The debranching enzyme complex missing in glycogen accumulating mutants of *Chlamydomonas reinhardtii* displays an isoamylase-type specificity

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Abstract

To investigate the functions of debranching enzymes in starch biosynthesis, we have partially purified and characterized these activities from wild type and mutant *sta7* *Chlamydomonas reinhardtii*. Mutants of the *STA7* locus substitute synthesis of insoluble granular starch by that of small amounts of glycogen-like material. The mutants were previously shown to lack an 88 kDa debranching enzyme. Two distinct debranching activities were detected in wild-type strains. The 88 kDa debranching enzyme subunit missing in glycogen-producing mutants (CIS1) is shown to be part of a multimeric enzyme complex. A monomeric 95 kDa debranching enzyme (CLD1) cleaved $\alpha$-1,6 linkages separated by as few as three glucose residues while the multimeric complex was unable to do so. Both enzymes were able to debranch amylopectin while the $\alpha$-1,6 linkages of glycogen were completely debranched by the multimeric complex only. Therefore CLD1 and the multimeric debranching enzyme display respectively the limit-dextrinase (pullulanase) and isoamylase-type specificities. Various mutations in the *STA7* locus caused the loss of both CIS1 and of the multimeric isoamylase complex. In contrast to rice and maize mutants that accumulate phytoglycogen owing to mutation of an isoamylase-type DBE, isoamylase depletion in Chlamydomonas did not result in any qualitative or quantitative difference in pullulanase activity. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Amylopectin; *Chlamydomonas reinhardtii*; Isoamylase; Pullulanase; Starch debranching enzyme

1. Introduction

Most archaeabacteria, eubacteria and non-photo-synthetic eukaryotes store glucose in the form of glycogen, an homogeneous water-soluble $\alpha$-1,4 linked polysaccharide with 8–10% $\alpha$-1,6 branches leading to a monomodal chain-length distribution (for review see [1]). Because of physical constraints, $\beta$ glycogen granules cannot exceed 25 nm in diameter, a size-limit that was clearly overcome in the plant kingdom. The solution to the problem caused by the large amounts of glucose to be stored within the plant cell came with the substitution of glycogen by insoluble macrogranular starch synthesis [2,3].

In contrast to glycogen, starch is an heterogeneous structure composed of two distinct polysaccharide fractions [4]. Amylopectin, the major
fraction of starch is composed of large molecules \((10^4 - 10^5 \text{ kDa})\) with 5% \(\alpha-1,6\) branches. Amylose is composed of smaller \((10 - 10^2 \text{ kDa})\) molecules with less than 1% \(\alpha-1,6\) branches. Amylopectin is a complex and ordered polysaccharide displaying an asymmetrical pattern of branches and a trimodal chain-length distribution.

The reasons that plants are able to synthesize such large glucans are not yet fully understood although amylpectin crystallization was lately proposed to be generated by a glucan trimming pathway [5]. This suggestion followed the discovery in maize, rice and *Chlamydomonas reinhardtii* of debranching enzyme-deficient mutants substituting starch synthesis by that of glycogen-like polymers [6--9]. In algae, this substitution was complete. Since an 88 kDa debranching enzyme (6-glucanohydrolase) was absent in the *C. reinhardtii* mutants, it was concluded that polysaccharide debranching is mandatory to obtain significant amylpectin synthesis in plants [9].

The model proposes the existence of pre-amyllopectin, a precursor whose surface is undergoing successive rounds of disorganized branching and ordered debranching [5]. Discontinuous branching and debranching cycles were first envisioned. Later refinements of this model propose that debranching enzyme merely assists the process of amylopectin crystallization by continuously cleaving off those chains which prevent proper alignment of the glucan chains. Selectivity of the debranching enzyme has been proposed to facilitate the pruning of those branched glucans that otherwise prevent polysaccharide crystallization [5].

Two types of debranching enzymes are known in plants. Limit-dextrinases (pullulanase-type) define debranching enzymes capable of cleaving pullulan, a bacterial homopolymer of maltotriosyl residues linked together by \(\alpha-1,6\) branches [10]. A limit dextrinase (EC 3.2.1.41) displays very slow and intermediate cleavage kinetics when acting respectively on glycogen and amyllopectin [10--12].

Isoamylase-type of debranching enzymes (EC 3.2.1.68) were first reported in bacteria. These are incapable of cleaving pullulan while displaying full cleavage of both amyllopectin and glycogen [12,13]. The *sul* locus of maize was shown to encode an isoamylase-type of debranching enzyme [7,14]. Despite this, the interpretation of phytoglycogen accumulation in the *sul* mutants is complicated by the observation of a simultaneous decrease of a limit-dextrinase activity not encoded by the *sul* gene [6]. Recent characterization of a rice sugary-1 allelic series hints that in cereals the severity of the sugary phenotype is equally related to that of the decrease in limit-dextrinase activity [15]. The reason for this decrease is presently not understood.

