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# In Vitro Biosynthesis of Phosphorylated Starch in Intact Potato Amyloplasts[1]

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**Intact amyloplasts from potato (*Solanum tuberosum* L.) were used to study starch biosynthesis and phosphorylation. Assessed by the degree of intactness and by the level of cytosolic and vacuolar contamination, the best preparations were selected by searching for amyloplasts containing small starch grains. The isolated, small amyloplasts were 80% intact and were free from cytosolic and vacuolar contamination. Biosynthetic studies of the amyloplasts showed that [1-$^{14}$C]glucose-6-phosphate (Glc-6-P) was an efficient precursor for starch synthesis in a manner highly dependent on amyloplast integrity. Starch biosynthesis from [1-$^{14}$C]Glc-1-P in small, intact amyloplasts was 5-fold lower and largely independent of amyloplast intactness. When [$^{33}$P]Glc-6-P was administered to the amyloplasts, radiophosphorylated starch was produced. Isoamylase treatment of the starch followed by high-performance anion-exchange chromatography with pulsed amperometric detection revealed the separated phosphorylated $\alpha$-glucans. Acid hydrolysis of the phosphorylated $\alpha$-glucans and high-performance anion-exchange chromatography analyses showed that the incorporated phosphate was preferentially positioned at C-6 of the Glc moiety. The incorporation of radiolabel from Glc-1-P into starch in preparations of amyloplasts containing large grains was independent of intactness and most likely catalyzed by starch phosphorylase bound to naked starch grains.**

Starches from tuberous plants such as potato (*Solanum tuberosum* L.) contain a small fraction of covalently bound phosphate in their amylopectin (Jane et al., 1996). Approximately 60% to 70% of the phosphate groups are linked to C-6 of the Glc residues, 30% to 40% to C-3, and a small fraction (1%) may be linked to C-2 (Hizukuri et al., 1970). Bay-Smidt et al. (1994) detected variations in the level of starch phosphorylation among different potato varieties, and Abel et al. (1996) and Lorbert and Kossmann (1997) observed similar variations among transgenic potato lines with altered levels of the enzymes involved in starch biosynthesis. However, the mechanism responsible for potato starch phosphorylation remains elusive. Nielsen et al. (1994) used a potato tuber disc system to show that starch phosphorylation proceeds concomitantly with the de novo biosynthesis of starch. Starch is synthesized and stored in amyloplasts; the availability of isolated intact amyloplasts thus constitutes an important tool for studies of the phosphorylation process.

Intact amyloplasts have been purified from storage organs of a number of important crops and used to study the regulation of the carbon flux into starch (Echeverria et al., 1988; Entwistle et al., 1988; Mohabir and John, 1988; Smith et al., 1990; Tetlow et al., 1993; Kosegarten and Mengel, 1994; Naeem et al., 1997). Biosynthetic studies using different crops (pea, cauliflower, maize, and wheat) showed that amyloplasts import carbon from the cytosol in the form of hexose-P (Tyson and ap Rees, 1988; Hill and Smith, 1991; Neuhaus et al., 1993). This conclusion was strengthened by determining the degree of labeling randomization in starch that was isolated after incubation with Glc isotopically labeled at the C-1 or C-6 position (Keeling et al., 1988; Hatzfeld and Stitt, 1990; Viola et al., 1991). Entwistle and ap Rees (1990) showed that potato tubers lack plastidic Fru-1,6-bisphosphatase activity; Kossmann et al. (1992) cloned the gene encoding the plastidic Fru-1,6-bisphosphatase from leaves and determined that it was not expressed in potato tubers. Therefore, hexose-P, not triose-P, must be imported and used directly for starch synthesis in potato tuber amyloplasts. Two reports presented evidence that hexose-P is imported into the amyloplasts of potato tubers in the form of Glc-1-P (Kosegarten and Mengel, 1994; Naeem et al., 1997). A third report demonstrated that Glc-6-P is the preferred transported metabolite in proteoliposomes derived from potato tuber amyloplasts (Schott et al., 1995).

We have examined the ability of different potato tuber amyloplast preparations to import hexose-P and to synthesize phosphorylated starch. We now document that intact amyloplasts containing small starch grains efficiently import Glc-6-P and are able to perform de novo synthesis of phosphorylated starch.

## MATERIALS AND METHODS

### Plant Material and Chemicals

Potato (*Solanum tuberosum* L. cv Dianella) plants were grown in a greenhouse as described by Nielsen et al. (1994), and tubers of approximately 25 g fresh weight were selected for the experiments. Unless otherwise stated, we

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Abbreviations: ADH, alcohol dehydrogenase; AGPase, adenosine-5′ diphosphoglucose pyrophosphorylase; HPAEC-PAD, high-performance anionic-exchange chromatography with pulsed amperometric detection; 3-PGA, 3-phosphoglyceric acid.

obtained all chemicals from Sigma. The [1-$^{14}$C]Glc-1-P and [1-$^{14}$C]Glc-6-P came from New England Nuclear and [$\gamma$-$^{33}$P]ATP from Amersham. [$^{33}$P]Glc-6-P was synthesized enzymatically from [$\gamma$-$^{33}$P]ATP, as described by Nielsen et al. (1995).

### Preparation of Homogenate and Isolation of Intact Amyloplasts

All steps were performed on ice using potato tubers harvested immediately before the start of the experiment. We obtained tuber tissue homogenates by processing 0.5 g of tuber tissue in l mL of isolation medium composed of 20 mM Hepes/KOH (pH 7.2), 500 mM sorbitol, 1 mM $MgCl_2$, 5 mM KCl, 1 mM $MnCl_2$, 1 mM EDTA, 10% (v/v) ethylene glycol, 1% (w/v) PVP, 0.1% (w/v) BSA, and 5 mM DTT using a glass homogenizer (Duall, Vineland, NJ). Only those tubers containing an AGPase activity above 600 nmol $min^{-1}$ $g^{-1}$ fresh weight were used for the experiments.

