Plant Physiol. (1990) 92, 615–621
0032-0889/90/92/0614/07/$01.00/0

Received for publication August 18, 1989
and in revised form October 17, 1989

Purification and Characterization of Pea Epicotyl β-Amylase[1]

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ABSTRACT

The most abundant β-amylase (EC 3.2.1.2) in pea (*Pisum sativum* L.) was purified greater than 880-fold from epicotyls of etiolated germinating seedlings by anion exchange and gel filtration chromatography, glycogen precipitation, and preparative electrophoresis. The electrophoretic mobility and relative abundance of this β-amylase are the same as that of an exoamylase previously reported to be primarily vacuolar. The enzyme was determined to be a β-amylase by end product analysis and by its inability to hydrolyze β-limit dextrin and to release dye from starch azure. Pea β-amylase is an approximate 55 to 57 kilodalton monomer with a pI of 4.35, a pH optimum of 6.0 (soluble starch substrate), an Arrhenius energy of activation of 6.28 kilocalories per mole, and a K_m of 1.67 milligrams per milliliter (soluble starch). The enzyme is strongly inhibited by heavy metals, *p*-chloromercuriphenylsulfonic acid and *N*-ethylmaleimide, but much less strongly by iodoacetamide and iodoacetic acid, indicating cysteinyl sulfhydryls are not directly involved in catalysis. Pea β-amylase is competitively inhibited by its end product, maltose, with a K_i of 11.5 millimolar. The enzyme is partially inhibited by Schardinger maltodextrins, with α-cyclohexaamylose being a stronger inhibitor than β-cycloheptaamylose. Moderately branched glucans (e.g. amylopectin) were better substrates for pea β-amylase than less branched or non-branched (amyloses) or highly branched (glycogens) glucans. The enzyme failed to hydrolyze native starch grains from pea and glucans smaller than maltotetraose. The mechanism of pea β-amylase is the multichain type. Possible roles of pea β-amylase in cellular glucan metabolism are discussed.

The physiological roles of β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) in plant cells are not well understood. Traditionally, β-amylase has been associated with starch degradation. Although the entire pathway of starch degradation has not been conclusively established in any plant tissue (28), it has long been attributed to various combinations of activities of α-amylase, β-amylase, starch debranching enzyme, starch phosphorylase, and α-glucosidase (1). Not all of these enzymes are active in all starch-containing tissues, and not all of these enzymes are located in the same cellular compartment as is particulate starch. Several studies with pea indicate that most of the cell's β-amylase activity is extrachloroplastic and that chloroplasts contain very low or no β-amylase activity (12, 13, and refs. contained therein). In contrast, one study with pea (34) indicates relatively high β-amylase activity in chloroplasts; however, even in this study most of the β-amylase was found to be extrachloroplastic with 50 to 60% localized in vacuoles. As higher plant particulate starch is contained within plastids, the role of vacuolar β-amylase in starch degradation is uncertain. Furthermore, the role of nonvacuolar β-amylase has not been established, and in some storage tissues β-amylase appears to be inessential for starch degradation (29).

To understand the possible roles β-amylase may have in cellular glucan metabolism, it is necessary to elucidate the physical and kinetic properties of the enzyme. While the characteristics of β-amylase from storage tissues such as barley (15) and wheat (30) grains, soybean seeds (17, 19), and sweet potato tubers (29) have been well documented, much less information is known about β-amylases from tissues containing transitory starch, such as leaves. We present here the purification and characterization of β-amylase from pea epicotyls.

MATERIALS AND METHODS

Plant Tissue

Pea (*Pisum sativum* L. cv Laxton's Progress No. 9) seeds were surface-sterilized, sown, and watered as previously described (2). Plants used for enzyme extraction were grown in a growth chamber in continuous darkness at 20°C and were harvested after 2 weeks. Plants used for starch grain extraction were grown for 9 d in a greenhouse at 20 to 22°C, with natural lighting.

Enzyme Extraction

All extraction and purification steps were done at 4°C and all buffers contained 0.04% (w/v) NaN_3. Frozen (−20°C) pea epicotyls were homogenized (1 g/2 mL buffer) with a Polytron[3] in buffer A (20 mM Hepes [pH 6.8], 3 mM DTT, 3

[1] This material was supported by the Cooperative State Research Service, U.S. Department of Agriculture, under agreement No. 87-CRCR-1-2324; the College of Agriculture and Life Sciences, University of Wisconsin-Madison; and the USDA Agricultural Research Service.

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mM $CaCl_2$, 20 μM leupeptin). The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 40 min. This crude extract supernatant was dialyzed overnight against buffer B (10 mM Mes [pH 5.0], 3 mM thiodiglycol, 0.5 mM $CaCl_2$). Protein was estimated by the Bradford method (3), using BSA as a standard, at all steps of purification.

Chromatography

The dialyzed, crude enzyme extract was centrifuged at 10,000*g* for 40 min and the supernatant was applied to a DEAE-cellulose column (2.5 × 20 cm) that was equilibrated with buffer B. The column was washed with 250 mL of buffer B and bound proteins were eluted with a linear 0 to 500 mM NaCl gradient in 300 mL buffer B. Fractions with β-amylase activity were pooled and dialyzed overnight against buffer C (20 mM Hepes [pH 7.0], 150 mM NaCl, 3 mM thiodiglycol, 1 mM $CaCl_2$).

