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A β-Amylase in Potato Tubers Is Induced by Storage at Low Temperature[1]

Tom Hamborg Nielsen*, Uta Deiting, and Mark Stitt

Plant Biochemistry Laboratory, Department of Plant Biology, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK–1871 Frederiksberg C, Denmark (T.H.N.); and Botanisches Institut, Heidelberg Universität, Im Neuenheimer Feld 360, 69120 Heidelberg, Germany (U.D., M.S.)

A new starch-degrading enzyme activity is induced by storage of potato (*Solanum tuberosum* L.) tubers at low temperatures (L. Hill, R. Reimholz, R. Schröder, T.H. Nielsen, M. Stitt [1996] Plant Cell Environ 14: 1223–1237). The cold-induced activity was separated from other amylolytic activities in zymograms based on iodine staining of polyacrylamide gels containing amylopectin. A similar band of activity was detected at normal growth temperatures in leaves, stems, and growing tubers but was present only at low activity in warm-stored tubers. The cold-induced enzyme was separated by ion-exchange chromatography from other amylolytic activities. It has a broad neutral pH optimum. Characterization of its hydrolytic activity with different substrates showed that the cold-induced activity is a β-amylase present at low activity in tubers stored at 20°C and induced progressively when temperatures are decreased to 5 and 3°C. The first clear induction of β-amylase activity was observed within 3 d of storage at 3°C, and the activity increased 4- to 5-fold within 10 d. The possible involvement of the cold-induced β-amylase in sugar accumulation during cold storage is discussed.

When potato (*Solanum tuberosum* L.) tubers are stored at low temperatures they accumulate sugars, primarily Glc, Fru, and Suc (Sowokinos, 1990), in a process that is termed "cold sweetening." Higher storage temperatures (e.g. above 10°C) prevent this sugar accumulation. Cold sweetening is of commercial interest, since high sugar content will result in a poor-quality product when the tubers are used for fries and chips. Recently, it was shown that the accumulation of sugars is due to a specific activation of Suc synthesis by up-regulation of Suc-P synthase (Hill et al., 1996). Along with the activation of Suc synthesis, a new amylolytic activity also induced in the cold-stored tubers was discovered. This activity was observed in zymograms, in which the amylolytic enzymes were degrading amylopectin. Since starch is the likely source of carbohydrates for the cold sweetening of tubers (Isherwood, 1976), this induction of new amylolytic activities could contribute to the sweetening process.

Starch degradation can occur via phosphorolytic and hydrolytic reactions and probably requires the concerted action of several enzymes (Beck and Ziegler, 1989). The degradation of starch granules in vivo is in most cases poorly understood. In leaf tissues, degradation of starch appears to involve the cooperative attack by phosphorolytic and hydrolytic activities (Steup et al., 1983; Beck and Ziegler, 1989). Recently, hexose transport over the chloroplast membrane was demonstrated as a prerequisite to retaining normal starch degradation rates in leaves of Arabidopsis (Trethewey and ap Rees, 1994), which would suggest an important role of α-glucosidase in the degradation of transitory starch. In cereal seeds, the mobilization of the starch reserves is triggered by the induction of α-amylases. These cereal seeds are the only tissues in which the starch degradation process has been characterized in detail, and several elements of a signal transduction pathway leading to the expression of specific α-amylase genes have been identified. However, the cereal seed is a highly specialized system, and it is not safe to deduce extensive similarity to other tissues such as potato tubers because both the physiology and the cellular organization are fundamentally different.

A range of starch-degrading enzyme activities have been reported in potato tubers, including α-amylase, isoamylase, β-amylase, α-glucosidase, and phosphorylase (Gerbrandy et al., 1975; Kennedy and Isherwood, 1975; Fan, 1975; Killilea and Clancy, 1978; Schneider et al., 1981; Cochrane et al., 1991a, 1991b; Cottrell et al., 1993). However, it is not clear whether the degradation is primarily amylolytic or phosphorolytic. Morrell and ap Rees (1986) concluded that the degradation of starch in cold-stored potato tubers was phosphorolytic because they detected no amylase activities in the tubers. However, several other authors (Sowokinos et al., 1985; Davies and Ross, 1987; Cochrane et al., 1991b; Cottrell et al., 1993) have detected substantial activities. This discrepancy may be due to the use of different assay procedures.

Several authors have looked for changes in starch-degrading activities after transfer of potato tubers to low temperature to promote sugar accumulation. Low storage temperature has been reported to result in increased activity of both α-amylase and β-amylase (Cochrane et al., 1991b; Cottrell et al., 1993), as well as phosphorylase (Claassen et al., 1993). On the other hand, Kennedy and

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* Corresponding author; e-mail thn@biobase.dk; fax 45–3–5283333.

