Plant Physiol. (1995) 108: 211–217

The Role of Pea Chloroplast α-Glucosidase in Transitory Starch Degradation[1]

Zhuotao Sun[2], Stanley H. Duke*, and Cynthia A. Henson

Department of Agronomy (Z.S., S.H.D., C.A.H.) and Cereal Crops Research Unit, United States Department of Agriculture, Agricultural Research Service (C.A.H.), University of Wisconsin, Madison, Wisconsin 53706

Pea chloroplastic α-glucosidase (EC 3.2.1.20) involved in transitory starch degradation was purified to apparent homogeneity by ion exchange, reactive dye, hydroxylapatite, hydrophobic interaction, and gel filtration column chromatography. The native molecular mass and the subunit molecular mass were about 49.1 and 24.4 kD, respectively, suggesting that the enzyme is a homodimer. The enzyme had a K_m of 7.18 mM for maltose. The enzyme's maximal activity at pH 7.0 and stability at pH 6.5 are compatible with the diurnal oscillations of the chloroplastic stromal pH and transitory starch accumulation. This pH modulation of the α-glucosidase's activity and stability is the only mechanism known to regulate starch degradative enzymes in leaves. Although the enzyme was specific for the α-D-glucose in the nonreducing end as the glycon, the aglycon moieties could be composed of a variety of groups. However, the hydrolysis rate was greatly influenced by the aglycon residues. Also, the enzyme could hydrolyze glucans in which carbon 1 of the glycon was linked to different carbon positions of the penultimate glucose residue. The ability of the α-glucosidase to hydrolyze α-1,2- and α-1,3-glucosidic bonds may be vital if these bonds exist in starch granules because they would be barriers to other starch degradative enzymes. This purified pea chloroplastic α-glucosidase was demonstrated to initiate attacks on native transitory chloroplastic starch granules.

Transitory starch in photosynthetic tissues is thought to be degraded by both hydrolases and phosphorylases (Preiss and Levi, 1980). One of the enzymes in the hydrolytic pathway is α-glucosidase (EC 3.2.1.20). The function of α-glucosidases in starch degradation has been traditionally considered to be hydrolysis of maltose produced by other starch-catabolizing enzymes (Dunn, 1974).

α-Glucosidases in leaves have received much less attention than starch-degrading enzymes such as amylases or phosphorylases, and very little is known about their characteristics. However, α-glucosidases from starch-storing organs such as seeds of barley (Sun and Henson, 1990), rice (Takahashi et al., 1971), buckwheat (Takahashi and Shomomura, 1968a, 1968b; Takahashi et al., 1969; Kanaya et al., 1976), green gram (Yamasaki and Konno, 1979), flint corn (Chiba and Shimomura, 1975a, 1975b), and beets (Suzuki and Uchida, 1985) have been purified and characterized. In contrast to other starch-degrading enzymes, α-glucosidases have a unique property: flexible substrate specificity. They are capable of hydrolyzing α-D-glucosides (*p*-nitrophenyl-α-D-glucoside and methyl-α-D-glucoside), oligosaccharides (maltodextrins), and polysaccharides (amylose, amylopectin, and glycogen). Also, the position of the glucosidic linkage to the glycon or the penultimate Glc residue from the nonreducing end is not critical. Most will hydrolyze 1,2-, 1,3-, 1,4-, and 1,6-α-D-glucosidic bonds at variable rates. This catalytic flexibility of α-glucosidases was recently used to expand the known function of barley seed α-glucosidases in granular starch hydrolysis. In vitro studies indicated that the barley enzymes initiated attacks on starch granules, and there was a significant synergistic hydrolysis of native starch granules by the combined action of α-glucosidases and α-amylases (Sun and Henson, 1990).

Transitory leaf starch degradation differs from that of cereal endosperm reserve starch in that it is compartmentalized within the chloroplasts of living cells. Therefore, a prerequisite for an enzyme to function in leaf starch degradation is that the enzyme must be found inside the chloroplasts, the site of starch accumulation. Thus far, leaf starch-degrading enzymes capable of hydrolyzing native transitory starch granules have been either localized outside chloroplasts (Steup and Schächtele, 1981) or inconclusively localized inside chloroplasts (Beck and Ziegler, 1989). Three isoforms of α-glucosidases were recently found in pea (*Pisum sativum*) leaves. The two acid isoforms were apoplastic and the neutral isoform was identified as a chloroplastic enzyme (Beers et al., 1990). In view of the newly found importance of α-glucosidases in cereal seed starch hydrolysis (Sun and Henson, 1990) and the localization of an α-glucosidase in pea leaf chloroplasts (Beers et al., 1990), we investigated the pea chloroplastic α-glucosidase in the context of starch degradation. In the present report we describe the purification and characterization of an α-glucosidase from pea leaf chloroplasts.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum*, cv Laxton's Progress No. 9) leaves and stipules were harvested from 3-week-old plants grown as previously described (Beers et al., 1990).