The dialyzed enzyme preparation resulting from anion exchange chromatography, concentrated by ultrafiltration with a YM10 membrane (Amicon Macrosolute Concentrator), was then applied to a Sephadex G-75 column (1.5 × 95 cm) and eluted with buffer C. Fractions containing β-amylase activity were glycogen-precipitated by the method of Silvanovich and Hill (26). Precipitates were resuspended in buffer C and centrifuged at 20,000*g* for 15 min. Pellets were washed in buffer C by resuspension and centrifugation several times. Pooled fractions were then stored at −20°C for further purification.

The native M_r of β-amylase was determined by column chromatography on a Sephacryl S-200 (Pharmacia) gel filtration column (1.5 × 90 cm) previously equilibrated with buffer C. Mol wt protein standards used to calibrate the column were the same as previously used (12).

Electrophoresis

Preparative (3 mm) native gel electrophoresis of glycogen-precipitated β-amylase was by a modification of the procedure previously described (11). The separation gel was a 7 to 15% (w/v) polyacrylamide linear gradient. Electrophoresis was at 46 mA through the stacking gel and at 60 mA thereafter. β-Amylase activity was located by incubating a small strip of gel in 2% (w/v) starch, in assay buffer, for 30 to 45 min. The gel was rinsed twice with H_2O and then stained with 100 mM KI/1 mM I_2 solution. β-Amylase activity appeared as a light band on a dark background. The area containing β-amylase activity was excised from the remainder of the gel and dialyzed against buffer C.

Analytical (1.5 mm) native gel electrophoresis and electrophoretic transfer of proteins through starch containing gels were performed as described by Kakefuda and Duke (11), with modifications described by Beers and Duke (2).

SDS-PAGE was modified from that described by Laemmli (14). Acrylamide stock solution consisted of 30% (w/v) acrylamide and 0.8% (w/v) *N,N'*-bis-methylene acrylamide. Final concentrations in the 1 cm stacking gels were 4% acrylamide, 100 mM Tris-HCl (pH 6.8), 0.08% SDS, and 2.6 mg/mL ammonium persulfate. Final concentrations in the 6 cm separating gels were 13.8% acrylamide, 750 mM Tris-HCl (pH 8.8), 0.2% SDS, and 0.75 mg/mL ammonium persulfate. Gels were polymerized by the addition of 0.075% (stacking gel) or 0.025% (separating gel) final concentration of *N,N,N',N'*-tetramethylethylenediamine. Sample buffer consisted of 0.2 mg/mL bromphenol blue, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, and 125 mM Tris-HCl (pH 6.8). Samples were made to 33% (v/v) sample buffer and placed in a boiling water bath for 10 min. Electrode buffer was 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 1 mg/mL SDS. Electrophoresis was at constant current (20 mA) until the dye front reached the gel bottom.

Isoelectric focusing was performed at 10°C on prefocused (30 min) LKB PAGPlates (pH range 4.0–6.5) as directed by the manufacturer. After focusing was completed, gels were either fixed and silver stained (20), or stained for activity (11). Gel pH gradients were determined by excising pieces from gels, eluting ampholines for 1 h, and then determining the pH of eluates.

Enzyme Assays

Production of reducing sugars from glucan substrates was measured using dinitrosalicylic acid reagent (10). Assays of column eluates were conducted in assay buffer (100 mM K-succinate [pH 6.0], 1 mM $CaCl_2$, 0.04% NaN_3) for 15 min (starch and β-limit dextrin) or 2 h (pullulan) at 30°C. Assays for total amylolytic activity contained 2% (w/v) Lintner soluble potato starch (Sigma No. S-2630) as the substrate. Debranching enzyme activity was measured with 2% (w/v) pullulan as the substrate. The combined activities of α-amylase and debranching enzyme were determined using β-limit dextrin, prepared by the method of Perten (23), as the substrate. When determining the rates of soluble starch hydrolysis in the presence of varying concentrations of maltose, the starch iodine method (7) was used.

Assays using maltodextrins as substrates were in buffer C, and 100 μg substrate was used per 200 μL assay. The hydrolysis products were quantitated after HPLC separation by the method of Henson (9).

Hydrolysis of inulin was assayed according to Henson (9) except the buffer was the same as that used for measuring β-amylase hydrolysis of soluble starch.

Starch Grain Extraction

Cotyledons from 9 d old seedlings were disinfected for 10 min with NaOCl and rinsed under running distilled water. Starch grains were then extracted in toluene as described by Chang (4) or in buffer as described below.

Cotyledons (100 g) were homogenized with a Polytron in 200 mL of buffer D (50 mM Hepes [pH 7.5], 3 mM DTT, 1 mM $CaCl_2$), filtered through four layers of cheesecloth and centrifuged at 3000*g* for 5 min. Pellets resuspended in 60 mL grinding buffer were centrifuged again, resuspended in 25 mL of buffer D, and layered onto a sucrose gradient (10 mL 60% sucrose over 10 mL 80% sucrose). Gradients were centrifuged at 150*g* for 5 min, 600*g* for 5 min, and then 1400*g* for 10 min. Pelleted starch was washed 3 times with storage buffer (50 mM Hepes [pH 6.0], 3 mM DTT, 1 mM $CaCl_2$, 0.04%

NaN_3, 20 μM leupeptin) and stored at 4°C in 100 mL storage buffer.

