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Starch Degradation in Synchronously Grown *Chlamydomonas reinhardtii* and Characterization of the Amylase[1]

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ABSTRACT

The activities of amylase and phosphorylase were monitored during the 12-hour light/dark synchronous cell cycle of autotrophically grown *Chlamydomonas reinhardtii* 11-32/90. The activity of amylase increased from 7.3 to 42 micromole reducing equivalents per 10^9 cells per hour while phosphorylase increased from 43 to 214 micromole glucose 1-phosphate released per 10^9 cells per hour between the midlight and middark periods. Cellular fractionation indicated that both enzymes were localized solely within the chloroplast. The pH optima for amylase and phosphorylase were 6.7 to 7.6 and 6.0 to 7.4, respectively. The amylase is a heat-labile α-amylase which is insensitive to ethylenetetraacetate but inhibited by *N*-ethylmaleimide.

Starch synthesis in both higher plants and in algae is tightly regulated by the activity of ADPglucose pyrophosphorylase (14, 15). However, little is known about the regulation of starch degradation in photosynthetic tissues. α-Amylase is thought to be the initial enzyme which is active on the starch granule, so if degradation is regulated, it should be at the level of α-amylase (14, 15). Kruger *et al.* (7) have reported that in pea leaves starch degradation is controlled, rather than simply persistent at low levels throughout periods of net starch synthesis and degradation. They found that starch turnover does not occur during periods of net starch synthesis in pea leaves held in continuous light. Their results do not distinguish between regulation of degradation by modulation of amylase activity by effectors (as is the case with ADPglucose pyrophosphorylase regulation of starch synthesis in leaves) and regulation by cell cycle control of total amylase activity.

Chlorophycean algae growing synchronously on a diurnal cycle accumulate starch during the light period and degrade it during the dark to satisfy the requirements of cell division (3). Such algae can provide a homogeneous cell population for measurement of enzymic activities which may regulate starch degradation and provide a useful tool for understanding the regulation of starch degradation in photosynthetic tissue. Wanka *et al.* (18) used synchronously grown *Chlorella* to show that α-amylase and phosphorylase activities varied throughout the cell cycle of this alga. They found that α-amylase was undetectable early in the period of starch accumulation but was active during net starch breakdown. Their result can be interpreted to indicate that starch degradation in the alga may be limited by developmentally regulated amylase levels.

Kombrink and Wöber (6) reported that amylase and phosphorylase of the green alga *Dunaliella marina* are restricted to the chloroplast. Thus, in contrast to higher plants, where these enzymes are chloroplastic and extrachloroplastic (15), any fluctuations in amylolytic and phosphorolytic function in the algal cell reflect changes in enzymic activity at the site of starch degradation. Recently, this laboratory (5) has reported a procedure for the isolation of photosynthetically competent chloroplasts from light/dark synchronously grown *Chlamydomonas reinhardtii* 11-32/90. It was of interest to determine if amylase and phosphorylase were localized in the chloroplast and to determine whether, as in *Chlorella* (18), diurnal variation in enzyme activity could serve to regulate starch degradation in *C. reinhardtii*.

The experiments reported here localize amylase and phosphorylase in the chloroplast of *C. reinhardtii* and follow their activities throughout the cell cycle. Additionally, *Chlamydomonas* amylase was partially characterized with regard to its similarity to leaf amylase.

MATERIALS AND METHODS

Cell Culture. *Chlamydomonas reinhardtii* (obtained from the Pflanzenphysiologisches Institut der Universität Göttingen, 11-32/90), was grown photoautotrophically in a 12-h light/dark synchronized regime by the procedure of Klein *et al.* (5).

Exponentially growing cells were harvested by centrifugation at 1500*g* for 10 min, washed twice with chilled water, and frozen in liquid N_2. Frozen cells were stored at −5°C. Cell number was determined with a hemocytometer. Zoospores enclosed in the mother cell wall were counted as one cell.

C. reinhardtii Dangeard 137c wt (obtained from Dr. R. K. Togasaki) and *Chlorella pyrenoidosa*, Culture Collection of Algae at the University of Texas at Austin 251, were grown photoheterotrophically as previously described (1). *C. reinhardtii* Dangeard 137c wt did not grow under the temperature regime we used for synchronous cultures and has not been used in chloroplast isolation. However, under the conditions in our laboratory, it was more convenient to use than strain 11-32/90 in larger, nonsynchronous cultures. Therefore, this strain was used for the determination of enzyme properties.

Preparation of Cell Extracts. Algae were broken in 50 mM Hepes-NaOH (pH 7.0), 2 mM EDTA, 2 mM DDT, and 2 mM $CaCl_2$ by 4 cycles of freezing in liquid N_2 followed by thawing rapidly by immersing in 30°C water. Following this, they were subjected to 4 to 6 cycles of sonication for 30 s of 50% pulses, alternated with 30-s cooling periods. Cell extracts were centrifuged at 5900*g* for 25 min. The supernatants were used for enzyme assays.

