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# Characterization of α-Amylase from Shoots and Cotyledons of Pea (*Pisum sativum* L.) Seedlings[1]

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## ABSTRACT

**The most abundant α-amylase (EC 3.2.1.1) in shoots and cotyledons from pea (*Pisum sativum* L.) seedlings was purified 6700- and 850-fold, respectively, utilizing affinity (amylose and cycloheptaamylose) and gel filtration chromatography and ultrafiltration. This α-amylase contributed at least 79 and 15% of the total amylolytic activity in seedling cotyledons and shoots, respectively. The enzyme was identified as an α-amylase by polarimetry, substrate specificity, and end product analyses. The purified α-amylases from shoots and cotyledons appear identical. Both are 43.5 kilodalton monomers with pIs of 4.5, broad pH activity optima from 5.5 to 6.5, and nearly identical substrate specificities. They produce identical one-dimensional peptide fingerprints following partial proteolysis in the presence of SDS. Calcium is required for activity and thermal stability of this amylase. The enzyme cannot attack maltodextrins with degrees of polymerization below that of maltotetraose, and hydrolysis of intact starch granules was detected only after prolonged incubation. It best utilizes soluble starch as substrate. Glucose and maltose are the major end products of the enzyme with amylose as substrate. This α-amylase appears to be secreted, in that it is at least partially localized in the apoplast of shoots. The native enzyme exhibits a high degree of resistance to degradation by proteinase K, trypsin/chymostrypsin, thermolysin, and *Staphylococcus aureus* V8 protease. It does not appear to be a high-mannose-type glycoprotein. Common cell wall constituents (*e.g.* β-glucan) are not substrates of the enzyme. A very low amount of this α-amylase appears to be associated with chloroplasts; however, it is unclear whether this activity is contamination or α-amylase which is integrally associated with the chloroplast.**

Typically, the degradation of storage starch in germinating seeds is studied as a unique process, unrelated to the degradation of transitory starch in photosynthetic tissues. Hence, few examples exist where seed amylases have been compared to shoot amylases. Chao and Scandalios (6) found that two genetically identical amylases were present in all organs and tissues (*i.e.* endosperm, scutellum, shoots, and roots) of maize seedlings. Jacobsen *et al.* (10) reported that an extrachloroplastic barley leaf amylase corresponded to a low pI group, aleurone α-amylase. Increased synthesis of this isozyme was induced in barley leaf tissue by imposition of water stress. No detailed interorgan comparisons of starch-degrading enzymes from starch-storing legumes, such as pea, have been reported.

Developing pea cotyledons share certain aspects of starch metabolism with mature photosynthetic organs. For example, during seed development, starch accumulates within cotyledon plastids (weakly granal chloroplasts) (1). It is unclear, however, whether starch grains remain within the plastid envelope during germination (22) or are in direct contact with the cytoplasm (1). In addition, during the early stages of germination, cotyledons appear to contain amylases identical to those present in pea leaves (EP Beers, SH Duke, unpublished observation).

It has been presumed that the most significant difference between starch degradation in cotyledons and that in leaves is that cotyledons lack the diurnal alternation between net synthesis and degradation of transitory starch (2). In pea cotyledons, amylase activity increases for at least the first 10 d of germination (17, 24). The majority of the cotyledon amylolytic activity appears to be due to α-amylase (24). In contrast, β-amylase is the major amylase in photosynthetic tissues of pea (3).

The purification and partial characterization of the major endoamylase from pea leaves has been reported (25). It shares several characteristics with the major α-amylase from cotyledons (21). These include: thermal stability, EDTA sensitivity, a pH optimum of approximately 6.0, and the ability to liberate glucose, maltose, maltotriose, and higher oligosaccharides from starch. These factors alone, however, are insufficient for determining the isozymic or identical nature of endoamylase isolated from each organ.

The major pea leaf endoamylase is an extrachloroplastic enzyme (13); although apparently, it is always present at detectable levels with purified chloroplasts (3). In addition, it is probably the same endoamylase recently localized to the apoplast of pea stems (3). Conclusive evidence concerning the identity of these enzymes, however, has not been presented. Thus, it was the objective of this study to determine whether the major endoamylases found in pea leaves, stems and cotyledons are identical or isozymic amylases. In addition, we have attempted to show whether the major endoamylase commonly associated with chloroplasts is the same endoamylase that is found in the apoplast.

## MATERIALS AND METHODS

### Plant Tissue

Pea (*Pisum sativum* [L.] cv Laxton's Progress No. 9) seeds (J. W. Jung Seed Co., Randolph, WI) were surface-sterilized

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and sown as described by Beers and Duke (3). Plants were grown in a glasshouse under natural light (enzyme purification) or natural light supplemented with fluorescent lamps (chloroplast isolation) placed 70 cm above the surface of the vermiculite. Plants were watered with tap $H_2O$ as needed and with nutrient solution (9) every 7 d.

### α-Amylase Purification

Cotyledons (102 g) used for α-amylase purification were from 16-d-old seedlings. Leaves plus stipules (145 g), stems (97 g), and shoots (*i.e.* all above ground tissue) (358 g) from 3- to 4-week-old seedlings were also used for α-amylase purification. Cotyledons were harvested, washed several times in tap $H_2O$, surface-sterilized in 0.5% (w/v) NaClO for 10 min, and washed in cold tap $H_2O$ for 30 min. Unblemished cotyledons were selected for α-amylase extraction. Leaves, shoots, and stems were washed several times in distilled $H_2O$ prior to homogenization. Tissues were homogenized with a Polytron PT 10-35 homogenizer (Kinematica, GmgH, Lucerne, Switzerland) equipped with a PTA 35 generator in cold (0–4°C) grinding buffer consisting of 40 mM Hepes-NaOH (pH 7.2), 200 mM NaCl, 3 mM $CaCl_2$, and 3 mM DTT (buffer A). Buffer A was used at a 2:1, buffer:tissue ratio and leupeptin (20 μM final concentration) was added just prior to homogenization. Homogenized tissue was filtered through four layers of cheesecloth and centrifuged at 20,000*g* for 20 min at 4°C. Amylose affinity chromatography was similar to that described by Zeigler (25), using Type II, practical grade potato amylose (Sigma). The supernatant was decanted and either stored at −20°C or loaded directly on an amylose column (2.6 × 10 cm). Fresh amylose was used for each preparation and the bed volume varied: 40 mL for cotyledon, 30 mL for shoot and leaf, and 15 mL for stem extracts. Fractions (7 mL) were collected at 4°C at a flow rate of 1.25 mL/min. The column was washed with 5 bed-volumes of buffer A, followed by at least 16 bed-volumes of 10 mM Na-acetate-NaOH, (pH 6.0), 1 mM $CaCl_2$ (buffer B). α-Amylase activity was eluted at room temperature, at maximum flow rate, with at least 5 bed-volumes of buffer B, made to 1% (w/v) potato dextrin. Dextrin-eluted fractions with α-amylase activity were pooled and concentrated to approximately 1% of original volume on a YM30 membrane (Amicon). The YM30 filtrate was concentrated on an YM10 membrane and applied to a Sephacryl S-200 (Pharmcia) gel filtration column (1.5 × 100 cm), equilibrated with 10 mM Hepes-NaOH (pH 7.2), 1 mM $CaCl_2$, 1 mM monothioglycerol. Fractions (3 mL) with α-amylase activity were pooled and concentrated on a YM10 membrane. α-Amylase from shoots, leaves and stems was used in this state of purification for partial characterization. Cotyledon α-amylase was further purified by CHA[2]-Sepharose retardation. The coupling of CHA to epoxy-activated Sepharose 6B (Sigma) was achieved using the method of Silvanovich and Hill (20). α-Amylase was loaded on a CHA-Sepharose column (1.5 × 28 cm), equilibrated with 50 mM Na-acetate-NaOH (pH 5.5), 1 mM $CaCl_2$ at 4°C. The column was washed with column buffer until no α-amylase activity was detected. Fractions (3.5 mL) containing α-amylase activity were pooled and concentrated on a YM10 membrane. Protein concentration was estimated using the method of Bradford (4), with BSA as standard.

