

# Novel, Starch-Like Polysaccharides Are Synthesized by an Unbound Form of Granule-Bound Starch Synthase in Glycogen-Accumulating Mutants of *Chlamydomonas reinhardtii*

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In vascular plants, mutations leading to a defect in debranching enzyme lead to the simultaneous synthesis of glycogen-like material and normal starch. In *Chlamydomonas reinhardtii* comparable defects lead to the replacement of starch by phytoglycogen. Therefore, debranching was proposed to define a mandatory step for starch biosynthesis. We now report the characterization of small amounts of an insoluble, amylose-like material found in the mutant algae. This novel, starch-like material was shown to be entirely dependent on the presence of granule-bound starch synthase (GBSSI), the enzyme responsible for amylose synthesis in plants. However, enzyme activity assays, solubilization of proteins from the granule, and western blots all failed to detect GBSSI within the insoluble polysaccharide matrix. The glycogen-like polysaccharides produced in the absence of GBSSI were proved to be qualitatively and quantitatively identical to those produced in its presence. Therefore, we propose that GBSSI requires the presence of crystalline amylopectin for granule binding and that the synthesis of amylose-like material can proceed at low levels without the binding of GBSSI to the polysaccharide matrix. Our results confirm that amylopectin synthesis is completely blocked in debranching-enzyme-defective mutants of *C. reinhardtii*.

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Mutants of maize, rice, and *Chlamydomonas reinhardtii* and, more recently, *Arabidopsis* have been reported to accumulate in place of or in addition to starch a novel type of WSP known as phytoglycogen (Sumner and Somers, 1944; Mouille et al., 1996; Nakamura et al., 1996; Zeeman et al., 1998). In corn, *Arabidopsis*, and algae, the mutants were shown to lack a specific form of DBE with isoamylase-

like specificity (James et al., 1995; Rahman et al., 1998; Zeeman et al., 1998). Both rice and maize were further reported to be missing a specific pullulanase-type of DBE (Pan and Nelson, 1984; Nakamura et al., 1996; Rahman et al., 1998). The major difference between algae and vascular plants consists of the severity of the phenotype recorded. Whereas maize, rice, and *Arabidopsis* substitute only part of their starch production by phytoglycogen, *C. reinhardtii* was reported to replace all of the starch by a low (5% of the wild type) amount of WSPs. The severity of this defect led us to suggest that debranching is mandatory to obtain significant amylopectin and, therefore, starch synthesis in plants.

The results obtained in maize, rice, and *C. reinhardtii* led us, together with a number of other authors, to propose a novel pathway for storage polysaccharide synthesis in plants (Ball et al., 1996). The latter consisted of trimming loosely spaced  $\alpha$ -1,6 branches from a precursor of amylopectin to generate the asymmetrical distribution of branches required for amylopectin crystallization. However, this was recently questioned because in *Arabidopsis* leaves the loss of isoamylase activity is not accompanied by a disappearance of starch but rather by its selective decrease and partial replacement by phytoglycogen (Zeeman et al., 1998). To check for the presence of normal starch in the *C. reinhardtii* mutants, we scaled up our algal cultures to detect and characterize tiny amounts of insoluble granular polysaccharide (less than 1% of the wild-type starch amount). We further introduced mutations leading to the absence of GBSS or SS to test the dependency of the polysaccharides accumulating in DBE-defective mutants toward the different starch synthases.

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Abbreviations: DBE, debranching enzyme; DP, degree of polymerization; GBSS, granule-bound starch synthase;  $\lambda_{\max}$ , wavelength of the maximal absorbance of the iodine-polysaccharide complex; SS, soluble starch synthase; WSP, water-soluble polysaccharide.

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<sup>1</sup> This work was supported by grants from the Ministère de l'Éducation Nationale, by the Centre National de la Recherche Scientifique (CNRS) (Unité Mixte de Recherche du CNRS no. 8576), by the European Union (FAIR grant no. CT95-0568), and by the National Institutes of Health (no. 5-T32-GM0287).

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We now report the presence of a novel type of insoluble, starch-like material entirely constituted of amylose-like chains in the phytoglycogen-producing mutants of *C. reinhardtii*. We show the dependence of this material on GBSSI activity. Moreover, in contrast to all of the results obtained to date, we show that GBSSI does not bind to the granular polysaccharide. We believe our results demonstrate that GBSSI requires crystalline amylopectin for binding *in vivo* and that phytoglycogen synthesis can proceed in the complete absence of insoluble polysaccharide synthesis. We therefore confirm that amylopectin synthesis is completely blocked in the *sta7* mutants of *C. reinhardtii*.

## MATERIALS AND METHODS

### Material

The starch determination kit was purchased from Boehringer Mannheim. Rabbit liver glycogen and maize amylopectin were supplied by Sigma. Percoll was from Pharmacia.

### Strains, Media, Incubation, and Growth Conditions

The genotypes of all of the strains of *Chlamydomonas reinhardtii* used in this work are listed in Table I. Starch and WSP were always prepared from nitrogen-starved media. Media and culture conditions used in our starvation experiments were as described by Ball et al. (1990). Formulas for Tris-acetate-phosphate and high salt-high acetate media and genetic techniques were according to the method of Harris (1989a, 1989b). All experiments were performed under continuous light ( $40 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in the presence of acetate at 24°C in liquid cultures that were shaken vigorously without CO<sub>2</sub> bubbling.

### Purification of the Insoluble Macrogranular Fraction

Pure native starch was prepared from nitrogen-starved cultures, inoculated at  $10^6$  cells mL<sup>-1</sup>, and harvested after 5 d of growth under continuous light ( $40 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in Tris-acetate-phosphate without nitrogen medium. Algal suspensions were passed through a French press at 10,000 p.s.i. A crude starch pellet was obtained by spinning the lysate at 10,000g for 20 min. The pellet was resuspended in 300  $\mu\text{L}$  of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA per 10<sup>9</sup> starting cells, and passed twice through a Percoll gradient (1.2 mL of Percoll for the 300  $\mu\text{L}$  of crude starch pellet). The purified starch pellet was rinsed in distilled water, centrifuged twice at 10,000g, and kept at 4°C for immediate use or dried for subsequent analysis. Starch amounts were measured using the amyloglucosidase assay as described by Delrue et al. (1992).