In Arabidopsis a mutant accumulating both phytoglycogen and starch was recently shown to lack a plastidial isoamylase [16] with no concomitant decrease in limit-dextrinase. In our initial characterization of the *sta7* mutants of *C. reinhardtii* we reported the absence of an 88 kDa debranching enzyme (CIS1) in all *sta7* carrying strains. Proof of the 6-glucanohydrolase (debranching) activity of the missing hydrolase was obtained through NMR characterization of the dextrin products eluted from starch filled PAGE zymogram gels [9]. However the results described do not distinguish between isoamylase and limit-dextrinase-types of enzymes. In potato, maize and rice [13,17,18] the isoamylase was shown to be part of a large multimeric complex whose precise composition remains unknown while the pullulanase behaved as a monomer in maize and rice.

We now report a more detailed investigation on the debranching enzymes of *C. reinhardtii*. A major multimeric enzyme complex \((500 \pm 100 \text{ kDa})\) is shown to contain the CIS1 88 kDa DBE subunit. In addition one minor 95 kDa debranching enzyme (CLD1) was found in these algae. The multimeric and 95 kDa DBEs were shown to harbor respectively isoamylase and limit-dextrinase-type of activities. The limit-dextrinase activity had been previously demonstrated to be entirely located within the algal plastid [19]. We report the absence of both the multimeric and CIS1 isoamylase subunit debranching enzymes in glycogen producing mutants defective for the *STA7* gene. In contrast to rice and maize mutants that accumulate phytoglycogen owing to mutation of an isoamylase-type DBE, the minor single limit dextrinase of Chlamydomonas was unaffected by the presence of a *sta7* mutation.

2. Materials and methods

2.1. Material

Sigma Chemical Co. (St. Louis, MO) supplied the apoferritin and thyroglobulin mass standards,
rabbit liver glycogen, potato amylose, pullulan from *Aureobasidium pullulans* and maize amylopectin. Phosphorylase limit dextrin was prepared from glycogen [20]. *Pseudomonas amylofera* isoamylase was from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). *Klebsiella pneumoniae* pullulanase was from Sigma Chemical Co. The catalase mass standard was from Boehringer (Mannheim, GmbH).

2.2. Strains, media, incubation and growth conditions

Our wild type (with respect to debranching enzyme activities) reference strains used in this work are IJ2 or A35 while strain S [9] was used as our reference sta7 mutant strain. Cultures were always prepared from nitrogen supplied TAP medium supplemented with yeast extracts and harvested in late log-phase (2.10^6 cells ml^-1). Media and culture conditions used in our experiments were as described in [21]. Recipes for TAP and HSA media and genetic techniques can be found in [22,23]. All experiments were performed under continuous light (40 μEm^-2 s^-1) in the presence of acetate at 24°C in liquid cultures that were shaken vigorously without CO₂ bubbling.

2.3. Enzyme purification

Crude extracts were prepared from 10 to 20 l of nitrogen-supplied cultures. Algae were ruptured by passing them in a French press (10 000 p.s.i) at a density of 10^6 cells ml^-1 and immediately frozen at −80°C. Protein content was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories GmbH, Munich). All purification steps were carried-out at 4°C. After thawing, the crude extract was subjected to a 10 000 g centrifugation for 20 min. The pellet was discarded and the supernatant was cleared by protamine sulfate precipitation (50 μl of a 10% w/v protamine sulfate solution added per ml of extract). After 15-min incubation, the supernatant obtained was precipitated with 35% saturation of ammonium sulfate and centrifuged at 10 000 g for 20 min. The precipitate was resuspended in 2 ml of Bis-Tris propane-HCl 20 mM, DTT 20 mM pH 7 buffer (buffer A) and immediately loaded on a 1 cm diameter, 60 cm long FPLC Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) gel permeation chromatography column pre-equilibrated in the same buffer. The chromatography was performed at a flow rate of 1 ml min^-1. The same S-300 column was precalibrated by subjecting 5 mg of thyroglobulin (669 kDa), apoferritin (443 kDa) and catalase (240 kDa) to the same chromatographic procedure. The position of each peak fraction was used to measure the apparent size of large-size *Chlamydomonas* proteins. Fractions (1 ml) were immediately subjected to analysis in starch containing and pullulan azure-containing zymograms (see below). The same fractions (50 μl) were tested for production of reducing ends from amylose, glycogen and pullulan (see enzyme assays). The purified fractions could be stored frozen at −80°C for up to 2 weeks without significant activity losses. The S-300 fractions containing the 88 kDa blue-staining DBE activity [9] free of amylose activity (fractions 52–65) were pooled and loaded on a FPLC UnoQ1 (Bio-rad) column equilibrated in buffer A. Elution was obtained with a linear gradient from 0 to 50% NaCl in 50 min. Again, fractions were checked for contaminating activities by zymograms and immediately frozen at −80°C. The S-300 fractions (66–89) containing pullulanase and amylose activities were subjected to the same procedure. The fractions containing the 88-kDa debranching enzyme subunit (fractions 19 and 20 of the first UnoQ1) and the pullulanase (fractions 7 and 8 of the second UnoQ1) free of contaminating activities were immediately used for debranching analysis or kept at −80°C for subsequent analysis. The pullulanase could be stored with no loss of activity for over 6 months while significant (30%) decreases of the 88-kDa debranching enzyme subunit containing fractions was evidenced after 2 weeks at −80°C. The amount of enzyme activity harvested from 20 l cultures was sufficient to perform all characterizations reported below. However this amount was insufficient to repeat the experiments from a unique cell extract.