Amyloplasts were isolated from potato tuber pieces (approximately 2 g), which were individually chopped using a razor blade while positioned on a stainless steel mesh (300 $\mu$m) mounted in a small glass beaker, and submersed in the isolation medium (45 mL). The material remaining on the mesh after chopping was carefully removed before a new piece of tissue was processed in the same medium. Finally, the mesh was removed and the small amyloplasts were collected as the upper 12 mL of the suspension in the beaker. After removal of larger starch grains by a gentle centrifugation step (30*g*, 5 min, 4°C), an aliquot (500 $\mu$L) of the supernatant (Table I, crude extract) was used for enzyme assays. Two aliquots (5 mL) of the crude extract were applied to 5 mL of the isolation buffer with 2% (w/v) Nycodenz (Sigma) layered on a 1% (w/v) Bacto agar pad (Difco Laboratories, Detroit, MI) and subjected to centrifugation (30*g*, 25 min, 4°C). The amyloplasts on the agarose pad were gently resuspended in 5 mL of isolation medium, layered on 5 mL of 2% (w/v) Nycodenz, and recentrifuged as before. The recovered amyloplasts (Table I, small amyloplasts) were resuspended in a 2.1-mL (low amyloplast concentration) or a 0.25-mL (high amyloplast concentration) buffer and held for additional experiments. Grain size distribution of the small amyloplasts was determined by transmission electron microscopy. Large amyloplasts were obtained using the same procedure except that the bottom part of the suspension was used, and the initial centrifugation step was omitted.

### Enzyme Assays

Enzyme assays (total volume of 1 mL) were carried out at 20°C using a DW-2000 UV-visible spectrophotometer (SLM-Aminco product line, Spectronic Instruments, Rochester, NY) operated in the dual-wavelength mode (340/400 nm) unless otherwise indicated. Except for the latency experiments, we subjected the samples to ultrasonication (3 × 5 s) to ensure organelle disruption and removed the starch grains from the sample by centrifugation (10,000*g*, 5 min). All of the assays were started by addition of the substrates.

We monitored the marker enzyme activities according to the following previously reported procedures. Amyloplasts: AGPase (EC 2.7.7.27) was determined as described by Sowokinos (1976), except for the addition of 500 mM sorbitol to the assay mixture, alkaline inorganic pyrophosphatase (EC 3.6.1.1) was quantified spectrometrically (600 nm) as described by Gross and ap Rees (1986); cytosol: ADH (EC 1.1.1.1) and pyrophosphate:Fru-6-P phosphotransferase (EC 2.7.1.90) were determined as described by MacDonald and ap Rees (1983) and Nielsen et al. (1991), respectively; vacuole: $\alpha$-mannosidase (EC 3.2.1.24) was quantified spectrometrically (405 nm) as described by Stitt et al. (1989); mitochondria: Cyt *c* oxidase (EC 1.9.3.1) was quantified spectrometrically (550 nm) in the presence of 0.025% Triton X-100 (Rasmussen and Møller, 1990); and other enzymes: ATPase activity was quantified as the liberation of radiolabeled orthophosphate from [$\gamma$-$^{33}$P]ATP. The reaction mixture (total volume of 24 $\mu$L) contained amyloplasts (8 $\mu$L of a high concentration of amyloplasts), 66.6 kBq [$\gamma$-$^{33}$P]ATP, 1 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM Mops/KOH, pH 7.3. The reaction was terminated by applying aliquots (1 $\mu$L) of the reaction mixture onto a TLC plate (silica gel 60, Merck, Darmstadt, Germany). The products formed were separated by developing the TLC plate for 45 min in a solvent containing 35 mL of methanol, 15 mL of water, and 0.5 g of NaCl. The distribution of radiolabel was monitored by autoradiography. The regions corresponding to the positions of ATP and Pi were excised, resuspended in 1 mL of water mixed with 10 mL of Ecoscint A (National Diagnostics, Manville, NJ), and the radioactivity was quantified by liquid-scintillation counting (model 1215 RackBeta, Pharmacia LKB). The reaction was linear for at least 20 min.

### Amyloplast Intactness

The percentage of amyloplast intactness was calculated as 100 − ([activity in intact amyloplasts × 100]/[activity in ruptured amyloplasts]) using AGPase as the marker enzyme. The intact amyloplasts were ruptured by adding 0.1% (v/v) Triton X-100, shaking vigorously for 10 s, and centrifuging (10,000*g*, 2 min, 20°C) before determining the activity of the AGPase released by the rupturing. We confirmed the intactness of the amyloplasts in each isolation experiment and verified that Triton X-100 did not alter the activity of the enzyme.

### Assessment of Conversion between Glc-6-P and Glc-1-P

To measure the degree of phosphoglucomutase-mediated conversion between Glc-6-P and Glc-1-P, we determined the content of each compound at the end of the incubation period. Glc-6-P was determined as described by Michal (1988). $NAD^+$ was added to a final concentration of 0.4 mM followed by 5 units of Glc-6-P dehydrogenase from *Leuconostec mesenteroides*. Glc-1-P was determined as Glc-6-P after the addition of 2 units of phosphoglucomutase. In some experiments, known amounts of Glc-6-P and Glc-1-P were included as internal standards at the end of the incubation period. Endogenous phosphoglucomutase activity

derived from the amyloplast preparation was insignificant and consequently did not disturb the assay.

