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Purification and characterisation of a novel starch synthase selective for uridine 5′-diphosphate glucose from the red alga *Gracilaria tenuistipitata*

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Abstract. Red algae (Rhodophyceae) are photosynthetic eukaryotes that accumulate starch granules in the cytosol. Starch synthase activity in crude extracts of *Gracilaria tenuistipitata* Chang et Xia was almost 9-fold higher with UDP[U-^{14}C]glucose than with ADP[U-^{14}C]glucose. The activity with UDP[U-^{14}C]glucose was sensitive to proteolytic and oxidative inhibition during extraction whilst the activity with ADP[U-^{14}C]glucose appeared unaffected. This indicates the presence of separate starch synthases with different substrate specificities in *G. tenuistipitata*. The UDPglucose: starch synthase was purified and characterised. The enzyme appears to be a homotetramer with a native M_r of 580 kDa and displays kinetic properties similar to other α-glucan synthases such as stimulation by citrate, product (UDP) inhibition and broad primer specificity. We propose that this enzyme is involved in cytosolic starch synthesis in red algae and thus is the first starch synthase described that utilises UDPglucose in vivo. The biochemical implications of the different compartmentalisation of starch synthesis in red algae and green algae/plants are also discussed.

Key words: *Gracilaria* (starch synthesis) – Rhodophyceae – Starch synthase – Starch synthesis – UDPglucose

Introduction

Intracellular storage polysaccharides are present in most living cells. The most common types are α-glucans which can be deposited as soluble or insoluble polymers. Bacteria, yeast, fungi and animal cells store glycogen, a polymer formed by α-1,4-linked D-glucose residues with numerous α-1,6-glucosidic branch points, deposited as amorphous granules in the cytoplasm. On the other hand, green algae and higher plants (Chlorophyta) accumulate starch granules in their plastids (chloroplasts). The granules are semi-crystalline, anhydrous structures formed by a mixture of an essentially unbranched α-1,4-linked D-glucose polymer (amylose) and of a larger polymer (amylopectin) with the same basic structure and frequent α-1,6 branch points. The growth of α-glucan molecules is based on enzyme-catalysed apposition of new glucose units to the nonreducing end of existing chains by α-1,4 bonds. Branches are introduced by the formation of α-1,6-bonds between existing chains by branching enzymes (EC 2.4.1.18). Chain elongation is catalysed by α-glucan synthases using the sugar nucleotides ADPglucose (ADPGlc) or UDPglucose (UDPGlc) as glucosyl donors and the EC classification of these enzymes is based on the selectivity for the glucosyl donor. For example, bacterial glycogen synthases are specific for ADPGlc (ADPGlc: α-1,4-glucan 4-α-D-glucosyltransferase; EC 2.4.1.21) and are distinct from eukaryote glycogen synthases which use UDPGlc (UDPGlc: glycogen 4-α-D-glucosyltransferase; EC 2.4.1.11). Plant starch synthases are generally specific for ADPGlc and for this reason are classified together with bacterial glycogen synthases. However, some forms of starch synthase associated with the starch granule can use both ADPGlc and UDPGlc as glucosyl donors in vitro and consequently are sometimes classified as EC 2.4.1.11. The physiological significance of UDPGlc utilisation by these enzymes has been questioned. Firstly, there is no evidence that UDPGlc is present (Gerhardt and Heldt 1984) or can be synthesised in the plastidic compartment (Nishimura and Beevers 1979; Usuda and Edwards 1980), or that it can be transported across the plastidic membrane (Pozueta-Romero et al. 1991). Secondly, granule-bound starch synthases utilise ADPGlc much more efficiently than UDPGlc (Preiss 1988 and references therein).

Abbreviations: ADPGlc = adenosine 5′-diphosphate glucose; CDPGlc = cytidine 5′-diphosphate glucose; [^{14}C]Glc1P = [^{14}C]glucose-1-phosphate; Glu6P = glucose-6-phosphate; GDPGlc = guanosine 5′-diphosphate glucose; PEG = polyethyleneglycol; PMSF = phenylmethylsulfonylfluoride; TCA = trichloroacetic acid; UDPGal = uridine 5′-diphosphate galactose; UDPGlc = uridine 5′-diphosphate glucose

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Thirdly, transgenic or naturally mutated plants which lack or have a greatly diminished ADPGlc synthesising capacity also show a severe reduction in starch content (Tsai and Nelson 1966; Müller-Röber et al. 1992). Moreover, separation of the enzyme from the starch granule and subsequent purification generally leads to the selective loss of activity with UDPGlc (Preiss 1988 and references therein). There is now a general consensus that in higher plants ADPGlc is the only glucosyl donor for starch synthases in vivo.

There are substantial structural and kinetic differences between ADPGlc- and UDPGlc-specific α-glucan synthases. No homology is found between amino acid sequences of mammalian UDPGlc: glycogen synthase and the ADPGlc: glycogen synthase of *Escherichia coli* (Leung and Preiss 1987), although some homology (ca. 30%) exists between the *E. coli* protein and plant starch synthases (Dry et al. 1992; Baba et al. 1993). Starch synthases are monomeric enzymes (Preiss 1988) and their activity is not regulated. Control of starch and bacterial glycogen synthesis resides on substrate (ADPGlc) production (Preiss and Romero 1994; Martin and Smith 1995). On the other hand, UDPGlc: glycogen synthases are oligomeric enzymes (Roach 1986) often found complexed with other enzymes (e.g. glycogenin, glycogenin synthase; Gosh et al. 1989; Nimmo et al. 1976). Moreover, UDPGlc: glycogen synthases are widely regarded as the rate-limiting enzyme in glycogen biosynthesis and their activity is modulated by allosteric mechanisms and through protein phosphorylation (Cohen 1982, 1986; Roach 1986). The many similar features showed by ADPGlc-dependent starch synthesis in the Chlorophyta and glycogen synthesis in bacteria can be attributed to the origin of chloroplasts from endosymbiotic cyanobacteria (Bhattacharya and Medlin 1995; Delwiche et al. 1995; Reith 1995).

Red algae represent a group of photosynthetic eukaryotes that, in contrast to green algae and higher plants, accumulate starch (floridean starch) in granules exclusively located outside the plastid (rhodoplast) (Pueschel 1990). Floridean starch is a major product of photosynthesis and the granules from most red algae contain only a highly branched amylopectin-like polymer (Craigie 1974 for review), although amylose has been detected in the granules of some primitive species (McCracken and Cain 1981). The pathway of floridean starch biosynthesis has received little attention. However, its unique cytosolic location raises important questions related to the nature of the α-glucan synthase involved and the glucosyl donor used for chain elongation in vivo. Zymograms of *Rhodymenia* sp. extracts have revealed two forms of α-glucan synthase utilising both ADPGlc and UDPGlc as substrates (Fredrick 1968). Nagashima et al. (1971) also reported that starch synthase in the calcareous red alga *Serraticardia maxima* was active with UDPGlc but that ADPGlc was the most efficient glucosyl donor. On the basis of their findings these authors argued that the floridean starch synthase resembles the granule-bound starch synthases of higher plants. However, these observations are based on studies with crude enzyme extracts and no detailed studies on purified starch synthase preparations from red algae are available. Here we present the results of an investigation on starch synthase activity in the red alga *Gracilaria tenuistipitata* which, under certain growth conditions, can accumulate starch granules accounting for over 80% of the cell volume (Ekman et al. 1989). We have found that UDPGlc is a considerably more effective glucosyl donor for starch synthase activity than ADPGlc in crude algal extracts. Furthermore, a unique UDPGlc: starch synthase has been purified and characterised from the alga and we suggest that it represents the first described starch synthase that utilises UDPGlc in vivo.