### Purification of the WSPs

WSPs were prepared from 20 L of 1 week-old nitrogen-starved cultures that were inoculated at  $10^6$  cells mL<sup>-1</sup>. Algae were harvested and ruptured by passing them in a French press (10,000 p.s.i) at a density of  $10^8$  cells mL<sup>-1</sup> in

the presence of pronase (1 mg mL<sup>-1</sup>). The crude extract was immediately frozen at -80°C and thawed after a minimum of 4 h of storage. Cell debris were discarded by spinning at 10,000g for 30 min. The supernatant was extracted three times with 2:0.1 (v/v) chloroform:methanol and centrifuged at 3000g for 15 min. Water was added between each extraction to keep the volume of the aqueous phase constant. Emulsions were obtained by intensive shaking. The water-methanol soluble fraction was concentrated by rotary evaporation and redissolved in 10% DMSO to be subsequently loaded onto a gel-filtration column (TSK-HW-50 [F], Merck, Darmstadt, Germany) eluted by 10% DMSO as described by Maddelein et al. (1994). The high-*M<sub>r</sub>* peak was desalted by dialysis at 4°C overnight and lyophilized. The low-*M<sub>r</sub>* peak was desalted by gel filtration onto a column (TSK-HW-40, Merck). The peak of dextrin was lyophilized and stored at room temperature.

### Debranching Analysis

Phytoglycogens (500  $\mu\text{g}$ ) were suspended in 10  $\mu\text{L}$  of water and debranched with 1  $\mu\text{L}$  of isoamylase (200 units/mL, Megazyme International, Bray, County Wicklow, Ireland) in 40  $\mu\text{L}$  of 50 mM sodium acetate buffer (pH 4.0). The reaction was incubated for 2 h at 37°C and terminated by heating in a water bath at 100°C for 5 min. Completion of the reaction was ascertained by assaying the amount of reducing ends through the standard dinitrosalicylate procedure. The latter consists of mixing 5  $\mu\text{L}$  of the sample with 45  $\mu\text{L}$  of water. The diluted sample was then added to 150  $\mu\text{L}$  of 1% dinitrosalicylate solution and the *A*<sub>540</sub> was read. Complete debranching was obtained when maximal and constant absorbencies that compared favorably with those of amylopectin, amylose, and glycogen standards were recorded. The samples were then evaporated to dryness in a centrifugal vacuum evaporator.

### Separation of Labeled Oligosaccharides

The debranched and undebranched samples were labeled with 8-amino-1,3,6-pyrenetrisulfonic acid and analyzed with a DNA sequencer, as fully described by O'Shea and Morell (1996).

### *trans* Complementation Tests

The segregants (IJS) obtained from the cross between strains IJ2 and S were subjected to *trans* complementation tests to characterize their deficiencies. Diploid clone selection was achieved by growth after 5 d of the sexual fusion products on appropriate selective media. The diploids obtained were purified three times, checked for cellular volume, and then amplified. Cell patches incubated for 7 d on solid, nitrogen-deprived medium were stained with iodine. Diploids homozygous for the *sta2* mutation appear red after staining (Delrue et al., 1992).

### Transmission Electron Microscopy

Cell suspensions prepared from nitrogen-starved media were harvested first and immediately embedded at 45°C in

**Table 1.** *Strains and genotypes*

Strain	Genotype	Defective Enzyme <sup>a</sup>
137C	<i>mt - nit1 nit2</i>	—
BAFR1	<i>mt + nit1 nit2 sta2-29::ARG7</i>	GBSSI
IJ2	<i>mt - nit1 nit2 sta2-29::ARG7 sta3-1</i>	GBSSI SSII
S	<i>mt + nit1 nit2 sta7-7::ARG7</i>	DBE
IJS15	<i>mt + nit1 nit2 sta7-7::ARG7</i>	DBE
IJS24	<i>mt - nit1 nit2 sta7-7::ARG7</i>	DBE
IJS2	<i>mt - nit1 nit2 sta3-1 sta7-7::ARG7</i>	DBE SSII
IJS13	<i>mt + nit1 nit2 sta3-1 sta7-7::ARG7</i>	DBE SSII
IJS21	<i>mt + nit1 nit2 sta3-1 sta7-7::ARG7</i>	DBE SSII
IJS8	<i>mt + nit1 nit2 sta2-29::ARG7 sta7-7::ARG7</i>	DBE GBSSI
IJS39	<i>mt + nit1 nit2 sta2-29::ARG7 sta7-7::ARG7</i>	DBE GBSSI
IJS50	<i>mt + nit1 nit2 sta2-29::ARG7 sta7-7::ARG7</i>	DBE GBSSI
IJS3	<i>mt - nit1 nit2 sta2-29::ARG7 sta3-1 sta7-7::ARG7</i>	DBE GBSSI SSII
IJS27	<i>mt + nit1 nit2 sta2-29::ARG7 sta3-1 sta7-7::ARG7</i>	DBE GBSSI SSII

<sup>a</sup> Names of the enzyme activities missing in the corresponding strains.

a 3% agar gel to prevent these highly mobile cells from adopting a heterogeneous spatial distribution. Small, solidified cubes of agar containing the samples were then cut and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) for 3 h at room temperature. A part of these samples was postfixed in 1% osmium tetroxide for 1 h. The fixed samples were then dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Epon. Sections of 80 nm thickness were mounted on copper or gold grids previously coated with carbon. Two complementary treatments were applied on the grids as follows: (a) sections of samples fixed by glutaraldehyde and osmium tetroxide were mounted on copper grids and then stained for 30 min in 2.8% uranyl acetate (50% methanol) at 48°C; and (b) sections of samples fixed by glutaraldehyde alone were mounted on gold grids and then stained according to the modified PATAg procedure previously described by Simon et al. (1995). The samples were first treated for 30 min in a saturated solution of 2–4-dinitrophenylhydrazine in 15% acetic acid to block free aldehydes. After several washes in deionized water, samples were oxidized for 30 min in 1% periodic acid aqueous solution, then treated for 18 h with 0.2% thiosemicarbazide in 20% acetic acid, and finally treated for 30 min with 1% silver proteinate aqueous solution.

The sections were examined with a transmission electron microscope (model 100S, Jeol) operated at 80 keV.

### Zymogram Analysis

Soluble crude extracts were prepared as previously described (Fontaine et al., 1993) and immediately stored at –70°C. The lysate was cleared by centrifuging at 10,000g for 10 min at 4°C. Proteins were measured using the Bio-Rad protein assay kit. In 100  $\mu$ L of 25 mM Tris glycine, pH 8.3, 1% SDS, 5%  $\beta$ -mercaptoethanol, 100 to 500  $\mu$ g of protein was denatured by boiling in a water bath for 4 min. The denatured proteins can be stored at 4°C without subsequent loss of enzyme activity. Starch synthase and DBE zymograms were as previously described by Mouille et al. (1996) and Buléon et al. (1997).