### Starch Synthesis Using [$^{14}$C]Glc-6-P or [$^{14}$C]Glc-1-P as a Precursor

Intact amyloplasts (300 $\mu$L) were gently transferred to 500-$\mu$L microcentrifuge tubes placed on ice. Components were added to the following final concentrations, as specified in "Results" (total volume of 325 $\mu$L): 2 mM Glc-1-P, 2 mM Glc-6-P, 0.1% (v/v) Triton X-100, 4 mM Mg-ATP, and 4 mM 3PGA, together with 6.3 kBq of either [1-$^{14}$C]Glc-6-P or [1-$^{14}$C]Glc-1-P. The reaction mixtures were incubated (1 or 2 h, 20°C) with slow rotation around the horizontal axis to prevent sedimentation, frozen in liquid nitrogen, and stored at −80°C. The starch grains were isolated, washed, gelatinized, and treated with $\alpha$-amylase, as described by Nielsen et al. (1994) before the radioactivity was determined by liquid-scintillation counting. For each experiment we included zero-time controls; transferring the sample to liquid nitrogen immediately terminated the amyloplast incubation.

### Starch Synthesis Using [$^{33}$P]Glc-6-P as a Precursor

Amyloplast preparations (approximately 100 mg of starch) from 8 to 10 tubers were combined, incubated as described above in an isolation buffer combined with 4 mM Mg-ATP, 4 mM 3PGA, and 9 MBq [$^{33}$P]Glc-6-P for a total volume of 2 mL. After a 1.5-h incubation, the transfer of the sample to liquid nitrogen terminated the reaction. After thawing, the starch was pelleted and washed extensively in water, 96% EtOH, 1 M NaCl, and 1 M KPi buffer, pH 7.5, until no further radioactivity could be detected.

### Isolation of Phosphorylated Linear Oligosaccharides

$^{33}$P-radiolabeled potato starch (approximately 50 mg) was gelatinized (2 min, 100°C) in 10 mL of 10 mM sodium acetate (pH 4.0), debranched with 1.2 units of iso-amylase (Megazyme, Sydney, Australia) (2 h, 40°C), and then the reaction was terminated (5 min, 100°C). The preparation was divided into two equally sized portions. The first portion was adjusted to pH 7.4 by the addition of 500 $\mu$L of 1 M Mops (pH 7.4) and treated with 2 units of $\beta$-amylase (Megazyme) (1 h, 40°C) after which the reaction was terminated by heat (5 min, 100°C). After a brief centrifugation, the sample was applied to a DEAE-Sepharose column (12 × 62 mm, Pharmacia) previously equilibrated with Hepes, pH 8.0, and extensively washed with distilled water. Neutral sugars were eluted with 5 mM Hepes, pH 8.0. Finally, the phosphorylated linear oligosaccharides were eluted in 6 mL of 0.1 M NaCl and 10 mM HCl. The samples were freeze-dried, resuspended in a small volume of distilled water, and further analyzed by HPAEC-PAD.

The second half of the amyloplast starch sample was hydrolyzed with $\alpha$-amylase (Termamyl, Novo, Copenhagen, Denmark) as described by Nielsen et al. (1994). After a short centrifugation, the phosphorylated glucans produced were isolated by passing the supernatant over a DEAE-Sepharose column and freeze-dried. Some phosphoglucan fractions were further hydrolyzed in 0.7 N HCl (2–4 h, 100°C) to cleave all of the $\alpha$-glucosidic bonds present. They were freeze-dried, resuspended, and neutralized in a small volume before the generated content of Glc and Glc phosphates was determined by HPAEC-PAD.

### HPAEC-PAD Analysis

The phosphorylated linear glucans were analyzed by HPAEC-PAD on a chromatographic system (model DX-500, Dionex, Sunnyvale, CA) fitted with a CarboPac PA-100 analytical column (4 × 250 mm, Dionex) and equipped with a pulsed amperometric detector (model ED40, Dionex). The phosphorylated glucans were separated (flow rate 1 mL min$^{-1}$) using the following sodium acetate gradient profile in 150 mM NaOH: (a) from 0 to 5 min, linear gradient of 0 to 170 mM sodium acetate; and (b) from 5 to 190 min, linear gradient up to 500 mM sodium acetate, as described by Blennow et al. (1997). The collected fractions were neutralized with 4 N HCl and the radioactivity quantified by liquid scintillation. $\alpha$-Amylase-hydrolyzed phosphorylated glucans were chromatographed using a CarboPac PA1 analytical column and eluted (flow rate 1 mL min$^{-1}$) with the following sodium acetate gradient profile in 10 mM NaOH: (a) from 0 to 15 min, 10 mM sodium acetate; (b) from 15 to 20 min, linear gradient from 10 to 200 mM sodium acetate; (c) from 20 to 40 min, linear gradient from 200 to 300 mM sodium acetate; (d) from 40 to 45 min, concave gradient from 300 to 800 mM sodium acetate (curve 7); and (e) 45 to 50 min, 800 mM sodium acetate. The $\alpha$-amylase-treated fractions that were subjected to additional acid hydrolysis were chromatographed using the same method and column as described above, and the radioactivity in the collected fractions was quantified by liquid-scintillation counting.