Materials and methods

Chemicals. Both ADP[U-^{14}C]Glc and UDP[U-^{14}C]Glc were purchased from Amersham (UK). The specific activity in experiments with [U-^{14}C]ADPGlc was adjusted for each batch to take into account the presence of a radioactive contaminant (Viola et al. 1999). Unlabelled sugar nucleotides and other chemicals were from Sigma (St. Louis, Mo., USA). Glycogen (oyster) was de-proteinised with trichloroacetic acid (TCA) prior to use (Dickey-Dunkirk and Killilea 1985). Products for column chromatography were purchased from Pharmacia (Uppsala, Sweden).

Algal material. *Gracilaria tenuistipitata* Chang et Xia (Gracilariales, Rhodophyceae) was originally collected from China in 1990 and has since been grown in unialgal culture in a cylinder system as described by Lignell et al. (1987). The culture was maintained in 17 ‰ salinity, at 22 °C with 16 h of light (150 μmol photons m^{-2} s^{-1}) and 8 h of darkness.

Enzyme extraction. Algae were ground in liquid nitrogen with a mortar and pestle and extracted in 3 volumes (w/v) ice-cold Bicine-KOH (0.1 M, pH 8) containing EDTA (1 mM), phenylmethylsulfonylfluoride (PMSF; 1 mM), benzamidine (0.5 mM), Na_2SO_4 (5 mM), DTT (5 mM) as well as other chemicals as stated. Cell walls and starch granules were pelleted by centrifugation at 16 000 *g*, 4 °C, for 20 min. The pellet was washed twice in extraction buffer and collected by centrifugation before it was used in assays. In some experiments, algae were extracted in 0.5 M Tris-HCl (pH 7.5) containing 0.5 mM cysteine and 0.5 M sucrose according to the method by Nagashima et al. (1971).

Starch granule isolation. Floridean starch granules were isolated from *G. tenuistipitata* according to the method of Yu (1992) with the exception that the starch synthase extraction buffer was used instead of distilled water during the procedure. The starch grains, forming a white band in the pellet, were collected, resuspended in the enzyme extraction buffer and filtered through two layers of Miracloth. The suspension was centrifuged at 4000 *g* for 20 min, the supernatant discarded and the pellet resuspended in enzyme extraction buffer. This procedure was repeated until the starch grain suspension appeared clean from cell wall polysaccharides and then it was passed through a Munktell filter paper No 3 (Grycksbo, Sweden) in a Büchner funnel. The starch grains were again collected by centrifugation (4000 *g*, 20 min), applied on top of a 60% sucrose solution and centrifuged at 10 000 *g* 10 min. The sucrose was discarded and the starch pellet washed with buffer and collected by centrifugation. Starch granules from tubers of *Solanum tuberosum* (cv. Record) were extracted by the method of Kuipers et al. (1992).

Radioactive assay of starch synthase activity. All assays were performed at 30 °C. Standard assays contained Bicine-KOH (0.1 M, pH 8.0), 0.5 M sodium citrate, 15 mg/mL de-proteinised glycogen, 2 mM UDPGlc or ADPGlc, 3.7 kBq UDP[U-^{14}C]Glc or

ADP[U-^{14}C]Glc and aliquots of enzyme preparation in a final volume of 0.2 mL. In some cases amylopectin (15 mg/mL) or starch granules (75 mg/mL) were used as reaction primer instead of glycogen. Alkaline phosphatase (2 U/assay; Sigma P-4252) was included in assays of crude extracts but not of purified preparations. At the end of the incubation glycogen was precipitated with 2 mL methanol-KCl (Smith 1990), incubated at −20 °C for 5 min and collected by centrifugation (3000 *g*, 5 min, 4 °C). The pellet was resuspended in 0.3 mL distilled water and the precipitation, centrifugation and resuspension procedure repeated twice more before the resuspended pellets were mixed with 5 mL scintillation cocktail and the incorporated radioactivity determined by liquid scintillation counting. For identification of the radioactive product in the washed pellets, these were resuspended into 0.2 mL of 50 mM acetate buffer (pH 4.8) containing 2 U amyloglucosidase (*Aspergillus niger*; Sigma A-7420) and incubated for 30 min at 30 °C to digest the glycogen. Subsequently, amyloglucosidase was heat-inactivated and glycogen (10 mg/mL) was added to the solution, immediately followed by 2 mL KCl/methanol. After standing at −20 °C for 15 min, the samples were centrifuged and washed as described for the standard assay, and the radioactivity in pellets and supernatants was quantified by liquid scintillation counting.

Aliquots of reaction mixtures were routinely tested for conversion of UDP[U-^{14}C]Glc into [^{14}C]glucose-1-phosphate ([^{14}C]Glc1P) by the method of Viola et al. (1994). Controls were performed with boiled extracts and all assays were performed in duplicate.

Assay of starch synthase activity by HPLC. For some kinetic determinations (e.g. primer requirement, substrate specificity) enzyme activity in purified preparations was quantified by the rate of nucleotide 5′-diphosphate (NDP) released into the reaction medium. The assay conditions were the same as for the standard assay (except for the identity of the NDPsugar and for the absence of radiolabel) and at the end of the incubation, aliquots of the reaction mixtures were boiled and NDPsugars and NDP were chromatographically separated by HPLC as described in Viola et al. (1999). The retention times of UDPGlc, ADPGlc, cytidine 5′-diphosphate glucose (CDPGlc), guanosine 5′-diphosphate glucose (GDPGlc) and uridine 5′-diphosphate galactose (UDPGal) varied under these conditions. However, baseline separation was observed between individual NDPsugars and the corresponding NDP which eluted between 1.1 and 1.8 min after the respective sugar nucleotide.

Purification of UDPGlc: starch synthase. Algae were rinsed briefly in distilled water, blotted dry and ground to a fine powder in liquid nitrogen. The powder was extracted in 3 volumes (w\v) Bicine-KOH (0.1 M, pH 8) containing DTT (5 mM), EDTA (1 mM), Na_2SO_4 (5 mM), PMSF (1 mM) and benzamidine (0.5 mM). The extract was filtered through double layers of Miracloth and centrifuged for 20 min at 16 000 *g* and 4 °C. The starch synthase activity was precipitated with glycogen (3 mg/ml) and polyethyleneglycol (PEG; MW 8000) as described by Dickey-Dunkirk and Killilea (1985) except that PEG was added to the extracts and not incorporated in the extraction medium. The glycogen pellet was resuspended in approximately 1/10 of the original volume of extraction buffer and was applied to a column (20 cm long, 2.5 cm i.d.) containing 30 mL Q-Sepharose equilibrated with buffer A (50 mM Hepes, pH 8.0; 5 mM DTT). The column was washed with buffer A until no glycogen was detected (A_{280}) in the eluant. Enzyme activity was eluted with 10 mL of buffer A containing 0.6 M KCl and subsequently diluted to 100 mL with buffer A to reduce salt strength. The starch synthase activity was once again precipitated with glycogen and PEG, resuspended, and diluted to 10 mL before application to a MonoQ column (HR 5/5) equilibrated with buffer A. Proteins were eluted with a linear gradient from 0 to 0.7 M KCl over 40 mL with a flow rate of 0.5 mL/min and 1-mL fractions collected. Enzyme activity eluted as a single peak between 18 and 31 min. Active fractions eluted from MonoQ were pooled and used for kinetic determinations.

Determination of molecular weight. Pooled MonoQ fractions were adjusted to 300 mM KCl and aliquots (200 µL) were applied to a Superose 12 column (HR 10/30), pre-equilibrated with 50 mM Hepes (pH 8), containing 5 mM DTT and 300 mM KCl. Proteins were eluted in the same buffer at a flow rate of 0.3 mL/min and 0.6-mL fractions were collected and assayed for activity. The Superose 12 column was calibrated with gel-filtration protein markers.