### SDS-PAGE Analysis of Granule-Bound Proteins, Western Blots, and GBSS Activity Assay

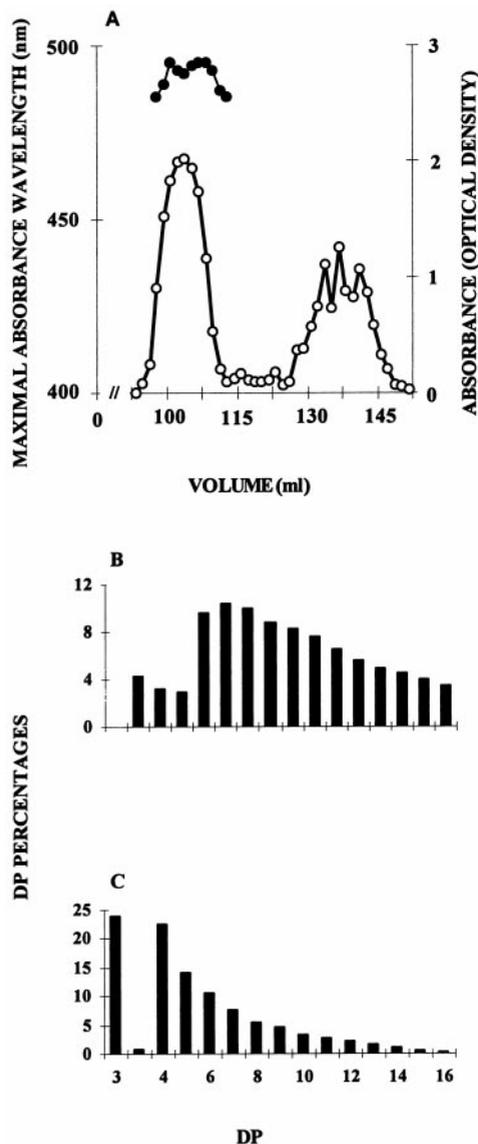
Purified starches (1–6 mg) were boiled for 5 min in 80  $\mu$ L of 2% SDS and 5%  $\beta$ -mercaptoethanol and then centrifuged for 20 min at 10,000g to extract proteins from the granule. Supernatants were loaded onto SDS-PAGE and stained with Coomassie brilliant blue R250.

Western-blot analysis was performed on proteins solubilized from the granule as described by Buléon et al. (1997). The specific antiserum PA55 was directed against the synthetic peptide GTGGLRDTVENC, representing a consensus sequence derived from the C-terminal part of the vascular plant starch synthases (Buléon et al., 1997). The GBSS activity assay was as described by Delrue et al. (1992).

## RESULTS

### Mutants Defective for the *STA7* Locus Synthesize up to 0.5% of the Wild-Type Amount of Starch in the Form of Insoluble, High-Amylose Types of Granular Polysaccharides

Because of the low amounts of polysaccharide found in the *sta7* mutants of *C. reinhardtii*, we scaled up our culture volumes to 20 L and measured the amounts of the WSP and high-density-insoluble material that sedimented in the bottom of Percoll gradients. As previously reported, in the mutants we found 5%  $\pm$  3% ( $n = 5$ ) of the amount of polysaccharide measured by the amyloglucosidase assay in wild-type reference strains in the form of WSP. Both high and low-molecular-mass WSPs could be separated through TSK-HW-50 (Fig. 1A) gel-permeation chromatography. Because of the high sensitivity of the technique, we used fluorescence labeling coupled to PAGE on a DNA-sequencing gel to analyze both the size distribution of the small oligosaccharide fraction and that of the high-molecular-mass polysaccharide subjected to selective debranching through bacterial isoamylase. Results displayed in Figure 1, B and C, together with those previously pub-



**Figure 1.** Determination of the chain-length distributions of both *sta7* high- and low-molecular-mass WSPs. A, TSK-HW-50 gel-permeation chromatography of *sta7* WSP. The  $\lambda_{\max}$  (in nanometers [●]) of the unbranched fractions is scaled on the left y axis and the amount of Glc (in micrograms per milliliter [○]) measured for each fraction is indicated on the right y axis. The x axis shows the elution volume scale (in milliliters). B and C, Histograms of chain-length distributions obtained through gel electrophoresis of fluorescent glucans with or without isoamylase-mediated enzymatic debranching. Both chain-length distributions of debranched high-molecular-mass (B) and unbranched low-molecular-mass (C) WSPs eluted from the TSK-HW-50 column (A) are displayed as percentages of chains of DP between 2 and 16. The x axis displays a DP scale, and the y axis represents the relative frequencies of the chains expressed as percentages. The peak seen between DPs 3 and 4 is consistent with the position of a branched trisaccharide.

lished (Mouille et al., 1996), clearly confirm the high-molecular-mass fraction as glycogen-like material, whereas the oligosaccharide fraction contained both branched and linear glucans. The high-mass glucans display an iodine

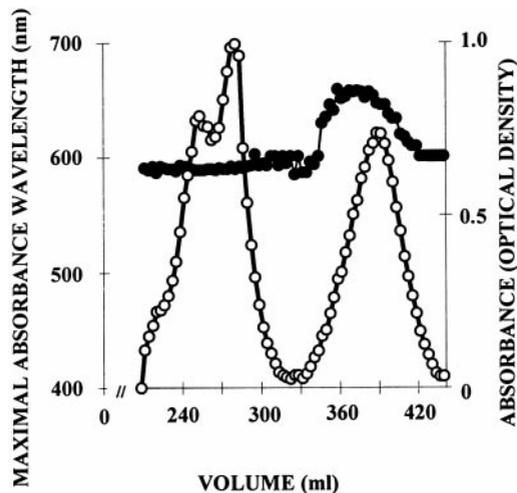
interaction (Fig. 1A), a branching ratio (Mouille et al., 1996), a chain-length distribution (Fig. 1B), and granule morphology (Fig. 8, C–F) similar to those reported for glycogen. The relative amounts of oligosaccharide to polysaccharide in the water-soluble fraction were between 20% and 60% ( $40\% \pm 20\%$ ). We believe that these variations reflect the presence of fluctuating amounts of  $\alpha$ -amylase in the extracts to which the soluble polysaccharides are highly sensitive.

That these polysaccharides are made of  $\alpha$ -1,4-linked  $\alpha$ -1,6-branched Glc residues is beyond doubt. First, this material is 100% sensitive to amyloglucosidase. Second, it is only partly sensitive to  $\beta$ -amylase (not shown). Third, the  $^{13}\text{C}$ -NMR spectra that are typical of glycogen were found for all WSPs described in this paper. These spectra were identical to those that we published previously (Mouille et al., 1996). Six major NMR signals corresponding to the six carbon atoms (numbered 1 through 6) of Glc could always be distinguished at approximately 100 ppm ( $\text{C}_1$ ), 71.95 ppm ( $\text{C}_2$ ), 72.44 to 73.16 ppm ( $\text{C}_3$ ), 78.9 ppm ( $\text{C}_4$ ), 71.64 ppm ( $\text{C}_5$ ), and 60.64 ppm ( $\text{C}_6$ ). These major signal chemical shifts correspond to carbons within Glc residues present in the middle of  $\alpha$ -1,4-linked glucans. In addition and because of the abundance of the branches, we were able to pick up two additional signals at 70.25 and 61.09 ppm assigned, respectively, to  $\text{C}_4$  and  $\text{C}_6$  of the non-reducing-end terminal Glc residues. Other signals dispersed around the  $\text{C}_1$  and  $\text{C}_4$  major peaks are due to those Glc residues at or around the branch points.