RESULTS AND DISCUSSION

Enzyme Purification

Etiolated tissues were used to avoid accumulation of ribulose-1,5-bisphosphate carboxylase/oxygenase, the presence of which makes β-amylase purification much more difficult. The presence of starch hydrolyzing enzymes other than β-amylase required screening of column fractions with several substrates until the contaminating glucanases were eliminated. Elimination of contaminating carbohydrases was usually achieved by DEAE-cellulose chromatography (Fig. 1). Fractions containing β-amylase activity eluting from the DEAE-cellulose column were identified by their ability to hydrolyze soluble starch and their failure to hydrolyze β-limit dextrin and pullulan (Fig. 1). No exoamylase other than β-amylase was detected in pea tissues. Occasionally, some β-amylase fractions pooled from anion exchange chromatography were contaminated with a small amount of α-amylase which was removed by gel filtration through Sephadex G-75. Unless otherwise stated, the β-amylase preparation obtained after anion exchange and gel filtration chromatography (Table I)

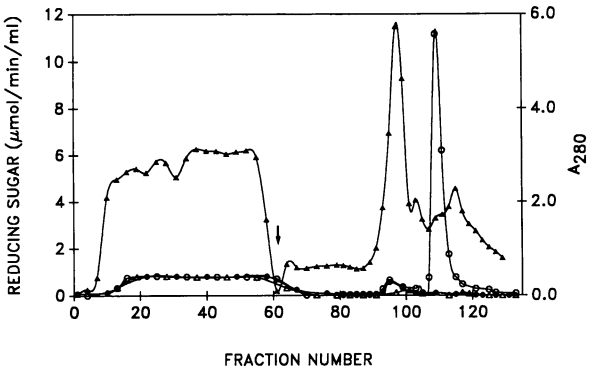

Figure 1. Elution profile of pea epicotyl proteins with amylolytic activities from a DEAE-cellulose column (2.5 × 20 cm) equilibrated with buffer B (see "Materials and Methods"). Unbound proteins were washed from the column with 250 mL of buffer B (fractions 1–60), and bound proteins were eluted with a 0 to 500 mM NaCl gradient (300 mL, fractions 60–135) applied at the arrow. Amylolytic activities were assayed by the hydrolysis of soluble starch (○), β-limit dextrin (●), and pullulan (△). Protein (▲) was monitored by A_{280}.

with the highest specific activity (286 μmol min^{-1} mg^{-1} protein) and free of contaminating amylases (Figs. 1 and 2) was used in the characterization studies. β-Amylase was further purified, >800-fold, by glycogen precipitation and preparative native gel electrophoresis. Although α-amylases have been purified by glycogen precipitation (26), this is the first reported use of the procedure in β-amylase purification.

Several affinity chromatographic techniques were tried during the purification process. Cyclodextrin affinity chromatography of both α- and β-amylases (10, 26, 32) and starch affinity chromatography of β-amylase (28) have been used to achieve high levels of purification and high yields with a single step. Our attempts using α-cyclodextrin, starch, amylose, and maltose-agarose did not give satisfactory results. Either the enzyme failed to bind to the ligand (cyclodextrin, starch, amylose) or selective elution from other proteins was not achieved (maltose-agarose). A 1% solution of α-cyclodextrin reduced activity of β-amylase to 27% of the control, indicating the enzyme can bind α-cyclodextrin, although the use of this ligand in an affinity column was not efficacious.

Physical Properties of β-Amylase

The failure of β-amylase to bind to concanavalin A indicated it was not a glycoprotein with a high content of α-mannose. Additional evidence that β-amylase was not a glycoprotein was that after isoelectrofocusing, β-amylase did not stain with periodic acid-Schiff reagent (24). Furthermore, its SDS-PAGE M_r wt did not change after overnight incubation with endoglycosidase H from *Streptomyces plicatus* (31). β-Amylases from several plant species (5, 15, 19, 29) and *Clostridium thermosulphurogenes* (25) have not been found to be glycoproteins. In contrast, the β-amylase from *Entamoeba histolytica* trophozoites may be a glycoprotein as it bound to concanavalin A (21).

The M_r weight of pea epicotyl β-amylase, determined from the mean of four experiments with a Sephacryl S-200 gel filtration column, was 55 kD. This is in good agreement with the mol wt (57 kD) determined by SDS-PAGE of the enzyme obtained after preparative, native gel electrophoresis (Fig. 3). Hence, pea epicotyl β-amylase is a monomer. The M_r of pea epicotyl β-amylase is similar to that of β-amylases from barley (15) and soybean (19). Larger, multimeric β-amylases have been reported from sweet potato (native = 197 kD, subunit = 64 kD) (29), *Vicia faba* leaves (native = 107 kD, subunit = 26 kD) (5), and *C. thermosulphurogenes* (native = 210 kD, subunit = 51 kD) (25).

Table I. *Purification Steps for Pea Epicotyl β-Amylase*

Fraction	Total Protein[a]	Total Activity[a]	Specific Activity[a]	Purification	Yield
	mg	*μmol min^{-1}*	*μmol min^{-1} mg^{-1} protein*	*-fold*	%
Crude	824	966	1.17	1.00	100
DEAE	26.2	748	28.5	24.4	77.5
G-75	3.89	452	116	99.3	46.8
Glycogen precipitation	0.65	361	553	473	37.4
Preparative native PAGE	0.28	284	1030	878	29.5

[a] Values are the means of four purifications.

