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Pyrophosphorylases in *Solanum tuberosum*[1]

IV. Purification, Tissue Localization, and Physicochemical Properties of UDP-Glucose Pyrophosphorylase

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The enzyme UDP-glucose pyrophosphorylase (UGPase) from potato (*Solanum tuberosum* L. cv Norchip) tubers was purified 177-fold to near homogeneity and to a specific activity of 1099 international units/mg of protein. The molecular mass of the purified enzyme was 53 kD as determined by SDS-PAGE and gel filtration. Immunological and activity assays detected UGPase at similar levels in potato stems, stolons, and tubers. Leaves and roots contained lower levels of UGPase activity and protein. Lineweaver-Burk plots for substrates inorganic pyrophosphate and UDP-glucose were linear in the pyrophosphorolytic direction, yielding K_m values of 0.13 and 0.14 mM, respectively. However, Lineweaver-Burk plots for the substrates glucose-1-P and UTP were biphasic in nature when UGPase was assayed in the direction of UDP-glucose synthesis. At physiological substrate concentrations (i.e. from 0.05–0.20 mM), K_m values of 0.08 mM (glucose-1-P) and 0.12 mM (UTP) were obtained. When substrate concentrations increased above 0.20 mM, K_m values increased to 0.68 mM (glucose-1-P) and 0.53 mM (UTP). These kinetic patterns of potato UGPase suggest a "negative cooperative effect" (A. Conway, D.E. Koshland, Jr. [1968] Biochemistry 7: 4011–4022) with respect to the substrates glucose-1-P and UTP. The biphasic substrate saturation curves were similar to the kinetics of the dimeric form of UGPase purified from *Salmonella typhimurium* (T. Nakae [1971] J Biol Chem 246: 4404–4411). The in vivo significance of the enzyme's "negative cooperativity" in the direction of UDP-glucose synthesis and potato sweetening is discussed.

An actively growing potato (*Solanum tuberosum* L.) tuber utilizes Suc imported from leaves to supply the carbon and energy for cellular growth and starch synthesis. Imported Suc is most likely degraded by cytosolic Suc synthase to form Fru and UDP-Glc (refs. in Mares et al., 1985; ap Rees and Morrell, 1990). If the two hexose moieties of Suc are to be routed to starch formation, they must first enter the hexose-phosphate pool. Fru can be readily phosphorylated via fructokinase or hexokinase. However, if UDP-Glc is to enter the hexose-phosphate pool, one of the α-β phosphodiester bonds of UDP-Glc must be cleaved. One enzyme capable of this activity is UGPase (UTP:α-D-glucose-1-phosphate uridylyl transferase, EC 2.7.7.9), which catalyzes the reversible reaction:

$$\text{UDP-Glc} + \text{PPi} \rightleftharpoons \text{Glc-1-P} + \text{UTP}$$

The Glc-1-P formed may then cross the amyloplast membrane directly en route to support starch synthesis (refs. in Sowokinos, 1990). Glc-1-P formed can also enter respiratory pathways following conversion by PGM and PHI (Mares et al., 1985).

Conversely, a sprouting tuber is in a sucroneogenic mode, degrading starch and exporting Suc. The pathway of sucroneogenesis from starch to Suc also invokes UGPase to produce the cytosolic intermediate UDP-Glc, which is a substrate for the rate-limiting SPS reaction (Sung et al., 1988; Sowokinos, 1990). UDP-Glc is also the glucosyl donor for the synthesis of oligosaccharides and extraplastidal polysaccharides. UDP-Glc can also be derivatized to other nucleoside diphospho-sugars that are used in the synthesis of pectic substances, hemicelluloses, glycolipids, and other assorted glycosylated molecules (Goodwin and Mercer, 1983).

The metabolic importance of UGPase in the direction of UDP-Glc formation is particularly evident in mutant strains of *Dictyostelium*, which have reduced activity levels of this enzyme. The morphogenesis of the mutant strains is arrested in stages of growth that require stored glycogen to be mobilized for use in cellulose formation (Dimond et al., 1976). Similarly, UGPase activity may determine, in part, a potato clone's ability to convert stored starch into sugars. The activity of this enzyme has been shown to be correlated with the

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Abbreviations: n, Hill coefficient; PGM, phosphoglucomutase; PHI, phosphohexoseisomerase; SA, specific activity; SPS, sucrose phosphate synthase; TPBS, Tween-20 phosphate buffered saline; UGPase, UDP-glucose pyrophosphorylase; V_{max}, maximum reaction velocity.

amount of Glc that tubers of diverse potato genotypes accumulate in storage (Sowokinos, 1990).

We undertook the purification and characterization of UGPase as a prelude to cloning and genetic manipulation of its activity level in potato tubers. UGPase has been purified from bacteria, yeast, and slime mold as well as from a number of other mammalian and plant tissues including potato (refs. in Turnquist and Hansen, 1973; Hondo et al., 1983; Nakano et al., 1989; Vella and Copeland, 1990). We report an alternative chromatographic schedule that yields a purified UGPase with kinetic and structural properties differing from those previously reported for plant UGPases. Immunological and activity localization of this enzyme in various potato tissues are also presented.