The amounts of high-density-insoluble material that could be purified through isopycnic Percoll gradient centrifugation were slightly greater than what we previously reported (Mouille et al., 1996) and were estimated at  $0.4\% \pm 0.2\%$  of what would have otherwise been the normal starch amount in wild-type algae. Although very low, this still amounted to 10% of the total WSP fraction present in the mutants and was characterized by a  $\lambda_{\max}$  of 590 to 600 nm, consistent with the presence of amylose-like chains within the polymer structure. This fraction was detected in all eight *sta7* mutants investigated. Because in *Arabidopsis* normal starch is reported to be present with phytylglucosylated amylose in isoamylase-defective mutants, we undertook a detailed, structural characterization of this material purified from 80-L algal cultures.

### The Amylose-Like Polysaccharide Displays an Entirely Novel Structure

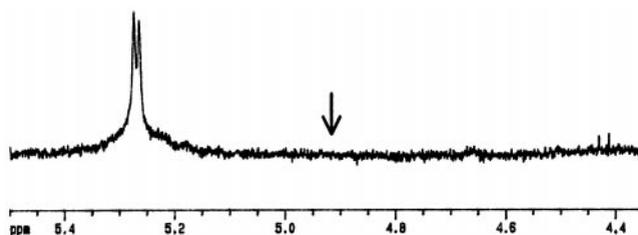
The high-amylose material consisted of both a high- (30%) and a low (70%)-molecular-mass component that could be separated through TSK-HW-75 gel-permeation chromatography (Fig. 2). The high-molecular-mass fraction displayed a  $\lambda_{\max}$  of 590 nm, comparable to that of amylopectin components found in high-amylose starches of *C. reinhardtii* and the *su-2* mutant of maize (Fontaine et al., 1993; Takeda and Preiss, 1993; Libessart et al., 1995). However, unlike all other wild-type or mutant amylopectins analyzed to date, we found less than 1% branches in this material by proton NMR (Fig. 3). The  $^{13}\text{C}$ -NMR spectra were indistinguishable from those published for amylose



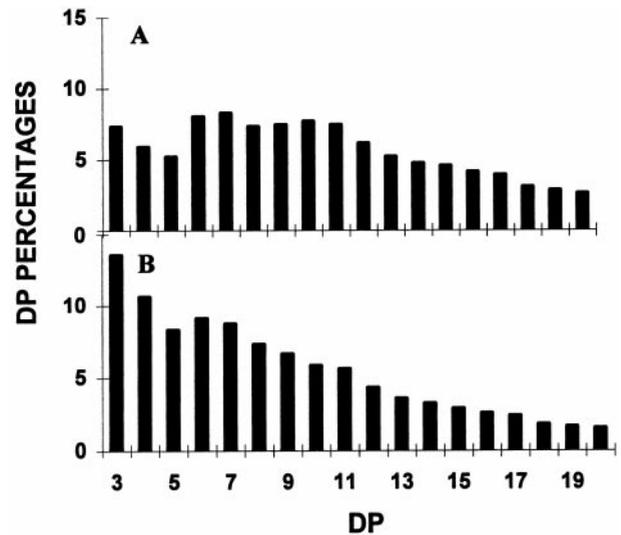
**Figure 2.** Separation of amylopectin-like material and amylose extracted by TSK-HW-75 gel-permeation chromatography. Absorbance ( $\circ$ ) was measured for each fraction at  $\lambda_{\max}$  (y axis on the right). The  $\lambda_{\max}$  (in nanometers) is displayed as an unbroken, thin line on the left y axis. The x axis represents the elution volume scale (in milliliters).

(Fontaine et al., 1993), further establishing this material as composed of  $\alpha$ -1,4-linked Glc residues. A low  $1\% \pm 0.5\%$  branching level was measured with greater precision through comparative fluorescence labeling of the debranched polysaccharide fraction. The size distribution of the debranched glucans displayed a pattern similar to that of the high-mass amylose present in wild-type starches (Fig. 4A). Because of both the high mass and the low  $\lambda_{\max}$  of this fraction, these results could not have been produced by contamination of low amounts of amylopectin by amylose. The size distribution, branching ratio ( $<1\%$ ), and  $\lambda_{\max}$  of the low-molecular-mass fraction were similar to those of amylose. However, upon debranching, this fraction yielded an unusual chain-length distribution that could distinguish it from standard amylose and amylopectin (Fig. 4B).

Because of the overall exceptional structure of this polysaccharide, we reasoned that it arose through a complete block in amylopectin synthesis and was probably due to the selective action of GBSSI, the amylose-biosynthetic enzyme. To test this hypothesis and to ascertain whether such a polymer is required to obtain phytoglycogen subse-



**Figure 3.** Proton NMR analysis of the *sta7* high-molecular-mass amylopectin-like component. The  $^1\text{H}$  spectrum was made in 90% deuterated DMSO, 10%  $^2\text{H}$  (Mouille et al., 1996). The position of the  $\alpha$ -1,6-specific signal is displayed by an arrow and is shown to be below the noise level ( $<2\%$ ).



**Figure 4.** Histograms of the chain-length distribution of starch-like polysaccharides obtained through gel electrophoresis of fluorescent glucans after isoamylase-mediated enzymatic debranching. Both chain-length distributions of debranched high-molecular-mass (amylopectin-like; A) and debranched low-molecular-mass (amylose-like; B) fractions are displayed as percentages of chains of DP ranging from 3 to 20. Separation of both fractions was achieved through TSK-HW-75 gel-permeation chromatography of granular polysaccharides (Fig. 2) produced in the *sta7* glycogen-synthesizing mutants.

quently, we introduced mutations inactivating selectively GBSSI and SSII in a *sta7* mutant background.