[1] Supported by the National Science Foundation under agreement No. DCB-9003937; the College of Agriculture and Life Sciences, University of Wisconsin-Madison; and the U.S. Department of Agriculture, Agricultural Research Service.

[2] Present address: Schreier Malting Company, P.O. Box 59, Sheboygan, WI 53082–0059.

* Corresponding author; fax 1–608–262–5217.

Enzyme Extraction

Pea leaves and stipules were homogenized with a Polytron PT 10–35 homogenizer[3] (Kinematica, GmgH, Lucerne, Switzerland) equipped with a PTA35 generator in prechilled grinding buffer (40 mM Hepes, 200 mM NaCl, 0.02% NaN_2, 3 mM monothioglycerol, pH 7.2). One gram of tissue was homogenized in 3 mL of buffer. The homogenate was filtered through five layers of cheesecloth and centrifuged at 30,000*g* for 30 min at 4°C. The supernatant was dialyzed overnight against DEAE-Sephacel column buffer (25 mM ethylenediamine, 0.02% NaN_2, 1 mM monothioglycerol, pH 7.0).

α-Glucosidase Purification

All enzyme purification steps were conducted at 4°C. The dialyzed supernatant was centrifuged at 30,000*g* for 30 min at 4°C and loaded onto a DEAE-Sephacel anion-exchange column equilibrated with column buffer. The column was then washed with at least 10 column volumes of column buffer, and the α-glucosidase of interest was eluted at approximately 0.35 mM NaCl with 500 mL of a 0 to 0.6 M NaCl gradient in column buffer.

A column of Reactive Brown 10 agarose was run with DEAE-Sephacel column buffer. The enzyme was not bound; however, some contaminating proteins were bound and eliminated. Fractions with α-glucosidase activity were pooled and dialyzed against hydroxylapatite column buffer (10 mM sodium phosphate, 0.02% NaN_2, 1 mM monothioglycerol, pH 6.5).

The dialyzed α-glucosidase pool from the Reactive Brown 10 column was loaded onto a hydroxylapatite column equilibrated with column buffer. The column was washed with 5 column volumes of column buffer, and the bound α-glucosidase was eluted with 400 mL of a 10 to 300 mM sodium phosphate gradient in column buffer. The most active fractions eluted at approximately 210 mM sodium phosphate.

Hydrophobic interaction column chromatography was conducted with phenyl agarose equilibrated with hydroxylapatite column buffer with 25% $(NH_4)_2SO_4$ saturation. The α-glucosidase pool eluted from the hydroxylapatite column was brought to 25% $(NH_4)_2SO_4$ saturation with 100% saturated $(NH_4)_2SO_4$ in hydroxylapatite column buffer and then loaded onto a phenyl agarose column. The column was washed with 5 column volumes of column buffer and eluted with 400 mL of a 25 to 0% saturated $(NH_4)_2SO_4$ hydroxylapatite column buffer. The active fractions [peak at approximately 5.5% $(NH_4)_2SO_4$] were pooled and concentrated with an Amicon (Beverly, MA) YM30 membrane.

A Sephacryl S-300 gel filtration column was equilibrated with 20 mM Hepes (pH 7.0) containing 150 mM NaCl, 0.02% sodium azide, and 1 mM monothioglycerol and calibrated with Cyt *c* (12.5 kD), egg albumin (45 kD), BSA (68 kD), catalase (240 kD), aldolase (158 kD), and ferritin (450 kD).

[3] Mention of a proprietary product does not constitute a guarantee or warranty of the producer by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other suitable products.

The protein concentration was measured according to the method of Bradford (1976) with BSA as the standard.