[2] Abbreviations: CHA, cycloheptaamylose; APS, ammonium persulfate; IEF, isoelectric focusing; PNPG, *p*-nitrophenyl α-D-glucopyranoside; $G_n$, maltodextrins with $n$ = number of glucose residues.

### Electrophoresis

Native polyacrylamide 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 (3).

SDS-PAGE was conducted using the buffer system of Laemmli (14) with 13.8% acrylamide separating gels. Samples were made to 33% (v/v) SDS sample buffer and placed in a boiling water bath for 5 min. Electrophoresis was carried out at a constant current of 20 mA. Silver staining of SDS gels was performed according to Morrissey (18).

Peptide fingerprints were produced according to method II of Cleveland (7), using endoproteinase Glu-C from *Staphylococcus aureus* V8 (EC 3.4.21.19) (Boehringer Mannheim). Shoot and cotyledon α-amylase samples subjected to proteolysis were obtained by excision of bands from SDS-polyacrylamide gels. Prior to SDS-PAGE, the samples were purified by amylase-affinity chromatography followed by YM30 filtration and YM10 concentration.

IEF of amylases was conducted with precast polyacrylamide gels (Pharmacia) (pH range 4.0–6.5). Electrophoresis was conducted on a cooling plate (10°C), using 8 × 11 cm gels. Following prefocusing at 8 W and 150 mA for 500 V-h, samples were streaked directly onto gels (samples of purified α-amylase contained 5 mg/mL BSA to improve focusing). Electrophoresis was then continued for 2,000 V-h. To determine the pH of IEF gels, 1 $cm^2$ segments from the center of the gel were excised along its entire length. Ampholine was extracted from each segment in 0.75 mL of double distilled $H_2O$ and pH measured. Amylases were detected by staining with KI-$I_2$ (382 mM KI-3.3 mM $I_2$), following incubation at 30°C for 30 min in buffer C (50 mM Na-acetate-NaOH [pH 6.0], 1 mM $CaCl_2$, 0.02% Na-$N_3$), made to 2% (w/v) soluble starch.

### Amylolytic Activity Assays

Production of reducing sugars from Lintner soluble starch, amylose, amylopectin, β-limit dextrin, pullulan, β-glucan, nigeran, polygalacturonic acid, crab shell chitin, and pea starch grains was measured using 3,5-dinitrosalicylic acid reagent (8). Solutions (20 mg/mL) of the above materials (starch grains excepted, see below) were prepared by boiling for 2 min in buffer C. One unit of activity releases of 1 μmol of reducing power (as maltose equivalents) per min. The reaction was initiated by the addition of 0.5 mL of the above substrates to enzyme in a final volume of 0.5 mL buffer C (final assay volume, 1 mL). The increase in $A_{540}$ was linear over the period of incubation (≤30 min). For starch grain hydrolysis, 0.1 mL of suspended, purified starch grains (27 mg/mL) was transferred to assay tubes containing α-amylase in a final volume of 0.9 mL of buffer C. Assays were conducted at 30°C for 30 min or less, unless otherwise noted.

To test for maltase activity, cotyledon and shoot α-amylases were incubated at 30°C with 5 mg maltose, isomaltose or panose in 0.2 mL of buffer C. After 4 h, a 0.1 mL aliquot was transferred to a 1 mL cuvette containing 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 4 mM ATP, 0.3 mM $NAD^+$, 1 IU glucose-6-phosphate dehydrogenase, and 1.8 IU hexokinase. After 30 min, the production of glucose from maltose, isomaltose and panose was determined from the change in $A_{340}$ (reduction of $NAD^+$). Maltase activity was also assayed with PNPG as substrate in a 1 mL reaction mixture containing 1 mg/mL PNPG, 50 mM K-succinate (pH 5.5), and 0.5 mM $CaCl_2$. The reaction was terminated with 0.1 mL of 1 N NaOH. Release of glucose from PNPG was determined from the change in $A_{420}$.

Detection of α-amylase during purification was based on the hydrolysis of starch azure (20 mg/mL), prepared in buffer C (8). Values for assays described above are presented in the text as the means of three replicates.

### End Product Analyses

The hydrolysis of boiled potato amylose by purified α-amylase (350 munits) was assayed in buffer C with 4 mg/mL amylose and 0.1 mM $CaCl_2$, in a final volume of 0.2 mL. Assays were conducted at 30°C for up to 4 h. Each assay was terminated by immersion of the assay tube in a boiling water bath for 15 min. Samples were then centrifuged for 5 min at maximum rpm in a microcentrifuge (Beckman, microfuge 11). Sugars, maltodextrins and oligosaccharides in 40 μL samples were separated by HPLC and detected as before (12), except that the column flow rate was 0.2 mL/min. Analysis of hydrolysis products of maltodextrins (maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) (Boehringer Mannheim) by α-amylase was conducted in the same manner, except that the concentration of substrates was 2 mg/mL. Maltodextrins with degrees of polymerization of $G_1$ through $G_4$ were effectively separated, whereas maltodextrins $\geq G_5$ were not totally separated.

### Polarimetry

The procedure was modified from that described by Swain and Dekker (21) using a Perkin-Elmer, model 241 polarimeter. Purified shoot (1 unit) or cotyledon (3 units) α-amylase was added to a 1 mL polarimeter tube containing 1% maltoheptaose in buffer C, maintained at 30°C. The optical rotation of the maltodextrin solution was recorded after 10, 30, 40, and 60 min. Equilibration of maltodextrin anomers and termination of the reaction was achieved by the addition of 0.5 mg of NaOH.