Electron microscopy. Fixation, embedding and electron microscopy were performed as described by Nyvall et al. (1999).

Protein determination. Proteins were measured using the BioRad Protein Assay Dye Reagent (BioRad, München, Germany) using bovine serum albumin as standard.

Electrophoresis. The protein extracts were mixed with double-strength sample buffer and boiled 5 min before application to SDS-polyacrylamide gels with a 5% stacking gel and a 10% running gel. The gels were run on a Mini-Protean II vertical gel electrophoresis cell (BioRad, München, Germany) with a discontinuous Tris-glycine buffer system (Laemmli 1970). Proteins were visualised by silver staining.

Results and discussion

The cell structure of Gracilaria tenuistipitata. The ultrastructure of *G. tenuistipitata* is characterised by a thick cell wall composed mainly by agar polymers. The rhodoplasts with unstacked, evenly spaced thylakoids, lie adjacent to the cell wall (Fig. 1). Starch granules with different shape and size are found free in the cytosol. The abundance of starch granules varies with the nitrogen status of the alga, being increased under nitrogen-limitation (Yu 1992).

Extraction and assay of starch synthase from Gracilaria tenuistipitata. The starch synthase activity of

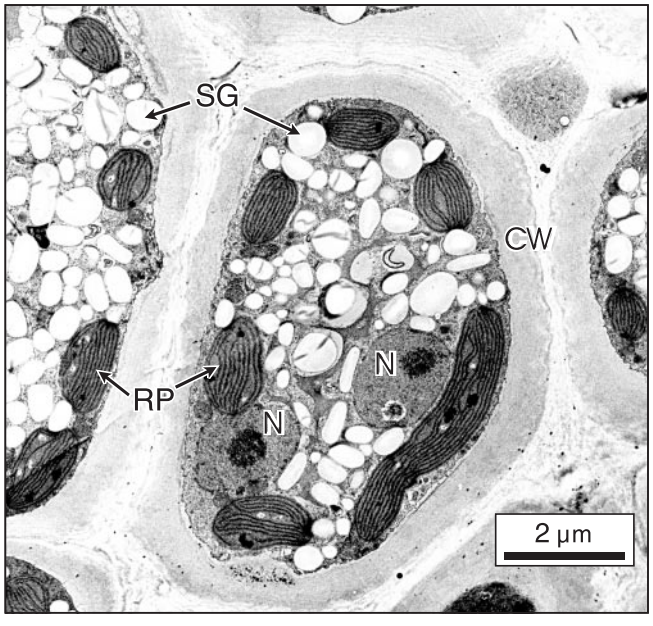

Fig. 1. Thin section of *G. tenuistipitata* thallus showing starch granules outside the rhodoplasts. *CW*, cell wall; *N*, nuclei; *SG*, starch granules; *RP*, rhodoplasts

Table 1. Effect of extraction medium on recovery of α-glucan synthase activity from *G. tenuistipitata*. Algal fronds were ground in liquid N_2 and extracted with 3 volumes of: 1) the standard medium used for plant starch synthase, i.e. 0.1 M Bicine, 1 mM EDTA, 5 mM DTT, 5 mM Na_2SO_4, 0.5 mM benzamidine, 1 mM PMSF; 2) the buffer of Nagashima et al. (1971), i.e. 0.5 M Tris-HCl, 0.5 M sucrose and 0.42 mM cysteine; 3) Nagashima's medium supplemented with 1 mM EDTA, 5 mM DTT, 5 mM Na_2SO_4, 0.5 mM benzamidine and 1 mM PMSF. Data ± SE are average of three separate extractions

Extraction medium	Fraction	Activity (nmol (g FW)$^{-1}$ h^{-1})			UDPGlc/ADPGlc activity ratio
		ADP[U-^{14}C]Glc	UDP[U-^{14}C]Glc	Total activity	
1	Supernatant	12.1 ± 3.4	104.3 ± 10.5	116.4	8.6
	Pellet	0.7 ± 0.5	10.5 ± 5.3	11.2	15.0
2	Supernatant	17.2 ± 6.1	5.7 ± 2.3	22.9	0.3
	Pellet	0.2 ± 0.2	0.1 ± 0.3	0.3	0.5
3	Supernatant	5.9 ± 2.3	96.1 ± 9.7	102.0	16.2
	Pellet	0.5 ± 0.4	8.2 ± 5.1	8.7	16.4

Table 2. Purification of UDPGlc: starch synthase from *Gracilaria tenuistipitata*

Step	Volume (ml)	Proteins (mg)	Activity (mU)	Specific activity (mU/mg protein)	Purification (-fold)	Yield (%)
Crude extract	373.0	384.2	238.2	0.6	1.0	100
Glycogen pellet 1	30.5	10.7	223.1	20.8	33.6	94
Q-Sepharose	10.0	3.8	135.5	35.5	57.2	57
Glycogen pellet 2	1.4	0.4	66.6	166.5	268.5	28
Mono Q	3.0	0.1	18.3	228.6	368.7	8

G. tenuistipitata was extracted and assayed using standard protocols for higher-plant starch synthase. Under these conditions, incorporation of UDP[U-^{14}C]Glc into α-glucan was 8.6-fold higher than with ADP[U-^{14}C]Glc and over 90% of enzyme activity was associated with the soluble fraction regardless of the substrate used (Table 1, extraction medium 1). These results contrast with a previous study with the red alga *Serraticardia maxima* in which starch synthase activity was found largely associated with the starch granules and where ADP[U-^{14}C]Glc was a better glucosyl donor than UDP[U-^{14}C]Glc (Nagashima et al. 1971). When *G. tenuistipitata* was extracted using the method of Nagashima et al. (1971), i.e. in 0.5 M Tris-HCl (pH 7.5) containing 0.5 mM cysteine and 0.5 M sucrose, total starch synthase activity (i.e. the sum of the activity with ADP[U-^{14}C]Glc and UDP[U-^{14}C]Glc) was less than 20% that obtained with our original method (Table 1, extraction medium 2). This decrease was entirely attributable to loss of enzyme activity with UDP[U-^{14}C]Glc and, as a result, the UDPGlc/ADPGlc activity ratio decreased from 8.6 to 0.3. Notably, addition of protease inhibitors and reducing agents to the Nagashima medium increased the recovery of starch synthase activity from *G. tenuistipitata* to levels comparable to our original method and restored a high UDPGlc/ADPGlc activity ratio (Table 1, extraction medium 3). These results indicate that incorporation of UDP[U-^{14}C]Glc or ADP[U-^{14}C]Glc into α-glucan is catalysed by separate enzymes in *G. tenuistipitata*. The enzyme active with UDPGlc was the most important starch synthase in this alga and appears sensitive to proteolytic and/or oxidative inactivation. The apparent instability of this UDPGlc: starch synthase during extraction suggests a substantial underestimation of its activity in previous studies with *S. maxima* (Nagashima et al. 1971) or other red algae (Fredrick 1967, 1968).