2.4. Enzyme assays

Isoamylase activity were assayed by incubating 50 μl of the enzyme preparation in Bis-Tris propane-HCl 20 mM, DTT 2 mM pH 7 buffer containing 5 mg ml^-1 substrate (pullulan, amylopectin, glycogen, amylose) at 30°C in a final volume of 1 ml. Aliquots (100 μl) were taken and
the reaction was stopped by incubation at 100°C for 2 min. The activity was determined by measuring the increase in reducing power on the basis of the method of Nelson [24] and Somogyi [25] using maltotriose as a standard. The same procedure was used for pullulanase activity but incubation was at 35°C. Debranching analysis with commercial enzymes was monitored using the same procedure except that pullulanase was assayed in Sodium Acetate 55 mM pH 5 at 25°C and isoamylase in Sodium Acetate 55 mM pH 3.5 at 45°C. After stopping the reaction, samples were neutralized by NH₄OH before measuring the amount of reducing ends produced. Isoamylase and pullulanase activities were assayed in the presence of glycogen and pullulan respectively by the procedure described above but in the presence of various concentrations of Hydrogen peroxide.

2.5. Zymogram analysis

Zymograms in starch containing gels allowing the detection of most starch hydrolases and branching enzymes have been described for under-natured samples by Kakefuda and Duke [26] and for denatured enzymes by Mouille et al. [9].

To detect limit-dextrinase, 70 μl final volume (up to 400 μg of crude extract protein) of sample in purification buffer was loaded on a 29:1 (acrylamide:bisacrylamide) 10% (1.5 mm thick) polyacrylamide gel (mini-protean II cell (Bio-Rad)) containing 0.6% pullulan azure (Sigma Chem. Co.) ran at 20 V cm⁻¹ for 90 min in 25 mM Tris glycine pH 8.3; 1 mM DTT. Gels were incubated for 1–12 h in the same buffer. The Chlamydomonas activity could also be monitored under denaturing conditions. 70 μl of extract in purification buffer was added to 15 μl of freshly prepared 10% SDS 50% β-mercaptoethanol and boiled in a water-bath for 5 min. The experimental conditions were as before except that 0.1% (w/v) SDS was included in the gel and the migration buffer. Electrophoresis was carried out at room temperature at 15 V cm⁻¹ for 120 min. At the end of the run, the gel was washed four times with gentle shaking for 30 min in 100 ml of 40 mM Tris at room temperature to remove SDS and renaturate proteins. The gel was then incubated for 1–12 h in 25 mM Tris glycine pH 8.3 1 mM DTT. The zymogram was immediately photographed. The molecular mass of the enzyme activity detected on zymogram was measured on the same polyacrylamide gel. Some sections of the gel were stained with Coomassie Brilliant Blue while others were renatured and incubated as described above.

2.6. NMR analysis

Nuclear magnetic resonance analysis was performed with the same set-up and conditions as those described in Fontaine et al. [27]. The level of branching was estimated by integration of the same regions of proton resonance of the monosubstituted and disubstituted glucose (δ, 5.2 and 4.9 ppm, respectively) [28]. Reducing end signals appeared in the dextrin samples. To estimate the percentage of reducing ends, we integrated the signals due to the α (5.1 ppm) and β (4.5 ppm) anomeric forms of the reducing ends with respect to same region of the monosubstituted glucose proton resonance.