### Tissue Infiltration-Extraction

Four 2 g replicates of pea stem tissue were subjected to six cycles of vacuum infiltration-extraction as described by Beers and Duke (3). Buffer A was used as the infiltration-extraction and grinding buffer.

### Starch Grain Extraction

Cotyledons (100 g) were harvested from 9-d-old seedlings as described for α-amylase purification and homogenized with a Polytron in 200 mL of 50 mM Hepes (pH 7.5), 3 mM DTT, 1 mM $CaCl_2$ (buffer D). Homogenized tissue was filtered through four layers of cheesecloth and centrifuged at 3000*g* for 5 min. Pellets were resuspended in 60 mL of buffer D, centrifuged again, and resuspended in 25 mL of buffer D. Aliquots were layered onto 60 to 80% (10 mL of each) (w/v) discontinuous sucrose gradients. The gradients were centrifuged at 150*g* for 5 min, 600*g* for 5 min, and 1400*g* for 10 min. Supernatants were discarded and starch grain pellets were washed three times by centrifugation in storage buffer (50 mM Hepes [pH 6.0], 3 mM DTT, 1 mM $CaCl_2$, 0.04% $NaN_3$, 20 μM leupeptin), and stored in 100 mL of storage buffer.

Pea leaf starch grains were extracted from 4-week-old plants. Pea leaves (180 g) were homogenized with a Polytron in 650 mL of 20 mM Hepes (pH 6.9), 3 mM DTT, 3 mM $CaCl_2$, (buffer E). Following filtration through a 105 μm mesh, the extract was centrifuged at 5000*g* for 10 min. Supernatants were decanted and pellets were resuspended in 60 mL of buffer E. Aliquots were layered onto 20 mL of 80% (w/v) sucrose and centrifuged at 20,000*g* for 15 min. Sample above the sucrose cushion was discarded. The starch grain pellet was combined with the sucrose cushion, which contained suspended starch grains, and made to 40% sucrose by dilution with buffer E. This suspension was again layered onto an 80% sucrose cushion (10 mL) and centrifuged at 2,000*g* for 3 min. Sample above the sucrose was discarded and the sucrose cushion was diluted 5-fold with buffer E and centrifuged at 20,000*g* for 5 min. The supernatant was discarded and the starch grains were resuspended in buffer C and centrifuged at 20,000*g* for 5 min. This was repeated twice after which starch grains were stored at 4°C in buffer C.

### Chloroplast Isolation

Chloroplasts were isolated from fully expanded leaves of 2- to 3-week-old plants by the method of Kakefuda *et al.* (13). The buffer used throughout the procedure consisted of 50 mM Hepes (pH 7.5), 330 mM sorbitol, 5 mM isoascorbate, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 μM leupeptin.

### Treatment of α-Amylases with Endoglycosidase H

The procedure was adapted from that of Trimble and Maley (23). Purified cotyledon (3 μg) and shoot (0.8 μg) α-amylases in 25 μL of 50 mM Na-acetate (pH 5.5), 0.013% SDS were placed in a boiling water bath for 1 min. After cooling to 30°C, 5 milliunits of endoglycosidase H (EC 3.2.1.96) (Boehringer Mannheim) were added to the sample. After incubation at 30°C for 20 h, samples were prepared for SDS-PAGE as described. SDS-PAGE and silver staining of the gel were conducted as described.

## RESULTS

### α-Amylase Purification

The specific amylolytic activity of a crude cotyledon extract was nearly 6-fold that of a crude shoot extract (Tables I and II). Amylose affinity chromatography was the single most

**Table I.** *Purification Steps for α-Amylase from Pea Shoots*

| Purification Step | Total Volume | Total Protein | Total Activity | Recovery | Specific Activity | Purification |
|---|---|---|---|---|---|---|
| | *mL* | *mg* | *μmol/min* | % | *μmol/min/mg protein* | *-fold* |
| Crude extract | 610 | 3,050 | 717 | 100 | 0.240 | 1 |
| Amylose column | 215 | 10.65 | 104 | 14.5 | 162 | 675 |
| YM30 filtration[a] | 1.4 | 0.29 | 65.5 | 9.1 | 744 | 3100 |
| Sephacryl S-200[a] | 0.386 | 0.015 | 24.8 | 3.5 | 1610 | 6710 |

[a] YM10 concentrate thereof.

**Table II.** *Purification Steps for α-Amylase from Cotyledons of Pea Seedlings*

| Purification Step | Total Volume | Total Protein | Total Activity | Recovery | Specific Activity | Purification |
|---|---|---|---|---|---|---|
| | *mL* | *mg* | *μmol/min* | % | *μmol/min/mg protein* | *-fold* |
| Crude extract | 260 | 1740 | 2410 | 100 | 1.4 | 1 |
| Amylose column | 331 | 11.3 | 1900 | 79.1 | 169 | 120 |
| YM30 Filtration[a] | 2.6 | 4.3 | 924 | 38.4 | 215 | 154 |
| Sephacryl S-200[a] | 2.4 | 0.3 | 317 | 13.2 | 1060 | 754 |
| CHA-Sepharose[a] | 0.608 | 0.09 | 109 | 4.5 | 1190 | 846 |

[a] YM10 concentrate thereof.

effective step in the purification of pea α-amylase; resulting in a 120- and 675-fold purification of cotyledon and shoot α-amylases, respectively. Recovery of α-amylase activity, as a percentage of total amylolytic activity, by amylose chromatography was 14.5% for shoot (Table I) and 79% for cotyledon (Table II) preparations. The pea shoot and cotyledon amylolytic activity recovered by amylose chromatography were >96 and 100%, respectively, α-amylase activity, as determined by EGTA sensitivity (preincubation with 10 mM EGTA for 15 min at 30°C followed by 10 min standard assay, see "Materials and Methods") and thermostability. Approximately 11% (shoots) and 18% (cotyledons) of the thermo-stable amylolytic activity found in crude extracts was recovered in the amylose column void and flow-through (data not shown). Following YM30 filtration, the specific activity of shoot α-amylase (Table I) was similar to the highest level of purity (670 units/mg protein) attained by Zeigler (25). Dextrins were removed from samples by Sephacryl S-200 gel filtration. Gel filtration of cotyledon and shoot α-amylases also resulted in significant increases in purity. Ultimately, shoot α-amylase was purified 6710-fold, while cotyledon α-amylase was purified 846-fold. The specific activity of the most purified enzyme was 1610 units/mg protein for shoot and 1190 units/mg protein for cotyledon α-amylase.