Enzyme purification. The UDPGlc: starch synthase of *G. tenuistipitata* was purified 369-fold with a final yield of 8% (Table 2). The enzyme showed strong affinity for glycogen, enabling the insolubilisation of the enzyme-glycogen complex in the presence of PEG. The yield after the second precipitation (28%) was comparable to that obtained for the purification of glycogen synthase from bovine heart with a similar method (Dickey-Dunkirk and Killilea 1985). These authors observed that the removal of glycogen from their enzyme preparations resulted in rapid loss of enzyme activity. These losses were attributable to a combination of hydrophobic adsorption of the enzyme to the ion exchanger and loss of enzyme stability upon separation of the protein from glycogen. Under our conditions almost 40% of the activity was lost in the glycogen-removing Q-Sepharose step between the two PEG precipitations. A further 70% loss of the remaining activity occurred during purification of the second PEG precipitate via analytical scale chromatography on MonoQ (Table 2). The active preparation obtained from MonoQ was subjected to size-exclusion chromatography on Superose 12 and the native molecular weight of the purified enzyme was estimated at 580 kDa (not shown). An SDS-PAGE analysis of the fractions eluted across the peak of activity revealed a correlation between enzyme activity and a polypeptide with an apparent M_r of 145 kDa (Fig. 2). This suggests a tetrameric conformation for the native enzyme. Although the enzyme preparation appeared homogeneous in some of the fractions, activity

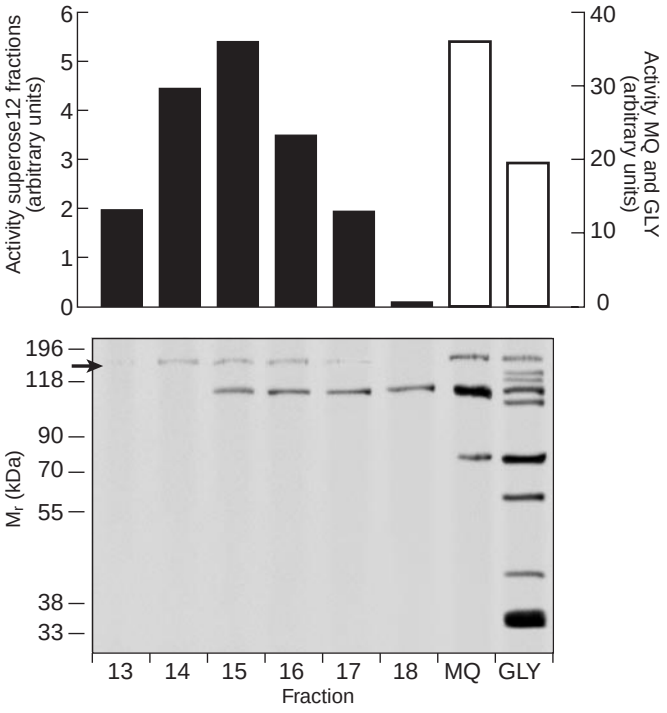

Fig. 2. Size-exclusion chromatography of partially purified UDPGlc: starch synthase from *G. tenuistipitata*. The active preparation obtained following MonoQ chromatography (see Table 1) was applied to a Superose 12 column and the fractions were assayed for enzyme activity and subjected to SDS-PAGE. For comparative purposes the enzyme activity and SDS-PAGE profile of the preparations obtained from the second PEG precipitation of the enzyme-glycogen complex (*GLY*) and of the pooled active fractions following MonoQ chromatography (*MQ*) are also shown. *Upper panel:* UDPGlc: starch synthase activity in 100 μL (fractions 13–18 from Superose 12) or 25 μL (MQ, GLY) of sample assayed for 60 min. *Lower Panel:* SDS-PAGE of fractions of upper panel. *Arrow* indicates the polypeptide band correlated with enzyme activity. Each track contains protein from 15 μL of the fraction except for the GLY and MQ lanes where 5 μL fraction was used. The M_r values (kDa) of standards are indicated

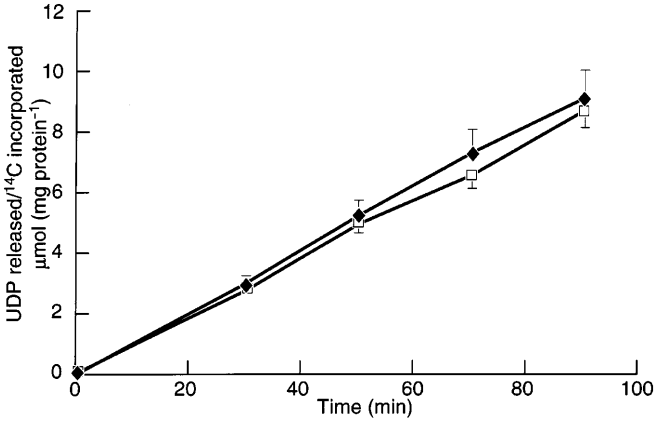

Fig. 3. Comparative analyses of the rate of UDP release (◆)and the rate of UDP[U-^{14}C]Glc incorporation into glycogen (□) during the assay of purified UDPGlc: starch synthase from *G. tenuistipitata*. The assay was carried out as described in *Materials and methods* (standard assay) using UDP[U-^{14}C]Glc as substrate and 50 μL of enzyme preparation (MonoQ fraction). At the intervals stated, the reaction was stopped by boiling for 2 min and aliquots (10 μL) applied to HPLC for analysis of UDP released. The remainder of the sample was precipitated with KCl/methanol for determination of label incorporated into glycogen. Data ± SE from 3 replicates

was always irreversibly lost within hours from elution and specific activity could not be determined accurately. For this reason the size-exclusion step has not been included in the purification schedule and kinetic characterisation of the enzyme was carried out on the pooled active fractions obtained from MonoQ chromatography.

The tetrameric conformation is also the most common of known UDPGlc: glycogen synthases (McVerry and Kim 1972). However, the molecular weight of the algal UDPGlc: starch synthase subunits is higher than that reported for UDPGlc: glycogen synthases of *Neurospora* (Takhara and Matsuda 1978), yeast (Huang and Cabib 1974), rabbit muscle (Nimmo et al. 1976), and rat liver (Rulfs et al. 1985) which range from 79 to 93 kDa. The subunit M_r of the algal enzyme is also higher than that of ADPGlc: α-glucan synthases from *E. coli* (50 kDa; Kumar et al. 1986) and a number of higher-plant starch synthases surveyed by Preiss (1988) which ranged between 60 and 95 kDa. More recently, starch synthase isoforms with M_r of 139.2 and 140 kDa have been reported in potato (Abel et al. 1996; Marshall et al. 1996) and a starch synthase cDNA with an estimated size of 188 kDa has been isolated from maize (Gao et al. 1998). All described ADPGlc: α-glucan synthases are monomeric in their native configuration.

Assay and authentication of reaction products. The potential interference by other UDPGlc-utilising enzymes during the assay of starch synthase activity in crude extracts was examined. Conversion of UDP[U-^{14}C]Glc into [^{14}C]Glc1P was observed and resulted in ^{14}C incorporation into polymeric glucan via α-glucan phosphorylase (not shown). This interference was eliminated by the inclusion of alkaline phosphatase in the assay mixture which prevented accumulation of [^{14}C]Glc1P during the course of the assay. Under these conditions, UDP[U-^{14}C]Glc incorporation into glycogen was linear with time (15–90 min) and extract volume (20–80 μL). Phosphatase was omitted in assays of purified preparations as no [^{14}C]Glc1P formation was detected. With these preparations, linearity was observed between the rate of UDP release and the rate of UDP[U-^{14}C]Glc incorporation into KCl/methanol-insoluble products (Fig. 3). The nature of the insoluble labelled products formed during the course of the reaction was further investigated. Amyloglucosidase digestion of the KCl/methanol-insoluble products released 95.9 ± 5% of the radioactivity. Addition of TCA (20% w/v) to assay mixtures at the end of incubations precipitated 8.5 ± 2% of the radioactivity insolubilised in replicates treated with KCl/methanol. These results indicate that the purified enzyme catalyses the incorporation of UDP[U-^{14}C]Glc into glycogen and not into proteins or β-glucans. It was also determined that the reaction was not affected by $MgCl_2$ or $MnCl_2$ (not shown) which are required for glycogenin-dependent