## RESULTS

### Amyloplast Characterization

Amyloplasts containing small starch grains constituted the best starting material to isolate amyloplasts with a maximal degree of intactness and the highest starch biosynthetic activity per milligram of starch present. In such amyloplast preparations, the average size of the starch grains was 7 ± 5 $\mu$m (mean ± SD, 135 amyloplasts) and the intactness of approximately 80% (mean of 23 experiments), as determined by the latency of AGPase. The degree of intactness did not change for at least 2 h after isolation. In each isolation experiment, the yield of amyloplasts was determined by measuring the activity of the amyloplast marker enzyme AGPase in (a) the tuber tissue, (b) the crude extract after removal of the large starch grains, and (c) the isolated, small amyloplasts obtained after density fractionation. The yield of AGPase activity in the crude extract was 12% ± 3% (mean ± SD, 19 preparations) of that measured in the tuber tissue. The amyloplasts contained 0.4% ± 0.1% (mean ± SD, 19 preparations) of the AGPase activity in the crude extracts.

**Table I.** *The activity of marker enzymes for different organelles and cellular compartments during the isolation of intact small amyloplasts from potato tubers*

The yield (in parentheses) is presented as the percentage of the enzymatic activity in the tuber tissue and was calculated for each separate marker enzyme. The data presented are results from one typical set of experiments.

| Compartment and Enzyme | Tuber Tissue | | Crude Extract | Small Amyloplasts |
|---|---|---|---|---|
| | *nmol min$^{-1}$ g$^{-1}$ fresh wt* | | *nmol min$^{-1}$ tuber$^{-1}$* | |
| Amyloplast | | | | |
| AGPase | 1116 | 27,900 | 3200 (100) | 11.7 (0.37) |
| PPase[a] | 520 | 13,000 | 1800 (100) | 5.0 (0.28) |
| Cytosol | | | | |
| ADH | 274 | 6,850 | 1870 (100) | 0.8 (0.04) |
| PFP[b] | 816 | 20,400 | 2550 (100) | 0.9 (0.04) |
| Vacuole | | | | |
| α-Mannosidase | 62 | 1,560 | 88 (100) | 0.0 (0.00) |
| Mitochondria | | | | |
| Cyt *c* oxidase | 550 | 13,800 | 1500 (100) | 3.0 (0.20) |

[a] PPase, Alkaline inorganic pyrophosphatase. [b] PFP, Pyrophosphate: Fru-6-P phosphotransferase.

The purity of the isolated amyloplasts with respect to the presence of other organelles was assessed by measuring the activity of marker enzymes for cytosol, mitochondria, and vacuoles. The data obtained in a typical set of experiments are shown in Table I. In this set, the total AGPase activity in the isolated, small amyloplasts was 11.7 nmol tuber$^{-1}$ min$^{-1}$. However, among individual preparations, the total AGPase activity varied from 3 to 30 nmol tuber$^{-1}$ min$^{-1}$, resulting in a mean value of 10 ± 7 nmol tuber$^{-1}$ min$^{-1}$ (mean ± SD, 23 preparations). A comparison of the amyloplast AGPase activity with that of other marker enzyme activities demonstrated that the isolated amyloplasts were not contaminated by other organelles except mitochondria. Compared with the preparations of small amyloplasts, the large amyloplast preparations had a much lower level of intactness (65%, mean of 14 experiments).

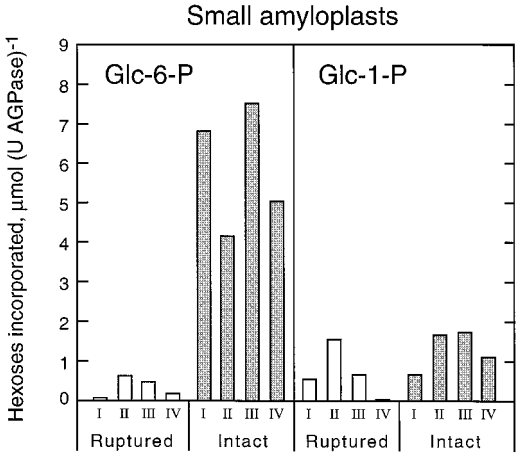


**Figure 1.** Starch biosynthesis from [1-$^{14}$C]Glc-6-P and [1-$^{14}$C]Glc-1-P in experiments with ruptured (□) and intact (■) potato tuber amyloplasts. Small amyloplasts were selected for the experiment. The amyloplasts were ruptured by treatment with 0.1% Triton X-100. The amyloplasts were incubated for 1 h with 4 mM exogenously added ATP and 3PGA and 2 mM metabolite (Glc-6-P or Glc-1-P) with 6.3 kBq [1-$^{14}$C]Glc-6-P or [1-$^{14}$C]Glc-1-P. The amount incorporated was normalized on the basis of the activity of AGPase in the plastid preparations. Each column represents a mean of three replicates. Each separate amyloplast preparation is numbered using roman numerals. U, Units.

## Starch Synthesis

Starch biosynthesis in the isolated small and large amyloplasts was studied using $^{14}$C-radiolabeled Glc-6-P or Glc-1-P as the substrate. Using small amyloplasts the amount of hexoses incorporated per unit of activity of AGPase was typically 3 times higher when using Glc-6-P compared with Glc-1-P (Fig. 1). Starch synthesis from Glc-6-P, but not from Glc-1-P, was dependent on amyloplast intactness. In the course of the biosynthetic experiments, Glc-6-P and Glc-1-P might have been interconverted through the action of phosphoglucomutase. In all of the experiments, the interconversion was less than 3% at the end of the incubation period, and the starch synthesized as a result of substrate isomerization accounted for less than 0.4% of the total amount of de novo-synthesized starch.

In contrast to the results obtained with the small amyloplasts, Glc-1-P was the preferred substrate for starch synthesis in the large amyloplasts. As observed for the small amyloplasts, the formation of starch from Glc-1-P was largely independent of amyloplast intactness (Fig. 2). The large amyloplasts showed a significantly higher rate of starch synthesis from Glc-1-P, whereas the synthesis from Glc-6-P was at a similar range as in the small amyloplasts.