3. Results

3.1. Wild-type strains of C. reinhardtii contain at least two kinds of debranching activities

Crude extracts from late log-phase algae (20 l) were subjected to protamine and ammonium sulfate precipitations, gel filtration on sepharose S-300 columns followed by UnoQ-mediated anion exchange. Since a limit-dextrinase activity had been reported previously by Levi and Gibbs [19], we followed pullulan degradation on pullulan azure containing gels. The latter always displayed a 95 kD limit dextrinase activity (shown in Fig. 8). Other zymograms performed in starch-containing gels subsequently stained with iodine allowed the detection of both an 88 kDa blue staining and a 53 kDa white staining band. These were previously shown to respectively contain debranching and α-amylase enzyme activities [9].

Results are summarized in Table 1 and Table 2 and Fig. 1. The zymogram data are not shown but are summarized by a schematic drawing at the bottom of Fig. 1. Position of size standards is highlighted (Fig. 1). It is clear that the limit-dextrinase (pullulanase) activity elutes with the bulk of the other starch hydrolases in a single wide peak (fractions 66–89) on the S-300 column precluding any significant enzymological characterization to
be made (Fig. 1). The activity containing the 88 kDa debranching enzyme (fractions 52–65) eluted as a large size complex whose apparent mass was estimated at 500 ± 100 kDa. The complex was purified 10-fold and can be considered free of other interfering activities with the exception of a small amount of trailing limit dextrinase that could be detected on pullulan azure containing gels but not on starch containing gels.

A second chromatographic step through the UnoQ column increased the purity of both activities (Table 1 and Table 2). Fig. 2A displays the level of purification of the 88 kDa debranching enzyme subunit that was achieved in these experiments. In zymograms performed without denaturation [24] we systematically observed three blue activity bands (Fig. 2B) co-eluting with the single 88 kDa band (CIS1) appearing after denaturation. It remains possible that some DBE subunits do not refold properly after denaturation and that several distinct DBE subunits are visualized under native conditions. Yields and purification factors were calculated for both the limit dextrinase and the CIS1-containing enzyme complex activities (see Section 2). A sum of 5.7% of the total limit-dextrinase activity was recovered after two-chromatographic steps. The activity was stable after freezing for up to 6 months. Yield and purification factors of 2.5% and 140-fold, respectively were measured for the 88 kDa debranching enzyme subunit-containing activity. This is probably an underestimate since the bulk of the activity measured in crude extracts is due to other hydrolases such as α-amylase. This debranching activity was lost within a few hours in buffers containing less than 1 mM DTT and could not be recovered even after prolonged incubation in up to 20 mM DTT. The enzyme proved to be very sensitive to oxidation.

The sensitivity of both enzymes towards hydrogen peroxide is compared in Fig. 3. Because of their distinctive elution patterns and behavior we could already conclude that the limit-dextrinase and the CIS1 DBE containing complex were distinct activities. The stable limit-dextrinase at best afforded for very faint bluish bands that could only be seen on starch-containing zymograms after purification and concentration but never in crude extracts.

### Table 1

<table>
<thead>
<tr>
<th>88 kDa Debranching enzyme purification</th>
<th>Yield (%)</th>
<th>Purification factor</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>100</td>
<td>0</td>
<td>322</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>65</td>
<td>1.3</td>
<td>210</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>40</td>
<td>1.6</td>
<td>130</td>
</tr>
<tr>
<td>Gel permeation (fractions 52–65)</td>
<td>17</td>
<td>9.9</td>
<td>55</td>
</tr>
<tr>
<td>Anion exchange (fractions 19–20)</td>
<td>2.5</td>
<td>140</td>
<td>8.2</td>
</tr>
</tbody>
</table>

* Purification was from $4 \times 10^{10}$ cells. Debranching activity corresponds to μmoles of maltotriose equivalents produced per hour from amylopectin for the whole fraction under study (see Section 2). The activity was measured without interference of other hydrolases after the gel permeation step. Debranching activity in crude extracts or after both precipitation steps is skewed by the presence of α amylase.