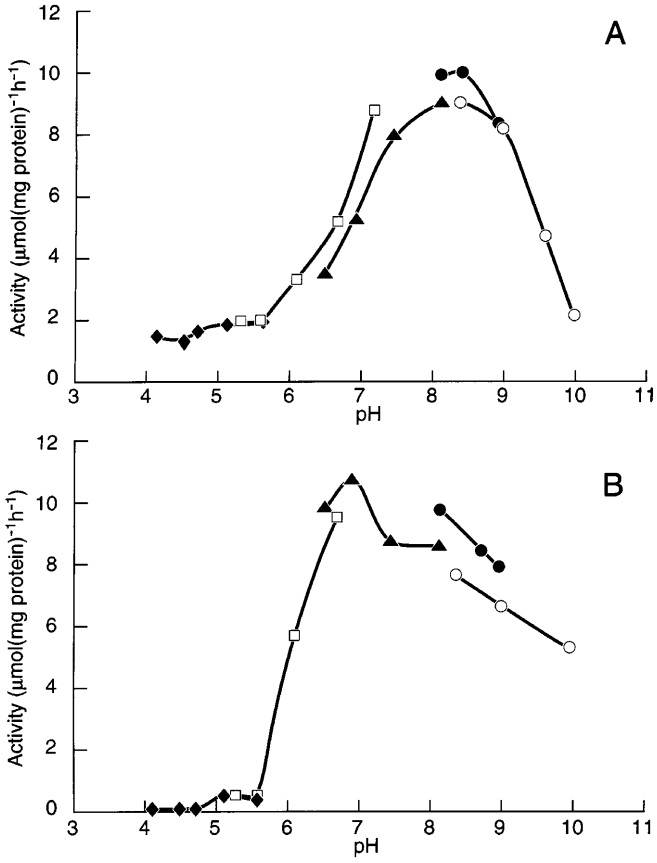

Fig. 4A,B. The effect of pH on purified UDPGlc: starch synthase activity from *G. tenuistipitata*. Assays (HPLC protocol) were carried out in the presence citrate (0.5 M) and amylopectin (**A**) or glycogen (**B**) as reaction primer. Buffers used were: acetate (◆), citrate (□), Mops (▲), bicine (●) and glycine (○) at 0.1 M final concentration. Assays were conducted in the presence of 50 μL of enzyme preparation and were stopped after 60 min. Activity is expressed as the rate of UDP released as determined by HPLC

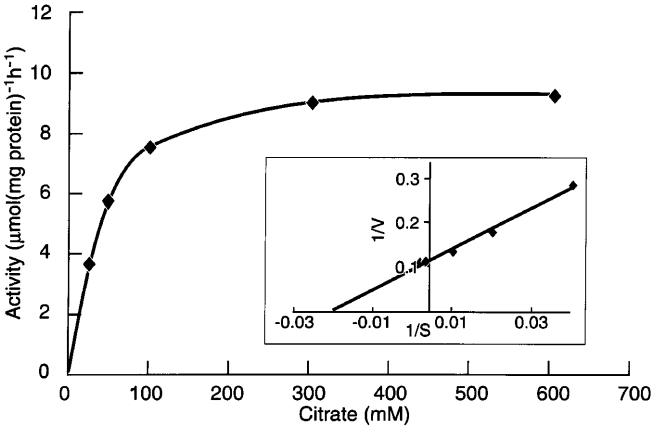

Fig. 5. The effect of sodium citrate on the algal UDPGlc: starch synthase activity. *Insert*: the corresponding double reciprocal plot. Assay conditions were the same as in Fig. 2

transglucosylation (Smythe and Cohen 1991; Ardila and Tandecarz 1992). These results indicate that the protocols used provided accurate determinations of UDPGlc: starch synthase activity of *G. tenuistipitata*.

Kinetic properties. Slight differences in optimum pH for activity were observed depending on whether glycogen or amylopectin was used as primer for the UDPGlc: starch synthase. With amylopectin, enzyme activity was maximal at pH 8.1 with sharp declines below pH 7 and above pH 9 (Fig. 4A). With glycogen, the optimum pH was broader (6.7–8.1) with activity falling rapidly below 6.5 and, more gradually, above 8 (Fig. 4B). Broadly similar pH optima have been reported for other enzymes known to be active in the cytosol of Gracilariales such as α-glucan phosphorylase (Yu and Pedersén 1991) and UDPGlc: epimerase (Prosselkov et al. 1996). For comparison, an optimal pH range between 8.5 and 10.3 has been reported for maize soluble starch synthases (Imparl-Radosevich et al. 1998) whilst optimum activity for tadpole UDPGlc: glycogen synthase was at pH 7.5 or 9.0, depending on the presence of glucose-6-phosphate (Glc6P) (Hannigan et al. 1985). The stimulation of starch synthase activity by citrate in the presence of saturating UDPGlc and glycogen concentrations is shown in Fig. 5. The concentration of citrate which resulted in half-maximal enzyme activation was 48.6 mM. For comparison, the values reported for glycogen synthase of rat liver and tadpole are 15 mM and 26.5 mM respectively (Sevall and Kim 1971; Magner and Kim 1973). In the absence of citrate, the enzyme displayed a sigmoidal saturation curve with respect to either amylopectin (Fig. 6) or glycogen (Fig. 6). In the presence of 0.5 M citrate, the saturation curve with either primer became hyperbolic. The enzyme displayed hyperbolic saturation with respect to UDPGlc with or without citrate with either glycogen or amylopectin (not shown). The K_m for UDPGlc in the presence of citrate was lower with glycogen than with amylopectin but V_{max} was similar for both primers. (Table 3). Addition of citrate reduced the K_m for UDPGlc and the concentration of primer (glycogen or amylopectin) required for half-maximal activity and increased the V_{max} of the reaction. These effects are broadly similar to those induced by citrate on UDPGlc: glycogen synthase (Sevall and Kim 1971; Magner and Kim 1973) and on plant starch synthases (Preiss 1988). The exact mode of action of citrate on α-glucan synthase is not known, but studies with UDPGlc: glycogen synthase from rat liver and tadpoles suggests that it may induce conformational changes in the enzyme (Sevall and Kim 1971; Magner and Kim 1973). The K_m for UDPGlc determined in the presence of citrate and glycogen was 0.17 mM. Similar K_m values are reported in the literature for ADPGlc: starch synthases and UDPGlc: glycogen synthases (Sevall and Kim 1971; François and Hers 1988; Preiss 1988). No information is available on the concentration of citrate or UDPGlc in *Gracilaria*.

The effect of a range of metabolites on the UDP[U-^{14}C]Glc incorporation into glycogen by purified enzyme preparations is shown in Table 4. None of the compounds tested stimulated enzyme activity, including Glc6P (up to 20 mM), a known allosteric activator of UDPGlc: glycogen synthases (Leloir et al. 1959). ATP, UTP and more effectively, UDP inhibited enzyme

