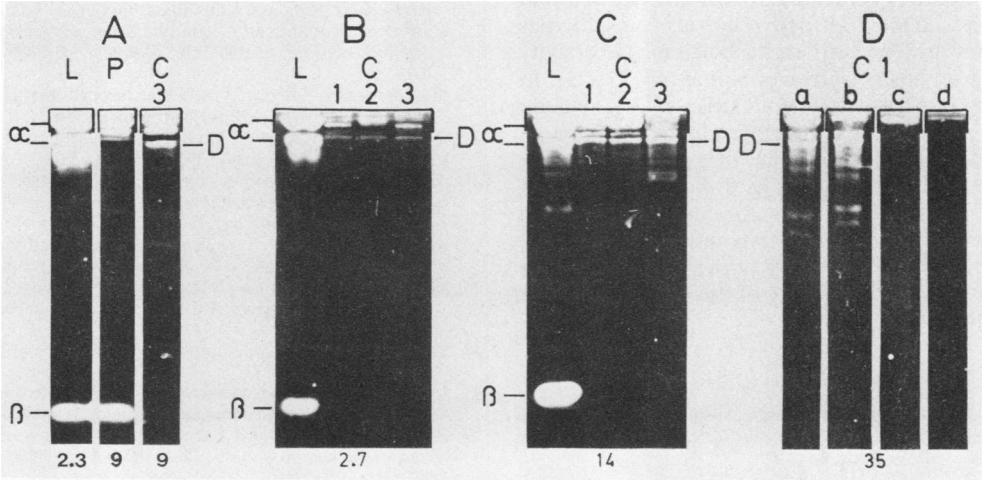

FIG. 6. Zymograms of amylolytic activity of extracts prepared from leaves, protoplasts, and chloroplasts. A, Comparison of extracts of leaves, protoplasts, and chloroplasts prepared from the protoplasts; gel incubated with 0.1 M sodium acetate/1 mM calcium acetate (pH 6.0). B, Comparison of extracts of leaves and all chloroplasts; gel incubated as in A. C, As in B, but gel incubated with 0.1 M sodium citrate (pH 6.0). D, A chloroplast extract untreated (a, b) and heated for 10 min at 70°C with 10 mM calcium (c, d); gel incubated as in A and B (a, c) and as in C (b, d). Lane designations: L, leaves; P, protoplasts; C, chloroplasts (C1–C3 correspond to the three modes of chloroplast preparation designated in the text and in "Materials and Methods"). D denotes debranching enzyme activity (light blue due to the presence of amylose against the violet gel background), and β indicates exoamylase activity (as in Fig. 1A). All other light bands correspond to endoamylase activity (see Fig. 1A, including the two bands corresponding to the purified enzyme denoted by α). Beginning of the separation gel marked by open frames at the top of the zymograms; μg of Chl equivalents applied to the gel are indicated beneath.

matography (41 kD) agrees well with the figures determined for cereal, bean, and sorghum α-amylases (45–48 kD: 26) and for the presumed monomeric form of extrachloroplastic spinach leaf endoamylase (40 kD: 18). In the latter study a dimeric form of the spinach leaf enzyme was also inferred to exist. Although the pea leaf endoamylase was a monomer, the two discrete bands of amylolytic activity of different electrophoretic mobility observed upon nondenaturing PAGE indicate that the enzyme comprises two different forms having an identical mol wt.

The action pattern, dependence on calcium for activity (and stability at high temperature and low pH) and insensitivity to sulfhydryl reagents of the purified endoamylase show this enzyme to possess catalytic properties very similar to those of cereal grain α-amylases (26) and quite different from those of the only endoamylase hitherto purified from mature leaves (18). Endoamylases in mature assimilatory plant tissue are thus by no means restricted to calcium-independent, heat-labile enzymes. Most leaves may not, however, possess such calcium-dependent, heat-stable endoamylases (7), and care must thus be taken to not inadvertantly select for or against a particular type of the enzyme by the indiscriminate use of selective treatment or assay conditions.

The absence of most of the endoamylase purified in the present study from the mesophyll cells and chloroplasts indicates that most of the endoamylolytic potential of pea leaves is not directly related to the degradation of assimilatory starch. This is also true respective of the majority of the exoamylase activity of the leaf, in addition to the enigma presented by the extrachloroplastic localization of pea leaf mesophyll exoamylase (29). Detailed studies of the tissue-specific localization of the major amylolytic activities in pea leaves must be undertaken in order to begin to understand the significance of this hydrolytic potential for the metabolism of the leaf. The localization of extrachloroplastic amylases in epidermal tissue has been suggested (5, 24).

The results of the present study confirm an earlier report (29) that pea leaf chloroplasts contain endoamylases. Their apparent lack of calcium requirement and heat stability demonstrate that various types of endoamylases having fundamentally different properties may be present in the same leaf, and may account for their not having been detected in other studies (25). It remains to be determined to which extent these endoamylases are actually involved in the degradation of assimilatory starch in pea leaf mesophyll chloroplasts, in view of their relatively low activities and the concommitant presence of debranching enzyme activity (see above) and known phosphorylytic potential (13, 23, 25). In addition to enabling a more complete comparison with the main endoamylolytic activity of the leaf, further characterization of the chloroplastic endoamylases along the lines described in the present study may provide information as to possible means of regulation of the *in situ* activity of these enzymes. Sensitivity to pH changes in the pH range of from 7 to 8 and to the presence of maltose as described above respective of the purified enzyme could well be of relevance to the activity of the endoamylases in chloroplasts (1, 2, 19).

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