α-Glucosidase Activity Assays

α-Glucosidase activity was determined with maltose as the substrate. The reaction mixture (200 μL) contained 50 mM citrate (pH 7.0), 50 mM maltose, and an α-glucosidase preparation up to 100 μL. The mixture was then incubated for 30 or 60 min depending on the amount of enzyme activity used. The reaction was terminated by boiling for 5 min. Glc released was measured by the reduction of NAD^+ by the coupled reactions of hexokinase and Glc-6-P dehydrogenase in the presence of ATP and NAD^+ (Henson et al., 1986). In the substrate-specificity study, liberated Glc was quantitated similarly, except that when *p*-nitrophenyl-α-D-glucoside was used as the substrate, *p*-nitrophenol was measured by A_{420}. One milliliter of *p*-nitrophenyl-α-D-glucoside (1 mg in 1 mL of 50 mM citrate at pH 7.0) was incubated with an enzyme preparation up to 100 μL. The reaction was terminated by addition of 100 μL of 1 N NaOH and placement on ice. One unit (IU) was defined as the amount of enzyme that hydrolyzed 1 μmol of α-D-glucosidic bonds at 30°C per min.

SDS-PAGE

SDS-PAGE (7.5% acrylamide) was run as described by Laemmli (1970). The gel was silver stained by the method described by Giulian et al. (1983).

Product Analysis of Maltoheptaose Hydrolysis

The reaction mixture (200 μL) contained 1.5 milliunits of α-glucosidase, 50 mM maltoheptaose, 0.02% sodium azide, and 50 mM citrate buffer, pH 7.0. The incubation was conducted at 30°C for up to 6 h. The reaction was terminated by boiling. The reaction mixture was then diluted 6-fold and filtered. HPLC separation of Glc and maltodextrins in 20 μL of the filtrate was on a Dionex (Sunnyvale, CA) CarboPac PA1 column (4 × 250 mm). Solvent A was 100 mM NaOH and 600 mM Na acetate. The gradient profile during the 30-min separation was from 100% A:0% B to 33% A:66% B over 20 min, followed by 10 min of 100% A. Separated Glc and maltodextrins were detected by a pulsed electrochemical detector with a gold electrode. Detection took place from 0.3 to 0.5 s at a potential of 0.1 V, during the 0.65-s pulsed amperometric cycle (E1 = 0.01 V [time = 0.5 s], E2 = 0.6 V [time = 0.1 s], E3 = −0.6 V [time = 0.05 s]).

Starch Granule Isolation and Hydrolysis

Pelleted starch granules from enzyme extraction were resuspended in DEAE-Sephacel column buffer. Starch granules were purified by repeated centrifugation (350*g*, 5 min) through a layer of 50% Percoll in DEAE-Sephacel column buffer.

Reaction mixtures containing 2.45% starch, 0.691 milliunit of α-glucosidase, 0.02% azide, and 66.7 mM citrate (pH 7.0) were incubated in Eppendorf microfuge tubes at 30°C

Table I. *Purification of pea leaf chloroplastic α-glucosidase*

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*milliunits*	*milliunits/mg*	*fold*	*%*
Crude extract	5584.00	529.6	0.095	1.0	100.0
DEAE-Sephacel	1309.72	323.5	0.247	2.6	61.1
Reactive Brown 10	432.24	211.8	0.490	5.2	40.0
Hydroxyl-apatite	36.70	121.7	3.320	34.9	23.0
Phenyl agarose	7.10	36.6	5.130	54.0	6.9
Sephacryl S-300	0.67	11.1	16.510	178.0	2.1

for up to 42 h. Control assays contained boiled α-glucosidase. Starch granules were kept suspended by constant shaking. Microfuge tubes were periodically removed and boiled for 5 min to terminate the reaction. The mixture was clarified by centrifugation, and Glc in the supernatant was measured.

Chloroplast Isolation

Chloroplasts were isolated from fully expanded leaves and stipules by the method of Kakefuda et al. (1986) as modified by Beers et al. (1990).

RESULTS

Purification and General Properties of Pea Leaf Chloroplastic α-Glucosidase

Whole leaves were used in the purification. To identify the chloroplastic α-glucosidase, purified chloroplast extract and whole-leaf extract for purification were chromatographed separately on a DEAE-Sephacel anion-exchange column. The identity of the chloroplastic α-glucosidase was determined by comparing chromatographic profiles of α-glucosidases extracted from purified chloroplasts and whole leaves (Beers et al., 1990). A representative purification scheme is summarized in Table I. The α-glucosidase was purified 178-fold with a yield of 1% by anion-exchange, reactive dye, hydroxylapatite, hydrophobic interaction, and gel filtration chromatography. α-Glucosidase in Sephacryl S-300 fractions was purified to homogeneity as determined by enzyme activity per unit protein, and the absence of contaminating proteins was determined by SDS-PAGE (Table I, Fig. 1). Prolonged incubation (1–6 h) of maltoheptaose with the purified enzyme did not produce any maltose, indicating that the enzyme preparation was free of contaminating starch-hydrolyzing enzymes (data not shown).