The high degree of purity attained by amylose chromatography of a crude shoot extract, followed by YM30 filtration, is evident with SDS-PAGE (Fig. 1). In addition to the 43.5 kD α-amylase, a high mol wt protein was observed (well above the 66 kD standard). This band was eliminated with

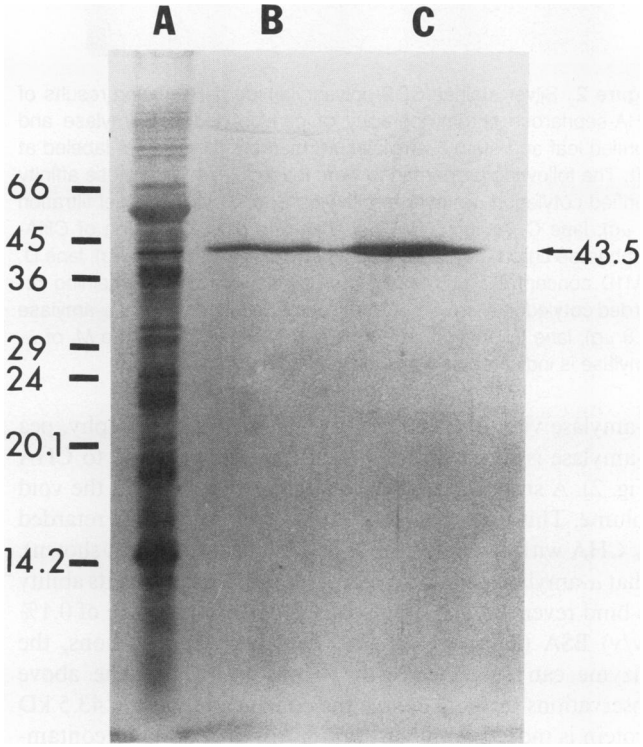


**Figure 1.** Silver stained SDS-polyacrylamide gel showing purification of pea shoot α-amylase. Positions of $M_r$ markers are indicated at left in kD. The following preparations were loaded: lane A, crude pea shoot extract (5 μg); lane B, amylose-affinity purified α-amylase subjected to Amicon YM30 filtration and YM10 concentration (3 μg); lane C, sample treated as in lane B followed by Sephacryl S-200 gel filtration and YM10 concentration (0.6 μg). The $M_r$ of α-amylase is indicated at right in kD.

gel filtration. Two faint, high $M_r$ bands (66,000 and 55,500) can be seen at an approximately uniform density in each lane (Figs. 1 and 2). These bands were present when SDS sample buffer alone was subjected to SDS-PAGE (data not shown). They are artifacts of the SDS buffer and are not present in α-amylase preparations.

Purification of cotyledon α-amylase required an additional step (Table II and Fig. 2). Following gel filtration of the YM30 filtrate, the sample was still contaminated with a 25 kD protein (Fig. 2). The 25 kD protein had no affinity for CHA and was eliminated by this step of purification.

Under conditions used to separate the 25 kD protein from

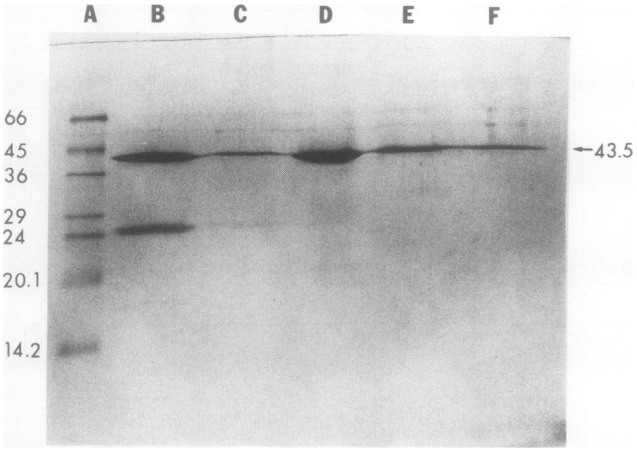


**Figure 2.** Silver stained SDS-polyacrylamide gel showing results of CHA-sepharose chromatography of pea cotyledon α-amylase and purified leaf and stem α-amylase. $M_r$ markers (lane A) are labeled at left. The following preparations were loaded: lane B, amylose affinity purified cotyledon α-amylase following Sephacryl S-200 gel filtration (2 μg); lane C, Amicon YM10 concentrate of void fraction of CHA-Sepharose chromatography of cotyledon α-amylase (0.4 μg); lane D, YM10 concentrate of CHA-Sepharose flow-through containing retarded cotyledon α-amylase (3 μg); lane E, purified pea leaf α-amylase (0.8 μg); lane F, purified pea stem α-amylase (0.3 μg). The $M_r$ of α-amylase is indicated at right in kD.

α-amylase via CHA-Sepharose affinity chromatography, pea α-amylase is retarded by, but not reversibly bound to CHA (Fig. 2). A small amount of α-amylase also elutes in the void volume. This α-amylase is identical to that which is retarded by CHA with respect to $M_r$ (Fig. 2) and pI (data not shown). That α-amylase has affinity for CHA is illustrated by its ability to bind reversibly to CHA-Sepharose in the presence of 0.1% (w/v) BSA (data not shown). Under these conditions, the enzyme can be eluted with 10 mg/mL CHA. The above observations further support the conclusion that the 43.5 kD protein is indeed α-amylase and not simply a major contaminant of α-amylase activity purified by amylose affinity chromatography.

## Mol Wt

Under conditions recommended for Sephacryl S-200 column calibration and determination of the $M_r$ of unknown proteins (*i.e.* neutral pH and high ionic strength), pea α-amylase has an apparent $M_r$ of 30,000 (data not shown). Under conditions of low ionic strength, however, Sephacryl S-200 chromatography of pea α-amylase reveals a $M_r$ of 45,000. This agrees more closely with that of a previously described pea shoot endoamylase (about 43,000; ref. 25) and our results from SDS-PAGE, indicating a $M_r$ of 43,500 (Fig. 1). The apparent retardation of α-amylase under high ionic strength may be due to an enhancement of hydrophobic interactions between α-amylase and the Sephacryl gel filtration medium. The gel filtration and SDS-PAGE data indicate that the major pea α-amylase is a ca. 43.5 kD monomer.

## Mol Wt, Peptide Map, and pI of α-Amylases from Various Organs

Pea leaf and stem α-amylases were purified according to the scheme presented for shoots in Table I. A comparison of α-amylases from leaves and stems with that from cotyledons reveals that all three organs yield apparently identical, 43.5 kD α-amylases (Fig. 2). Two additional lines of evidence support this conclusion. First, identical peptide fingerprints were produced following the partial digestion of shoot and cotyledon α-amylase by *S. aureus* V8 protease (Fig. 3). Second, KI-$I_2$ stain is absent at pH 4.5 in an IEF zymogram of purified α-amylase from stem, leaf and cotyledon (Fig. 4).

## Kinetic Properties and Substrate Specificity

The optimum pH for enzyme activity with soluble starch was a rather broad plateau of 5.5 to 6.5 (data not shown). The apparent $K_m$ of soluble starch was 1.6 mg/mL for enzyme from both shoots and cotyledons.