### Table 2

<table>
<thead>
<tr>
<th>95 kDa Limit dextrinase purification</th>
<th>Yield (%)</th>
<th>Purification factor</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>100</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>93</td>
<td>1.9</td>
<td>13</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>86</td>
<td>3.4</td>
<td>12</td>
</tr>
<tr>
<td>Gel permeation (fractions 66–89)</td>
<td>79</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>Anion exchange (fractions 7–8)</td>
<td>5.7</td>
<td>180</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Purification was from $4 \times 10^{10}$ cells. Debranching activity corresponds to μmoles of maltotriose equivalents produced per hour from pullulan for the whole fraction under study (see Section 2).
Fig. 1. Purification of Chlamydomonas debranching enzymes. A. 400 mg of crude protein was loaded on a S-300 HR (1 × 60 cm) gel filtration FPLC. Fractions of 1 ml were collected at a rate of 1 ml min⁻¹. Proteins were assayed using the Bio-Rad determination kit (broken thin line). We have drawn the chromatograph section containing the relevant DBE activities. The α-amylase activity peak tails off at fraction 110. Estimates of DBEs and α-amylase were obtained by assaying the reducing ends (nmoles maltotriose produced per hour and for the whole fraction studied) upon incubation of 20 µl of each fraction with pullulan (●), glycogen (▲) and amylose (○) (see Section 2). Note that glycogen and amylose will generate reducing ends from both amylase and isoamylase activities. Therefore the beginning of the amylose digestion will not necessarily precisely coincide with the beginning of the α-amylase peak. Enzyme activities were localized by loading 50 µl of each fraction on both native and denaturing starch containing PAGE zymogram gels and on pullulan azure containing PAGE gels. The nature of the 88 kDa and 53 kDa activity bands activity bands had been previously ascertained by proton NMR of the eluted dextrins [9]. The nature of the pullulanase band has been ascertained by correlation to standard activity assays (such as production of reducing ends from pullulan) on the peak fractions. The position of each activity as revealed by zymograms is illustrated by lines whose thickness reflects the intensity of the zymogram bands. The 95 kDa limit dextrinase band intensity cannot be compared to the DBE, or amylase bands since it was revealed on pullulan-azure gels. The column was pre-calibrated with the 669, 443 and 240 kDa mass standards corresponding respectively to thyroglobulin, apoferritin and catalase. The arrows correspond to the peak fraction position for each of the proteins.

3.2. Characterization of the 88 kDa subunit containing debranching enzyme complex

We tested the activity of the 88 kDa subunit containing debranching enzyme complex towards four different substrates. These included pullulan, amylopectin, amylose and glycogen. The pooled peak fractions from the UnoQ1 anion exchange step were used for these studies (fractions 19 and 20 in Table 1). Results obtained with the debranching enzyme are displayed in Fig. 4A and can be compared to those obtained with the *Pseudomonas amylofermentans* isoamylase (Fig. 4C) and the *Klebsiella pneumoniae* pullulanase (Fig. 4D). It is evident that because of both the limiting amounts of enzyme activity available from 20–40 L cultures and because of the sensitivity of the enzyme to oxidation we have obtained only 50–75% of the level of polysaccharide debranching obtained through commercial enzymes. To ensure that the Chlamydomonas enzyme was able to digest over 80% of the branches hydrolyzed by the commercial enzyme references, we have devoted a 40 l culture to the sole digestion of 5 mg of glycogen, pullulan and amylopectin in 12 h. The

Fig. 2. Zymogram analysis of the semi-pure 88 kDa polypeptide-containing DBE complex. A. Denatured purified (fraction 19 of the anion exchange) (lane 1) and denatured crude extract (lane 2) were loaded on a starch-containing gel according to the procedure detailed in [9]. After renaturation, the gels are flooded with an iodine-containing buffer. Those enzymes that are able to modify the structure of starch within the gel will alter the iodine staining properties accordingly. B. The undenatured samples (80 µl) from fraction 52–59 of the S300 GPC separated by PAGE under native conditions were blotted onto starch-containing gels according to Kakefuda [26].
Fig. 3. Hydrogen peroxide inhibition of Chlamydomonas DBEs 5 mg of either pullulan or glycogen were digested with 50 µl (the activities increased proportionally when 10–950 µl peak fraction volumes were used) of fraction containing, respectively the 95 kDa limit dextrinase (●, fraction 7 from the anion exchange (Table 2) and the semi-pure 88 kDa polypeptide-containing DBE complex (▲, fraction 20 from the anion-exchange (Table 1)) in a final volume of 1 ml. If the hydrogen peroxide concentration was raised to 2 mM the limit dextrinase and the semi-pure 88 kDa polypeptide-containing DBE complex displayed respectively 100 and 8% residual activity.

unincubated polysaccharides were compared to the debranched products through proton NMR analysis (Fig. 5) which directly demonstrated elimination of α-1,6 linkages. It is clear that the 88 kDa enzyme activity hydrolyses the branches of amylopectin to near completion (over 80%) whereas pullulan is not a substrate. Similar results were obtained with glycogen (data not shown). We therefore conclude that the CIS1 containing debranching enzyme complex displays an isoamylase-type of specificity. Glycogen phosphorylase limit dextrin was also tested because the Escherichia coli debranching enzyme purified by Jeanningros et al. [29] cleaves the α-1,6 branches of glycogen phosphorylase limit dextrin 100-fold faster than that of intact glycogen. However no significant differences were found in the initial rates of polysaccharide debranching when these substrates were compared (Fig. 6).