The subunit molecular mass of the enzyme was about 24.4 kD based on SDS-PAGE (Fig. 1a). The native molecular mass of the α-glucosidase was estimated to be about 49.1

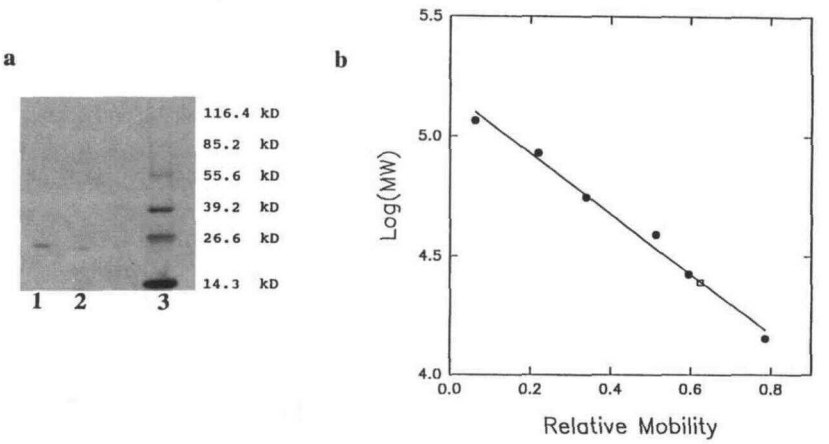

Figure 1. a, Subunit molecular mass (MW) of α-glucosidase determined by SDS-PAGE. Lanes 1 and 2, Purified α-glucosidase (0.1 μg/lane from the two fractions from a Sephacryl S-300 column that had the highest protein concentration and α-glucosidase activity). α-Glucosidase in lane 1 was purified to homogeneity and that in lane 2 to near homogeneity. Lane 3, Protein molecular mass standards. The molecular mass of the α-glucosidase subunit is 24.4 kD. Molecular mass standards used were lysozyme (14.3 kD), triose-phosphate isomerase (26.6 kD), aldolase (39.2 kD), glutamate dehydrogenase (55.6 kD), Fru-6-P kinase (85.2 kD), and β-galactosidase (116.4 kD). b, Native molecular mass of α-glucosidase determined by Sephacryl S-300 gel filtratrion. ■, Native α-glucosidase (49.1 kD). The column was calibrated with Cyt *c* (12.5 kD), hen egg albumin (45.0 kD), BSA (68.0 kD), aldolase (158.0 kD), catalase (240.0 kD), and ferritin (450.0 kD). The amount of enzyme protein added to the Sephacryl S-300 column and the percentage of recovery are indicated in Table I.

kD with Sephacryl S-300 (Fig. 1b). Therefore, the native enzyme is probably a homodimer.

Hydrolysis of maltose by the purified α-glucosidase at different pHs is shown in Figure 2A. The optimal pH is about 7.0. The enzyme had a narrow range of pH stability (Fig. 2B). Its activity decreased quickly when the pH was less than or greater than 6.5. The α-glucosidase lost 72% of its activity at pH 7.0 after 18 h (Fig. 2).

The K_m value for maltose was estimated from a Lineweaver-Burk plot (data not shown). The α-glucosidase had a K_m of 7.18 mM for maltose. The reaction with maltose exhibited first-order kinetics.

The α-glucosidase hydrolyzed all six maltodextrins tested (Table II). The enzyme hydrolyzed maltose, maltotriose, and maltotetraose at comparable rates. The hydrolysis of the other three maltodextrins was 19 to 28% faster.

Glucobioses with all possible α-D-glucosidic bonds were used as substrates (Table III). These disaccharides were hydrolyzed to different extents. Nigerose and kojibiose were much better substrates than maltose. However, the hydrolysis rates of isomaltose and trehalose were only 59 and 17% of that of maltose, respectively.