## Small Amyloplasts de Novo Synthesize Phosphorylated Starch

We investigated the ability of small amyloplasts to catalyze de novo synthesis of phosphorylated starch by using a highly concentrated preparation of intact, small amyloplasts and $^{33}$P-radiolabeled Glc-6-P as a precursor. Of the [$^{33}$P]Glc-6-P (10 nmol, 9 MBq) initially administered to the amyloplast preparation, 0.005% was incorporated into

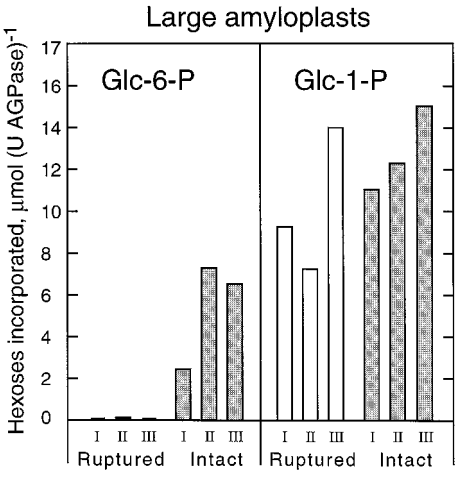


**Figure 2.** Starch biosynthesis from [1-$^{14}$C]Glc-6-P and [1-$^{14}$C]Glc-1-P in experiments with ruptured (□) and intact (■) plastids prepared from large amyloplasts. The amyloplasts were incubated as described in the legend to Figure 1. Each set of experiments is numbered. The amount of hexose residues incorporated is calculated as described in the legend to Figure 1. U, Units.

starch. To verify that the $^{33}$P radiolabel was covalently bound to the Glc residues in the starch, the gelatinized starch was subjected to enzymatic and acid hydrolysis. After isoamylase treatment and DEAE anion-exchange chromatography, 83% of the radioactivity was recovered as phosphorylated α-glucans. Upon chromatography on a HPAEC column, 92% of this radioactivity was recovered in the fractions containing phosphorylated glucans. The elution profile obtained after isoamylase treatment and isolation appears in Figure 3A. Pi and Glc-6-P eluted at 2 to 4 mL and 8 to 10 mL, respectively, followed by the phosphorylated α-glucans in order of increasing chain length (Blennow et al., 1997). The linear phosphorylated α-glucans were then treated with β-amylase, which resulted in the repeated release of maltose units from the nonreducing end of each glucan chain until a phosphorylated Glc residue was reached and further hydrolysis was blocked. Figure 3B illustrates the elution profile of the shortened phosphorylated glucans. As expected, the quantity of glucans eluting between 40 and 140 mL was significantly reduced, whereas the quantity eluting between 6 and 40 mL was increased, and 31% of the radioactivity recovered in elution volume (20–160 mL) was shifted to elution volumes less than 20 mL.

The elution profile obtained after α-amylase treatment of the $^{33}$P-labeled phosphorylated α-glucans and HPAEC-PA1 column chromatography is shown in Figure 4A. Using the PA1 column, Glc-6-P eluted at 25 mL (Fig. 5C) and Glc-3-P at 27 mL (Fig. 5B), whereas the short phosphorylated α-glucans eluted between 25 and 33 mL (Fig. 5A). When the preparation of phosphorylated short α-glucans was acid hydrolyzed to cleave all α-glucosidic linkages, we expected the incorporated $^{33}$P to be recovered in Glc-6-P and Glc-3-P, with Glc-6-P as the predominant component (Bay-Smidt et al., 1994). The radioactivity profile obtained was in agreement with this interpretation (Fig. 4B). After α-amylase degradation, acid hydrolysis, and PA1 chromatography, 79% of the radioactivity was recovered as hexose-P. Radiolabeled Glc-6-P was the major component; low amounts of radioactivity co-eluted with Glc-3-P.

## DISCUSSION

Potato tuber amyloplasts with an intactness of 80% were isolated using mechanical disruption of the tuber tissue in medium containing 0.5 M sorbitol followed by density gradient purification. The amyloplasts remained intact for several hours at room temperature. Amyloplast preparations were essentially free of cytosolic and vacuolar enzyme activities (Table I).

The isolation of potato amyloplasts has previously been reported based on tissue culture material derived from tubers (Mohabir and John, 1988; Kosegarten and Mengel, 1994) or on the use of tuber tissue as the starting material (Mohabir and John, 1988; Schott et al., 1995; Naeem et al., 1997), as in this study. The yield determined from the relative activity of the amyloplast marker enzyme AGPase (Table I) was low (0.4% of the amyloplasts released from the tissue) compared with the 10% yield reported by Naeem et al. (1997). However, all attempts to increase the