Abbreviations: BPNPG7, end-blocked *p*-nitrophenyl-maltoheptaose; PNPG5, *p*-nitrophenyl-maltopentaose.

Isherwood (1975) and Hill et al. (1996) found no effect of storage temperature on phosphorylase activity, and Sowokinos et al. (1985), who did report a selective increase in phosphorylase in the sweetening tuber tissues, found no changes in amylase activity. These inconsistencies might be due to differences between cultivars. However, another explanation is that the assays used to measure amylolytic activities do not always fully distinguish between the different types of enzymes, e.g. α- and β-amylase activities, and will not detect separately potential isoforms of each of these types. This complicates the interpretation of previous experiments on potato tubers, in which amylases have not been separated and characterized. In the following study, we have separated the cold-induced enzyme detected by zymograms (Hill et al., 1996) from the other amylolytic activities in cold-sweetening tubers of the potato cv Desirée. We show that it is a β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2.), whereas α-glycosidase and endoamylolytic activities are not influenced by low storage temperature.

MATERIALS AND METHODS

Tubers of potato (*Solanum tuberosum* cv Desirée) were harvested from a field during August and stored in darkness at 20°C. During cold treatment the tubers were transferred to cabinets in which the temperature was regulated within ±0.2°C. Unless otherwise indicated the tubers were stored for 7 d at 3°C. Tuber tissue was harvested by punching a rod with a 10-mm cork borer and cutting 2-mm slices directly into liquid nitrogen. The frozen tissue was stored at −80°C. For the larger preparations used for chromatographic separation of enzymes, fresh tissue was used.

Native Gel Electrophoresis and Detection of Amylolytic Activities

Frozen tuber discs were extracted in 2 volumes of a buffer containing 50 mM Mops-KOH (pH 7.5), 20 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EDTA, and 0.1% (v/v) β-mercaptoethanol (buffer A). The extraction medium also contained 30 g L^{-1} PEG-8000 and 20 g L^{-1} polyvinylpolypyrrolidone. Twenty microliters of 50% (v/v) glycerol containing 0.1 mg L^{-1} bromphenol blue was added to 60 μL of crude extract. Then 10 to 35 μL of this mixture was loaded in each lane of the gels.

The enzymes were separated by discontinuous PAGE using separating gels containing amylopectin, as described by Hill et al. (1996). Separating gels (0.75 mm) contained 7.5% (w/v) polyacrylamide (30:0.8 acrylamide:bisacrylamide), 0.6% (w/v) potato amylopectin (Merck, Darmstadt, Germany), and 375 mM Tris-HCl (pH 8.8). Stacking gels contained 3% (w/v) polyacrylamide and 63 mM Tris-HCl (pH 6.8). The gels were electrophoresed at 0 to 4°C (Mini Protean 2 system, Bio-Rad) for 1 to 1.5 h at a constant current of 30 mA (two gels). After electrophoresis the gels were washed twice for 10 min in water and incubated (1.5 h at 20°C) in buffer containing 0.1 M Mes-KOH (pH 6.2), 2 mM $CaCl_2$, and 0.1% (v/v) β-mercaptoethanol (buffer B). Gels were then washed for 10 min in water and stained with iodine to detect degradation of the amylopectin.

For determination of the pH optimum only the incubation buffer was changed. All incubations contained 0.1 M of the indicated buffer (pH set with KOH or HCl) and 0.1% β-mercaptoethanol.

Assay of Starch-Hydrolyzing Enzymes

Activities of endo-amylase and exo-amylase were determined by detecting the degradation of maltooligosaccharides linked to a *p*-nitrophenyl group by a glucosidic bond at the reducing end, using assay kits purchased from Megazyme (Sydney, Australia). These assays were designed for specific detection of cereal grain enzymes. The endo-amylase substrate is a *p*-nitrophenyl-maltoheptaose chemically blocked at the nonreducing end (BPNPG7) to prevent degradation by exo-amylase. The exo-amylase substrate is a nonblocked *p*-nitrophenyl-maltopentaose (PNPG5) that is too short to allow significant activity of cereal α-amylases. Enzyme extract (50 μL) was mixed with 225 μL of buffer containing 100 mM Mes-KOH, pH 6.2, 1 mM EDTA, and 0.1% (v/v) β-mercaptoethanol. Assays were started by adding 25 μL of substrate and coupling enzyme (final concentration 0.4 mM oligosaccharide and 2.5 units of α-glucosidase). Assays were stopped by adding 2.5 volumes of 10 g L^{-1} Trizma-base (Sigma), and the activity was determined as liberated *p*-nitrophenolate detected spectrophotometrically at 405 nm. α-Glucosidase was determined in the same way using *p*-nitrophenyl-glycoside (5 mM) as substrate.