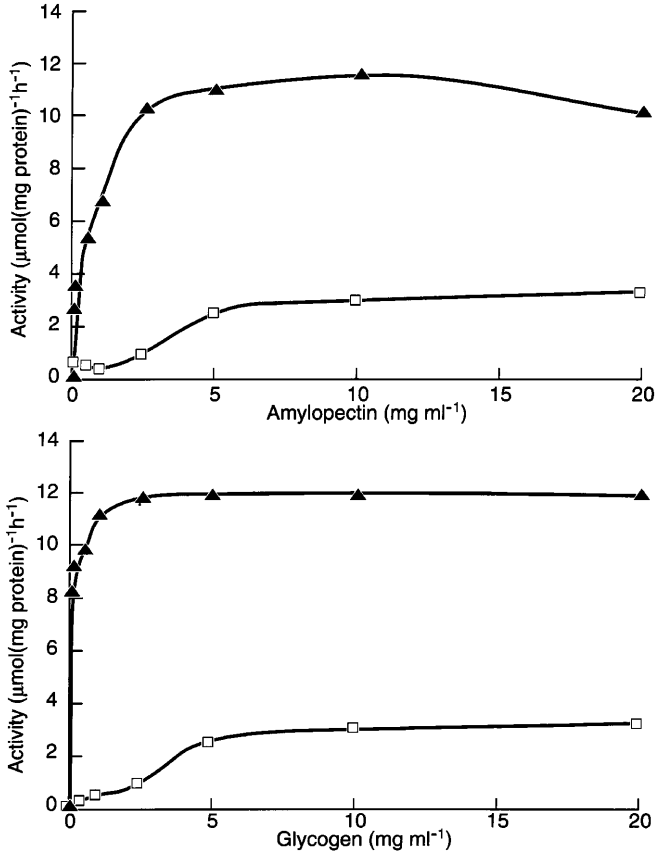

Fig. 6. The effect of citrate on the algal UDPGlc: starch synthase activity at different concentrations of amylopectin or glycogen. The partially purified enzyme was assayed with 0.5 M citrate (▲) or without citrate (□). Assay conditions were the same as in Fig. 2

activity. Product inhibition (ADP) is also observed with ADPGlc-dependent starch synthases (Preiss and Levi 1980). Importantly, the algal enzyme activity was unaffected by the presence of ADP or ADPGlc in equimolar amounts to UDP[U-^{14}C]Glc. The substrate specificity of the enzyme was determined by the rate of NDP release from a range of sugar nucleotides (Table 5). The highest rates of NDP release were observed with UDPGlc even though significant activity was also observed with GDPGlc and, to a lesser extent, with CDPGlc. These results may imply a relatively low specificity of the algal enzyme. However, inclusion of GDPGlc or CDPGlc in equimolar concentration to UDP[U-^{14}C]Glc had no effect on enzyme-catalysed ^{14}C incorporation into glycogen (see Table 4). This suggests that the observed NDP release from these sugar nucleotides by the partially purified preparation may not be attributable to starch synthase activity. Further characterisation of substrate specificity will require the use of labelled sugar nucleotides and the characterisation of the labelled product formed.

Primer requirement for enzyme activity was determined also from the rate of UDP release rather than that by UDP[U-^{14}C]Glc incorporation as only products with >25 glucose residues are insolubilised by KCl/methanol treatment (Denyer et al. 1996). Glycogen, amylopectin, soluble starch and, to a lesser extent, amylose could act

Table 4. Effect of various metabolites on partially purified UDPGlc: α-glucan synthase activity from *G. tenuistipitata*. Activity was estimated as UDP[U-^{14}C]Glc incorporation into glycogen in the presence of 0.5 M citrate. Routine assays contained 2 mM UDPGlc and metabolites at the concentrations stated. Data are expressed as a percentage of the activity observed in the absence of the metabolite and are means of three separate determinations

Substrate	Concentration (mM)	Relative activity (%)
UTP	2	79
	5	45
UDP	2	16
	5	2
ATP	2	78
	5	86
ADP	2	88
ADPglucose	2	99
GTP	2	94
GDP	2	108
GDPglucose	2	109
CDPglucose	2	100
3-Phosphoglycerate	2	89
Fructose-1,6-bisphosphate	2	104
Glucose-1,6-bisphosphate	2	111
Glucose-6-phosphate	20	101
Glucose-1-phosphate	20	88
Glycerol	5	100

Table 5. Substrate specificity of partially purified UDPGlc: starch synthase from *G. tenuistipitatata*. Activity was determined as the rate of NDP release after 1 h incubation in the presence of glycogen, 0.5 M citrate and 2 mM of each NDPsugar. Quantification of NDP released was carried out by HPLC. Data are means ± SE from three separate determinations

Substrate	Relative activity (%)
UDPglucose	100
UDPgalactose	10.1 ± 5.5
GDPglucose	28.7 ± 4.2
CDPglucose	2.5 ± 1.0
ADPglucose	3.8 ± 0.9

Table 3. Effect of citrate on kinetic parameters of partially purified UDPGlc: starch synthase activity from *G. tenuistipitata*. Data are means ± SE of three separate determinations

Primer	Citrate (0.5 M)	K_m UDPGlc (mM)	Primer concentration (μg/mL) required for half-maximal rate	V_{max} (μmol (mg protein)$^{-1}$ h^{-1})
Glycogen	–	0.69 ± 0.07	4700 ± 520	3.7 ± 0.3
	+	0.17 ± 0.02	21 ± 4	12.3 ± 1.4
Amylopectin	–	n.d.	3020 ± 180	3.0 ± 0.4
	+	0.52 ± 0.03	122 ± 15	11.7 ± 2.1

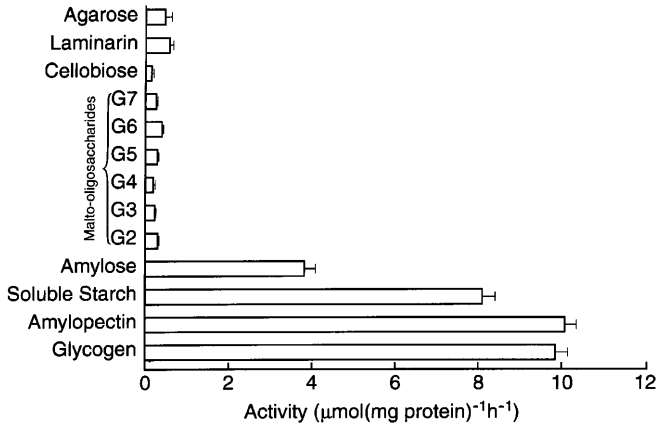

Fig. 7. Activity of UDPGlc: starch synthase in the presence of maltodextrins (G2 to G7), glucans or galactans. The concentration of primer was 10 mg mL^{-1}. Assay conditions were the same as in Fig. 2. Data are means ± SE of three replicates

as primers for the reaction while little or no activity was detected with short linear glucan chains, β-glucans or β-galactan (Fig. 7). The lack of activity with linear glucans with degree of polymerisation up to 7 suggests a requirement for an additional primer for chain initiation in vivo. Notably, native starch granules were also effective as reaction primers. Table 6 shows the incorporation of UDP[U-^{14}C]Glc and ADP[U-^{14}C]Glc into starch granules purified from *G. tenuistipitata* or potato tubers. Substantial label incorporation into the potato starch granules with both substrates was detected in the absence of the algal enzyme with both labelled substrates used. This is attributable to activity of the granule-bound starch synthase. Inclusion of algal enzyme preparation significantly increased the amount of UDP[U-^{14}C]Glc incorporated into potato or algal starch granules. The algal enzyme-dependent incorporation of UDP[U-^{14}C]Glc incorporation into algal or potato starch granules was 3.9% and 18% respectively of that into glycogen. The presence of algal enzyme stimulated ADP[U-^{14}C]Glc incorporation into floridean starch but not into potato starch granules. Incorporation of ADP[U-^{14}C]Glc into floridean starch granules was less than 50% that of UDP[U-^{14}C]Glc.