The α-glucosidase could hydrolyze a variety of α-D-glucosides (Table IV). The aglycon moieties of the glucosides included aryl, alkyl, sulfate, glucitol, and nucleotide moieties. The hydrolysis rate of *p*-nitrophenyl-α-D-glucoside was 57.5%

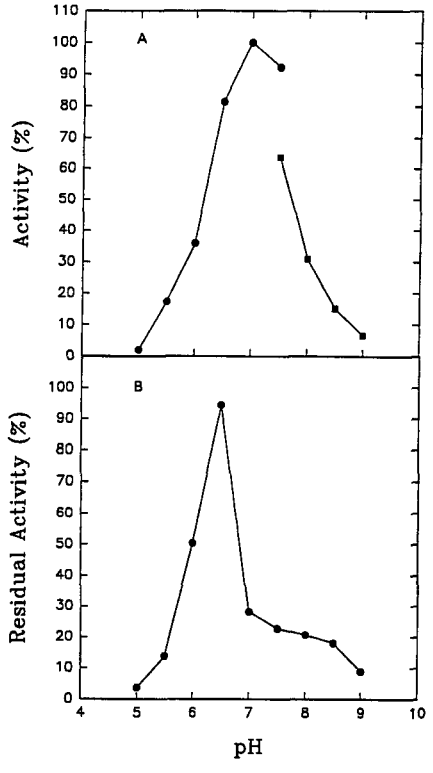

Figure 2. A, pH-activity profile of α-glucosidase. Activity was measured with maltose as the substrate. ●, Citrate buffer; ■, tricine buffer. B, pH-stability profile of α-glucosidase. The enzyme solution was dialyzed against buffers of different pHs for 18 h. The residual activity was then assayed with maltose as the substrate and expressed as a percentage of the activity before dialysis.

Table II. *α-Glucosidase activity toward maltodextrins*

All substrate concentrations were 50 mM, and assays were at 30°C for 1 h.

Substrate	Activity	
	milliunits min^{-1} mL^{-1}	%
Maltose	15.14	100.0
Maltotriose	14.68	97.0
Maltotetraose	15.53	102.6
Maltopentaose	18.82	124.3
Maltohexaose	18.03	119.1
Maltoheptaose	19.46	128.5

higher than that of maltose, whereas other α-D-glucosides were hydrolyzed at slower rates.

α-D-Glucans tested contained α-1,4-linkages (amylose), α-1,4- and α-1,6-linkages (soluble starch, amylopectin, pullulan, glycogen, and dextran), and α-1,4- and α-1,3-linkages (nigeran) (Table V). Hydrolysis of these polysaccharides was far slower than that of maltose. Amylopectin was the preferred Glc polymer among the glucans, whereas nigeran was the least-favored substrate. However, there is no clear relationship between hydrolysis rate and the type of α-D-glucosidic bonds in the glucan.

The action of α-glucosidase on starch granules isolated from pea leaves was followed by measuring the release of Glc (Fig. 3). The activity toward granular starch was limited but was easily detectable.

DISCUSSION

Purification of the pea chloroplastic α-glucosidase was difficult because the enzyme activity in the crude extract was very low and the enzyme was not stable during purification. This is reflected in the amount of enzyme (0.011 IU) obtained. Once purified, the α-glucosidase was stable for at least a few weeks at 4°C in the presence of 25% glycerol.

The maximal activity at pH 7.0 and stability at pH 6.5 of the chloroplastic α-glucosidase are physiologically significant for the following reasons. The pH of chloroplast stroma oscillates diurnally (Werdan et al., 1975). Starch-hydrolyzing enzymes that contribute to starch degradation would be expected to have a pH optimum compatible with the stromal pH in the dark. The pH optimum of the α-glucosidase is in the range of chloroplast stromal pH, pH 6.93 to 7.28 in the dark (Werdan et al., 1975). Transitory starch

Table III. *α-Glucosidase activity toward glucobioses*

All substrate concentrations were 50 mM, and assays were at 30°C for 1 h.

Substrate	Linkage	Activity	
		milliunits min^{-1} mL^{-1}	%
Maltose	α-1,4	15.14	100.0
Nigerose	α-1,3	25.93	171.3
Kojibiose	α-1,2	21.73	143.5
Isomaltose	α-1,6	8.88	58.7
Trehalose	α-1,1	2.60	17.2
Cellobiose	β-1,4	0.00	0.0

Table IV. *α-Glucosidase activity toward α-D-glucosidase*

All substrate concentrations were 50 mM except for 6-bromo-2-napthyl α-D-glucoside, which was 0.5 mg/mL, and *p*-nitrophenyl α-D-glucoside, which was 1 mg/mL. Assays were at 30°C for 1 h.