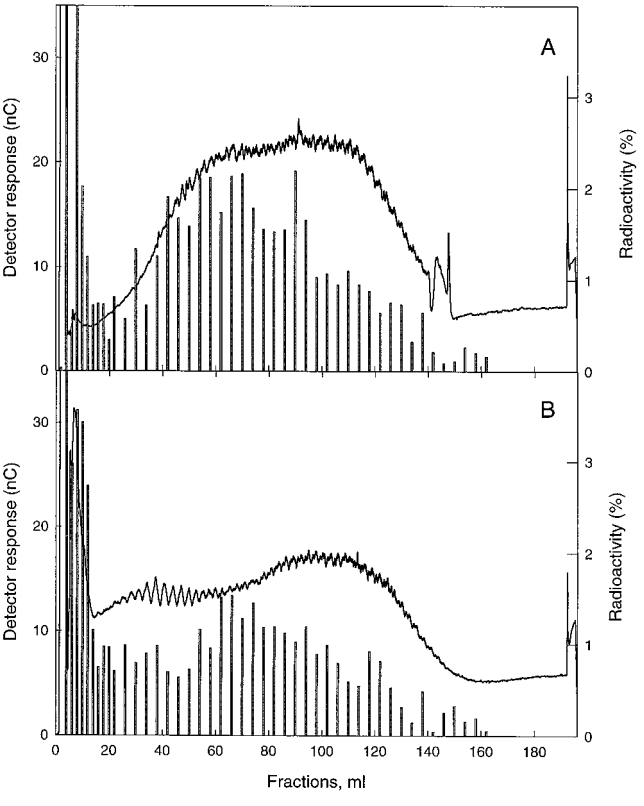


**Figure 3.** Verification of incorporated phosphate into starch in experiments with small amyloplasts using [$^{33}$P]Glc-6-P and effect of β-amylase. A, HPAEC elution profile of linear phosphorylated oligosaccharides together with the corresponding radioactivity. B, Detector response and corresponding radioactivity after β-amylase treatment of the linear phosphorylated oligosaccharides. In both panels peaks of radioactivity were integrated and normalized to 100%. nC, Nanocoulombs.

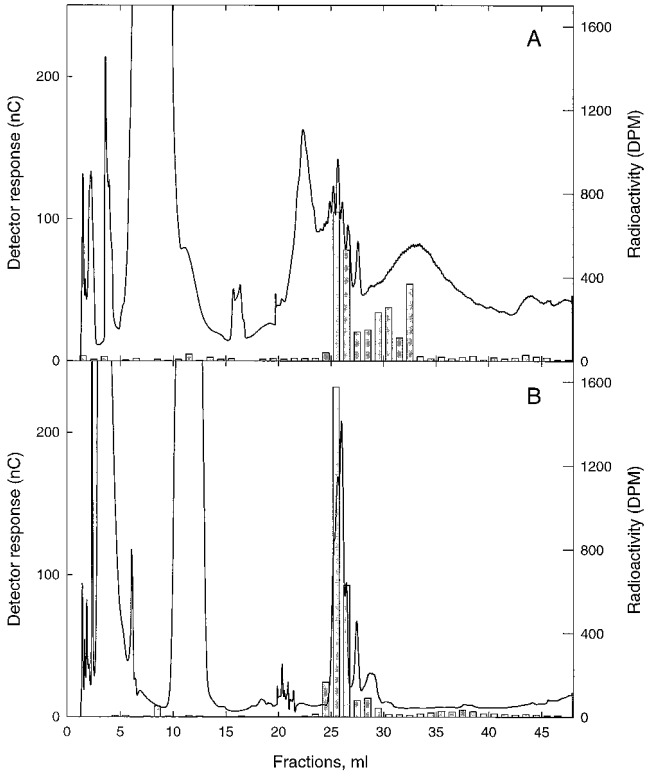


**Figure 4.** Verification of phosphate incorporated into starch at C-6 and C-3 in the Glc residues in experiments with small amyloplasts using [$^{33}$P]Glc-6-P. A, HPAEC elution profile of phosphorylated dextrins treated with α-amylase and corresponding radioactivity. B, Detector response and radioactivity after acid hydrolysis: 2 h in 0.7 N HCl at 100°C. nC, Nanocoulombs.

yield compromised the ability to obtain amyloplasts with high intactness and purity and reflected the selection for small amyloplasts.

Because triose-P cannot be converted to hexose-P inside potato tuber amyloplasts and therefore cannot be used directly for starch synthesis (Entwisle and ap Rees, 1990; Kossmann et al., 1992), we studied starch synthesis using Glc-6-P and Glc-1-P as the substrates and demonstrated that intact, small potato amyloplasts could import and use Glc-6-P for the synthesis of starch (Fig. 1). In proteoliposomes reconstituted from potato amyloplasts isolated from transgenic potato with a reduced starch content, Glc-6-P, but not Glc-1-P, was translocated by counterexchange with Pi, dihydroxyacetonephosphate, and 3PGA (Schott et al., 1995). Others have reported that potato amyloplasts preferentially took up Glc-1-P for starch synthesis (Naeem et al., 1997). Kosegarten and Mengel (1994) also showed starch synthesis from Glc-1-P, but did not test Glc-6-P as a substrate. The differences concerning the preferred precursor for import and subsequent synthesis of starch may be explained by the different methods used for plastid isolation.

The data in this study clearly demonstrated that Glc-6-P was the precursor for starch synthesis in small amyloplasts and that only intact amyloplasts contributed to the incorporation. In contrast to these results, Glc-1-P was the most efficient precursor for starch synthesis when large amyloplasts were used (Fig. 2). The incorporation from Glc-1-P proceeded independently of amyloplast intactness. The preparation of large amyloplasts contained a large fraction of "naked" starch grains. Several of the enzymes involved in starch biosynthesis, including starch phosphorylase, are known to be associated with starch grains (Martin and Smith, 1995). The high rate of starch biosynthesis observed upon administration of Glc-1-P to large amyloplasts, as in the present study, may therefore be explained by the activity of starch phosphorylase bound to contaminating naked starch grains. This is not a plausible explanation, however, for the conflicting results obtained by Naeem et al. (1997), who found that starch biosynthesis from Glc-1-P was increased by the addition of ATP and was dependent on intactness. An alternative explanation is that small potato amyloplasts use Glc-6-P and large potato amyloplasts use Glc-1-P as a precursor for starch synthesis, although no physiological arguments are available to support such a difference.