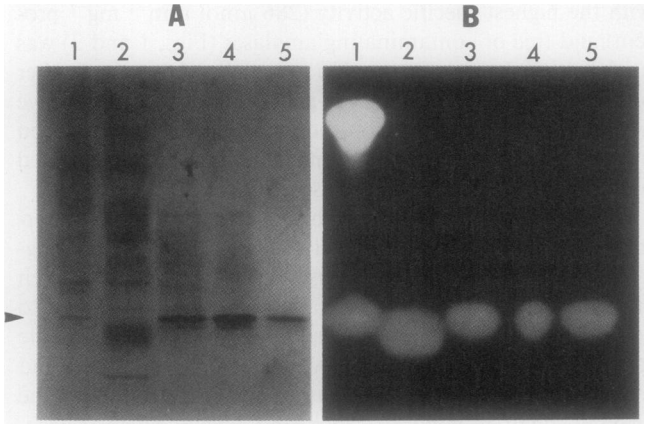

Figure 2. Native PAGE of purification steps of pea epicotyl β-amylase. Gels were either silver stained for protein (A) or stained for amylolytic activity (B). The arrow indicates β-amylase. In both gels, lane 1 contains crude extract and other lanes contain protein from purification steps: lane 2, DEAE-cellulose anion exchange chromatography; lane 3, Sephadex G-75 gel filtration; lane 4, glycogen precipitation; lane 5, preparative native PAGE. Protein (in μg) loaded on gels was as follows: lanes A1 (2.25); B1 (6.00); A2 (2.18); B2 (1.45); A3 (1.16); B3 (0.20); A4 (1.56); B4 (0.10); A5 (0.20); B5 (0.04). The large clear area in lane 1 in gel b is due to the presence of the primarily apoplastic α-amylase found in pea (*cf.* ref 2).

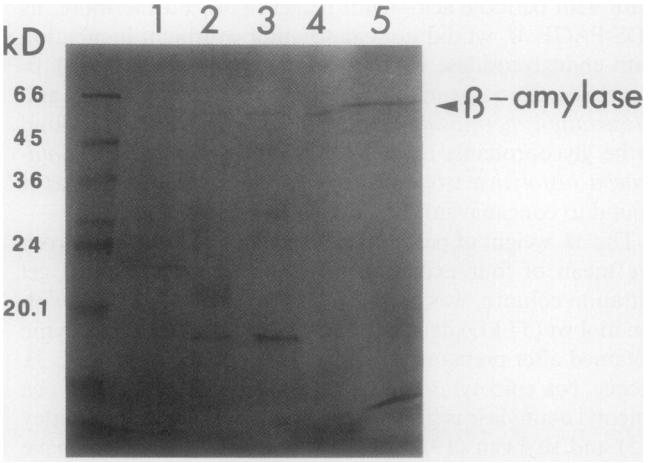

Figure 3. Silver staining of SDS-PAGE separated pea epicotyl β-amylase at different purification steps. The left lane contains SDS low mol wt standards (Sigma). Lane 1 contains crude extract and other lanes contain protein from purification steps: lane 2, DEAE-cellulose anion exchange chromatography; lane 3, Sephadex G-75 gel filtration; lane 4, glycogen precipitation; lane 5, preparative native PAGE. Protein (in μg) loaded on the gel was as follows: lane 1 (0.30); 2 (0.29); 3 (0.27); 4 (0.26), 5 (0.26).

When subjected to isoelectric focusing and subsequent staining of the gels for either amylase activity or protein content, pea epicotyl β-amylase appeared as a single band at pH 4.35 (Fig. 4). This was in sharp contrast to the multiple charge isoforms of β-amylase found in barley (four isoforms) (15), soybean (seven isoforms) (17), and wheat (four isoforms) (30). The pea β-amylase pI was near that of the most acidic

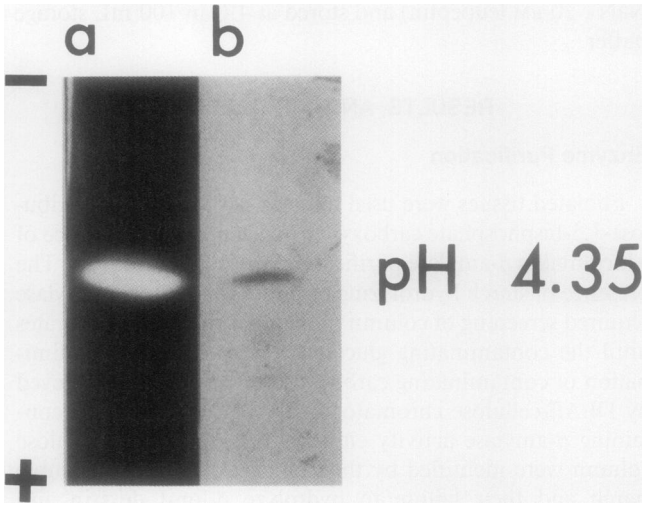

Figure 4. Isoelectrofocusing gel of purified pea epicotyl β-amylase. The left half of the gel was infiltrated with starch and stained for amylolytic activity with KI/I_2 solution and the right half of the gel was silver stained. The pH gradient of the gel was from 6.5 (top) to 4.0, as determined with a pH electrode.