Substrate	Activity	
	milliunits min^{-1} mL^{-1}	%
Maltose	15.14	100.0
p-Nitrophenyl α-D-glucoside	23.85	157.5
Maltitol	5.59	36.9
UDPG	5.38	35.5
Glc-6-sulfate	3.79	25.0
6-Bromo-2-naphthyl α-D-glucoside	2.43	16.1
Methyl α-D-glucoside	1.85	12.2
ADPG	1.71	11.3

accumulation fluctuates diurnally. Although starch mobilization has been shown to proceed in the light, it is slower than in the dark (Stitt and Heldt, 1981). Hence, the activity of chloroplastic starch-degrading enzymes must be subject to control. However, very little is known about the regulation of the potential degradative enzymes α-amylase, starch phosphorylase, and starch-debranching enzyme (Preiss, 1982). Pea chloroplast α-glucosidase is the only transitory starch degradative enzyme demonstrated to have an inherent regulatory property. Proton concentration in the stroma can modulate both its activity and state of activation. During the day when the stromal pH increases to approximately 8.0, the enzyme will not be active because high pH inhibits and destabilizes the enzyme (Fig. 2). At night, the enzyme is active and could, therefore, be involved in starch degradation, but its activity will first increase as the stromal pH is decreased and then decrease because of inactivation by neutral pH. This instability at neutral pH is in agreement with the endogenous rhythm of starch degradation. In other words, starch degradation by this α-glucosidase will not be sustained even if the skotoperiod (dark period) is lengthened for a few cycles.

As with other glycosidases, the purified pea leaf chloroplastic α-glucosidase was found to have very wide substrate specificity. Although the enzyme was specific for the α-D-Glc in the nonreducing end as the glycon, wide tolerance was observed for changes in the aglycon. The aglycon moieties could be composed of a variety of groups. These included Glc, Glc oligomer, Glc polymer, sugar alcohol, and alkyl, aryl, sulfate, and nucleotide moieties. However, the hydrolysis rate was greatly influenced by the aglycon residues. Also, the enzyme could hydrolyze substrates in which carbon 1 of the glycon was linked to different carbon positions of the penultimate Glc residue. Generally, maltodextrins and glucobioses with α-D-glucosidic linkages were better substrates than α-D-glucans and α-D-glucosides, except that the hydrolysis rate of *p*-nitrophenyl-α-D-glucoside was higher and that of trehalose was lower relative to the hydrolysis rate of maltose. α-1,2 and α-1,3-glucosidic bonds in disaccharides were hydrolyzed faster than α-1,4-, α-1,6-, and α-1,1-glucosidic bonds. The flexible substrate specificity and the transglucosylation activity of the α-glucosidase can be explained by the mechanism proposed for the action of glycosidases (Nisizawa and Hashimoto, 1970).

Table V. *α-Glucosidase activity toward α-D-glucans*

All substrate concentrations were 1.0% (w/v), and assays were at 30°C for 4 h.

Substrate	Linkage	Activity	
		milliunits min^{-1} mL^{-1}	%
Maltose		15.14	100.0
Amylopectin	α-1,4; α-1,6	1.28	8.5
Dextran	α-1,4; α-1,6	0.83	5.5
Pullulan	α-1,4; α-1,6	0.39	2.6
Amylose	α-1,4	0.30	2.0
Glycogen	α-1,4; α-1,6	0.26	1.7
Soluble starch	α-1,4; α-1,6	0.22	1.5
Nigeran	α-1,3; α-1,4	0.10	0.7
Native granule	α-1,4; α-1,6	0.36	2.4

The physiological significance of α-glucosidase in transitory starch degradation has not been clearly established (Killilea and Clancy, 1978). However, the major function of α-glucosidase has been assumed to be hydrolysis of maltose in the final step of the hydrolytic pathway (Dunn, 1974). The results obtained in this study indicated that the function of α-glucosidase in transitory starch degradation might be more important than previously recognized. The pea chloroplastic α-glucosidase hydrolyzed maltodextrins at rates no lower than that at which maltose was hydrolyzed. It is likely that maltodextrins produced by α-amylase's action are also physiological substrates.