3.3. Characterization of the pullulanase

Fractions 7–8 of the UnoQ1 column, which were used for further characterizations, still contained 5.7% of the measurable crude extract activity and were purified 180-fold. We repeated the analysis performed on the CIS1 containing debranching enzyme complex (Fig. 4A) with fractions 7–8 containing the pullulanase activity (Fig. 4B) and compared this to the results obtained with the P. amylofermentum isoamylase (Fig. 4C) and the K. pneumoniae pullulanase (Fig. 4D). It is clear that the Chlamydomonas enzyme can debranch pullulan (Fig. 4B). It also cleaved amylopectin (Fig. 4B), as expected for a plant limit-dextrinase. It is remarkable that both the Chlamydomonas limit-dextrinase and the Klebsiella pullulanase were able to cleave some of the branches of glycogen. However this digestion remained limited and glycogen was never debranched to completion. Cleavage of pullulan was obtained and yielded the appearance of numerous reducing ends detected by proton NMR (Fig. 7). Selective hydrolysis of the α-1,6 linkage was evidenced by a specific decrease of the α-1,6 proton NMR signals although the absolute level of pullulanase activity in the fractions used in this study proved insufficient to cleave 5 mg of pullulan, amylopectin or glycogen to completion within 12 h (Fig. 7). We further characterized the pullulanase from crude or purified extracts by performing zymograms in pullulan azure-containing gels. We found a perfect correlation during purification between the zymogram and pullulan debranching enzyme assays. We used both native or denatured extracts with subsequent PAGE respectively without or with SDS. The denatured proteins were allowed to renature before incubation. This denaturation-renaturation step induced a two to threefold reduction in enzyme activity. Under native conditions a unique major band was always observed after purification, while in crude extracts a very faint additional slower migrating smear was irregularly observed. Under denaturing conditions and upon renaturation a single sharp activity band was systematically observed. The high quality of these zymograms enables us to report a 95 ± 2 kDa mass for the C. reinhardtii pullulanase-type of debranching enzyme. This activity will be referred to as CLD1.

3.4. CLD1 is unaffected by the absence of a functional STA7 gene

The data shown here and our previous studies demonstrate that STA7 is required for the pres-
ence of multisubunit isoamylase-type 6-glucanohydrolase enzyme complex. Because maize and rice mutants with such a mutation exhibit pleiotropic defects in the pullulanase-type of activity [6,8,17], we proceeded to characterize the debranching enzymes from all our sta7 defective mutants. In addition to the seven previously described alleles that were generated by insertional mutagenesis we have selected a novel (sta7-8) allele through standard UV mutagenesis. The mutants displayed identical phenotypes and lacked both the CIS1 subunit detected through denaturing zymograms and the large size multimeric debranching enzyme complex as evidenced by zymograms from undenatured extracts blotted according to Kakefuda et al. [26]. Zymogram analysis performed on pullulan azure-containing PAGE gels failed to display any modification of CLD1. In addition the meiotic progeny of a cross involving strain S (containing the sta7-4::ARG7 disruption) and a wild-type strain was subjected to zymogram analysis under native or denaturing conditions. No marked quantitative or qualitative difference could be detected between the sta7- and the STA7 segregants (Fig. 8). Moreover the faint slow-migrating smear of pullulanase activity was detected irregularly in both wild type and mutants. To ensure that no small quantitative activity modifications existed between wild type and mutant populations we confirmed the zymogram results by quantitative pullulanase assays. We measured pullulanase activities in a population of 10 wild-type (with an

![Fig. 4. Substrate specificity of debranching enzymes. A. 5 mg of substrate polysaccharide were digested with 50 μl of fraction containing the semi-pure 88 kDa polypeptide-containing DBE complex of Chlamydomonas (fraction 19–20 from the anion exchange (Table 1)) in a final volume of 1 ml. 50 μl of sample was subjected at various time to the reducing end assay as described in Section 2. Substrate polysaccharide include pullulan (●), maize amylopectin (■), potato amylose (○) and rabbit liver glycogen (▲). B. Five miligrams of substrate polysaccharide was digested with 50 μl of fraction (the activity increased proportionally when 50–750 μl peak fraction volumes were used) containing the semi-pure 95 kDa Chlamydomonas limit-dextrinase (fractions 7–8 from the anion exchange (Table 2)) in 1 ml final volume. Fifty microlitres of sample was subjected at various times to the reducing end assay. Substrate polysaccharide symbols are as described above. C. Five miligrams of substrate polysaccharide was digested with 20 units of reference Pseudomonas amyloferans isoamylase in 1 ml final volume. Fifty microlitres of sample was subjected at various time to the reducing end assay as described in Section 2. D. Five miligrams of substrate polysaccharide was incubated with 0.3 units of reference K. pneumoniae pullulanase in a 1 ml final volume. Fifty microlitres of sample was subjected at various time to the reducing end assay as described in Section 2. Substrate polysaccharide symbols are as described above.]
Fig. 5. Proton NMR spectra of amylopectin digested with the 88 kDa debranching enzyme. Part of the $^1$H NMR spectra of amylopectin in dimethyl-sulfoxide-$d_6$/D$_2$O (80:20) at 80°C is displayed. The chemical shifts for the $\alpha$ and $\beta$ anomers of the reducing end are respectively at 5.1 and 4.5 ppm and are displayed as H1$_\alpha$ and H1$_\beta$. The arrow display the signal corresponding to the anomeric proton of carbons engaged in an $\alpha$-1,6 linkage. Results obtained are analogous to those previously reported [9]. A. Undigested amylopectin; B. Amylopectin subjected to overnight digestion with 20 units of commercial Pseudomonas isoamylase; C. Amylopectin subjected to overnight digestion with 200 ml of the 88 kDa DBE from fraction 19–20 of the UnoQ as described in Section 2.