Table II. *Effects of Heavy Metals, Sulfhydryl Reagents, and Cyclodextrins on Pea Epicotyl β-Amylase Activity*

All concentrations of potential activators or inhibitors were 1 mM except where noted. Except for iodoacetamide and iodoacetic acid, all potential inhibitors were preincubated for 5 min with β-amylase at the assay pH before the addition of substrate. Iodoacetamide and iodoacetic acid were preincubated with β-amylase at pH 8.8 for 5 min (control also preincubated at pH 8.8 for these compounds), after which an aliquot was added to the reaction mixture at pH 6.0 containing substrate.

Compound	Activity
	μmol min^{-1} mg^{-1} protein (% starch control)
Control (boiled Lintner potato starch)	286 (100)
$CuCl_2$	11.5 (4.0)
$HgCl_2$	7.44 (2.6)
$FeCl_3$	296 (103)
NaCl	279 (97.7)
$CaCl_2$	277 (96.7)
$MgCl_2$	274 (95.6)
$ZnCl_2$	269 (94.0)
Iodoacetamide	263 (92.0)
Iodoacetic acid	242 (84.5)
N-Ethylmaleimide	12.0 (4.2)
p-Chloromercuriphenylsulfonic acid	0 (0)
β-Cycloheptaamylose (10 mg/mL)	232 (81.2)
α-Cyclohexaamylose (10 mg/mL)	77.5 (27.1)

wheat isoform and was more acidic than any isoform found in barley or soybean.

Plant β-amylases have been reported to require free sulfhydryl groups for activity and are inhibited by heavy metals as well as other thiol binding reagents (16, 29, 30). Pea β-amylase was inhibited by relatively high concentrations of Hg^{2+} and Cu^{2+} ions whereas other metals had little or no affect on activity (Table II). The sulfhydryl reagents *N*-ethylmaleimide

and *p*-chloromercuriphenylsulfonic acid greatly reduced or totally inhibited pea β-amylase activity. In contrast, iodoacetamide and iodoacetic acid had only marginal inhibitory effects on the activity of pea β-amylase. This pattern of sulfhydryl reagent selectivity is similar to that for *Bacillus polymyxa* β-amylase and not like that reported for other plant β-amylases which are largely inhibited by low concentrations of any sulfhydryl reagent (16). These data indicate that pea β-amylase sulfhydryls are not directly involved in catalysis. All exposed β-amylase cysteinyl sulfhydryls would be reactive to the concentrations of sulfhydryl reagents used in this study and all sulfhydryl reagents should have totally inhibited activity if cysteinyl sulfhydryls were necessary for catalysis. Pea β-amylase inhibition by sulfhydryl reagents could be due to binding of noncatalytic cysteinyl sulfhydryls, causing changes in the alignment of catalytic amino acids, as is proposed for sweet potato β-amylase (29), or by causing steric hinderance.

Kinetic Properties of β-Amylase

Pea epicotyl β-amylase hydrolysis of soluble starch was maximal at pH 6.0. Most β-amylases have an acidic pH optimum, ranging from pH 4.5 to 6.2 (15, 17, 30).

The Arrhenius energy of activation (E_a), calculated from 20 to 30°C, was 6.28 kcal/mol maltose produced with soluble starch as substrate. This is similar to the values reported for barley malt and wheat β-amylases; 5.5 to 9.3 kcal/mol (8), but about half the values reported for β-amylases from alfalfa tap roots (7). The data for pea β-amylase were linear from 5 to 40°C indicating that the same catalytic step was rate limiting at all temperatures. Thermal denaturation was evident at temperatures over 40°C (data not shown). Nonlinear Arrhenius plots have been reported for several β-amylases (7, 8), indicating that in some species the substrate binding and product release steps of the reaction mechanism may be separable by temperature, or that the enzyme goes through a temperature induced phase transition.

As determined by the Lineweaver-Burk method, the apparent Michaelis constant (K_m) for soluble starch was 1.67 mg/mL. Starch hydrolysis by pea β-amylase was competitively inhibited by the end product, maltose, with a K_i of 11.5 mM at pH 6.0. Nomura *et al.* (22) reported competitive inhibition by maltose of soybean β-amylase with K_i values of 5.8 mM at pH 5.4 and 4.1 mM at pH 8.0. Sweet potato β-amylase was also competitively inhibited by maltose with a K_i of 6 mM (18). In contrast, Thoma *et al.* (29) reported that maltose is a noncompetitive inhibitor of sweet potato β-amylase.

Pea β-amylase is inhibited by Schardinger dextrins (cyclodextrins), with α-cyclohexaamylose being a much stronger inhibitor (about 73% inhibition) than β-cycloheptaamylose (about 19% inhibition) (Table II). This is similar to sweet potato and *B. polymyxa* β-amylases, which are also more susceptible to cyclohexaamylose (16).

Substrate Specificity and Enzyme Mechanism

The amylase purified in this study was determined to be an exoamylase by its inability to hydrolyze β-limit dextrin (Fig. 1; Table III) and to release Remazol brilliant blue dye from starch azure (*cf.* ref. 6). It was determined to be a β-amylase by its inability to hydrolyze glucose residues from maltodextrins (Table IV) or starch (data not shown), its apparent ability to only hydrolyze maltose from α-1,4-linked dextrins (Table IV), and its production of a limit dextrin that stains lavender with KI/I_2 (Fig. 2B) (*cf.* refs. 2 and 11).