The ability of pea chloroplastic α-glucosidase to hydrolyze more than one type of α-D-glucosidic bonds could be vital if α-1,2 and α-1,3-glucosidic bonds are in starch (Abdel-Akher et al., 1952; Wolfrom and Thompson, 1956). α-Glucosidase is the only starch degradative enzyme known to be capable of hydrolyzing these uncommon bonds. Indeed, the pea α-glucosidase preferred α-1,3- and α-1,2-glucosidic bonds to α-1,4-glucosidic bonds (Table III). The existence and localization of un-

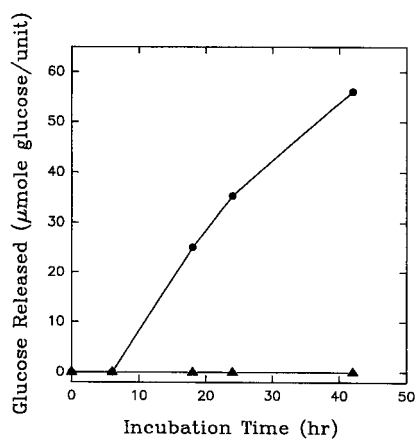

Figure 3. Native transitory starch granule hydrolysis by α-glucosidase. ●, α-Glucosidase (0.691 milliunit) with 2.45% (w/v) native starch granules, 0.02% (w/v) NaN_3, and 66.7 mM sodium citrate (pH 7.0) incubated at 30°C. ▲, Boiled enzyme with starch granules.

common bonds in starch granules have not been confirmed. If such bonds are on the surface of native starch granules, the ability of α-glucosidase to remove these would be essential, because they would constitute barriers to α-amylase, starch-debranching enzyme, and starch phosphorylase.

Although the pea chloroplastic α-glucosidase hydrolyzed native transitory starch granules only to a limited extent, the importance of the enzyme may be that this limited attack may render the starch granules accessible or more accessible to other starch-degrading enzymes. There may be synergistic degradation of starch granules by the concurrent action of α-glucosidase and other enzymes, as was found in cereal seeds (Sun and Henson, 1990). In barley, only slight hydrolysis of native starch granules was observed with α-glucosidase alone. However, the ratio of native starch granule degradation by the combination of an α-glucosidase and an α-amylase to the sum of degradations by the two enzymes acting separately ranged from 2:1 to 10.7:1.

Although hydrolysis of native transitory starch granules by the pea chloroplastic α-glucosidase is limited, to our knowledge this is the first report of a chloroplastic starch degradative enzyme that is capable of attacking native transitory starch granules, since no chloroplastic endoamylase has been isolated in a pure form (Beck and Ziegler, 1989) and the phosphorylase purified from chloroplasts was inactive on native transitory starch granules that were not preattacked by an endoamylase (Steup et al., 1983). In addition, there is no convincing evidence that chloroplastic endoamylase alone is responsible for the initial attack of transitory starch granules (Beck and Ziegler, 1989). Previous in vitro studies were conducted almost exclusively with a mixture of chloroplastic hydrolases (Beck et al., 1981), an endoamylase not shown to be free of other hydrolytic activities (Chang, 1982), or an endoamylase from a nonplant source (Steup et al., 1983). Another caveat is that soluble, not native granular, starch has been used as a substrate in most amylase studies. It is precarious to extrapolate results from such experiments to the identity, properties, and mode of action of the factor or factors acting upon native starch granules in vivo.

In summary, activity and pH-stability profiles of the α-glucosidase purified from pea leaf chloroplasts are consistent with chloroplast stromal pH oscillations and, therefore, provide a regulatory mechanism for diurnal rhythm of the enzyme activity. The wide substrate specificity of the α-glucosidase suggests that the enzyme may hydrolyze maltodextrins as well as maltose, and the enzyme may be responsible for cleaving α-1,2- and α-1,3-glucosidic bonds in starch. The ability of the enzyme to attack native transitory starch granules makes it, to our knowledge, the first enzyme purified from chloroplasts that can do so.

ACKNOWLEDGMENT

We are grateful to Dr. Seiya Chiba (Department of Applied Bioscience, Faculty of Agriculture, Hokkaido University, Sapporo, Japan) for his generous gift of kojibiose.

Received September 28, 1994; accepted January 10, 1995.