Table 6. Incorporation of labelled sugar nucleotides into native starch granules by partially purified starch synthase from *Gracilaria tenuistipitata* (+). Starch granules were purified from *G. tenuistipitata* or tubers of *Solanum tuberosum* (cv. Record) as described in *Material and methods* and used as a primer for enzyme activity. Label incorporation in the absence of algal extracts (−) and attributable to granule-bound enzyme activities was also determined. The reaction mixtures contained 60 mg starch granules/assay and were kept gently shaking during the course of incubation. Enzyme activity in the presence of glycogen as a reaction primer are also shown for comparative purposes. Data are means ± SE from three separate determinations

Primer	Enzyme preparation	Activity (μmol (mg protein) $^{-1}$ h $^{-1}$)	
		ADP[U-^{14}C]Glc	UDP[U-^{14}C]Glc
Glycogen	+	0.6 ± 0.11	11.3 ± 2.11
	−	0.1 ± 0.02	0.1 ± 0.01
Floridean starch	+	0.3 ± 0.12	0.5 ± 0.11
	−	0.1 ± 0.05	0.1 ± 0.02
Potato starch	+	27.5 ± 2.10	5.8 ± 0.91
	−	27.1 ± 1.99	3.5 ± 0.51

Multiplicity of starch synthases in red algae. Our investigations with crude extracts and purified enzyme preparations of *G. tenuistipitata* indicate the presence of separate starch synthases with different substrate specificity. There is additional evidence for the presence of ADPGlc: starch synthase in other red algae (Fredrick 1968; Nagashima et al. 1971; Sheath et al. 1979). It would be important to establish whether these enzymes operate in the same compartment. In *Porphyridium purpureum* cultures, chloramphenicol causes a decline of ADPGlc: starch synthase activity but has no effect on α-glucan phosphorylase whilst opposite results were obtained with cycloheximide (Sheath et al. 1981). Thus, it is tempting to speculate that the rhodoplast may be the site for functional assembly and activity of ADPGlc: starch synthase where it may be involved in the production of soluble α-glucans. Notably, α-glucan lyase, a novel enzyme with starch-hydrolysing activity isolated from *Gracilariopsis lemaneiformis* (Yu et al. 1993a), has also been shown to be located in the rhodoplast (Yu and Pedersen 1993). On the other hand, α-glucan phosphorylase has been immuno-localised to the surface of the starch granules in the cytosol of *Gracilaria sordida* (Yu et al. 1993b). Thus, net floridean starch biosynthesis may be controlled by the relative activity of UDPGlc: starch synthase and α-glucan phosphorylase in the cytosol of red algae. Interestingly, α-glucan phosphorylase activity from *G. sordida* is equally inhibited by low concentrations of UDPGlc and ADPGlc (Yu and Pedersen 1991), unlike the enzyme from higher plants which requires non-physiological levels of the sugar nucleotides (Steup 1988) or the enzyme from *Chlorella vulgaris* which is more sensitive to inhibition by ADPGlc than UDPGlc (Nakamura and Imamura 1983).

The different properties of the enzymes involved in α-glucan synthesis in red algae and green algae/plants may be explained by the different compartmentalisation of starch synthesis in these organisms. Phylogenetic analyses indicate an early evolutionary emergence of red algae that preceded that of plants, animals or fungi (Stiller and Hall 1997). Distinct biochemical relationships between the host and the plastid may have arisen in Chlorophyta and Rhodophyta if, as suggested by Stiller and Hall (1997), these organisms do not share a common host ancestor nor perhaps a common endosymbiont. Recently a gene encoding a starch-branching enzyme was cloned from *Gracilaria gracilis* (Lluisma and Ragan 1998). The gene sequence lacks transit peptide coding regions and phylogenetic analyses indicate that it groups with eukaryotic and not prokaryotic genes. This in turn indicates that the gene may be derived from the eukaryotic ancestor rather than from an endosymbiotic cyanobacterium.

Concluding remarks. We have shown that UDPGlc is a much better substrate than ADPGlc for starch synthesis in the floridean starch-accumulating red alga *G. tenuistipitata*. We have also provided evidence that questions the purported similarities between starch synthases of red algae and green algae/plants (Nagashima et al. 1971). The proposal that UDPGlc may be the natural substrate for starch synthesis in *G. tenuistipitata* is consistent with the cytosolic location of this pathway in red algae and underlines fundamental differences with the pathway in green algae/plants. The enzyme implicated in cytosolic starch synthesis in red algae (floridean starch synthase) apparently shares kinetic properties with both starch and glycogen synthases but has a native conformation and substrate specificity typical of UDPGlc: glycogen synthases. Thus, this enzyme may represent the first reported starch synthase that utilises UDPGlc in vivo. Further work is planned to identify other components of the starch-synthesising pathway in *Gracilaria* and to characterise the components at the molecular level.

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References

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

Ardila FJ, Tandecarz JS (1992) Potato tuber UDP-glucose:protein transglucosylase catalyzes its own glucosylation. Plant Physiol 99: 1342–1347

Baba T, Nishihara M, Mizuno K, Kawasaki T, Shimada H, Kobayashi E, Ohnishi S, Tanaka K-I, Arai Y (1993) Identification, cDNA cloning, and gene expression of soluble starch synthase in rice (*Oryza sativa* L.) immature seeds. Plant Physiol 103: 565–573

Bhattacharya D, Medlin L (1995) The phylogeny of plastids: a review based on comparisons of small-subunit ribosomal RNA coding regions. J Phycol 31: 489–498

Cohen P (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature 296: 613–620

Cohen P (1986) Muscle glycogen synthase. In: Boyer PD, Krebbs EG (eds) The enzymes, vol 17. Academic Press, Orlando, USA, pp 461–497

Craigie JS (1974) Storage products. In: Stewart WDP (ed) Algal physiology and biochemistry. Botanical monographs, vol 10. Blackwell Scientific Publications, Oxford, pp 206–235

Delwiche CF, Kuhsel M, Palmer JD (1995) Phylogenetic analysis of tufA sequences indicates a cyanobacterial origin of all plastids. Mol Phylogenet Evol 4: 110–128

Denyer K, Clarke B, Hylton C, Tatge H, Smith AM (1996) The elongation of amylose and amylopectin chains in isolated starch granules. Plant J 10: 1135–1143

Dickey-Dunkirk S, Killilea SD (1985) Purification of bovine heart glycogen synthase. Anal Biochem 146: 199–205

Dry I, Smith A, Edwards E, Bhattacharya M, Dunn P, Martin C (1992) Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. Plant J 2: 193–202

Ekman P, Lignell Å, Pedersén M (1989) Localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in *Gracilaria secundata* (Rhodophyta) and its role as a nitrogen storage pool. Bot Mar 32: 527–534

François J, Hers H-G (1988) The control of glycogen metabolism in yeast. 2. A kinetic study of the two forms of glycogen synthase and of glycogen phosphorylase and an investigation of their interconversion in a cell-free extract. Eur J Biochem 174: 561–567

Fredrick JF (1967) Glucosyltransferase isozymes in algae. Phytochemistry 6: 1041–104

Fredrick JF (1968) Biochemical evolution of glucosyl transferase isozymes in algae. Ann NY Acad Sci 151: 413–423

Gao M, Wanat J, Stinard PS, James MG, Myers AM (1998) Characterisation of *dull1*, a maize gene coding for a novel starch synthase. Plant Cell 10: 399–412

Gerhardt R, Heldt HW (1984) Measurement of subcellular metabolite levels in leaves by fractionation of freeze-stopped material in nonaqueous media. Plant Physiol 75: 542–547

Gosh P, Heath AC, Donahue MJ, Masaracchia RA (1989) Identification of a glycoprotein complex containing glycogen synthase isozyme in *Ascaris suum* obliquely-striated muscle. Comp Biochem Physiol 94B: 715–721

Hannigan LL, Donahue MJ, Masaracchia RA (1985) Comparative purification and characterization of invertebrate muscle glycogen synthase from the porcine parasite *Ascaris suum*. J Biol Chem 260: 16099–16105

Huang K-P, Cabib E (1974) Yeast glycogen synthetase in the glucose 6-phosphate-dependent form. J Biol Chem 249: 3851–3857

Imparl-Radosevich JM, Li P, Zhang L, McKean AL, Keeling PL, Guan H (1998) Purification and characterization of maize starch synthase I and its truncated forms. Arch Biochem Biophys 353: 64–72

Kuipers AG, Jacobsen E, Visser RGF (1994) Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule bound starch synthase gene expression. Plant Cell 6: 43–52