MATERIALS AND METHODS

Chemicals

Phosphoglucomutase, Glc-6-P dehydrogenase, UDP-Glc dehydrogenase, EDTA, Tris, glycylglycine, NAD, NADP, ADP-Glc, UDP-Glc, 3-P-glycerate, hexose-phosphate substrates, Glc-1,6-diphosphate, and other enzyme assay components were obtained from Sigma. The chromatography materials phenyl-Sepharose CL-4B, DEAE-Sephacel, and CM-Sepharose were purchased from Pharmacia. Bio-Gel A-0.5M and bovine gamma-globulin were purchased from Bio-Rad Laboratories.[5]

Preparation of Acetone Powders

The potato variety Norchip (*Solanum tuberosum* L.) was used as the source of enzyme during the course of this study. Acetone powders were prepared from immature tubers (200 g average size) as previously described (Sowokinos, 1976). Two hundred grams of fresh tissue, selected at random from five healthy tubers, were thinly sliced and immediately frozen. Each sample was blended at slow speed for three 1-min intervals in a 4-L explosion-proof Waring blender containing 1.5 L of cold acetone (−20°C). The residue was washed five times with 150 mL of cold acetone and then dried overnight in a vacuum desiccator. The dried samples were stored in airtight glass jars at −20°C over desiccant.

Extraction of UGPase from Various Potato Tissues

Tissues (i.e. stolons, tubers, stems, leaves, and roots) were removed from immature field-grown plants (cv Norchip). Each sample was briefly washed, diced, and frozen in liquid N_2. All extraction steps were conducted at 4°C unless otherwise indicated. One-half gram of each tissue was homogenized in a mortar and pestle with 1 mL of buffer consisting of 100 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM GSH, and 0.1% Na_2HSO_3 (w/v). One percent PVP (w/v) was added to the leaf extraction buffer. After a 10-min grinding period, the suspensions were centrifuged at 27,000*g* for 15 min. Precipitates were washed with 0.7 mL of buffer and recentrifuged, and the two supernatants from each tissue type were combined. Each extract was dialyzed overnight against the buffer (40 volumes) without sodium bisulfite or PVP, with one change. The dialysate was centrifuged to remove inactive protein. Extracts were immediately measured for enzyme activity.

[5] Mention of company or trade name does not imply endorsement by the U.S. Department of Agriculture over others not named.

Measurement of Pyrophosphorylase Activity

Pyrophosphorolysis of UDP-Glc was assayed using the one-step method previously described (Sowokinos, 1976). Reaction mixtures contained in 1 mL, pH 8.5, 80 μmol of glycylglycine, 1 μmol of UDP-Glc, 5 μmol of $MgCl_2$, 1 unit each of PGM and Glc-6-P dehydrogenase, 20 μmol of Cys, 0.02 μmol of Glc-1,6-diP, 0.6 μmol of NADP, and 0.01 to 0.05 unit of diluted enzyme. The reactions were initiated by the addition of 2.5 μmol of PPi. The formation of NADPH (340 nm) was recorded continuously at 37°C with a Beckman model DU-40 spectrophotometer, until a loss of initial linear reaction rate occurred. One unit of activity in the pyrophosphorolysis direction is defined as that amount of enzyme that catalyzes the formation of 1 μmol of Glc-1-P/min. SA is defined as IU/mg of protein.

Synthesis of UDP-Glc was determined using a two-step assay system. In the first part, reaction mixtures, pH 8.5, contained 80 μmol of glycylglycine, 8 μmol of UTP, 10 μmol of $MgCl_2$, 300 μg of BSA, and 0.01 to 0.02 unit of the purified enzyme. After a 2-min incubation period at 37°C, the reactions were initiated by the addition of 10 μmol of Glc-1-P to give a total volume of 1 mL. Following a 20-min reaction period, the formation of UDP-Glc was terminated by placing all tubes into a boiling water bath for 2 min. The mixtures were centrifuged for 10 min at 2500*g*, and the supernatants were collected.

In the second part, the amount of UDP-Glc formed was quantified using a modification of the methods described by Franke and Sussman (1971). Each reaction mixture, pH 8.5, contained 130 μmol of glycylglycine, 6 μmol of NAD, 0.05 unit of UDP-Glc dehydrogenase, and 200 to 500 μL of supernatant from the first part to give a total volume of 1.0 mL. The formation of NADH was recorded at 37°C until a maximum change in optical density occurred at 340 nm. The absorbance difference before and after the addition of the supernatant from the first part was used to calculate the amount of UDP-Glc formed. One unit of activity in the synthesis direction is defined as that amount of enzyme necessary to catalyze the formation of 1 μmol of UDP-Glc/min.

Substrates and other assay components were saturating so that the rate of each reaction measured was linear with respect to enzyme concentration and time under the experimental conditions used.