Total amylolytic activity was determined as the degradation of soluble starch (Kowalsky starch, Merck, Darmstadt, Germany). Enzyme (50 μL) and buffer B (200 μL) with soluble starch (final concentration 10 g L^{-1}) were mixed, and degradation products were determined colorimetrically according to the method of Bernfeld (1955).

Separation of Hydrolytic Activities

Starch-degrading enzymes were extracted from cold- and warm-stored tubers. All steps during separation were performed at 0 to 4°C. Tuber tissue (80 g) was homogenized in a blender (Waring; three times for 30 s each time) in 200 mL of buffer A with 30 g L^{-1} PEG-8000 and 20 g L^{-1} polyvinylpolypyrrolidone. The homogenate was centrifuged (15 min at 20,000*g*). The pellet was discarded and the supernatant was filtered through two layers of Miracloth (Calbiochem). Protein was precipitated from 150 mL of the homogenate with PEG-8000 (final concentration 170 g L^{-1}). The precipitate was pelleted by centrifugation (15 min at 20,000*g*). The supernatant was discarded, and the pellet was redissolved in 35 mL of buffer A. The redissolved protein was cleared by centrifugation (15 min at 20,000*g*) and separated over a 60-mL DEAE column (Fast Flow, Pharmacia) with a linear gradient of 0 to 0.4 M KCl in buffer A developed over 250 mL. Fractions (8 mL) were collected and analyzed for starch-degrading enzyme activities.

Detection of Hydrolysis Product

Fractions from the DEAE column were mixed with an equal volume of buffer B and 10 g L^{-1} soluble starch and incubated for 0 to 20 h at 20°C. The reaction was stopped at different times by applying 1 μL of the reaction mixture to a TLC plate (Silicagel F60, Merck). The plate was developed twice with an eluent containing isopropanol:butanol:water (12:3:4). Mixtures of Glc and malto-oligosaccharides (two to seven Glc residues) were used as standards. Products formed were visualized by charring of the plates wetted with 10% (v/v) H_2SO_4 in ethanol.

RESULTS

Amylolytic enzyme activities were detected in zymograms, where they appeared as light bands on a darker-brown background. As previously observed (Hill et al., 1996), tubers stored at low temperatures (3°C) contained an amylolytic activity that was absent or very weak in tubers stored at higher temperatures (20°C) (Fig. 1, lanes 1 and 2, respectively). We denote this the "cold-induced" band.

The same activity was present in growing tubers (Fig. 1, lane 3), although much weaker than in the cold-stored tubers. A similar band was observed in mature and developing leaves (Fig. 1, lanes 4 and 5). The activity was relatively high in the leaf tissues compared with the cold-stored tubers (note amounts of extracts loaded). The activity detected in mature leaves had a slightly higher mobility than that detected in the other tissues. A similar activity was observed in crude extracts of stem tissue (Fig. 1, lane 6). Thus, the cold-induced band is the predominant amylolytic activity in several nondormant tissues that were not cold-treated.

In a recent study, amylolytic activities from potato leaves were separated by ion-exchange chromatography (Viksø-Nielsen et al., 1997). To separate the activities in the tubers and identify which type of amylolytic enzyme the cold-induced band represents, we again used ion-exchange chromatography of the activities extracted from cold-stored and warm-stored tubers. Crude extracts of 80 g of tuber material were precipitated with 17 g L^{-1} PEG. The

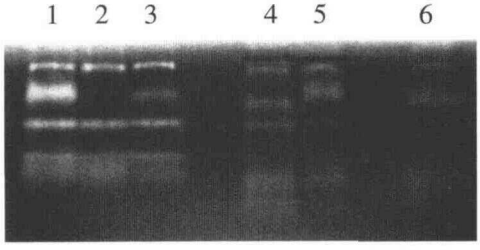

Figure 1. Zymogram of amylolytic activities in different tissues from potato plants. Crude extracts of tuber stored at 3°C (15 μL, lane 1), tuber stored at 20°C (15 μL, lane 2), growing tuber (15 μL, lane 3), source leaves (10 μL, lane 4), sink leaves (5 μL, lane 5), and stem (30 μL, lane 6). The enzymes were separated by electrophoresis in polyacrylamide gels containing 6 g L^{-1} amylopectin. The gels were incubated for 1 h in buffer (100 mM Mes-KOH, pH 6.2, 2 mM $CaCl_2$, 0.1% β-mercaptoethanol) at 20°C and then stained with iodine. Amylolytic activities are seen as light bands on a dark background.