α-Amylase was examined with respect to its ability to hydrolyze a variety of starch-like substrates (Table III). Similar results were obtained for α-amylase from both shoots and cotyledons, with all substrates tested. α-Amylase hydrolyzed amylose at rates similar to those obtained with soluble starch; but it was considerably less active with amylopectin and β-limit dextrin as substrates. Data concerning the hydrolysis of starch azure is not presented because the increase in $A_{595}$ was not linear under the assay conditions used for the substrate survey. Starch azure, however, was used routinely to follow α-amylase throughout purification, as no other amylase produced by pea hydrolyzes starch azure releasing soluble dye to a detectable extent. The ability of this enzyme to hydrolyze

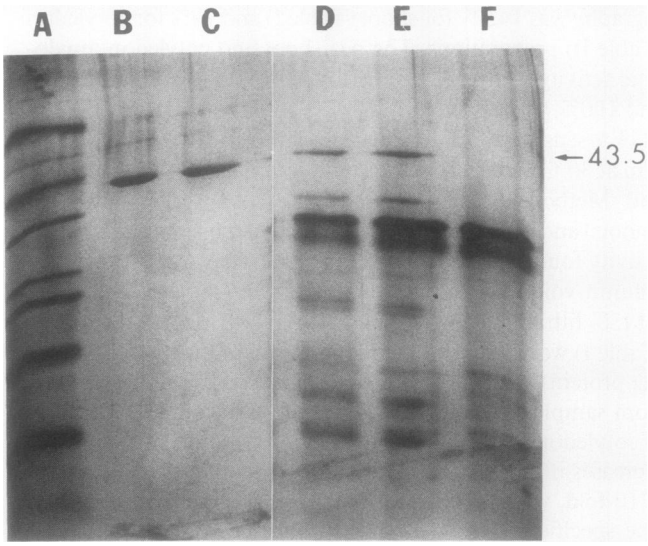


**Figure 3.** One-dimensional peptide mapping of purified shoot and cotyledon α-amylase. $M_r$ markers (lane A) are as shown in Figure 2. Lanes B and D were loaded with 2.0 μg of SDS-PAGE-purified shoot α-amylase. Lanes C and E were loaded with 2.0 μg of SDS-PAGE-purified cotyledon α-amylase. Lanes D and E were also loaded with 200 ng of *S. aureus* V8 protease. Lane F contains 200 ng of *S. aureus* V8 protease alone.

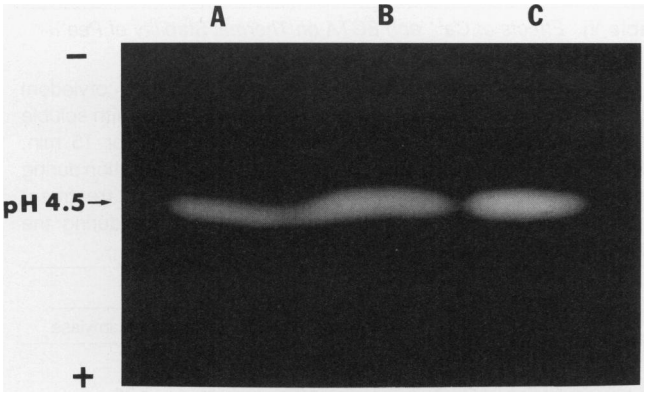


**Figure 4.** KI-$I_2$-stained starch infiltrated IEF polyacrylamide gel showing highly purified pea α-amylase. Samples loaded were as follows: lane A, stem α-amylase (0.5 μg); lane B, leaf α-amylase (1.5 μg); lane C, cotyledon α-amylase (0.9 μg). Inert protein (200 μg BSA) was added to each sample to stabilize α-amylase activity. The pI of the pea α-amylase is indicated at left.

β-limit dextrin indicates that it is not a β-amylase. In addition, pea α-amylase had no activity with pullulan, indicating that it is not a starch debranching enzyme. No glucose was released during 4 h of incubation with maltose, isomaltose (α-D-Glc-[1→6]-α-D-Glc) and panose (α-D-Glc-[1→6]-α-D-Glc-[1→4]-α-D-Glc); and PNPG was not hydrolyzed by the enzyme. Together, these results indicate that the enzyme is not an α-glucosidase (maltase). The above substrate specificity data are consistent with that of an α-amylase. Pea α-amylase had no detectable activity with the cell wall constituents, β-glucan or polygalacturonic acid, or with nigeran (α-D-Glc-[1→3]-α-D-Glc-[1→4])$_n$ or chitin. The enzyme also had no activity with either cotyledon or leaf starch grains, during short-term (≤30 min) assays (Table III). During long-term assays at 37°C, however, α-amylase liberated small quantities of maltodextrins from intact cotyledon starch grains. For example, during a 16 h assay, purified intact starch grains incubated without added α-amylase released 124 nmol of maltose equivalents. In the same experiment, the presence of 600 munits of α-amylase resulted in the release of an additional 211 nmol of maltose equivalents from pea cotyledon starch grains, a rate of hydrolysis equal to 0.021% of that observed with soluble starch (Table III). We do not know whether the observed increased hydrolysis is due solely to a direct attack on starch grains by α-amylase or is dependent upon the participation of other amylases associated with starch grains. Boiled cotyledon starch grains were hydrolyzed at rates similar to those found when soluble starch was the substrate.

## End Product Analysis

Of the maltodextrins tested ($G_3$ to $G_7$), all but maltotriose were hydrolyzed to some extent by shoot or cotyledon α-amylase during 4 h of incubation (Table IV). Only when maltoheptaose was the subtrate, however, was there a significant accumulation of all detectable maltodextrins. The maltodextrins produced during the hydrolysis of maltoheptaose were identified as α-anomers based on a decrease in the optical rotation of the maltodextrin solution as the equilibrium mixture of α and β forms was reached (Table V). The magnitude of this decrease was dependent upon the duration of the incubation. There was no disproportionation of maltodextrins, indicating that this α-amylase has no α-glucanotransferase activity (*cf.* ref. 12).