Table III. *Pea Epicotyl β-Amylase Substrate Specificity*

All substrates were at an initial concentration of 2% (w/v). All α-glucan substrates, except for maltodextrins, inulins, and panose, and where noted, were boiled prior to use. Assay times were 5 to 10 min, except for those with nonboiled starch grains, which were assayed both for 30 min at 30°C and for 23 h at 37°C, and pullulan and inulins, which were incubated for 2 h. Activity values are for production of reducing sugars, except for inulin and maltodextrin hydrolysis, which were quantitated by HPLC.

Substrate	Activity
	μmol min⁻¹ mg⁻¹ protein (% starch control)
Control (Lintner potato starch)	286 (100)
Nonboiled Lintner potato starch	0 (0)
Amylopectin (potato)	304 (106)
Glycogen type III (rabbit)	146 (51.0)
Glycogen type VII (mussel)	124 (43.2)
Glycogen type II (oyster)	118 (41.3)
Glycogen type VIII (limpet)	99 (34.5)
Amylose (potato)	143 (50.0)
Amylose (maize)	116 (40.7)
Maltoheptaose (G_7)	292 (102)
Maltotriose (G_3)	0 (0)
Maltose (G_2)	0 (0)
β-Limit dextrin (potato starch)[a]	0 (0)
Pullulan (*Aureobasidium pullulans*)	0 (0)
Nigeran (α-D-Glc-[1→3]-α-D-Glc-[1→4])$_n$	0 (0)
Panose (α-D-Glc-[1→6]-α-D-Glc-[1→4]-α-D-Glc)	0 (0)
Nonboiled starch grains (pea)[b]	0 (0)
Starch grains (pea)[b]	426 (149)
Nonboiled starch grains (pea)[c]	0 (0)
Starch grains (pea)[c]	698 (244)
Inulin (chicory)	0 (0)
Inulin (dahlia)	0 (0)
β-Glucan (barley)	0 (0)

[a] Prepared by the method of Perten (23). [b] Buffer extracted (see "Materials and Methods"). [c] Toluene extracted by the method of Chang (4).

In general, pea epicotyl β-amylase preferred a branched substrate (*i.e.* soluble starch or amylopectin) over a less branched or nonbranched (amylose) substrate (Table III). Glycogens, highly branched glucans, were not hydrolyzed as rapidly as starch and amylopectin, presumably due to steric hinderance. The glycogen hydrolysis rates were equivalent to rates of amylose hydrolysis. Amylose hydrolysis rates were probably limited by the availability of nonreducing ends, which are the sites of β-amylase attack. Pea β-amylase failed to cleave the α-1,6-, α-1,3-, and α-1,4-linkages found in pullulan, panose, and nigeran, and was incapable of hydrolyzing β-linked carbohydrates such as barley β-glucan (β-1,3- and β-1,4-linkages).

Products of hydrolysis of the oligosaccharide series malto-

Table IV. *Product Analysis of Maltodextrin Hydrolysis by Pea Epicotyl β-Amylase*

Assays were for 5 min with 500 μg substrate/mL and 1.13 units β-amylase (sufficient activity to completely hydrolyze G_7 to G_2 and G_3 in 5 min, based on the initial rate of activity [1 min]) in a final assay volume of 200 μL. Products were separated by HPLC.

Substrate	Final Concentration of Product				
	G_1	G_2	G_3	G_4	≥G_5
			μM		
G_3	0	0	990	0	0
G_4	0	865	0	280	0
G_5	0	660	620	0	0
G_6	0	980	0	220	0
G_7	0	840	320	0	≥44

triose (G_3)[4] through G_7 were examined to determine if the enzyme was a β-amylase and if the enzyme mechanism was a single catalysis or multiple catalysis per enzyme-substrate encounter. β-Amylase failed to cleave glucans smaller than G_4 (Tables III and IV). It did cleave G_4 through G_7, and it always produced G_2 but never G_1. If the enzyme was an endoamylase, even if G_2 was the product with the lowest possible degree of polymerization, G_4 should have been one of the products of G_7 hydrolysis. This was not the case, indicating that this enzyme is indeed an exoamylase and a β-amylase.

After the addition of sufficient β-amylase activity to completely hydrolyze maltodextrin substrates in about 2 min (based on initial rates with G_7), glucan chains of G_5 were totally hydrolyzed and G_7 were almost completely hydrolyzed. In contrast, G_4 and G_6 were only partially hydrolyzed, presumably due to the more rapid accumulation of G_2, the end-product competitive inhibitor of β-amylase, with the hydrolysis of G_4 and G_6 (Table IV). For every hydrolysis of G_4, two G_2s are formed and with complete G_6 hydrolysis, three G_2s per two cleavages. In contrast, only one G_2 is formed for each possible hydrolysis reaction with G_5 and G_7. Maltodextrins with degrees of polymerization greater than the initial substrate were not formed, indicating that this β-amylase had no glucanotransferase activity. Pea tissues contain high levels of D-enzyme (glucanotransferase) activity (12, 13).

The maltodextrin hydrolysis data (Table IV) indicate that the active site had four subsites which must be filled for catalysis to occur and that hydrolysis occurs between the second and third subsites. More than four subsites capable of binding glucose may be present but apparently are not required to be occupied for catalysis to occur.