Maize endosperm amyloplasts incorporated both Glc-1-P and Glc-6-P at comparable rates when supplied at high concentrations (above 2 mM) (Neuhaus et al., 1993). However, only Glc-6-P was imported via a counterexchange in proteoliposomes from maize endosperm (Möhlmann et al., 1997). Schott et al. (1995) obtained similar results with proteoliposomes derived from a potato plastid envelope

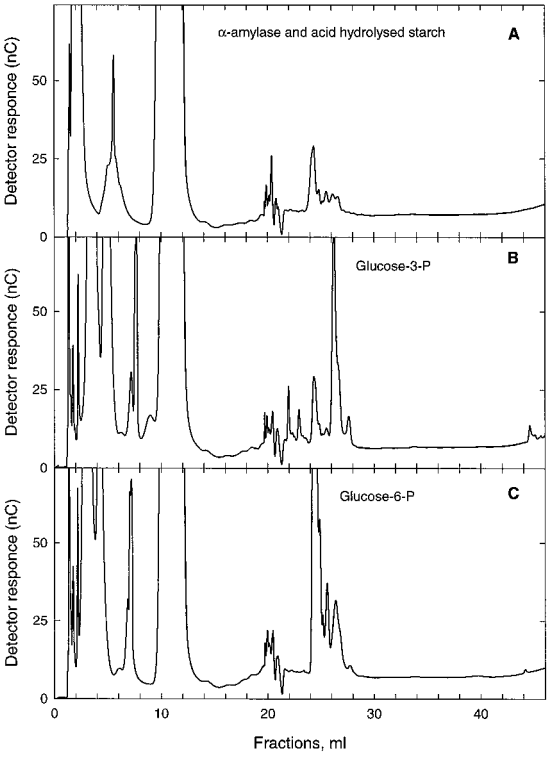


**Figure 5.** HPAEC elution profile of phosphorylated acid-hydrolyzed dextrins, as in Figure 4B, showing the elution time of internal standards of Glc-3-P and Glc-6-P. A, No internal standard added; B and C, Glc-3-P and Glc-6-P added as internal standards, respectively. nC, Nanocoulombs.

where counterexchange with Pi was tested. The rate of starch synthesis from Glc-6-P obtained with the small amyloplasts used in this study varied from experiment to experiment. The rates (Fig. 1) are comparable to the rate of starch synthesis (4.9 $\mu$mol hexoses incorporated $h^{-1}$ $unit^{-1}$ AGPase) reported by Naeem et al. (1997) using Glc-1-P as a precursor.

A remarkable feature of potato starch is its content of phosphate esterified to C-6 and C-3 in the Glc moieties of the amylopectin molecules. Here we show that starch synthesized in vitro by isolated potato amyloplasts is phosphorylated.

The elution of the phosphorylated glucans obtained by the debranching of amylopectin depends on the length of the glucan chain, the number of phosphate groups esterified to each glucan chain, the position of the phosphate in the chain, and probably also on the position of the phosphate on the Glc residues (Blennow et al., 1997). $\beta$-Amylolysis of phosphorylated glucans releases maltose from the nonreducing end of the glucan (Takeda and Hizukuri, 1981). The $\beta$-amylase cannot bypass Glc residues esterified with phosphate at the C-6 or C-3 position, and the $\alpha$-glucans recovered after $\beta$-amylase treatment are therefore expected to be shortened to chains representing the distance from the former amylopectin branchpoint to the phosphate group on the glucan chain. In agreement with this assumption, $\beta$-amylolysis produced a shift toward shorter glucans. Similarly, $\alpha$-amylase-treated and debranched phosphorylated glucans eluted within the first 20 mL (B. Wischmann, unpublished results).

Analysis using HPAEC-PAD programs that were optimized to separate Glc-6-P from Glc-3-P showed that the radioactivity obtained after acid hydrolysis was concentrated at the position of Glc-6-P and to a lesser degree at the position of Glc-3-P (compare Figs. 4B and 5). We therefore conclude that the de novo-synthesized starch is phosphorylated at the C-6 position but only secondarily at the C-3 position.

In conclusion, we have isolated intact amyloplasts containing small starch grains. These intact amyloplasts use Glc-6-P as an efficient precursor for starch synthesis, and the de novo-synthesized starch is phosphorylated at the C-6 position and to some extent also at the C-3 position of the Glc residues.

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## LITERATURE CITED

**Abel GJW, Springer F, Willmitzer L, Kossmann J** (1996) Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). Plant J **10:** 981–991

**Bay-Smidt AM, Wischmann B, Olsen CE, Nielsen TH** (1994) Starch bound phosphate in potato as studied by a simple method for determination of organic phosphate and $^{31}$P-NMR. Starch Stärke **46:** 167–172

**Blennow A, Bay-Smidt AM, Wischmann B, Olsen CE, Møller BL** (1998) The degree of starch phosphorylation is related to the chain length distribution of the neutral and the phosphorylated chains of amylopectin. Carbohydr Res **307:** 45–54

**Echeverria E, Boyer CD, Thomas PA, Liu K-C, Shannon JC** (1988) Enzyme activities associated with maize kernel amyloplasts. Plant Physiol **86:** 786–792

**Entwistle G, ap Rees T** (1990) Lack of fructose-1,6-bisphosphatase in a range of higher plants that store starch. Biochem J **271:** 467–472

**Entwistle G, Tyson RH, ap Rees T** (1988) Isolation of amyloplasts from wheat endosperm. Phytochemistry **27:** 993–996