Purification of UGPase

Extraction and $(NH_4)_2SO_4$ Precipitation

All steps were conducted at 4°C unless indicated otherwise. Forty grams of acetone powder were mixed with 400 mL of 1× extraction buffer (pH 7.5) without Na_2HSO_3. The slurry was stirred slowly for 40 min and centrifuged at 27,000*g* for 20 min. The pellet was washed once with 40 mL of buffer,

centrifuged, and the two supernatants were combined. After solid $(NH_4)_2SO_4$ addition (36.1%, w/v), the precipitate was discarded and $(NH_4)_2SO_4$ (20.1%, w/v) was added to the supernatant. The resulting precipitate (60–90% ammonium sulfate fraction) was collected after centrifugation and redissolved in 30 mL of buffer.

Column Chromatography

The protein solution was loaded (0.1 mL/min) onto a hydrophobic interaction column (phenyl-Sepharose CL-4B, 2.6 × 30 cm) previously equilibrated with 1.3 M ammonium sulfate. The protein was eluted (0.8 mL/min) with a reverse linear gradient ranging from 1.3 to 0 M ammonium sulfate in 10 mM Hepes (pH 7.0). Fractions containing the activity peak were pooled (70 mL) and concentrated to 2.4 mL using an Amicon ultrafiltration system with a YM 30 membrane. The concentrate was loaded (0.1 mL/min) onto a gel-filtration column (Bio-Gel A-0.5 M, 1.6 × 66 cm) previously equilibrated with the 1× extraction buffer without Na_2SO_3 but containing 100 mM NaCl. Fractions containing the peak activity (3 tubes/12 mL) were collected and dialyzed for 20 h against 40 volumes (480 mL) of 50 mM Tris-HCl buffer, pH 7.5, with one change at 10 h. The dialysate was loaded (0.2 mL/min) onto an anion-exchange column (DEAE-Sephacel, 1 × 16 cm) equilibrated with 50 mM Tris-HCl, pH 7.5. The protein was eluted in the same buffer with a linear gradient from 0 to 0.2 M NaCl. The activity peak was collected in a 12-mL volume and dialyzed for 6 h against 40 volumes (480 mL) of 10 mM sodium phosphate buffer, pH 6.0, with one change at 3 h. The dialysate was loaded (0.25 mL/min) onto a cation-exchange column (CM-Sepharose, 1 × 10 cm) equilibrated in 10 mM sodium phosphate buffer, pH 6.0. UGPase protein did not bind to the column and was collected in the first few fractions using the same buffer. The final 20-mL fraction was concentrated to 6.5 mL using Amicon ultrafiltration.

Protein Determination

Protein content of chromatographic isolates was quantitated according to the method of Bradford (1976). Protein content of fresh tissue extracts prepared for immunoblotting was determined according to the method of Rubin and Warren (1977). Bovine gamma-globulin was used as the protein standard.

Gel-Filtration Chromatography

A Bio-Gel column (1.6 × 66 cm) was used to determine the molecular mass of the purified UGPase from potato tuber. The column was equilibrated with the 1× extraction buffer without Na_2HSO_3 but containing 100 mM NaCl. Molecular mass standards run simultaneously with the potato protein were β-amylase (200 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

SDS-PAGE

SDS-PAGE was run with 0.75-mm thick slab gels containing 10% acrylamide according to the procedure of Laemmli (1970) as described previously (Sowokinos and Preiss, 1982). Molecular mass standards used were myosin (205 kD), β-galactosidase (116 kD), phosphorylase *b* (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). Proteins were identified using silver stain (Integrated Separation Systems, Hyde Park, MA).

Nondenaturing PAGE

A native polyacrylamide gel was run with 1.0-mm thick slab gels containing 7.5% acrylamide according to the procedure of Laemmli (1970). Proteins were identified using silver stain.

Antibody Production

UGPase isolated during the final CM-Sepharose purification step was further purified with preparative SDS-PAGE. Protein was electroeluted from the gel into dialysis tubing (mol wt cutoff 12,000–14,000), concentrated using a Centricon-30 microconcentrator, and mixed with complete Freund's adjuvant for the initial immunization (300 μg) of a New Zealand White rabbit. Two subsequent immunizations (75 μg, at 2-week intervals) were given using incomplete Freund's adjuvant. Final bleeding occurred after two additional weeks.