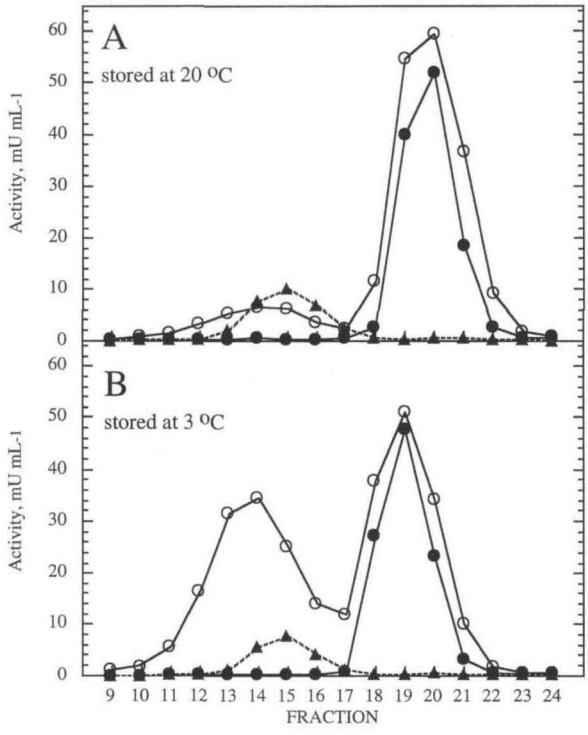

Figure 2. Separation of amylolytic activities by ion-exchange chromatography. Protein concentrated by precipitation with PEG-8000 was separated over a DEAE-Sepharose column with a linear salt gradient. ●, Activity with BPNPG7; ○, activity with PNPG5; ▲, α-glucosidase. A, Tuber stored at 20°C; B, tuber stored at 3°C. mU, Milliunits.

redissolved precipitate was applied to a DEAE column and eluted with a gradient of KCl. The collected fractions were analyzed by assays (see "Materials and Methods") designed to distinguish between the activities of endo-amylase, α-glucosidase, and exo-amylase and total amylolytic activity (Fig. 2). The same fractions were also analyzed by zymograms (Fig. 3) to compare the banding pattern in the gels with the activity of the different types of amylolytic enzymes.

An early-eluting activity in fractions 10 to 16 accepted PNPG5 but not BPNPG7 as a substrate, indicating that it was an exo-amylase (Fig. 2B). The column chromatography gave a good separation of this exo-amylase activity from the activities that accepted BPNPG7 as substrate, presumably endo-amylases. These activities eluted in later fractions. Unexpectedly, the fractions containing endo-amylases also cleaved PNPG5 (see below for discussion).

The early-eluting exo-amylase activity was specifically induced by low-temperature storage of the tubers (Fig. 2, compare A and B for the profile in warm- and cold-stored material, respectively). The other slow-eluting amylolytic activities showed no significant response to storage temperature. α-Glucosidase eluted slightly later than the cold-induced exo-amylase and was considerably less active than the exo-amylase. It was also unaffected by storage temperature.

Total amylolytic activity, detected as the degradation of soluble starch, corresponded in activity profile to the sum

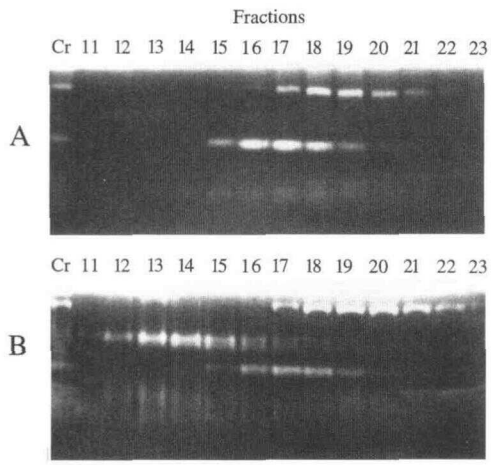

Figure 3. Zymogram of amylolytic activities (as in Fig. 1) in fractions from ion-exchange chromatography (same as in Fig. 2). A 25-μL fraction was loaded in each lane. A, Tuber stored at 20°C; B, tuber stored at 3°C. Cr, Crude extract; numbers indicate fractions 11 to 23 from DEAE columns shown in Figure 2.

of the individual amylases. It showed a strong cold induction in the fractions containing the early-eluting exo-amylase activity but not in the slow-eluting fractions (data not shown). The total amylolytic activity was 2 to 3 times higher than the sum of the individual hydrolytic activities, indicating that soluble starch is a better substrate for potato amylolytic activities than the malto-oligosaccharides.