Kumar A, Larsen CE, Preiss J (1986) Biosynthesis of bacterial glycogen. Primary structure of *Escherichia coli* ADP-glucose:α-1,4-glucan, 4-glucosyltransferase as deduced from the nucleotide sequence of the glgA gene. J Biol Chem 261: 16256–16259

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Leloir LF, Olavarría JM, Goldenberg SH, Carminatti H (1959) Biosynthesis of glycogen from uridine diphosphate glucose. Arch Biochem Biophys 81: 508–520

Leung PSC, Preiss J (1987) Biosynthesis of bacterial glycogen: primary structure of *Salmonella typhimurium* ADPglucose synthetase as deduced from the nucleotide sequence of the *glgC*$_s$gene. J Bacteriol 169: 4355–4360

Lignell Å, Ekman P, Pedersén M (1987) Cultivation technique for marine seaweeds allowing controlled and optimized conditions in the laboratory and on a pilot scale. Bot Mar 30: 417–424

Lluisma AO, Ragan MA (1998) Cloning and characterization of a nuclear gene encoding a starch-branching enzyme from the marine red alga *Gracilaria gracilis*. Curr Genet 34: 105–111

Magner LN, Kim K-H (1973) Regulation of rat liver glycogen synthase. Carboxylic acid activation of liver glycogen synthetase. J Biol Chem 248: 2790–2795

Marshall J, Sidebottom C, Debet M, Martin C, Smith AM, Edwards A (1996) Identification of the major starch synthase in the soluble fraction of potato tubers. Plant Cell 8: 1121–1135

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

McCracken DA, Cain JR (1981) Amylose in floridean starch. New Phytol 88: 67–71

McVerry PH, Kim KH (1972) Dephosphorylation and aggregation of rat liver glycogen synthetase. Biochem Biophys Res Commun 48: 1636–1640

Müller-Röber B, Sonnewald U, Willmitzer L (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO 11: 1229–1238

Nagashima H, Nakamura S, Nisizawa K, Hori T (1971) Enzymic synthesis of floridean starch in a red alga, *Serraticardia maxima*. Plant Cell Physiol 12: 243–253

Nakamura Y, Imamura M (1983) Characteristics of α-glucan phosphorylase from *Chlorella vulgaris*. Phytochemistry 22: 835–840

Nimmo HG, Proud CG, Cohen P (1976) The purification and properties of rabbit skeletal muscle glycogen synthase. Eur J Biochem 68: 21–30

Nishimura M, Beevers H (1979) Subcellular distribution of gluconeogenetic enzymes in germinating castor bean endosperm. Plant Physiol 64: 31–37

Nyvall P, Pedersén M, Longcore JE (1999) Thalassochytrium gracilariopsidis (Chytridiomycota), gen. et sp. nov., endosymbiotic in Gracilariopsis sp. (Rhodophyceae). J Phycol 35: 176–185

Pozueta-Romero J, Frehner M, Viale AM, Akazawa T (1991) Direct transport of ADPglucose by an adenylate translocator is linked to starch biosynthesis in amyloplasts. Proc Natl Acad Sci 88: 5769–5773

Preiss J (1988) Biosynthesis of starch and its regulation. In: Preiss J (ed) The biochemistry of plants, vol 14. Academic Press, New York, USA, pp 181–254

Preiss J, Levi C (1980) Starch biosynthesis and degradation. In: Preiss J (ed) The biochemistry of plants, vol 3. Academic Press, New York, USA, pp 371–423

Preiss J, Romero T (1994) Molecular biology and regulatory aspects of glycogen synthesis in bacteria. Prog Nucleic Acid Res Mol Biol 47: 299–329

Prosselkov PV, Gross W, Igamberdiev AU, Schnarrenberger C (1996) Purification and characterization of UDP-D-galactose 4-epimerase from the red alga *Galderia sulphuraria*. Physiol Plant 98: 753–758

Pueschel CM (1990) Cell structure. In: Cole KM, Sheath R (eds) Biology of the red algae. Cambridge University Press, Cambridge, pp 7–41

Reith M (1995) Molecular biology of rhodophyte and chromophyte plastids. Annu Rev Plant Physiol Plant Mol Biol 46: 549–575

Roach PJ (1986) Liver glycogen synthase. In: Boyer PD, Krebbs EG (eds) The enzymes, vol 17, Academic Press, Orlando, USA, pp 499–539

Rulfs J, Wolleben CD, Miller TB, Johnson GL (1985) Immunological identification of a glycogen synthase 93,000-Dalton subunit from rat heart and liver. J Biol Chem 260: 1203–1207

Sevall JS, Kim KH (1971) Regulation of hepatic glycogen synthetase of *Rana catesbeiana*. Significance of citrate activation with reference to insulin activation of glycogen synthetase. J Biol Chem 246: 7250–7255

Sheath RG, Hellebust JA, Sawa T (1979) Floridean starch metabolism of *Porphyridium purpureum* (Rhodophyta) I. Changes during ageing of batch culture. Phycologia 18: 149–163

Sheath RG, Hellebust JA, Sawa T (1981) Floridean starch metabolism of *Porphyridium purpureum* (Rhodophyta) III. Effects of darkness and metabolic inhibitors. Phycologia 20: 22–31

Smith AM (1990) Enzymes of starch synthesis. In: Lea PJ (ed) Methods in plant biochemistry, vol 3. Academic Press, San Diego, USA, pp 93–102

Smythe C, Cohen P (1991) The discovery of glycogenin and the priming mechanism for glycogen biogenesis. Eur J Biochem 200: 625–631

Steup M (1988) Starch degradation. In: Preiss J (ed) The biochemistry of plants, vol 14, Academic Press, New York, USA, pp 255–296

Stiller JW, Hall BD (1997) The origin of red algae: implications for plastid evolution. Proc Natl Acad Sci 94: 4520–4525

Takhara H, Matsuda K (1978) Biosynthesis of glycogen in *Neurospora crassa*. Purification and properties of the UDPglucose:glycogen 4-α-glucosyltransferase. Biochim Biophys Acta 522: 363–374

Tsai C-Y, Nelson OE (1966) Starch-deficient maize mutant lacking adenosine diphosphate glucose pyrophosphorylase activity. Science 151: 341–343

Ususda H, Edwards GE (1980) Localization of glycerate kinase and some enzymes for sucrose synthesis in C_3 and C_4 plants. Plant Physiol 65: 1017–1022

Viola R, Davies HV, Sommerville LB (1994) An HPLC method for the separation of hexose monophosphates and UDPglucose from plant extracts and its use to determine specific activity in radiotracer experiments. Phytochem Anal 5: 10–14

Viola R, Nyvall P, Foster J, Sommerville LB (1999) An HPLC method for the assay of starch synthase. Phytochemistry 50: 947–951

Yu S (1992) Enzymes of floridean starch and floridoside degradation in red alga. Purification, characterization and physiological studies. Acta Univ Ups 372: 1–47

Yu S, Pedersén M (1991) Purification and properties of α-1,4-glucan phosphorylase from the red seaweed *Gracilaria sordida* (Gracilariales). Physiol Plant 81: 149–155

Yu S, Pedersen M (1993) α-1,4-glucan lyase, a new class of starch / glycogen-degrading enzyme II. Subcellular localization and partial amino-acid sequence. Planta 191: 137–142

Yu S, Kenne L, Pedersén M (1993a) α-1,4-glucan lyase, a new class of starch/glycogen degrading enzyme. I. Efficient purification and characterization from red seaweeds. Biochim Biophys Acta 1156: 313–320

Yu S, Gómez-Pinchetti J-L, Ek B, Garcia-Reina G, Pedersén M (1993b) Subcellular immuno-localization, amino acid composition and partial amino acid sequences of α-1,4-glucan phosphorylase of *Gracilaria* spp. (Rhodophyta). Physiol Plant 89: 11–20