Immunoblotting

Protein was extracted from various tissues of glasshouse-grown potato plants (i.e. leaf, stem, stolon, tuber, and root) and from cultured potato cells (i.e. callus and suspension). Tissues (0.3–0.5 g) were homogenized in four volumes of SDS-PAGE buffer (v/w), sonicated, heated in boiling water for 3 min, centrifuged at 12,000*g*, and filtered through an Amicon disk membrane (0.45 μm). After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes according to the method of LeGendre and Matsudaira (1988). The membranes were blocked in 5% (v/v) chicken egg white in TPBS (0.05% [v/v] Tween-20, 10 mM Na_2HPO_4/NaH_2PO_4 [pH 7.5], and 0.9% [w/v] NaCl), washed in TPBS, blocked with 0.01% (w/v) biotin in TPBS, washed in TPBS, incubated in whole rabbit antisera (1:1000) in TPBS, incubated in horseradish peroxidase-labeled avidin-complexed biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA), washed in PBS, and incubated in a substrate solution (0.05% [w/v] 4-chloro-1-napthol, 17% [v/v] methanol, 0.025% [v/v] H_2O_2 in PBS) and rinsed with water.

RESULTS AND DISCUSSION

Purification of UGPase

Table I summarizes the purification of UGPase from potato tubers. The yield of activity was low (7%), but the purified enzyme demonstrated a high SA of 1099 units/mg of protein.

Figure 1 shows the results of the SDS-PAGE analysis of each of the purification steps. It is noted that the final purified enzyme preparation was near homogeneity (lane 6 contains 1 μg of the final CM-Sepharose isolate). Two lightly stained, faster-migrating bands appear on the SDS-PAGE gel. Based on the sensitivity of the silver stain used (0.3–0.5 ng), enzyme

Table I. *Purification of UGPase from S. tuberosum L. (cv Norchip)*

Fraction	Total Activity	Total Protein	Specific Activity	Yield	Purification
	units[a]	*mg*	*units mg*$^{-1}$ *protein*	%	*fold*
Acetone powder	17,208	2,782	6.2	100	
Ammonium sulfate (60–90%)	11,150	437	25.5	65	4.1
Phenyl-Sepharose CL-4B	10,028	61	164	58	26.5
Bio-Gel A-0.5 M	7,374	29	254	43	41.0
DEAE-Sephacel	1,382	1.58	875	8	141
CM-Sepharose	1,220	1.11	1,099	7	177

[a] Unit, The amount of enzyme necessary to catalyze the formation of 1 μmol of Glc-1-P per min.

purity appears to be greater than 99.8%. It is also possible that the faint, faster-migrating bands are artifacts from partial breakdown of the basic subunit that can occur during electrophoresis. This is suggested by the fact that the sum of the mass of the two lower bands equals the molecular mass of the upper UGPase subunit.

Properties of UGPase

Subunit Molecular Mass

The molecular mass of the potato UGPase subunit was determined to be 53 kD by denaturing SDS-PAGE (Fig. 1) and gel-filtration chromatography (data not shown). Polyclonal antisera produced against the purified enzyme revealed the presence of only a single band on the immunoblot against total tuber protein separated by SDS-PAGE (Fig. 2, lane 4). This band aligned with the 53-kD subunit of the purified enzyme (lane 3). Preimmune serum showed no reaction with either purified or total protein samples (lanes 1 and 2, respectively). The antisera inhibited 98% of the activity of purified preparations of UGPase (data not shown). Collectively, these results indicate that potato UGPase has a subunit molecular mass of about 53 kD. Preliminary evidence with nondenaturing PAGE of the purified enzyme suggested that potato UGPase can have a dimeric structure in vitro (data not shown).

The potato tuber monomeric subunit mass agrees with the reported values of 53 and 55 kD for the enzyme from *Typha latifolia* pollen (Hondo et al., 1983) and slime mold (Franke and Sussman, 1971), respectively. Previously, Nakano et al. (1989) indicated that potato UGPase probably exists as a monomeric polypeptide with an approximate molecular mass of 50 kD. Based on the coding sequence of a cDNA for UGPase isolated from a cDNA library of immature potato tuber, Katsube et al. (1990) predicted a subunit mass of 51,783. Although a monomeric nature for potato UGPase is certainly possible, a multimeric nature would be consistent with the multimeric forms reported for UGPase from slime mold (Franke and Sussman, 1971) and animal tissues (Turnquist and Hansen, 1973). The enzyme from slime mold, which shows extensive amino acid homology to UGPase

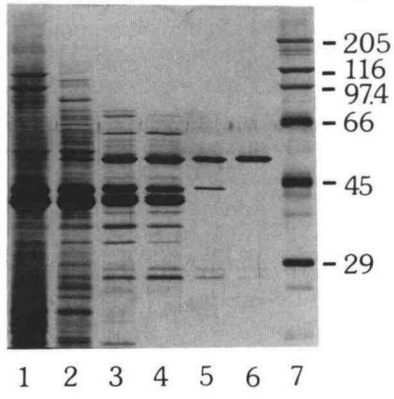

Figure 1. SDS-PAGE of purified potato tuber UGPase stained with silver. Lanes contain the following fractions: 1, acetone powder (25 μg); 2, ammonium sulfate precipitate (15 μg); 3, phenyl-Sepharose (10 μg); 4, Bio-Gel (10 μg); 5, DEAE-Sephacel (1 μg); 6, CM-Sepharose (1 μg); and 7, molecular mass standards (8 μg each).