The combination of ion-exchange chromatography and native gel electrophoresis (Fig. 3) separated several (at least seven) amylolytic activities in the cold-stored tubers. These may represent multiple forms, isozymes, or different types of hydrolytic enzymes. The cold-induced amylolytic activity detected in the zymograms eluted from the ion-exchange column in the same fractions as the early-eluting activity that cleaved PNPG5 (compare Figs. 2B and 3B). In warm-stored tubers this band was weak on the zymograms, and the measured activity in the early-eluting fractions with PNPG5 was also low (Figs. 2A and 3A). Closer inspection of the zymograms in Figure 3 reveals that the ion-exchange column fractions with a sharp, low-mobility, cold-induced band also contain some less-focused activities with a higher mobility. These less-focused bands were also induced by cold treatment (Fig. 3, compare A and B). This was not evident in the crude extracts (Fig. 1; Hill et al., 1996) but is evident in Figure 3 because other overlapping and noninduced bands are separated by the previous ion-exchange chromatography step.

To identify which of the two observed cold-induced bands represent the exo-amylase activity measured in the PNPG5 assay, the gels with electrophoresed enzymes were sliced into 2.5-mm pieces and each slice was analyzed for activity with PNPG5 (Fig. 4). Both the upper band and the less-focused lower bands reacted with the substrate, indicating that both bands represent exo-amylases. These may represent different forms of the same enzyme. Similar analysis of the second peak containing the endo-amylase (fraction 19) revealed that activities detected by both PNPG5 and BPNPG7 were accounted for by just one of the bands on the zymograms (data not shown), which is likely to be an endo-amylolytic activity that also accepts short-chain malto-oligosaccharides.

To provide further evidence that the cold-induced hydrolytic activities are β-amylases, the products formed during incubation with soluble starch were analyzed by TLC. Fractions 10 to 13, in which the cold-induced activity was the only predominant amylolytic activity, produced almost exclusively maltose, as would be expected from a β-amylase activity (Fig. 5). Only small amounts of oligosaccharides and Glc were formed by these fractions. In contrast, the slow-eluting fractions produced a range of saccharides (Fig. 5). Fractions 14 to 16 contained α-glucosidase and produced significant amounts of Glc, whereas later fractions predominantly formed oligosaccharides.

To characterize the pH dependence of the cold-induced β-amylase activity, the early-eluting activity was assayed with soluble starch as substrate at different pH values, and these measurements were supplemented with zymogram analysis of pH dependence (Fig. 6). Both methods indicated a broad pH optimum curve that peaked between 6.0 and 7.5.

To gain more information about the temperature dependence of the β-amylase induction, tubers were transferred from 20°C to different temperatures between 3 and 11°C. Crude extracts of these tubers were analyzed by zymograms and assayed for exo-amylase and endo-amylase activity. The foregoing analysis showed that the PNPG5 assay detected the cold-induced β-amylase and a second peak of amylase activity that eluted later from the ion-exchange column (Fig. 2). This second peak is mainly due

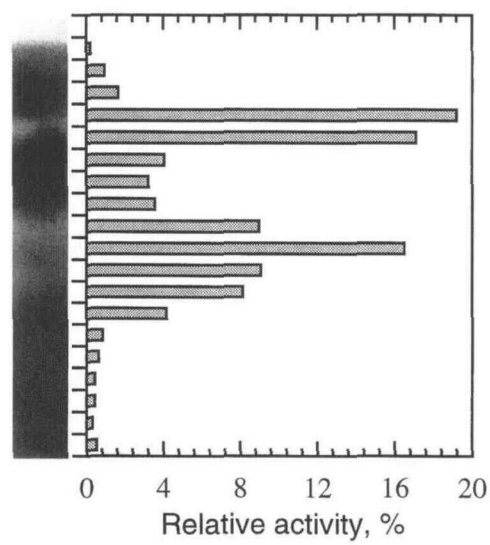

Figure 4. Location of exo-amylase activities in zymograms of fractions containing the cold-induced amylolytic activity. After electrophoresis, the gel was sectioned in individual lanes. One lane was sliced into 2.5-mm pieces. Amylolytic activity was measured for each slice with PNPG5 as substrate. The gel slices were incubated with substrate for 2 h with vigorous stirring and the liquid phase was analyzed for liberated *p*-nitrophenolate. The distribution of activity is shown (sum of all slices set to 100%). A neighboring lane was stained with iodine as in Figure 1.

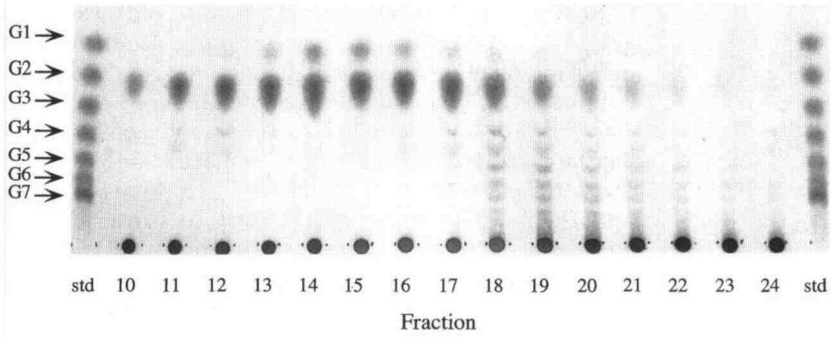

Figure 5. Identification of hydrolytic products formed by the separated amylolytic enzymes in cold-stored tubers. The indicated fractions correspond to Figure 2B. A sample of each fraction was incubated with 10 mg L^{-1} soluble starch for 6 h. The products formed were separated by TLC and stained by charring with sulfuric acid. std, Standard compounds; Gl, Glc; G2–G7, malto-oligosaccharides with chain lengths of two to seven Glc residues.