With amylose as the substrate, a rapid accumulation of glucose and all detectable maltodextrins was observed (Fig. 5). The level of maltodextrins ≥$G_5$ increased nearly two and one-half-fold in the first 30 min of the assay but remained at approximately the 30 min level thereafter (data not shown). Similarly, the most rapid accumulation of $G_1$ through $G_4$ occurred during the first 30 min of incubation. Unlike the larger maltodextrins, however, $G_1$ through $G_4$ continued to accumulate, albeit much more slowly, for the remainder of

**Table III.** *Pea α-Amylase Substrate Specificity*

Values reported are relative to 1562 (shoot) or 598 (cotyledon) μmol of reducing power/min/mg protein

| Substrate | Activity | |
|---|---|---|
| | Shoot amylase | Cotyledon amylase |
| | *% of control* | |
| Soluble starch | 100 | 100 |
| Amylopectin | 63 | 65 |
| Amylose (maize) | 98 | 94 |
| Amylose (potato) | 86 | 96 |
| β-Limit dextrin | 44 | 42 |
| Pullulan | 0 | 0 |
| PNPG | 0 | 0 |
| Maltose | 0 | 0 |
| Isomaltose | 0 | 0 |
| Panose | 0 | 0 |
| Barley β-glucan | 0 | 0 |
| Nigeran | 0 | 0 |
| Polygalacturonic acid | 0 | 0 |
| Crab shell chitin | 0 | 0 |
| Pea cotyledon starch grains[a] | 0 | 0 |
| Pea cotyledon starch grains[b] | ND[c] | 0.021 |
| Pea cotyledon starch grains[d] | 90 | 83 |
| Pea leaf starch grains[a] | 0 | 0 |

[a] Incubated for 20 min at 30°C. [b] Incubated for 16 h at 37°C. [c] Not determined. [d] Starch grains boiled prior to incubation for 20 min at 30°C.

**Table IV.** *End Product Analysis of Hydrolysis of Maltodextrins by Pea α-Amylase*

Assays were for 4 h with 2 mg substrate/mL and 350 munits α-amylase in a final assay volume of 0.2 mL.

| Substrate | Product | | | | |
|---|---|---|---|---|---|
| | $G_1$ | $G_2$ | $G_3$ | $G_4$ | ≥$G_5$ |
| | *final concentration (mM)* | | | | |
| $G_3$ | 0 | 0 | 3.79 | 0 | 0 |
| $G_4$ | 0 | 0.18 | 0 | 2.74 | 0 |
| $G_5$ | 0 | 0.26 | 0.19 | 0 | 2.81[a] |
| $G_6$ | 0 | 0.54 | Trace | 0.39 | ≤2.11 |
| $G_7$ | 1.17 | 0.46 | 0.27 | 0.23 | ≤2.03 |

[a] $G_5$ only.

**Table V.** *Optical Rotation of Products of Hydrolysis of Maltoheptaose by Pea α-Amylase*

Values in parentheses are for pea shoot α-amylase; all others are for pea cotyledon α-amylase.

| Incubation Time | Optical Rotation | | |
|---|---|---|---|
| | Incubation end-point | Following addition of NaOH | NaOH-Induced change |
| *min* | | *degrees* | |
| 0 | 1.764 | 1.751 | −0.013 |
| 10 | 1.790 | 1.782 | −0.008 |
| 30 | 1.776 | 1.757 | −0.019 |
| 40 | 1.766 | 1.717 | −0.049 |
| 60 | 1.753 | 1.692 | −0.061 |
| | (1.753) | (1.723) | (−0.030) |

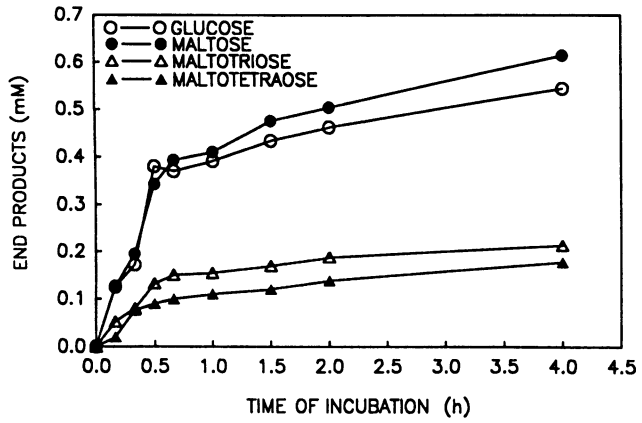


**Figure 5.** End products of hydrolysis of amylose by pea α-amylase. Values are means of two separate experiments for each time point. Maltodextrins were separated by HPLC using a μ-Spherogel carbohydrate column.

the assay. Glucose and maltose were the most abundant products of amylose hydrolysis, with maltose increasing at the same rate as glucose (Fig. 5). Since, in addition to glucose and maltose, maltotriose appears to be an end product of α-amylase activity, it is surprising that its accumulation does not parallel that of maltose and glucose. Maltotetraose is a substrate, albeit a poor one, for pea α-amylase, producing only maltose (Table IV). Thus, maltotetraose would be expected to contribute to the accumulation of maltose, and furthermore would not be expected to accumulate to the same extent as glucose and maltose. The change in the rate of hydrolysis of amylose which occurred after 30 min of incubation, and in the presence of ca. 0.4 mM glucose and maltose (Fig. 5), was not due to inhibition of α-amylase by the end products glucose, maltose, or maltotriose; *i.e.* the hydrolysis of amylose by α-amylase was not affected when 0.5 mM glucose, maltose or maltriose was present in the reaction mixture at the start of a 60 min assay (data not shown).

## EGTA and Heat Sensitivity

α-Amylase from both shoots and cotyledons was very heat-labile in the absence of $Ca^{2+}$ (Table VI). Preincubation at 70°C prior to the addition of starch effectively inactivated pea

**Table VI.** *Effects of $Ca^{2+}$ and EGTA on Thermal Stability of Pea α-Amylase*

Values reported are relative to 1590 (shoot) or 725 (cotyledon) μmol reducing power/min/mg protein. Prior to incubation with soluble starch, samples were incubated at either 70 or 30°C for 15 min, followed by a 10 min equilibration at 30°C. $CaCl_2$ concentration during the preincubation was 3 mM, except for the EGTA, $+Ca^{2+}$ treatment where it was 32 mM. EGTA concentration was 8 mM during the preincubation.

| Treatment | Activity | |
|---|---|---|
| | Shoot amylase | Cotyledon amylase |
| | *% of control* | |
| No preincubation, $+Ca^{2+}$ | 100 | 100 |
| 70°C, $+Ca^{2+}$ | 64 | 77 |
| 70°C, $-Ca^{2+}$ | 2 | 1 |
| 30°C, $+Ca^{2+}$ | 91 | 86 |
| 30°C, $-Ca^{2+}$ | 37 | 19 |
| 30°C, +EGTA | 7 | 10 |
| 30°C, +EGTA, $+Ca^{2+}$ | 40 | 48 |
| No preincubation, +EGTA | 90 | 76 |

α-amylase from shoots and cotyledons. The presence of $CaCl_2$ during this preincubation, however, was sufficient to preserve 64 and 77% of α-amylase activity from shoots and cotyledons, respectively. Even preincubation at 30°C for 25 min without $CaCl_2$ significantly reduced the activity of α-amylase from both sources (Table VI). The $Ca^{2+}$-chelator EGTA further reduced shoot and cotyledon α-amylase activity to 7 and 10% of the control, respectively. The presence of excess $CaCl_2$ during the 30°C preincubation, however, partially protected the enzyme. Without a preincubation period (*i.e.* when substrate was added immediately after EGTA) activity of shoot and cotyledon α-amylase was reduced by only 10 and 24%, respectively.