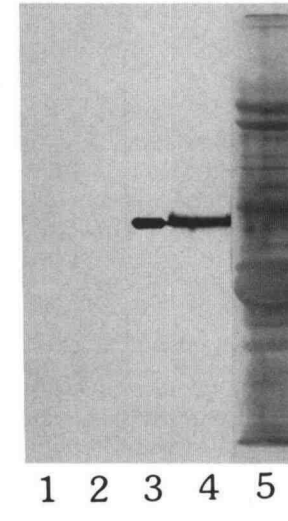

Figure 2. Specificity of anti-UGPase serum as demonstrated by reaction with a western blot developed from SDS-PAGE of the final CM-Sepharose and total tuber protein fractions. Lanes 1 and 3 contain 0.2 μg of purified UGPase. Lanes 2 and 4 contain 100 μg of total tuber protein. Lanes 1 and 2 were probed with preimmune serum (1:1000) and lanes 3 and 4 were probed with whole rabbit anti-UGPase (1:1000). Lane 5 contains 100 μg of total tuber protein stained with Coomassie blue R-250.

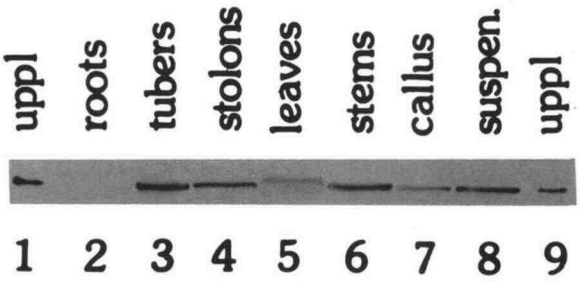

Figure 3. Potato tissue distribution of UGPase (upp1). Whole rabbit anti-UGPase (1:1000) was used to probe a western blot containing 200 μg of each of the tissues as labeled. Lanes 1 and 9 contain 50 and 10 ng of the purified enzyme, respectively.

from potato (Katsube et al., 1990), demonstrates a multimeric nature. Both monomeric and dimeric forms of the enzyme, which are capable of interconversion, have been isolated from *Salmonella typhimurium* (Nakae, 1971). Preliminary evidence, which suggested that potato UGPase can exist as a dimer in vitro, introduces the possibility that multimeric forms of the basic 53-kD subunit might exist in vivo during potato tuber growth and/or storage.

Localization

Immunoblots of protein from various tissues revealed the presence of UGPase in tubers, stolons, stems, callus, and suspension-cell cultures (Fig. 3). Less protein was present in leaves and only a trace of antigen was detected in roots. No larger or smaller immunoreactive polypeptides were present in the blots of any of the potato extracts. The physiological age and environment of the plants used as a source of tissue for Figure 3 may have contributed to the trace levels of UGPase in root tissue. Tissues were selected from greenhouse-grown plants (5 months of age) that were being used for the production of true seed. Characteristically, these plants develop extensive foliage but their tuber and root growth are minimal. To verify the immunoblot results, tissues were selected from actively growing potato plants and subjected to enzymic analysis.

Results in Table II indicate that UGPase is indeed present in potato roots but at a level one-sixth of that found in the tuber on a unit/g fresh weight basis. The enzyme level appeared the same in stolons, stems, and tubers based on SA determinations (i.e. range 3.25–3.88 IU/mg of protein), but the SA in roots and leaves was markedly lower. Recovery of enzyme units following addition of purified UGPase to root tissue prior to homogenization indicated unequivocally that the enzyme was not destroyed during the extraction procedure.

Kinetics and Effectors of UGPase in the Pyrophosphorolysis Direction

Enzyme, UDP-Glc, PPi, and Mg^{2+} were absolutely necessary for the reaction to occur in the pyrophosphorylase direction. ADP-Glc did not serve as a substrate for the reaction. Maximum reaction velocity (V_{max}) occurred in the presence of 5 mM Mg^{2+}, whereas 50% of the V_{max} was noted with 0.8 mM Mg^{2+}. The pH curve for UGPase was fairly broad between pH 7.5 and 9.0. A pH optimum of 8.5 was obtained when glycylglycine, Bicine, Tris, Hepes, Pipes, and sodium acetate buffers (80 mM) were used. UGPase was inhibited 50% with 6 mM Pi, 40% with 2 μM Fru-2,6-bisP, 27% with 4 mM Fru-6-P, and 20% with 0.4 mM 3-P-glycerate. A further increase in the concentration of these metabolites and effectors did not enhance their percent inhibition.

Double reciprocal plots were linear over a range of substrate concentrations from 0.05 to 2.0 mM (data not shown). K_m values in the pyrophosphorylase direction were 0.13 and 0.14 mM for PPi and UDP-Glc, respectively (Table III). A maximum velocity of between 800 and 900 IU/mg of protein was calculated. Given these low K_m values, the high SA, and the K_{eq} of the UDPG-PPase reaction in plants, which lies in the direction of Glc-1-P synthesis (Turnquist and Hansen, 1973), the formation of Glc-1-P from UDP-Glc must occur rapidly in potato tubers.