to an endo-amylase that reacts at approximately the same rate with BPNPG7 and PNPG5. To estimate the activity of cold-induced β-amylase in crude extracts, we therefore subtracted the activity measured with the BPNPG7 from the activity measured with PNPG5 (Fig. 7).

The induction of the exo-amylase depends strongly on the temperature. The most efficient induction was at 3°C (Fig. 7A). Increasing the temperature to 5°C gave a significantly lower induction, and the activity hardly changed at 7 to 11°C. These data were confirmed by zymograms, in which induction was highest at 3 and 5°C, and the first clear induction was seen within 3 d at low temperature (Fig. 8; Hill et al., 1996). A similar time dependence was also found in activity measurements (Fig. 7B).

The accumulation of sugars (Suc, Glc, and Fru) largely corresponded with the response of the exo-amylase to storage temperatures. After 10 d the sugar content, expressed as hexose equivalents, had increased from 12 to 41 μmol g^{-1} fresh weight at both 3 and 5°C. At 7, 9, and 11°C only small changes in sugar content were observed (sugar contents 16, 14, and 14 μmol g^{-1} fresh weight, respectively). At 3°C the first significant sugar accumulation occurred between 3 and 6 d of cold storage (data not shown; Hill et al., 1996).

DISCUSSION

The maximum rates of sugar accumulation during cold storage in Desirée tubers reported by Hill et al. (1996) are about 0.2 to 0.3 μmol g^{-1} fresh weight h^{-1} (hexose equivalents). After chromatographic separation of enzymes in cold-stored tuber material we measured activities of 1.4 and 1.1 μmol g^{-1} fresh weight h^{-1} in the assays with PNPG5 and BPNPG7 for exo- and endo-amylolytic activities, respectively. These are minimum values since losses during the partial enzyme purification were not corrected for. Calculations based on measurements on the unseparated crude extracts (Fig. 7B, d 10) give higher values (3, 6, and 1.9 μmol g^{-1} fresh weight h^{-1}, respectively). Furthermore, it should be considered that the amylases produce malto-oligosaccharides rather than Glc and will release more Glc units per cleavage action. The measured amylolytic activities in crude extracts would therefore be sufficient to account for the carbohydrate accumulation during cold sweetening. These results indicate that starch degradation in potato tubers is not exclusively phosphorolytic. However, to reach a final conclusion, subcellular compartmentation also needs to be considered. This will require further investigation, since the localization of these activities in potato tuber tissue is not yet known.

Most previous reports of β-amylase in potato tubers have been based on indirect evidence (Davies and Ross, 1987; Cochrane et al., 1991a, 1991b; Cottrell et al., 1993; Viksø-Nielsen et al., 1997). Only Fan (1975) demonstrated directly that tubers contain a β-amylase. Using a combination of chromatographic separation, specific assay procedures, and zymograms we have now shown that cold-induced activity in tubers indeed represents β-amylase (Figs. 2–4). This was confirmed by identifying the hydrolysis product as maltose (Fig. 6), although minor short-chain oligosaccharides were also noted. Viksø-Nielsen et al. (1997) purified and characterized a β-amylase from potato leaves and demonstrated that it is a dominant amylolytic activity in this tissue. This corresponded well to our zymograms (Fig. 1), in which a band similar to the cold-induced tuber enzyme was evident in both leaf and stem tissues.

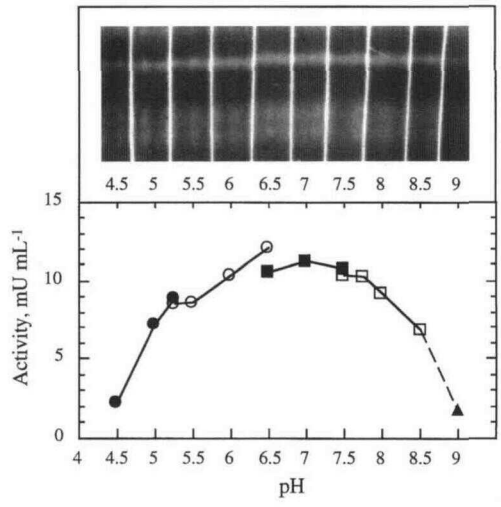

Figure 6. pH dependence of the activity of cold-induced β-amylase. The activity was determined by the assay measuring total amylolytic activity (degradation of soluble starch) using the early-eluting fraction of a cold-stored tuber preparation (fraction 12, Fig. 2B). Buffers used were: ●, acetate; ○, Mes; ■, Mops; □, Hepes; ▲, Tris. The top panel shows the corresponding effect of pH on the β-amylase activity in zymograms (same fraction as used for activity curve). The pH value of the incubation buffer is indicated below each lane. mU, Milliunits.