Pea epicotyl β-amylase releases its substrate after a single catalysis, and thus the mechanism is of the multichain type. This was determined by identifying end products resulting from G_7 hydrolysis (Table IV and data not shown). When end products were examined after 1 to 5 min of hydrolysis, G_5 was detected, although it could not be accurately quantified due to some overlap with the G_7 peak. If the enzyme had completely hydrolyzed a single molecule of G_7 without release of the substrate (single chain mechanism), only G_2 and G_3 would have been detected. If β-amylase functions only by the multiple attack mechanism (>1 hydrolysis occurs per enzyme-substrate encounter), only G_2 and G_3 would result from complete hydrolysis of G_7. No G_5 would be released. In time courses of G_7 hydrolysis, G_5 was detected; hence, pea β-amylase must function via the multichain attack mechanism. With this mechanism, β-amylase releases substrate and product after a single catalysis and the enzyme is then free to attack any other substrate glucan. This accounts for the production of G_5, G_3, and G_2 from the hydrolysis of G_7.

Both α- and β-amylases have been found, albeit only a small percentage of the cell's total amylase activity, in pea chloroplasts (2, 13). However, it is not totally clear as to which isoforms of pea β-amylase are chloroplastic. Ziegler and Beck (34) have found that the only β-amylase associated with pea chloroplasts is the same isoform as that found in the vacuole. This primarily vacuolar and rapidly migrating β-amylase is the most active isoform of β-amylase in pea and is the isoform purified and characterized in the work presented here (*cf.* Fig. 2 and with ref. 34). In contrast, Beers and Duke (2) and Kakefuda *et al.* (13) did not detect the vacuolar β-amylase in pea chloroplasts but did find a second apparent isoform of β-amylase in plastids. This least active isoform of β-amylase has not been characterized. Consequently, some of the β-amylase in pea epicotyl cells may function in hydrolysis of chloroplastic, particulate starch. Incubation of pea β-amylase with nonboiled, buffer-extracted or with nonboiled, toluene-extracted starch grains did not result in the production of any detectable reducing sugars (Table III). No reducing sugars were detected when nonboiled, soluble Lintner potato starch was incubated with β-amylase. When either buffer-extracted starch grains, toluene-extracted starch grains, or soluble starch were boiled before incubation with β-amylase, reducing sugars were detected. The pea epicotyl β-amylase that was in etiolated tissues used in this study apparently cannot initiate attack upon pea starch grains. No β-amylase has been demonstrated to attack starch grains that were not already degraded by other carbohydrases.

What remains to be determined is the identity of the *in vivo* substrate(s) of β-amylase. Steup (27) has found low mol wt glucans in pea and spinach leaf extracts that might be available for hydrolysis by β-amylase, as could be the larger α-glucans found by Yamada *et al.* (33) in other plant species. Identification of the *in vivo* substrates of these amylases is a prerequisite to assigning a physiological role in cellular glucan metabolism.

Potential Vacuolar Substrates, Inhibitors, or Activators

Since the primary β-amylase in pea has been reported to be located in the vacuole (34), and that is the β-amylase that we have purified in this study, pea β-amylase activity with potential vacuolar substrates was determined and the effects of vacuolar compounds on β-amylase activity were tested. Pea β-amylase had no activity with chicory or dahlia inulins (linear β-2, 1-polyfructosyl sucrose) (Table III), which are vacuolar storage carbohydrates in many species. Malate and fumarate additions (up to 50 mM) to assays had little or no effect on starch hydrolysis rates (data not shown). Sucrose did not

[4] Abbreviation: G_n, maltodextrins with n = number of glucose residues.

greatly influence starch hydrolysis rates, although concentrations of 20 to 50 mM sucrose did result in about 15% inhibition of β-amylase activity (data not shown).

Possible Roles of Pea β-Amylase

The ≃56 kD β-amylase characterized here is the major β-amylase in vegetative tissues of pea (Figs. 1 and 2; refs. 2, 34) and is primarily vacuolar in location (34). Two studies have failed to find this isoform of β-amylase in the chloroplast (2, 13), whereas another two studies have found this enzyme associated with chloroplasts (34; EP Beers, SH Duke, unpublished data). This β-amylase is incapable of hydrolyzing large glucans (*e.g.* soluble starch) or starch grains (Table III) unless these substrates are gelatinized by boiling. However, the enzyme readily hydrolyzes maltodextrins with a relatively low degree of polymerization (Table IV). These data indicate that if this enzyme is involved in chloroplast starch grain degradation, it must be secondary and would involve the hydrolysis of relatively small maltodextrins produced directly from starch grain degradation or from the disproportionating activity of chloroplast D-enzyme (11). In that the major α-amylase in pea (Fig. 2B) is primarily apoplastic (2; M Saeed, SH Duke, unpublished data), and only a very low level of its activity has been reported to be associated with chloroplasts, the role of any amylase in pea chloroplast starch grain attack is unconfirmed at this time.

The vacuolar location (34) of the β-amylase characterized here, and the substrate specificity of this enzyme, could lead one to speculate that the role of this β-amylase is in the hydrolysis of maltodextrins stored in the vacuole. Until a rigorous report of the presence or absence of α-glucans in vacuoles is published, such a role remains speculative.

ACKNOWLEDGMENT

We thank Robert D. Vogelzang for helping with the inhibitor studies.