Thermal stability, and protease resistance (see below) are common characteristics of secreted glycoproteins (15). Pea α-amylase, however, had no affinity for concanavalin A and was resistant to hydrolysis by endoglycosidase H (data not shown). Thus, it does not appear to be a high-mannose-type glycoprotein.

## Protease Sensitivity

Nondenatured pea α-amylase exhibited a high degree of resistance to proteolysis (as determined by *in vitro* starch hydrolysis) (Table VII), even when proteases were present at concentrations at least 500-fold greater than those used to produce the α-amylase peptide fingerprint (Fig. 3). Of the proteases tested, pea α-amylase was most sensitive to *S. aureus* V8 protease, but still retained 71% of its activity after 30 min of incubation at room temperature. In the presence of proteinase K, α-amylase exhibited a similar degree of resistance to proteolysis, retaining 75% of its activity. β-Amylase was almost completely inactivated by proteinase K and a chloroplast extract was only 18% as active. Both α- and β-amylase from pea were highly resistant to inactivation by trypsin/chymotrypsin and thermolysin. Seventy, 60, and 48% of chloroplast amylase activity was lost following treatment with

**Table VII.** *Sensitivity of Pea Amylases to Protease Treatment*

Values reported are relative to 567 (α-amylase), 170 (β-amylase), or 0.007 (chloroplast) μmol reducing power/min/mg protein. Prior to incubation with soluble starch, purified pea shoot α-amylase (0.10 μg) and β-amylase (0.59 μg) and chloroplast protein (450 μg) were incubated with proteinase K (100 μg), thermolysin (100 μg), trypsin/chymotrypsin (150 μg of each), V8 protease (100 μg), or buffer A alone (control), for 30 min at room temperature.

| Enzyme Preparation | Activity | | | |
|---|---|---|---|---|
| | Proteinase K | Thermolysin | Trypsin/Chymotrypsin | *S. aureus* V8 |
| | | | *% of control* | |
| Pea shoot α-amylase | 75 | 102 | 80 | 71 |
| Pea shoot β-amylase | 5 | 80 | 92 | 68 |
| Pea chloroplast extract | 18 | 40 | 30 | 52 |

trypsin/chymotrypsin, thermolysin, and *S. aureus* V8 protease, respectively.

### Subcellular Localization of α-Amylase

In Figure 6, a crude stem extract prepared from stems which had been subjected to six cycles of infiltration-extraction (3) is compared to an equivalent sample of apoplastic extract. It is clear that the majority of the pI 4.5 α-amylase has been extracted from the apoplast. A small amount of the vacuolar β-amylase (16) (pI 4.3) is present as a cellular contaminant of the apoplastic extract (*cf.* ref. 3). Purified cotyledon α-amylase is included for comparison; with it, a faint band can be seen at pH 4.7. This band was also observed in some shoot α-amylase samples subjected to IEF (data not shown). The pI 4.7 amylase is not a contaminant of BSA used to stabilize α-amylase during IEF and was never observed as a separate band following SDS-PAGE of purified α-amylase. Neither the pI 4.5 nor the pI 4.7 amylase was detected on KI-$I_2$-stained, IEF gels following incubation in starch solution containing 5 mM EDTA (data not shown).

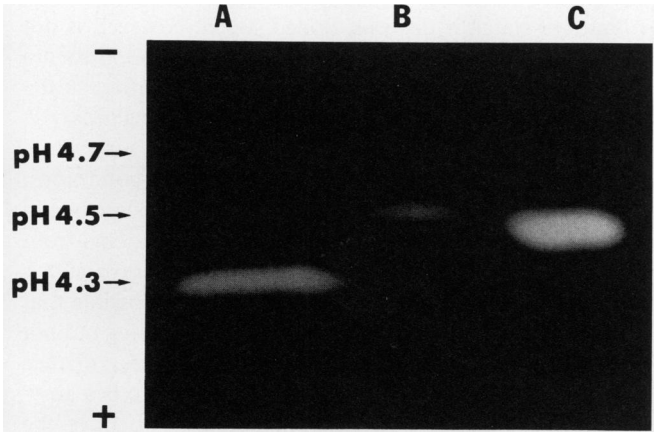


**Figure 6.** KI-$I_2$-stained starch infiltrated IEF polyacrylamide gel showing localization of the major amylases in pea stems. Lane A was loaded with 7% of the protein extracted by homogenization from stems (8 g) that had been vacuum infiltration extracted. Lane B was loaded with 7% of apoplastic proteins extracted from 8 g of pea stems via vacuum infiltration-extraction (*cf.* ref. 3). Lane C was loaded with 0.9 μg of purified cotyledon α-amylase and 200 μg of BSA. The pIs of β-amylase (4.3), α-amylase (4.5), and an unidentified amylase (4.7) are indicated at left.

Pea produces two major extrachloroplastic amylases: a vacuolar β-amylase (26) and the α-amylase described in this and other reports (13, 25). In spite of the fact that pea chloroplasts are routinely prepared which contain no detectable level of the cytosolic marker enzyme phospho*enol*pyruvate carboxylase (3), the major α-amylase alone (3) or in combination with vacuolar β-amylase (Fig. 7) was apparently present in extracts of purified chloroplasts.

To further characterize chloroplastic amylases with respect to the presence of the major α-amylase, the effects of EGTA on electrophoretic mobility and activity of amylases present in chloroplast extracts and purified shoot and cotyledon α-amylase were compared (Fig. 7). Only one amylase present in chloroplast preparations comigrates with purified pea α-amylase during native PAGE. It is the only amylase affected by incubation with EGTA prior to electrophoresis. Chelation of $Ca^{2+}$ appears to convert this amylase to a more rapidly migrating form, perhaps due to an increase in net negative charge and/or a conformational change. EGTA treatment would be expected to inactivate the enzyme. However, the presence of $Ca^{2+}$ during the blot-transfer of proteins resulted in a partial restoration of activity, thus permitting the detection of the

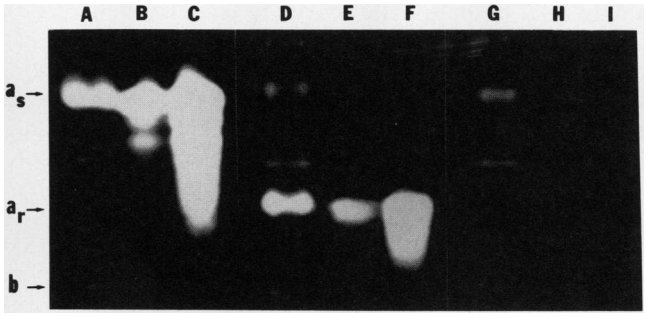


**Figure 7.** KI-$I_2$-stained blot-transfer polyacrylamide gel showing the effect of EGTA on the slowly ($a_s$) and more rapidly ($a_r$) migrating forms of α-amylase. Extracts from purified chloroplasts (972 μg protein; lanes A, D, and G), purified shoot α-amylase (0.010 μg protein; lanes B, E, and H) and purified cotyledon α-amylase (0.013 μg protein; lanes C, F, and I) were subjected to the following treatments prior to native PAGE and blot-transfer: lanes A, B, and C, incubation at room temperature for 30 min; lanes D, E, and F, as above in 5 mM EGTA; lanes G, H, and I, as in lanes D, E, and F, followed by blot-transfer through a starch-containing gel made to 2.5 mM EGTA. Extrachloroplastic β-amylase (b) was reported previously (3).

more rapidly migrating form. Subjecting an EGTA-treated sample to blot-transfer through an EGTA-containing gel effectively eliminated any activity of the $Ca^{2+}$-requiring $\alpha$-amylase in either the slowly or more rapidly migrating form (Fig. 7).