### The Amylose-Like Polysaccharide Is Not Required for Phytoglycogen Synthesis

Strain S (*sta7-4::ARG7*) was crossed with IJ2 (*sta2-29::ARG7 sta3-1*). The interesting double- or triple-mutant genotypes were detected with *trans* complementation tests (see "Materials and Methods"). We selected three genotypes for each of the following classes (Table I): wild type, *sta7*; *sta7 sta2*, *sta7 sta3*, *sta7 sta2 sta3*. The genotypes were double-checked through zymogram analysis of DBE and SS. We found no differences in behavior of the SS in a mutant *sta7* background. SSII disappeared, as expected, upon introduction of the *sta3* mutation. For each genotype class, three measurements of WSP and granular starch levels were taken on three randomly selected recombinants ( $n = 9$ ; Table II). For each class a complete WSP characterization was made, including a determination of the chain-length distribution of the novel types of phytoglycogens (Figs. 5 and 6). These were compared with the phytoglycogen produced by our reference *sta7* strains (Fig. 1, A and B).

It is clear that the absence of GBSSI because of the presence of the *sta2* mutation (*sta7 sta2*) is sufficient to lead to the disappearance of granular polysaccharides (Table II). Surprisingly, selective disappearance of SSII also led to an 85% quantitative decrease of the granular polysaccharide fraction but not to its disappearance. The residual material still displayed a high (600 nm)  $\lambda_{\max}$  upon complexation with iodine. The WSP fraction was qualitatively and quan-

**Table II.** Determination of the amounts of granular and soluble polysaccharides produced

Values are the averages of three different experiments involving three distinct segregants with the exception of the *sta2Δ1 sta3 sta7Δ7* class, which involved two distinct strains (in  $\mu\text{g}$  per  $10^6$  cells).

Genotype <sup>a</sup>			Granular Material	WSP
+	+	<i>sta7Δ7</i>	$0.12 \pm 0.01$	$0.43 \pm 0.03$
<i>sta2Δ1</i>	+	<i>sta7Δ7</i>	$0.007 \pm 0.0003$	$0.48 \pm 0.03$
+	<i>sta3</i>	<i>sta7Δ7</i>	$0.014 \pm 0.001$	$0.14 \pm 0.01$
<i>sta2Δ1</i>	<i>sta3</i>	<i>sta7Δ7</i>	$0.003 \pm 0.0002$	$0.20 \pm 0.01$

<sup>a</sup> *sta2Δ1*, *sta2-29::ARG7*; *sta3*, *sta3-1*; and *sta7Δ7*, *sta7-7::ARG7*.

titatively unaffected by the absence of GBSSI and, consequently, by that of the high-amylose granular fraction (Table II; Figs. 5A and 6A). On the other hand, the phytyloglycogen structure, the molecular mass distribution of the WSP fraction, and the amount of WSP were significantly affected by a defect in SSII (Table II; Figs. 5, B and C, and 6, B and C). A mutation in *STA3* leading to a defective SSII always resulted in reduction by one-half of the WSP content in the *C. reinhardtii* DBE-defective strains. Therefore, we conclude that *sta3* mutations are epistatic on *sta2* defects with respect to WSP amount, whereas the reverse proved to be true for the presence of the amylose-like material.

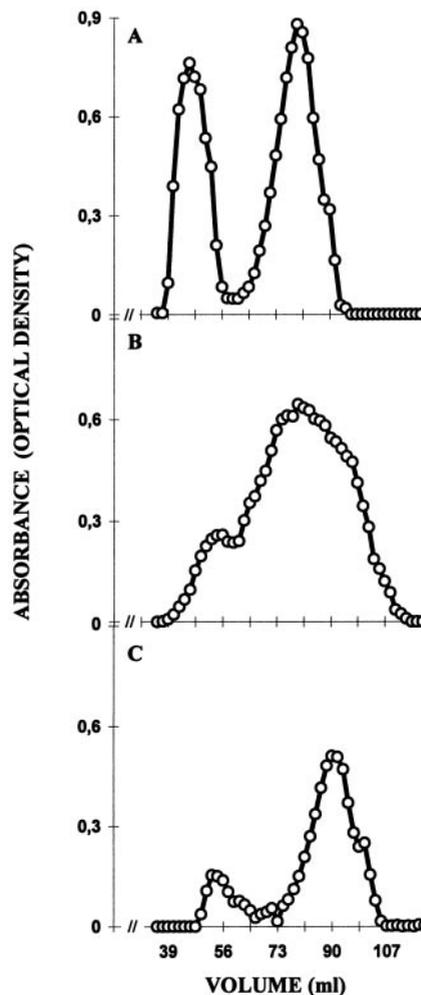
#### GBSSI Can Synthesize Amylose-Like Granular Material in the Absence of Polysaccharide Binding

In all cases in which starch synthesis was impaired in the presence of a wild-type *STA2* gene, there was a net increase of GBSSI-specific activity within the granule (Libessart et al., 1995; Van den Koornhuyse et al., 1996). A dramatic example of such an increase is provided in Figure 7A, comparing the amount of granule-bound proteins present after high or low starch synthesis occurring, respectively, under nitrogen-starvation-induced growth arrest (storage starch) or nitrogen-supplied growing conditions. These differences can be explained by the fact that an equivalent amount of GBSSI protein binds to a restricted polysaccharide amount. Because of both the low levels of granular material (0.5% of the wild-type amount) and the overrepresentation of long glucans, we expected this fraction to be filled with GBSSI protein and enzyme activity. We were surprised to find SDS-PAGE profiles generated from granule-associated proteins that could not be distinguished from those of strains completely lacking GBSSI (Fig. 7B). Moreover, the amount of GBSS activity monitored under these conditions decreased to less than 1% of the wild-type activity and was also less than those measured for strains carrying a gene-disrupted GBSSI structural gene (Fig. 7C). These results were confirmed by western blotting using an antibody directed against a C-terminal peptide sequence of higher-plant starch synthases (Fig. 7D). Western blots demonstrated the presence of a small amount of 75-kD cross-reacting polypeptide. Because SSI displays a 75-kD mass (Buléon et al., 1997), we suspect that this protein represents the residual 0.5% to 1% GBSS enzyme activity in a fashion

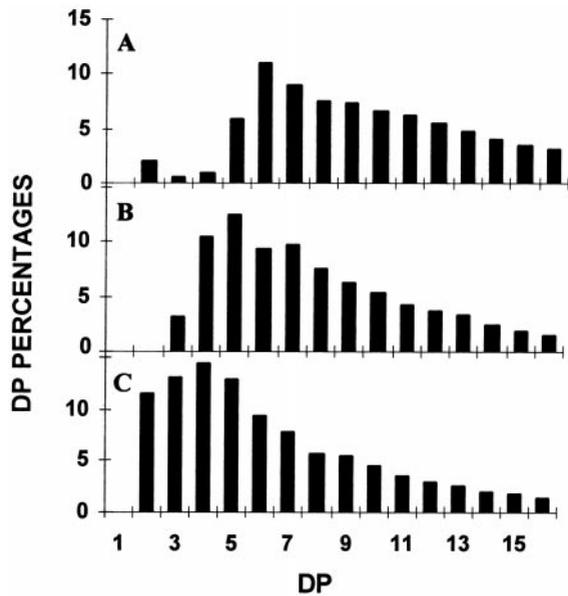
analogous to that described for the 77-kD GBSSII in potato and pea (Edwards et al., 1996). Mutants lacking both GBSSI and SSII behaved in an identical fashion. Because GBSSI and SSI display identical masses in *C. reinhardtii*, we were unable to discriminate between these two proteins and saw no convincing differences between western blots of soluble proteins extracted from wild-type and *sta7* mutants.