Kinetics and Effectors of UGPase in the UDP-Glc Synthesis Direction.

Enzyme, UTP, Glc-1-P, and Mg^{2+} were absolutely necessary for the synthesis of UDP-Glc to occur. The nucleotides ATP, CTP, and GTP could not substitute for UTP in the reaction. Maximum reaction velocity occurred in the presence of 10 mM Mg^{2+}, whereas 50% of the reaction was observed with 5.4 mM Mg^{2+}. Optimum reaction rate was obtained at pH 8.5 using the buffer glycylglycine (80 mM). High concentrations of Fru-2,6-bisP or 3-P-glycerate did not activate or inhibit the synthesis of UDP-Glc. These compounds, therefore, were selective by partially inhibiting the reaction only

Table II. *UGPase activity in various tissues selected from immature potatoes (cv Norchip)*

Tissue	Units[a]/g	Protein/g	Specific Activity
		mg	*units mg^{-1} protein*
Tubers	34.77 ± 1.29[b]	9.00 ± 0.25	3.86 ± 0.14
Roots	5.84 ± 0.09	4.92 ± 0.27	1.18 ± 0.02
Leaves	6.48 ± 0.08	31.64 ± 0.44	0.21 ± 0.01
Stolons	16.20 ± 0.51	4.94 ± 0.11	3.28 ± 0.10
Stems	11.58 ± 0.39	2.87 ± 0.12	4.04 ± 0.14

[a] Unit, The amount of enzyme necessary to catalyze the formation of 1 μmol of Glc-1-P per min. [b] Mean ± SE for five experiments.

Table III. *Kinetic constants of purified UGPase from S. tuberosum L. (cv Norchip)*

Reaction	Substrate	Concentration Range	K_m	K_m[a]	V_{max}
		mM	mM	mM	units[b] mg^{-1} protein
Pyrophosphorolysis	PPi	0.05–2.0	0.13	0.11	827
	UDP-Glc	0.05–2.0	0.14	0.12	890
Synthesis	UTP	0.05–5.0	0.18	0.17	94
		0.05–0.2	0.12		72
		>0.25	0.53		131
	Glc-1-P	0.05–5.0	0.17	0.18	69
		0.05–0.2	0.08		44
		>0.25	0.68		138

[a] Nakano et al., 1989. [b] Unit, The amount of enzyme necessary to catalyze the formation of 1 μmol of Glc-1-P (pyrophosphorolysis) or 1 μmol of UDP-Glc (synthesis) per min.

in the pyrophosphorolysis direction. Inorganic phosphate maximally inhibited the synthesis reaction only 30% at a high concentration of 12 mM.

Although the K_m values for the pyrophosphorolytic substrates (i.e. PPi and UDP-Glc) were similar to the values reported for the potato UDPG-PPase by Nakano et al. (1989) (see Table III), kinetics in the synthesis direction were distinctly different. It must be initially emphasized that the cultivars used to purify the enzyme were different in both cases. Figure 4 shows the Lineweaver-Burk plot obtained when the concentration of Glc-1-P was varied from 0.05 to 5.0 mM. The equation for the line, $y = 0.2521 + 0.0437x$ ($r = 0.932$, $F_{1,32} = 208$) appeared to adequately explain all the data points represented ($P < 0.0001$). The K_m for Glc-1-P (0.17 mM) was similar to the value reported by Nakano et al. (1989) (Table III).

To test the validity of the regression equation, standardized residuals were analyzed according to the procedure of Sokal and Rohlf (1981). A residual plot of the errors of the observed velocities from those predicted by the regression equation suggested that errors from the mean velocity were dependent on the concentration of Glc-1-P (data not shown). The curved residual distribution violated the assumption of random errors in a regression analysis. This could result if the relationship between the rate of UGPase activity and Glc-1-P concentration was actually nonlinear or biphasic in nature. Regression analysis indicated a change in kinetic behavior at a Glc-1-P concentration near 0.20 mM. To investigate this further, two separate regressions were calculated based on either low (0.05–0.20 mM) or high (>0.20 mM) concentrations of Glc-1-P (Fig. 4). As Glc-1-P concentration increased above 0.25 mM, the K_m of UGPase for Glc-1-P increased 8.5-fold from 0.08 to 0.68 mM (Table III). Corresponding n values increased from 0.40 to 0.92 as the concentration of Glc-1-P increased (Fig. 5). There appeared to be an increased interaction between Glc-1-P binding sites as the concentration of the substrate increased.

A similar pattern of kinetics and distribution of residuals was observed when UTP was used as a substrate (data not shown). Calculations from two separate regression equations revealed that the K_m for UTP increased from 0.12 to 0.53 mM as the concentration of UTP increased above 0.25 mM.