## DISCUSSION

The purified $\alpha$-amylases from shoots and cotyledons appear identical. Both are 43.5 kD monomers with pIs of 4.5, broad pH activity optima from 5.5 to 6.5, and nearly identical substrate specificities. They produce identical peptide maps following partial proteolysis by *S. aureus* V8 protease. Calcium is required for activity and thermal stability of this amylase. Both $CaCl_2$ and soluble starch protect pea $\alpha$-amylase from inactivation by EGTA. In addition, $CaCl_2$ protects $\alpha$-amylase from thermal inactivation. The effects of $Ca^{2+}$, $Ca^{2+}$-chelators, and thermal treatments on pea $\alpha$-amylase are consistent with those reported by Zeigler (25) and Swain and Dekker (21). Based solely on its sensitivity to $Ca^{2+}$-chelators, pea $\alpha$-amylase was assumed to be of the B type (high pI) (10). Unlike the EDTA sensitive barley amylases, however, the major pea $\alpha$-amylase is a low pI amylase (Fig. 4).

Pea cotyledons are starch-storing organs which continually increase their starch-degrading activity during the early stages of germination (17, 24). It is not surprising, therefore, that they would contain higher levels of $\alpha$-amylase activity than photosynthetic organs involved in the degradation of relatively low levels of transitory starch. The sum of the activity of thermo-stable shoot amylase which was reversibly bound to amylose and that which was recovered in the void and flow-through of the amylose column indicates that approximately 25% of total shoot amylolytic activity is due to the $\alpha$-amylase described in this report. In contrast, the sum of the corresponding values for thermo-stable cotyledon amylase reveals that approximately 97% of cotyledon amylolytic activity is due to $\alpha$-amylase. Based on these estimates, the specific activity of thermo-stable $\alpha$-amylase in shoots is equal to only 4.4% of the specific activity of that enzyme in cotyledons. It is important to note that total amylolytic activity of crude extracts includes the combined activity of all amylases, not just $\alpha$-amylase. Hence, while values for fold purification and percent recovery of cotyledon $\alpha$-amylase are representative of $\alpha$-amylase almost exclusively, those reported for shoot $\alpha$-amylase probably significantly underestimate the recovery and purification of $\alpha$-amylase.

Evidence was presented in this and a previous report (3) that pea shoot $\alpha$-amylase is a secreted enzyme, localized in the apoplast. Starch-like glucans have not been previously identified as components of cell walls, and it remains to be shown whether an endogenous substrate exists for this apoplastic $\alpha$-amylase. Other hydrolytic enzymes, for which there exists no apparent endogenous substrate, have been localized to the cell wall (5). It is unclear whether pea cotyledon cells also secrete $\alpha$-amylase.

Preparations of $\alpha$-amylase which produce a single band following SDS-PAGE or IEF often yield multiple bands or broadly diffuse areas of $\alpha$-amylase activity during native PAGE (Fig. 7). These areas are generally delimited by the slowly migrating $\alpha$-amylase, on the cathode side of the gel, and the rapidly migrating $\alpha$-amylase, on the anode side of the gel. Since $Ca^{2+}$ is not included in the PAGE buffers, it is possible that $Ca^{2+}$ is lost from $\alpha$-amylase throughout electrophoresis, allowing some $\alpha$-amylase to migrate farther than others; their activity is subsequently restored during electrophoretic transfer through the $Ca^{2+}$-containing blot gel. The rapidly migrating (*i.e.* minus $Ca^{2+}$) form of $\alpha$-amylase described here may account for the rapidly migrating ($a_3$) $\alpha$-amylase reported previously by Beers and Duke (3).

When compared to purified $\alpha$-amylase, extracts of purified chloroplasts contain one amylase with an identical mobility during native PAGE (Fig. 7). This amylase is also EGTA sensitive. Assuming this amylase is identical to the purified $\alpha$-amylase described in this report, two factors argue against its presence as a stromal amylase rather than a cellular/apoplastic contaminant of purified chloroplasts. First, activity of this amylase increases in organs where plastids are either senescent (*e.g.* cotyledons of germinating seedlings) or photooxidized (19). Second, there are no reports of a single protein being targeted both for secretion and for import by an organelle. Assuming, therefore, that $\alpha$-amylase is a secreted enzyme, present only as a contaminant of purified chloroplasts, it is unlikely that it plays a role in the initiation of transitory starch degradation. However, as extrachloroplastic contaminants, both $\alpha$-amylase and $\beta$-amylase may contribute a significant percentage of the amylolytic activity recovered with purified chloroplasts.

In contrast to extrachloroplastic $\alpha$-amylase in pea shoots, $\alpha$-amylase in cotyledons of seedlings may have direct access to starch grains (1). At 21°C, starch is hydrolyzed at a rate of 5.3 mg/day/seed in Progress No. 9 cultivar pea seedlings (17). With soluble starch as substrate, there is nearly 600-fold more $\alpha$-amylase activity (12.8 $\mu$mol/min/cotyledon pair, calculated from Table I assuming a $Q_{10}$ of two) than is necessary for *in vivo* rates of starch hydrolysis. Boiled starch, however, is not a natural substrate and starch grains, in their native form, are highly resistant to $\alpha$-amylase attack (Table III). Although the conditions existing within cotyledons of germinating peas (*e.g.* pH, starch concentration, compartmentation of $\alpha$-amylase, and starch) remain undefined, under our assay conditions, which appeared to be optimal, 12.8 units of $\alpha$-amylase would be expected to liberate 1.3 mg of maltose equivalents from starch grains in 24 h, or 13% of the daily *in vivo* starch hydrolysis reported by Monerri *et al.* (17). It is possible that during germination some unknown factors, including but not limited to synergistic interaction with other amylases, enhance the ability of $\alpha$-amylase to attack starch grains. No pea amylases other than $\alpha$-amylase have been shown to have the ability to initiate degradation of intact starch grains.

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