#### Electron Microscopy

Because both the glycogen found in animal cells and the maize phytyloglycogen were reported to display some level of organization, including rosette-like structures (Lavintman, 1966), we embarked on cytochemical observations made by transmission electron microscopy using the PATAg stain. This procedure enables the selective staining of starch granules and WSP in the wild-type and single or double mutants (Fig. 8). The wild-type cells prepared from nitrogen-starved media (Fig. 8, A and B) showed a signif-



**Figure 5.** Separation of WSP through TSK-HW-50 gel-permeation chromatography. A, *sta2 sta7* WSP. B, *sta3 sta7* WSP. C, *sta2 sta3 sta7* WSP. The x axis shows the elution volume (in milliliters), and the y axis represents the amount of Glc ([O]; in micrograms per milliliters) measured for each fraction.



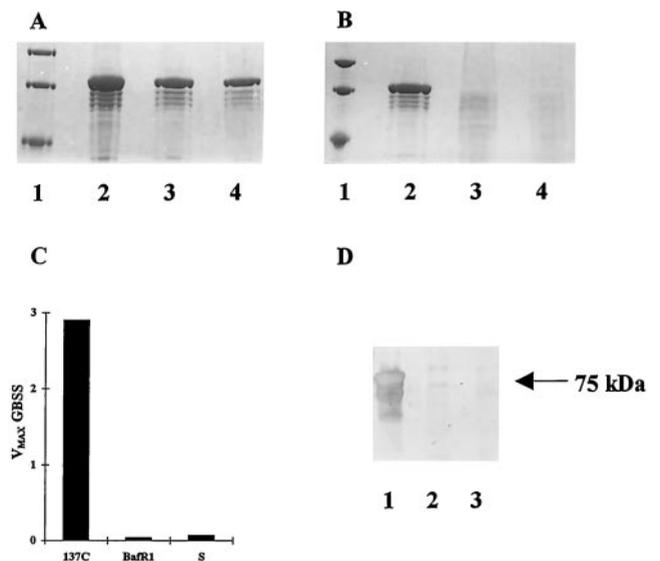
**Figure 6.** Histograms of chain-length distributions obtained through gel electrophoresis of fluorescent glucans after isoamylase-mediated enzymatic debranching of phytyglycogens produced by double or triple *C. reinhardtii* mutants. Chain-length distributions of debranched phytyglycogens from *sta2 sta7* (A), *sta3 sta7* (B), and *sta2 sta3 sta7* (C) mutant strains are displayed as percentages of chains of DP between 2 and 16. Phytyglycogen purification was achieved by taking the high-mass fraction after TSK-HW-50 gel-permeation chromatography, as displayed in Figure 5.

icant amount of starch granules, which were electron dense with the PATAg staining (Fig. 8B) and electron transparent (inverted contrast) with osmium-uranyl staining (Fig. 8A). The latter, which is used for contrasting unsaturated lipids and for proteins and cellular membranes, has no contrasting effect on polysaccharides. In both the single *sta7*-carrying mutant reference (Mouille et al., 1996) and in the double-mutant strains (Fig. 8, C–F), starch granules disappeared, whereas other polysaccharide structures were selectively revealed in the mutants through the PATAg staining procedure. These polysaccharides displayed no detectable morphological organization at this scale. According to both the biochemical and staining results, these structures correspond to phytyglycogen. This material appeared in the form of either small granular units, as in the single *sta7* mutant or in the double *sta2 sta7* mutant (Fig. 8, E and F), or in the form of elongated groups of units in the double *sta3 sta7* mutants (Fig. 8, C and D). Moreover, the size distribution of the WSP fit that which was expected for glycogen polymers. No analogous structures were ever detected in the wild-type algae. As usual, many lipid droplets were observed in all cells undergoing nitrogen starvation.

## DISCUSSION

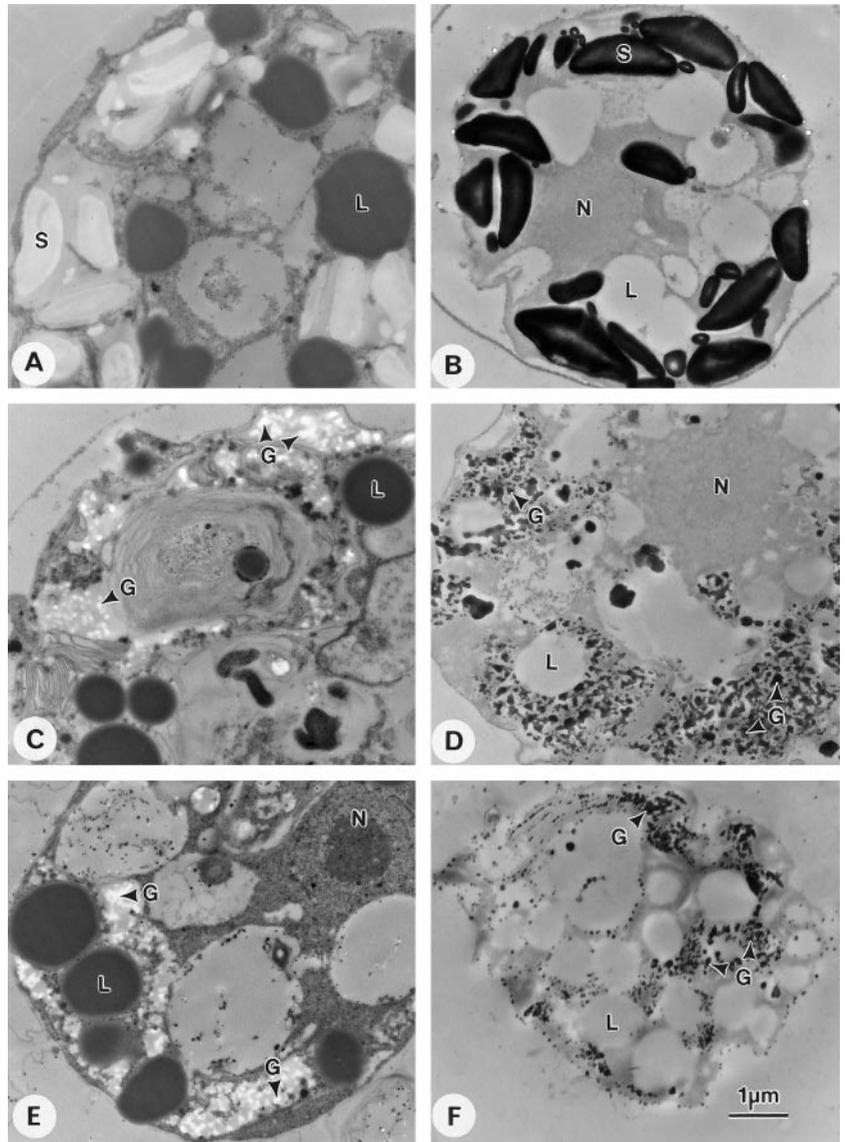
In this work we report the purification and characterization of an insoluble and dense polysaccharide that is synthesized in very low amounts in DBE mutants of *C. rein-*