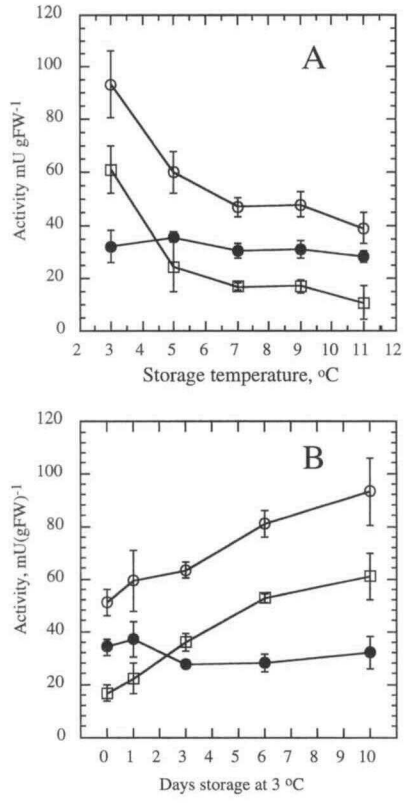

Figure 7. Activities of endo-amylase and β-amylase in stored potato tubers. A, Tubers stored for 10 d at 3, 5, 7, 9, and 11°C. B, Potato tubers stored for different times at 3°C. The tubers were transferred from 20°C at d 0. The curves show activity using BPNPG7 as substrate (●, endo-amylase activity) and using PNPG5 as substrate (○). The activity of β-amylase (□) was calculated as the activity with PNPG5 minus the activity with BPNPG7. Results are means ± SE of three individual enzyme extracts. mU, Milliunits; FW, fresh weight.

The zymograms separated at least two β-amylase bands that are cold-induced in tubers (Fig. 4). These may represent isozymes, different forms of the same enzyme, or the presence of active proteolytic products. Clarification of this must await further analysis. β-Amylase has been identified in a number of different plant species, and the molecular properties of the different β-amylases are varied. The reported masses of subunits range from 53 to 64 kD, and the holoenzyme may be a monomer (Monroe and Preiss, 1990), a homodimer (Fan, 1975; Viksø-Nielsen et al., 1997), or a homotetramer (Takeda et al., 1988).

Our finding that β-amylase is induced by cold treatment of the stored tubers is in agreement with data presented by Cottrell et al. (1993) and Cochrane et al. (1991b). These earlier studies relied on the specificity of PNPG5 as substrate for β-amylolytic activity. This convenient assay was developed by Mathewson and Seabourn (1983) and was optimized for cereal enzymes. However, our data indicate that it may be of limited value for analysis of crude homogenates of potato tuber. Cochrane et al. (1991a) concluded that, since β-amylase is the dominant amylase in the tubers, the assays can be used for crude homogenates. In contrast, our data show that endo-amylase activity dominates in warm-stored tubers and, even in cold-treated tubers, endo-amylase activity may contribute significantly to total amylolytic activity (Fig. 2). Endo-amylase interferes with the PNPG5 assay for β-amylase in tubers, because the endo-amylase in potato tubers apparently accepts short oligosaccharides such as PNPG5 as substrates (Fig. 2). To estimate the activity of β-amylase in a crude potato tuber homogenate, it is therefore necessary to correct for the interfering endo-amylase activity, as shown in Figure 7. The values achieved are approximate and rely on the observation that interfering endo-amylase has similar activity in the assays with BPNPG7 and PNPG5 (Fig. 2).

By combining chromatographic separation and zymograms, we could demonstrate that the cold-induced activity in tubers is indeed a β-amylase. When we compensate for endo-amylase activity (discussed in the previous paragraph), we observe an induction of β-amylase activity during cold treatment that was clearly indicated within 3 d at low temperatures (Fig. 7). This correlates well with the appearance of a new activity in zymograms (Fig. 8). Cottrell et al. (1993) and Cochrane et al. (1991b) also observed a considerable increase in α-amylase activity during cold storage of tubers. We did not observe a similar response in our plant material (Figs. 2 and 3), in which only β-amylase activity was induced. This may result from cultivar differences.