average and standard deviation respectively of 16.9 and 1.6 nmoles maltotriose equivalents produced per hour per mg protein) and 10 mutant (with an average and standard deviation, respectively of 15.9 and 1.1 nmoles maltotriose equivalents produced per hour per mg protein) segregants from the same cross in three series of experiments. No significant differences were found. To further prove the absence of subtle modifications of CLD1, we analyzed the behavior of this activity under our semi-purification conditions and found no significant modification. We therefore conclude that in *C. reinhardtii* the pullulanase activity is not significantly affected by the presence of a defect leading to phytoglycogen production.

4. Discussion

As was shown first in potato tubers [30], in the rice and maize endosperm [31,32] and more recently in pea embryos [12] and Arabidopsis leaves [16], at least two distinct types of starch debranching enzymes were found in *C. reinhardtii*. We provide evidence for the existence of a multimeric enzyme containing the CIS1 DBE subunit while the behavior of the CLD1 DBE is consistent with that of a monomeric protein. We show that the multimeric enzyme displays an isoamylase-type of cleavage specificity while the CLD1 minor activity displays the classical limit-dextrinase-type of activity. This situation is reminiscent of that initially reported for potato tubers [13] and more recently for maize and rice [17,18]. In the case of potato and rice the activity was purified to homogeneity and the protein composition of the complex analyzed in detail [13,18]. In potato two proteins of 95 and 83 kDa were separated from the complex but were not further characterized [13]. However the apparent mass of the isoamylase complex was estimated at 520 kDa. In rice one major and one minor protein was purified from the complex by

![Fig. 6. Relative activities of the Chlamydomonas isoamylase with respect to rabbit-liver glycogen and to glycogen phosphorylase limit dextrin. Glycogen (5 mg ml$^{-1}$) (▲) and glycogen phosphorylase limit dextrin (●) were incubated with 50 μl of the 88 kDa glucanohydrolase from fraction 19–20 of the UnoQ as in Fig. 4A.](image-url)
Fig. 7. Proton NMR spectra of pullulan digested partially or to completion with the Chlamydomonas 95-kDa glucanohydrolase and the *Klebsiella pneumoniae* pullulanase. Part of the $^1$H NMR spectra of amylpectin in dimethyl-sulfoxide-$d_6$/D$_2$O (80:20) at 80°C is displayed. The chemical shifts for the $\alpha$ and $\beta$ anomers of the reducing end are respectively at 5.1 and 4.5 ppm and are displayed as H$_1\alpha$ and H$_1\beta$. The $\alpha$-1,6 linkage anomic proton is displayed by an arrow at 4.85 PPM. The integration of the $\alpha$ and $\beta$ anomers reducing end signals matched closely the decrease recorded in the 4.85 ppm signal area. The origin of the small signals around 4.78 ppm and 4.47 ppm is not known and could reflect specific partially digested products. A. Undigested pullulan. B. Pullulan digested to completion with the *Klebsiella pneumoniae* pullulanase (0.3 units in 2 ml containing 10 mg pullulan) as described in Section 2. C. Pullulan subjected to partial (12 h) digestion with the 95-kDa Chlamydomonas glucanohydrolase as described in materials and methods.

Fig. 8. Limit-dextrinase activity in wild-type and mutant *sta7* offspring. 100 µg of crude extract were loaded in a pullulan azure-containing PAGE gel and subjected to electrophoresis in native conditions as described in Section 2. The smear of activity on top of the gel results from substrate degradation during migration. Lanes 1, 3, 5 and 7 and lanes 2, 4, 6 and 8 display the activities respectively from 4 wild-type and mutant recombinants from a cross between a wild-type (strain A35) and a mutant strain (strain S containing the *sta7*-4::*ARG7* gene disruption) lacking the 88 kDa isoamylase.