*hardtii*. To account for the low  $\lambda_{\max}$  of the high-mass fraction present within this polysaccharide, we propose the existence of a high-mass network of 80 to 100 Glc residue long chains hooked together through  $\alpha$ -1,4 linkages, with occasional small branches comparable to those defined for amylose. To probe the origin of this fraction, we have introduced, in addition to the DBE defect, mutations leading to the selective inactivation of GBSSI or SSII or both of these elongation enzymes. The disappearance of the insoluble fraction correlated perfectly with that of the GBSSI activity. Because GBSSI is known to synthesize long glucans in wild-type cells, we believe that this strongly suggests that GBSSI is responsible for the synthesis of this anomalous, amylose-like material. Similar fractions have been previously observed in DBE mutants of maize. It must



**Figure 7.** Detection of GBSS activity and protein from starch-like granules of phytyglycogen-producing strains. A and B, Coomassie brilliant blue R-250 stained 5% to 7.5% SDS-acrylamide gels of starch-bound proteins. In A and B, lanes 1 display molecular-mass size standards (from top to bottom 94, 67, and 43 kD). A, Lane 4 represents starch-bound proteins extracted from 6 mg of polysaccharide purified from the wild-type strain 137C after nitrogen starvation. Lanes 2 and 3 display starch-bound proteins extracted from 6 and 3 mg, respectively, of starch purified from nitrogen-supplied wild-type cultures. The major 76-kD band corresponds to the GBSS protein and displays the typical GBSS N-terminal sequence (Delrue et al., 1992). B, Lane 2 represents starch-bound proteins extracted from 2 mg of starch purified from nitrogen-supplied wild-type cultures (strain 137C). Lanes 3 and 4 display starch-bound proteins extracted from two samples of starch purified, respectively, from nitrogen-supplied *sta7-7::ARG7* (strain S) and *sta2-29::ARG7* (strain BafR1). C, Histograms of GBSSI activity are expressed as nanomoles of ADP-Glc incorporated into glucan per minute and per milligram starch. Starch was extracted from nitrogen-starved algae. Strains BafR1 and S contain gene disruptions inactivating GBSSI and DBE, respectively. 137C is the wild-type strain. D, Western blot of starch-bound proteins extracted from equal amounts of polysaccharide. Proteins cross-reacting with antibodies directed against the C-terminal consensus peptide sequence found on vascular plant starch synthases (see “Materials and Methods”) were selectively revealed. Lanes 1, 2, and 3, Strains 137C, BAFR1, and S, respectively.

**Figure 8.** Electron microscopy of nitrogen-starved wild-type and double-mutant cells. **A**, Strain containing wild-type *STA7* showing part of the cell filled with starch granules (S) seen as electron-transparent structures of about 2  $\mu\text{m}$  in length. Lipid bodies (L), approximately 1 to 2  $\mu\text{m}$  in diameter, appear as structures highly contrasted after a double fixation of the cells by glutaraldehyde osmium and uranyl acetate post-staining. **B**, Same strain as in **A**. In comparison with **A**, contrast appears inverted because of PATAg staining of the sections used after a single fixation of the cells with glutaraldehyde. Selectively, the starch granules are deeply black and the lipid bodies are transparent. **N**, Nucleus. **C**, Nitrogen-starved double-mutant strain *sta3-1 sta7-7::ARG7*. Starch is not synthesized by this strain. Storage carbohydrate is phytoglycogen (G), which appears as very small electron-transparent inclusions (<0.2  $\mu\text{m}$  in diameter) when the cells are fixed by glutaraldehyde osmium and the sections are poststained by uranyl acetate. **D**, Same nitrogen-starved double mutant as in **C**. Phytoglycogen, when contrasted with PATAg staining, appears more clearly as very dense granular particles, either unit-like particles or associated with more or less elongated strands. **E**, Nitrogen-starved double-mutant strain *sta2-29::ARG7 sta7-7::ARG7*. Again, starch is not synthesized by this strain and the storage carbohydrate is phytoglycogen, which appears similar to that in **C** when the cells are fixed by glutaraldehyde osmium and the sections are poststained by uranyl acetate. **F**, Same nitrogen-starved double mutant strain as in **E**. Phytoglycogen appears more clearly than in **E** when the cells are contrasted with PATAg staining. However, phytoglycogen particles are only in the form of small granular units and are smaller than in **D**.



be stressed, however, that the interpretation is complicated in sweet corn by the presence of the incomplete block that is recorded on the phenotype. Therefore, in this case, it is not known whether the anomalous material comes from the solubilization of preexisting granules. This could result from the presence of a progressively more severe defect in DBE activity during kernel development. Such a temporal delay in the expression of the mutant phenotype is expected to lead to the appearance of hybrid amylose-phytoglycogen structures that were reported by Boyer et al. (1981) and by Matheson (1975). In *C. reinhardtii* the situation is much simpler in that there is no starch to start with in the mutant and none to end with after subjecting the cells to polysaccharide accumulation conditions. In this case, the anomalous, amylose-like material and the phytoglycogen are discontinuous, because we find no evidence of amylose chains in the water-soluble fraction and no traces of phytoglycogen in the insoluble material.

Mutants defective for GBSSI (and consequently with no amylose) have been described in many different plant systems. These mutants build wild-type amounts of seemingly normal organized starch granules. However, amylopectin-less mutants accumulating wild-type amounts of starch granules solely made of amylose have never been reported to our knowledge. This can be interpreted simply by stating that amylose synthesis requires the presence of amylopectin, whereas synthesis of the latter is independent of amylose.

Our data imply that this requirement comes from both the need for a primer and the need for an organized crystalline amylopectin matrix to activate the normally granule-bound enzyme. In the *sta7* (DBE-defective) mutant background, the crystallization of amylopectin is prevented and GBSSI cannot bind to the polysaccharides. In the *sta7 sta3* (DBE- and SSII-defective) double-mutant background, the synthesis of the anomalous amylose-like ma-

terial is further limited by the availability of a primer substrate that is suitable for GBSSI. Recently, we demonstrated that amylose synthesis proceeds through extension and cleavage from a preexisting amylopectin primer (van de Wal et al., 1998).