LITERATURE CITED

Abdel-Akher M, Hamilton JK, Montgomery R, Smith F (1952) A new procedure for the determination of the fine structure of polysaccharides. J Am Chem Soc **74:** 970–971

Beck E, Pongratz P, Reuter I (1981) The amylolytic system of isolated spinach chloroplasts and its role in the breakdown of assimilatory starch. *In* G Akoyunoglou, ed, Proceedings of the Fifth International Congress on Photosynthesis, Vol 4. Balaban International Science Service, Philadelphia, pp 529–538

Beck E, Ziegler P (1989) Biosynthesis and degradation of starch in higher plants. Annu Rev Plant Physiol Plant Mol Biol **40:** 95–117

Beers EP, Duke SH, Henson CA (1990) Partial characterization and subcellular localization of three α-glucosidase isoforms in pea (*Pisum sativum* L.) seedlings. Plant Physiol **94:** 738–744

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem **72:** 248–254

Chang CW (1982) Enzymic degradation of starch in cotton leaves. Phytochemistry **21:** 1263–1269

Chiba S, Shimomura T (1975a) Purification and some properties of flint corn α-glucosidase. Agric Biol Chem **39:** 1033–1040

Chiba S, Shimomura T (1975b) Substrate specificity of flint corn α-glucosidase. Agric Biol Chem **39:** 1041–1047

Dunn G (1974) A model for starch breakdown in higher plants. Phytochemistry **13:** 1341–1346

Giulian GG, Moss RL, Greaser M (1983) Improved methodology for analysis and quantitation of proteins on one-dimensional silver-stained slab gels. Anal Biochem **129:** 277–287

Henson CA, Duke SH, Koukkari WL (1986) Rhythmic oscillations in starch concentration and activities of amylolytic enzymes and invertase in *Medicago sativa* nodules. Plant Cell Physiol **27:** 233–242

Kakefuda G, Duke SH, Hostak MS (1986) Chloroplast and extrachloroplastic starch-degrading enzymes in *Pisum sativum* L. Planta **168:** 175–182

Kanaya K, Chiba S, Shimomura T, Nishi K (1976) Improved method for purification of buckwheat α-glucosidase and some kinetic properties. Agric Biol Chem **40:** 1929–1936

Killilea SD, Clancy MJ (1978) Properties of potato α-glucosidase. Phytochemistry **17:** 1429–1431

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277:** 680–685

Nisizawa K, Hashimoto Y (1970) Glycoside hydrolases and glycosyl transferases. *In* W Pigman, D Horton, eds, The Carbohydrates: Chemistry and Biochemistry, Vol II. Academic Press, New York, pp 241–300

Preiss J (1982) Regulation of the biosynthesis and degradation of starch. Annu Rev Plant Physiol **33:** 431–454

Preiss J, Levi C (1980) Starch biosynthesis and degradation. *In* JB Pridham, ed, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp 371–423

Steup M, Robenek H, Melkonian M (1983) *In-vitro* degradation of starch granules isolated from spinach chloroplasts. Planta **158:** 428–436

Steup M, Schächtele C (1981) Mode of glucan degradation by purified phosphorylase forms from spinach leaves. Planta **153:** 351–361

Stitt M, Heldt HW (1981) Simultaneous synthesis and degradation of starch in spinach chloroplasts in the light. Biochim Biophys Acta **638:** 1–11

Sun Z, Henson CA (1990) Degradation of native starch granules by barley α-glucosidases. Plant Physiol **94:** 320–327

Suzuki Y, Uchida K (1985) An α-glucosidase from endosperm of germinating edible beet seeds. Agric Biol Chem **49:** 863–865

Takahashi M, Shimomura T (1968a) Biochemical studies on α-glucosidase from buckwheat (*Fagopyrum esculentum* Mönch). Part I. Screen of α-glucosidase from plant sources and changes of α-glucosidase activity during the germination stage of buckwheat. Agric Biol Chem **32:** 923–928

Takahashi M, Shimomura T (1968b) Biochemical studies on α-glucosidase from buckwheat (*Fagopyrum esculentum* Mönch). Part II. Purification of buckwheat α-glucosidase and some properties on maltose and soluble starch. Agric Biol Chem **32:** 929–939

Takahashi M, Shimomura T, Chiba S (1969) Biochemical studies on buckwheat α-glucosidase. Part III. Transglucosylation reaction of the enzyme and isolation of the reaction products. Agric Biol Chem **33:** 1399–1410

Takahashi M, Shimomura T, Chiba S (1971) Studies on α-glucosidase in rice. Part I. Isolation and some propertiesof α-glucosidase I and α-glucosidase II. Agric Biol Chem **35:** 2015–2024

Werdan K, Heldt HW, Milovancev M (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochim Biophys Acta **396:** 276–292

Wolfrom ML, Thompson A (1956) Occurrence of the (1–3)-linkage in starches. J Am Chem Soc **78:** 4116–4117

Yamasaki Y, Konno H (1979) Purification and properties of three α-glucosidases from germinated green gram (*Phaseolus vidissimus* Ten.) Agric Biol Chem **43:** 481–489