**Gross P, ap Rees T** (1986) Alkaline inorganic pyrophosphatase and starch synthesis in amyloplasts. Planta **167:** 140–145

**Hatzfeld WD, Stitt M** (1990) A study of the rate of recycling of triose phosphates in heterotrophic *Chenopodium rubrum* cells, potato tubers, and maize endosperm. Planta **180:** 198–204

**Hill L, Smith AM** (1991) Evidence that glucose 6-phosphate is imported as the substrate for starch synthesis by the plastids of developing pea embryos. Planta **185:** 91–96

**Hizukuri S, Tabata S, Nikuni Z** (1970) Studies on starch phosphate. Part 1. Estimation of glucose-6-phosphate residues in starch and the presence of other bound phosphate(s). Starch Stärke **22:** 330–343

**Jane J, Kasemsuwan T, Chen JF, Juliano BO** (1996) Phosphorus in rice and other starches. Cereal Foods **41:** 827–832

**Keeling PL, Wood JR, Tyson RH, Bridges IG** (1988) Starch biosynthesis in developing wheat grains. Plant Physiol **87:** 311–319

**Kosegarten H, Mengel K** (1994) Evidence for a glucose 1-phosphate translocator in storage tissue amyloplasts. Physiol Plant **91:** 111–120

**Kossmann J, Müller-Röber B, Dyer TA, Raines CA, Sonnewald U, Willmitzer L** (1992) Cloning and expression analysis of the plastidic fructose-1,6-bisphosphatase coding sequence from potato: circumstantial evidence for the import of hexoses into chloroplasts. Planta **188:** 7–12

**Lorbert R, Kossmann J, inventors.** March 20, 1997. Pflanzen die eine modifizierte stärke synthetisieren, sowie verfahren zu ihre herstellung. German patent DE 195 34 759 A1

**MacDonald FD, ap Rees T** (1983) Enzymic properties of amyloplasts from suspension cultures of soybean. Biochim Biophys Acta **755:** 81–89

**Martin C, Smith AM** (1995) Starch biosynthesis. Plant Cell **7:** 971–985

**Michal G** (1988) D-Glucose 6-phosphate and D-fructose 6-phosphate. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Ed 3, Vol VI. Metabolites 1: Carbohydrates. VCH Verlagsgesellschaft mbH, Weinheim, Germany, pp 191–198

**Mohabir G, John P** (1988) Effect of temperature on starch synthesis in potato tuber tissue and in amyloplasts. Plant Physiol **88:** 1222–1228

**Möhlmann T, Tjaden J, Henrichs G, Quick WP, Häusler R, Neuhaus HE** (1997) ADP-glucose drives starch synthesis in isolated maize endosperm amyloplasts: characterization of starch synthesis and transport properties across the amyloplast envelope. Biochem J **324:** 503–509

**Naeem M, Tetlow IJ, Emes MJ** (1997) Starch synthesis in amyloplasts purified from developing potato tubers. Plant J **11:** 1095–1103

**Neuhaus HE, Batz O, Thom E, Scheibe R** (1993) Purification of highly intact plastids from various heterotrophic plant tissues: analysis of enzymic equipment and precursor dependency for starch biosynthesis. Biochem J **296:** 395–401

**Nielsen TH, Skjærbæk HC, Karlsen P** (1991) Carbohydrate metabolism during fruit development in sweet pepper (*Capsicum annuum*) plants. Physiol Plant **82:** 311–319

**Nielsen TH, Wischmann B, Enevoldsen K, Møller BL** (1994) Starch phosphorylation in potato tubers proceeds concurrently with de novo biosynthesis of starch. Plant Physiol **105:**111–117

**Nielsen TH, Wischmann B, Møller BL** (1995) A convenient method for enzymatic synthesis of radiolabeled glucose-1,6-bisphosphate. J Label Compd Radiopharm **XXXVI**: 679–684

**Rasmussen AG, Møller IM** (1990) NADP-utilizing enzymes in the matrix of plant mitochondria. Plant Physiol **94:** 1012–1018

**Schott K, Borchert S, Müller-Röber B, Heldt HW** (1995) Transport of inorganic phosphate and C3- and C6-sugar phosphates across the envelope membranes of potato tuber amyloplasts. Planta **196:** 647–652

**Smith AM, Quinton-Tulloch J, Denyer K** (1990) Characteristics of plastids responsible for starch synthesis in developing pea embryos. Planta **180:** 517–523

**Sowokinos JR** (1976) Pyrophosphorylases in *Solanum tuberosum* 1. Changes in ADP-glucose and UDP-glucose pyrophosphorylase activities associated with starch biosynthesis during tuberization, maturation, and storage of potatoes. Plant Physiol **57:** 63–68

**Stitt M, Lilley RM, Gerhardt R, Heldt HW** (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. Methods Enzymol **174:** 518–552

**Takeda Y, Hizukuri S** (1981) Re-examination of the action of sweet-potato beta-amylase on phosphorylated (1→4)-α-D-glucan. Carbohydr Res **89:** 174–178

**Tetlow IJ, Blisset KJ, Emes MJ** (1993) A rapid method for the isolation of purified amyloplasts from wheat endosperm. Planta **189:** 597–600

**Tyson RH, ap Rees T** (1988) Starch synthesis by isolated amyloplasts from wheat endosperm. Planta **175:** 33–38

**Viola R, Davies HV, Chudeck AR** (1991) Pathways of starch and sucrose biosynthesis in developing tubers of potato (*Solanum tuberosum* L.) and seeds of faba bean (*Vicia faba* L.). Planta **183:** 202–208