In the course of searching for the determinants of biosynthesis of the amylose-like material, we constructed double- and triple-mutant genotypes that enabled us to ascertain the origin of the phytoglycogen fraction. We were not surprised to find that GBSSI had no role in the synthesis of this fraction, which was entirely under the control of SS. Thus, synthesis of phytoglycogen is completely independent of the presence of a preexisting insoluble polysaccharide.

#### ACKNOWLEDGMENTS

We would like to thank André Decq and Jocelyn Celen for their excellent technical assistance.

Received July 21, 1998; accepted October 16, 1998.

#### LITERATURE CITED

- Ball SG, Dirick L, Decq A, Martiat JC, Matagne RF (1990) Physiology of starch storage in the monocellular alga *Chlamydomonas reinhardtii*. *Plant Sci* **66**: 1–9
- Ball S, Guan H-P, James M, Myers A, Keeling P, Mouille G, Buléon A, Colonna P, Preiss J (1996) From glycogen to amylopectin: a model explaining the biogenesis of the plant starch granule. *Cell* **86**: 349–352
- Boyer CD, Damewood PA, Simpson EKJ (1981) The possible relationship of starch and phytoglycogen in sweet corn. I. Characterization of particulate and soluble polysaccharides. *Starch/Staerke* **33**: 125–130
- Buléon A, Gallant DJ, Bouchet B, Mouille G, D'Hulst C, Kossman J, Ball SG (1997) Starches from A to C. *Chlamydomonas reinhardtii* as a model microbial system to investigate the biosynthesis of the plant amylopectin crystal. *Plant Physiol* **115**: 949–957
- Delrue B, Fontaine T, Routier F, Decq A, Wieruszkeski JM, Van Den Koornhuysse N, Maddelein M-L, Fournet B, Ball SG (1992) Waxy *Chlamydomonas reinhardtii*: monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. *J Bacteriol* **174**: 3612–3620
- Edwards A, Marshall J, Denyer K, Sidebottom C, Visser R, Martin C, Smith A (1996) Evidence that a 77-kilodalton protein from the starch of pea embryo is an isoform of starch synthase that is both soluble and granule bound. *Plant Physiol* **112**: 89–97
- Fontaine T, D'Hulst C, Maddelein M-L, Routier F, Marianne-Pepin T, Decq A, Wieruszkeski JM, Delrue B, Van Den Koornhuysse N, Bossu J-P, and others (1993) Toward an understanding of the biogenesis of the starch granule. Evidence that *Chlamydomonas* soluble starch starch synthase II controls the synthesis of intermediate size glucans of amylopectin. *J Biol Chem* **268**: 16223–16230
- Harris EH (1989a) Culture and storage methods. In E Harris, ed, *The Chlamydomonas Sourcebook. A Comprehensive Guide to Biology and Laboratory Use*. Academic Press, San Diego, CA, pp 25–63
- Harris EH (1989b) Genetic analysis. In E Harris, ed, *The Chlamydomonas Sourcebook. A Comprehensive Guide to Biology and Laboratory Use*. Academic Press, San Diego, CA, pp 399–446
- James MG, Robertson DS, Meyers AM (1995) Characterization of the maize gene *sugary1*, a determinant of starch composition in kernels. *Plant Cell* **7**: 417–429
- Lavintman N (1966) The formation of branched glucans in sweet corn. *Arch Biochem Biophys* **143**: 1–8
- Libessart N, Maddelein M-L, Van Den Koornhuysse N, Decq A, Delrue B, Mouille G, D'Hulst C, Ball SG (1995) Storage, photosynthesis and growth: the conditional nature of mutations affecting starch synthesis and structure in *Chlamydomonas reinhardtii*. *Plant Cell* **7**: 1117–1127
- Maddelein M-L, Libessart N, Bellanger F, Delrue B, D'Hulst C, Van den Koornhuysse N, Fontaine T, Wieruszkeski J-M, Ball SG (1994) Toward an understanding of the biogenesis of the starch granule: determination of granule-bound and SS functions in amylopectin synthesis. *J Biol Chem* **269**: 25150–25157
- Matheson NK (1975) The  $\alpha(1-4)(1-6)$  glucans from sweet and normal corns. *Phytochemistry* **14**: 2017–2021
- Mouille G, Maddelein M-L, Libessart N, Talaga P, Decq A, Delrue B, Ball SG (1996) Preamylopectin processing: a mandatory step for starch biosynthesis in plants. *Plant Cell* **8**: 1353–1366
- Nakamura Y, Umemoto T, Takahata Y, Komae K, Amano E, Satoh H (1996) Changes in structure of starch and enzyme activities affected by sugary mutations. Possible role of starch debranching enzyme (R-enzyme) in amylopectin biosynthesis. *Physiol Plant* **97**: 491–498
- Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* **153**: 375–380
- O'Shea MG, Morell MK (1996) High resolution slab gel electrophoresis of 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) tagged oligosaccharides using a DNA sequencer. *Electrophoresis* **17**: 681–688
- Pan O, Nelson OE (1984) A debranching enzyme deficiency in endosperms of the *Sugary-1* mutants of maize. *Plant Physiol* **74**: 324–328
- Rahman A, Wong K-S, Jane J-L, Myers AM, James MG (1998) Characterization of SU1 isoamylase, a determinant of storage starch structure in maize. *Plant Physiol* **117**: 425–435
- Simon L, Bouchet B, Caye-Vaugien C, Gallant DJ (1995) Pullulan elaboration and differentiation of the resting forms in *Aureobasidium pullulans*. *Can J Microbiol* **40**: 35–45
- Sumner JB, Somers GF (1944) The water-soluble polysaccharides of sweet corn. *Arch Biochem* **4**: 7–9
- Takeda Y, Preiss J (1993) Structures of B90 (*sugary*) and W64A (normal) maize starches. *Carbohydr Res* **240**: 265–275
- Van den Koornhuysse N, Libessart N, Delrue B, Zabawinski C, Decq A, Iglesias A, Preiss J, Ball S (1996) Control of starch composition and structure through substrate supply in the monocellular alga *Chlamydomonas reinhardtii*. *J Biol Chem* **271**: 16281–16288
- van de Wal M, D'Hulst C, Vincken J-P, Buléon A, Visser R, Ball S (1998) Amylose is synthesized in vitro by extension of and cleavage from amylopectin. *J Biol Chem* **273**: 22232–22240
- Zeeman SC, Umemoto T, Lue WL, Au-Yeung P, Martin C, Smith AM, Chen J (1998) A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell* **10**: 1699–1712