LITERATURE CITED

1. **Beck E** (1985) The degradation of transitory starch granules in chloroplasts. *In* R Heath, J Preiss, eds, Regulation of Carbon Partitioning in Photosynthetic Tissue. American Society of Plant Physiologists, Rockville, MD, pp 27–44
2. **Beers EP, Duke SH** (1988) Localization of α-amylase in the apoplast of pea (*Pisum sativum* L.) stems. Plant Physiol **87:** 799–802
3. **Bradford M** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
4. **Chang CW** (1979) Isolation and purification of leaf starch components. Plant Physiol **64:** 833–836
5. **Chapman GW Jr, Pallas JE Jr, Mendicino J** (1972) The hydrolysis of maltodextrins by a β-amylase isolated from leaves of *Vicia faba*. Biochim Biophys Acta **276:** 491–507
6. **Doehlert DC, Duke SH** (1983) Specific determination of α-amylase activity in crude plant extracts containing β-amylase. Plant Physiol **71:** 229–234
7. **Doehlert DC, Duke SH, Anderson L** (1982) Beta-amylases from alfalfa (*Medicago sativa* L.) roots. Plant Physiol **69:** 1096–1102
8. **French D** (1960) β-Amylases. *In* PD Boyer, H Lardy, K Myrbäck, eds, The Enzymes, Ed 2, 4, Academic Press, New York, pp 345–368
9. **Henson CA** (1989) Purification and properties of barley stem fructan exohydrolase. J Plant Physiol **134:** 186–191
10. **Henson CA, Stone JM** (1989) Rapid high-performance chromatographic separation of barley malt α-amylase on cyclobond columns. J Chromatogr **469:** 361–367
11. **Kakefuda G, Duke SH** (1984) Electrophoretic transfer as a technique for the detection and identification of plant amylolytic enzymes in polyacrylamide gels. Plant Physiol **75:** 278–280
12. **Kakefuda G, Duke SH** (1989) Characterization of pea chloroplast D-enzyme (4-α-D-glucanotransferase). Plant Physiol **91:** 136–143
13. **Kakefuda G, Duke SH, Hostak MS** (1986) Chloroplast and extrachloroplastic starch-degrading enzymes in *Pisum sativum* L. Planta **168:** 175–182
14. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
15. **Lundgard R, Svensson B** (1987) The four major forms of barley β-amylase. Purification, characterization and structural relationship. Carlsberg Res Commun **52:** 313–326
16. **Marshall JJ** (1975) Inhibition of plant and bacterial α-amylases. Mol Cellul Biochem **7:** 127–129
17. **Mikami B, Aibara S, Morita Y** (1982) Distribution and properties of soybean β-amylase isozymes. Agric Biol Chem **46:** 943–953
18. **Misra UK, French D** (1960) Inhibition and action pattern of β-amylase in presence of maltose. Biochem J **77:** 1p
19. **Morita Y, Yagi F, Aibara S, Yamashita H** (1976) Chemical composition and properties of soybean β-amylase. J Biochem **79:** 591–603
20. **Morrissey JH** (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem **117:** 307–310
21. **Nebinger P** (1986) Separation and characterization of four different amylases of *Entamoeba histolytica*. I. Purification and properties. Biol Chem Hoppe-Seyler **367:** 161–167
22. **Nomura K, Mikami B, Morita Y** (1986) Interaction of soybean β-amylase with glucose. J Biochem **100:** 1175–1183
23. **Perten H** (1966) A colorimetric method for the determination of alpha-amylase activity (ICC method). Cereal Chem **43:** 336–342
24. **Segrest JP, Jackson RL** (1972) Molecular weight determinations of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Methods Enzymol **28:** 54–63
25. **Shen G-J, Saha BC, Lee Y-E, Bhatnagar L, Zeikus JG** (1988) Purification and characterization of a novel thermostable β-amylase from *Clostridium thermosulphurogenes*. Biochem J **254:** 835–840
26. **Silvanovich MP, Hill RD** (1976) Affinity chromatography of cereal α-amylase. Anal Biochem **73:** 430–433
27. **Steup M** (1988) Starch degradation. *In* J Preiss, ed, The Biochemistry of Plants, Vol 14, Carbohydrates. Academic Press, New York, pp 255–296
28. **Subbaramaiah K, Sharma R** (1985) Affinity chromatography of mustard β-amylase on starch columns. J Biochem Biophys Methods **10:** 315–320
29. **Thoma JA, Spradlin JE, Dygert S** (1971) Plant and animal amylases. *In* PS Boyer, ed, The Enzymes, Ed 3, Vol 5. Academic Press, New York, pp 115–189
30. **Tkachuk R, Tipples KH** (1966) Wheat β-amylases. II. Characterization. Cereal Chem **43:** 62–79
31. **Trimble RB, Maley F** (1984) Optimizing hydrolysis of n-linked high-mannose oligosaccharides by endo-β-n-acetylglucosaminidase H. Anal Biochem **141:** 515–522
32. **Vretblad P** (1974) Immobilization of ligands for biospecific affinity chromatography via the hydroxyl groups. The cyclohexaamylose-β-amylase system. FEBS Lett **47:** 86–89
33. **Yamada H, Yanahira S, Kiyohara H, Cyong J-C, Otsuka Y** (1986) Water soluble glucans from the seed of *Coix lacryma-jobi* var. ma-yuen. Phytochemistry **25:** 129–132
34. **Ziegler P, Beck E** (1986) Exoamylase activity in vacuoles isolated from pea and wheat leaf protoplasts. Plant Physiol **82:** 1119–1121