2D-PAGE. Both proteins displayed the same N-terminal amino acid sequences and similar peptide maps upon digestion with *Staphylococcus aureus* V8 protease. The apparent mass of the rice complex was estimated between 340 to 490 kDa depending on the GPC column used. Both the values published for potato or those estimated for rice are within the size-range we now report for Chlamydomonas. The pure rice isoamylase complex systematically yielded three to four blue bands upon analysis in starch containing zymogram gels [18]. We have observed the very same behavior for the algal isoamylase complex although the enzyme was not pure enough to investigate precisely the subunit composition. Also shared by other vascular plant enzymes is the exquisite sensitivity of the isoamylases to oxidation. This sensitivity could explain the special behavior and the multiple activity bands seen in zymogram gels. On the other hand the redox state of the isoamylase might be of functional relevance. Indeed Fu et al. [33] have recently demonstrated that potato tuber ADP-glucose pyrophosphorylase can be activated by thioredoxin thereby increasing the sensitivity of the enzyme to 3-PGA activation. Such a regulation could be at work at least in the chloroplast of leaf cells and in the Chlamydomonas plastid. We have been however unable to recover activity after oxidation of the isoamylase complicating thus future investigations on this issue. Of particular significance in this respect is the recent finding that transgenic barley expressing potato thioredoxin have increased levels of limit dextrinase activity [34].

It also remains possible that in rice and other plants the multimeric enzyme is composed of distinct yet related isoamylase subunits. As was demonstrated for plant ADP-glucose pyrophosphorylases, subunits with related primary sequences can take over a predominantly catalytic or regulatory function. In maize the *stu-1* gene product is able to function on its own since activity was successfully recovered upon expression in the *E. coli* cell [14].

The work reported here establishes that the 88 kDa 6-glucanohydrolase (CIS1) lacking in the *sta7* mutants of Chlamydomonas is part of a multimeric enzyme belonging to the isoamylase-type class of activity. If the *STA7* gene codes for a protein within this complex then the latter would have to be a component essential for catalysis
since all of the zymogram bands disappear in the presence of a \textit{sta7} mutation. All \textit{sta7} mutants have replaced starch synthesis by that of a small amount of glycogen-like material [9]. This defines debranching through isoamylase as a mandatory step of starch biosynthesis. The picture that is now emerging from the study of the glycogen-producing mutants of plants is that they all lack a particular form of isoamylase-type of debranching enzyme. However the interpretation of the phenotype recorded in cereals is further complicated by the observation of a concomitant decrease of limit-dextrinase activity. Recent evidence gathered from the analysis of \textit{sugary-1} mutants of rice suggest that the limit-dextrinase might play an important role in the expressivity of the sugary phenotype [15]. In \textit{C. reinhardtii} our data suggest the presence of a single pullulanase isoform with a mass similar to those reported for the vascular plant enzymes [8,17]. The insensitivity of this activity to the presence of a mutated \textit{STA7} locus distinguishes \textit{C. reinhardtii} from both maize and rice. Zeeman et al. [16] also failed to detect decreases of limit-dextrinase activity in the isoamylase-defective mutants of Arabidopsis. In vascular plant mutants, the appearance of phytoglycogen is not accompanied by the disappearance of starch but rather by its quantitative decrease. It is interesting however that from the zymograms published by Zeeman et al. [16] the levels of limit-dextrinase assayed with starch or amylopectin are comparable to those of the Arabidopsis isoamylase while in Chlamydomonas they differ nearly by two orders of magnitude. This major quantitative difference in the balance between isoamylase and limit-dextrinase in the two organisms could be related to the severity of the phenotype recorded in algae. If we assume that debranching enzymes are required to trim pre-amylopectin [5], it is very possible that in this respect some functional overlap exists between isoamylases and limit-dextrinases. Limit-dextrinase, if particularly abundant, might allow for a low rate of amylopectin crystallization in the absence of isoamylase. It is particularly striking to note that in Arabidopsis the already abundant limit-dextrinase is further enhanced by the presence of an isoamylase defect [16]. In addition such a functional overlap offers a logical explanation for the influence of the residual pullulanase activity in the expressivity of the rice sugary phenotype [15]. It would thus be of interest to increase the amount of the Chlamydomonas limit-dextrinase by, for instance, changing some of the physiological growth conditions. This might in turn lead to the limited synthesis of semi-crystalline starch. Another option would be to identify mutants lacking limit-dextrinase activity in plants such as maize or Arabidopsis where specific structural gene mutations can be identified by methods of transposon insertion screening.

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