β-Amylase is also inducible in several other plant systems, measured either as activity or by monitoring expression of the gene for β-amylase. Feeding sugars or polygalacturonic acid to sweet potato cuttings (Otho et al., 1995, and refs. therein) or to leaves of *Arabidopsis thaliana* (Mita et al., 1995), defective starch metabolism in leaves of *A. thaliana* (Caspar et al., 1989), application of stress to barley leaves (Dreier et al., 1995), and light treatment of mustard cotyledons (Sharma and Schopfer, 1987) have all been demonstrated to induce β-amylase in these tissues. Thus, the β-amylase gene is already known as a regulatory gene, although it is unclear to what extent any analogies to these systems can be made for potato tubers.

The temperature dependence for induction of amylase activity (Fig. 7A) corresponds well with the temperature dependence of sugar accumulation. The cold induction of β-amylase activity also appears to be an early response to low temperatures. We observed an increase in β-amylase activity within 3 d at 3°C (Fig. 7), which precedes any significant sugar accumulation (Hill et al., 1996). However, other metabolic fluxes change quickly as a response to altered temperatures (Hill et al., 1996), and we cannot

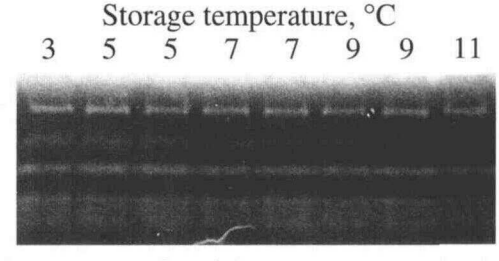

Figure 8. Zymograms of amylolytic activities (as in Fig. 1) in crude extracts of tubers stored for 3 d at 3, 5, 7, 9, and 11°C.

exclude the possibility that the induction of β-amylase could be a consequence of altered metabolism rather than a primary effect of temperature.

Our results provide correlative evidence for a role of the cold-induced β-amylase in cold sweetening. However, it is also possible that the induction of β-amylase represents a more general stress response that occurs in parallel with cold sweetening but is not directly involved in mobilization of the starch. Three arguments can be raised against a role for β-amylase in cold sweetening.

First, if the cold-induced β-amylase is involved in starch mobilization during cold sweetening, a possible consequence could be the accumulation of maltose, and this has not to our knowledge previously been documented in sweetening tubers. However, Hill et al. (1996) observed a significant transient increase in labeling of maltose and glucans in response to cold treatment of tubers supplied with [^{14}C]Glc. Krause (1994) has also achieved a major accumulation of maltose and short-chain glucans at 4°C in transgenic tubers with decreased expression of Suc-P synthase. In wild-type tubers cold treatment results in a specific stimulation of Suc-P synthase activity, and this most likely prevents the accumulation of amylolytic products. The absence of maltose accumulation should therefore not be taken as a strong indication against increased hydrolytic starch degradation.

The second argument against a role for β-amylase in cold sweetening is that the enzyme was present in nondormant tubers as well as in leaf and stem tissues that were not cold-treated (Fig. 1). However, this does not preclude its role in cold sweetening and starch mobilization. Even during the massive starch accumulation in nondormant tubers, a turnover of starch molecules may occur, although this remains to be demonstrated.

The third argument concerns the subcellular location of the cold-induced β-amylases. In Arabidopsis β-amylases appear to reside outside the plastids (Lin et al., 1988), and a specific localization of the protein in phloem tissue has been demonstrated (Wang et al., 1995). In wheat and pea leaves, β-amylases are found in the vacuoles (Ziegler and Beck, 1986). In view of this extraplastidial localization, it can be questioned whether β-amylase can act as a starch-degrading enzyme in these tissues. However, there is no general consensus about the localization in other tissues, and the neutral pH optimum of the potato tuber enzyme is evidence against a vacuolar function of this enzyme. Disintegration of the amyloplast membrane has been investigated as a possible mechanism for the onset of starch degradation in potato tubers (Sowokinos et al., 1985, 1987) and for the possible involvement of phosphorylases thought to reside in the cytosol at later stages of maturation (Brisson et al., 1989). A similar argument could apply to β-amylase. However, there is no indication of disruption of the membrane systems over the short storage periods required for the onset of cold sweetening (Isherwood, 1976).

Using a combination of column chromatography and zymograms we demonstrated in potato tubers the presence of a range of different enzymes with amylolytic or starch-modifying activity (Fig. 3). To evaluate the significance of all of these activities for starch metabolism, the purification and further characterization of substrate specificities and the occurrence of isoforms of the individual activities in potato tubers will be necessary. To finally establish whether the cold-induced β-amylase plays a role in cold-induced starch degradation, it will also be essential to localize the enzyme at the cellular and subcellular level.

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