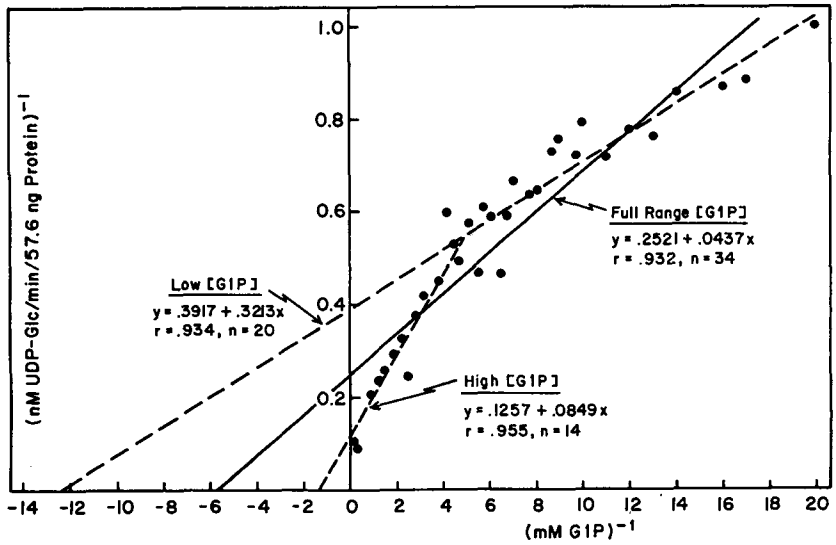

Figure 4. A Lineweaver-Burk plot of purified UGPase reaction in the direction of UDP-Glc synthesis. The reciprocal of each reaction velocity was plotted against the reciprocal of Glc-1-P (concentration range 0.05–5.0 mM).

Deviation of the synthesis reaction from Michaelis-Menten kinetics did not appear to be an artifact of the assay system used. Each experiment was conducted several times with basically the same result. Initially, a one-step continuous spectrophotometric assay was used to determine the rate of UDP-Glc synthesis. Because another enzyme was present in this coupled system (i.e. UDP-Glc dehydrogenase), it was suspected that some other aspect of the reaction mixture might be responsible for the nonlinear kinetics. To eliminate this possibility, the assay was adapted to a two-step process. Step one involved simply the formation of UDP-Glc with purified potato UGPase. All assay components were saturating so that the velocity of the reaction was limited solely by substrate concentration rather than by incubation time or enzyme concentration. Step two involved the quantitation of the UDP-Glu formed via the UDP-Glc dehydrogenase system. This latter step was verified with UDP-Glc standards and was found to be linear from 0.01 to 2.0 μmol of UDP-Glc per tube. Aliquots of the step-one extracts were adjusted so that the level of UDP-Glc introduced into step two fell within this linear concentration range.

Data obtained at this time, however, do not explain the molecular basis for these kinetics. One possibility is that increased substrate concentration causes a change in the three-dimensional structure of the basic 53-kD polypeptide subunit, which in turn alters its catalytic properties. Fitzgerald et al. (1969) showed a change in the catalytic properties of a single polypeptide species of UGPase from rat mammary glands. This conversion was a function of pH, temperature, and enzyme concentration. They indicated that although the K_m for UTP and the molecular mass stayed the same, its V_{max} increased markedly. The V_{max} of the potato enzyme also increased 2- to 3-fold as the concentration of either UTP or Glc-1-P increased, respectively (Table III). Additionally, a higher multimeric form of the 53-kD polypeptide could be formed, which exhibits different catalytic properties.

In *S. typhimurium*, Nakae and Nikaido (1971) observed multiple forms of UGPase that demonstrated real differences in their catalytic properties. They demonstrated an enzyme form IIIb (a dimer, molecular mass 80 kD) that gave nonlinear Lineweaver-Burk plots of $1/v$ versus $1/[S]$ when the concentrations of UTP and Glc-1-P were varied in the assay mixture. They attributed this deviation from Michaelis-Menten kinetics to the conversion of one of the monomers (IIIa) to a dimer. They explained the increased K_m values observed as a "negative cooperative effect" as described by Conway and Koshland (1968), where the binding of one substrate molecule makes the binding of another molecule of the same substrate at a second binding site more difficult. This dimer also yielded strictly linear kinetics when the reaction was measured in the pyrophosphorolysis direction. These kinetic characteristics of the bacterial dimer were similar to the kinetic pattern described with UGPase purified from cv Norchip potatoes. The conversion of the bacterial monomer to a dimer was shown to be temperature sensitive, stimulated by the presence of NaCl, and irreversible in nature (Nakae, 1971). Hill coefficients less than 1.0 (Fig. 5) supported a negative cooperative effect with the potato enzyme with respect to the binding of Glc-1-P. Whether multimeric forms of UGPase occur in vivo in the intact potato tubers remains to be determined.

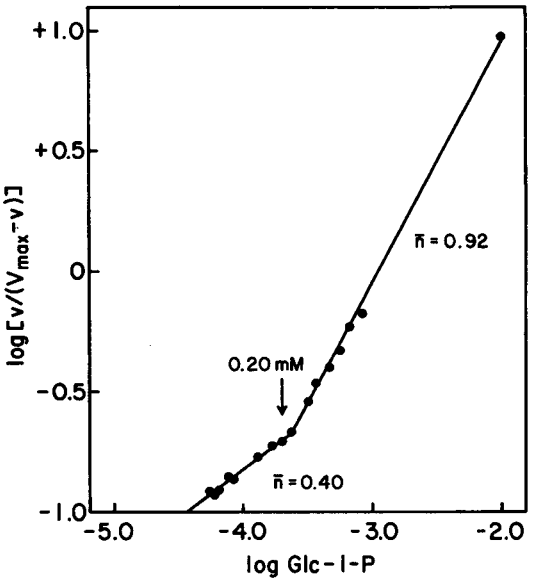

Figure 5. A Hill equation plot of the data in Figure 4.

Role of UGPase in Potato Sweetening

Due to the differences in the substrate saturation kinetics between the synthetic and pyrophosphorolytic reactions, it is tempting to suggest a regulatory function for UGPase during tuber sweetening. Studies of potato carbohydrate metabolism have suggested that cold sensitivity of glycolytic enzymes diverts products of starch breakdown (i.e. Glc-1-P) toward Suc formation (ap Rees et al., 1981). Altered temperature coefficients of key glycolytic enzymes in the cold (3–5°C), however, do not explain why certain potato clones sweeten at warmer temperatures (10–15°C) and others do not. Potato sweetening is a complex metabolic process and it is obvious that key regulatory steps also exist directly between the hexose-P's and free sugars. It was previously found that levels of UGPase activity were correlated ($r = 0.92$) with the amount of Glc (i.e. product of Suc hydrolysis) that was accumulated by genetically diverse potato clones in the cold (Sowokinos, 1990). SPS requires high concentrations of UDP-Glc (K_m = 2.5 mM) to achieve 50% of its maximum catalytic activity in potatoes (Murata, 1972). This level is approximately 10-fold higher than the concentration of UDP-Glc in mature tubers (Morrell and ap Rees, 1986). During periods when hexose-Ps are elevated in potato tubers, a coarse metabolic control via UGPase could supply varying levels of UDP-Glc and directly affect the velocity of the rate-limiting SPS reaction. Sicher (1986) also indicated that availability of UDP-Glc may be limiting the SPS reaction in leaf tissues.

There may also be a fine metabolic control where change in substrate concentration alters the catalytic properties of UGPase (Table III and Fig. 4). In stored tubers, cytosolic Glc-1-P may be directed toward respiration via PGM or toward sucroneogenesis via UGPase (Fig. 6). When the concentration of Glc-1-P is low (0.20 mM or less), PGM with a K_m for Glc-1-P of 0.06 mM (Takamiya and Fukui, 1978) preferentially cycles carbon toward respiratory pathways and energy pro-

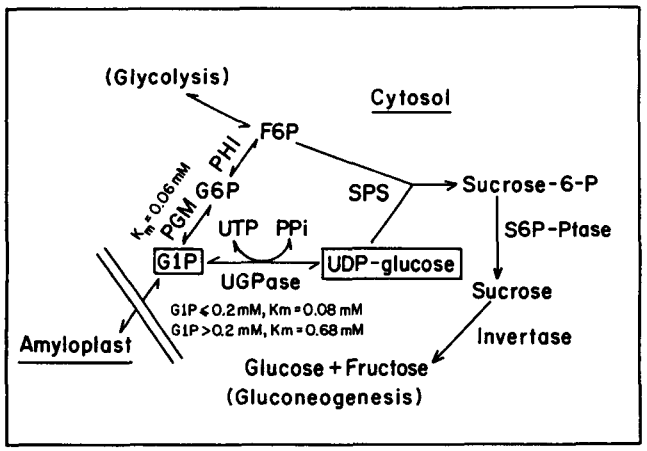

Figure 6. Competing enzyme systems for cytosolic Glc-1-P (G1P) directing carbons toward glycolytic or gluconeogenic pathways. Enzymes represented include UGPase, PGM, PHI, SPS, Suc-6-P phosphatase (S6P-Ptase), and invertase.

duction. Under situations when Glc-1-P is increased, as it is during low temperature stress (Pollock and ap Rees, 1975; Isherwood, 1976), the negative cooperativity would increase the K_m of potato UGPase for Glc-1-P 9-fold from 0.08 to 0.68 mM. Through this fine metabolic control, PGM would then demonstrate a 10-fold greater affinity for Glc-1-P than UGPase. Because glycolysis is also restricted, this could help explain the increase in Glc-1-P, Glc-6-P, and Fru-6-P observed in the cold-stored potatoes (Pollock and ap Rees, 1975). Because the efficiency of glycolysis is decreased at cold temperatures, decreasing the relative activity of potato UGPase may counter this effect by limiting the accumulation of free sugars.

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