Enzyme Assays. Amylolytic activity was assayed in a 0.5-ml reaction mixture containing 100 mM Hepes-NaOH (pH 7.0), 2 mg/ml potato amylopectin, and cell extract. The reactions were terminated after incubation at 30°C for 60 to 120 min by heating

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in a boiling water bath or by addition of 0.5 ml of Nelson Cu reagent (11). Reducing sugars generated were assayed by the method of Nelson (11). Maltase was assayed in the same reaction mixture, substituting 10 mM maltose for amylopectin. The reaction was stopped by heating in a boiling water bath and the glucose produced was assayed as described by Levi and Preiss (8).

Limit dextrinase was assayed in the same reaction mixture as amylolytic activity, substituting 2 mg/ml pullulan for amylopectin, and assayed for reducing sugars as described above.

Phosphorylase was assayed in 200 mM K-phosphate (pH 7.0), 2 mg/ml amylopectin, and extract in a 0.5-ml total volume. Phosphorolysis was stopped by heating in a boiling water bath. Glucose 1-P was assayed as described by Levi and Preiss (8).

Hexokinase was assayed in a reaction mixture of 50 mM Hepes-NaOH (pH 7.8); 0.1 mg/ml BSA; 0.2 mg/ml NADP, 2 mM $MgCl_2$, 50 mM glucose, 1 mM ATP, 0.25 unit/ml glucose-6-P dehydrogenase. Activity was followed by reduction of NADP at 340 nm.

Amylase type (α or β) was determined by monitoring the decline in 600-nm absorption of the iodine/amylopectin complex using the assay of amylase activity described above, except that the concentration of amylopectin was reduced to 0.6 mg/ml. Reactions were terminated by heating in a boiling water bath. Absorption at 600 nm of the glucan/iodine complex was measured on 0.1 ml of each sample by mixing with 1.0 ml of iodine reagent prepared as described by Boyer and Preiss (2). The degree of amylopectin hydrolysis in the remainder of the sample was measured by assay of the reducing sugars generated using the method of Nelson (11).

Protein was measured by the method of Lowry *et al.* (9).

Intracellular Localization. Cell fractionation was by the method of Klein *et al.* (5). Intact chloroplasts and protoplasts from *C. reinhardtii* 11-32/90 were suspended in 20 mM Tris-Cl pH 7.5, 75 mM KCl, 1 mM EDTA, 1 mM $CaCl_2$, 2.5 mM DTT, 1 mM $MgCl_2$, 10% (v/v) ethylene glycol, and 0.1% (v/v) Triton X-100 and used for assays.

Table I. *Distribution of Enzyme Activities in C. reinhardtii 11-32/90*

Enzyme	Protoplasts	Chloroplasts	In Chloroplasts
	nmol/mg Ch·h		%
Experiment 1			
Amylase	46.3	43.5	94
Phosphorylase	306	265	87
Experiment 2			
Amylase	38	40	105
Phosphorylase	284	328	116
Glyceraldehyde 3-P dehydrogenase (NADP)[a]	0.62	0.67	106
Isocitrate dehydrogenase (NADP)[a]	0.055	0.006	10

[a] From Klein *et al.* (5).

Partial Purification of Amylase. Amylase was partially purified from photoheterotrophically grown *C. reinhardtii* Dangeard 137c by co-precipitation with glycogen (10). Frozen algae (8.6 g) were broken in 20 ml buffer containing 50 mM Hepes-NaOH (pH 7.0), 70 mM KCl, 2 mM EDTA, 2 mM $CaCl_2$, 5 mM DTT, and 10% (v/v) ethylene glycol, as described above. The broken cell suspension was centrifuged for 20 min at 16,000*g*. The pellet was washed with an additional 5 ml of buffer, centrifuged at 16,000*g* for 20 min, and the supernatant fractions were combined and stored in liquid N_2.

The frozen supernatants were thawed, centrifuged at 30,000*g* for 30 min, and were brought to 40% ethanol (v/v) by the dropwise addition of 95% ethanol. The suspension was centrifuged 16,000*g*. The supernatant was then brought to 0.18% glycogen (w/v) by the addition of 2% rabbit liver glycogen. After 10 min, the glycogen-containing solution was centrifuged at 16,000*g* for 20 min. The glycogen precipitate was resuspended in a dialysis buffer of 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 5 mM mercaptoethanol, 1 mM $CaCl_2$, 10% (v/v) ethylene glycol, and dialyzed in this buffer for 1 h at room temperature followed by dialysis against three buffer changes over 48 h at 4°C. All procedures were at 4°C, except the first dialysis.

The specific activity of the amylase from *C. reinhardtii* Dangeard 137c was increased by glycogen precipitation from 0.58 to 1.91 μmol reducing equivalents/mg protein·h. Limit dextrinase and phosphorylase activities were removed.

Reagents. Potato amylopectin, maltose, pullulan, hexokinase, glucose-6-P dehydrogenase, *N*-ethylmaleimide, sweet potato β-amylase, and rabbit liver glycogen type III were purchased from Sigma.

RESULTS AND DISCUSSION

Enzymes of Starch Utilization. The activities of amylase, limit dextrinase, maltase, hexokinase, and phosphorylase in crude extracts of synchronously grown *C. reinhardtii* 11-32/90 after 1 h light were 8.2, 1.0, 3.0, 34.4, and 59.5 μmol product/10^9 cells·h, respectively, indicating the presence of all the enzymes necessary for hydrolytic and phosphorolytic utilization of starch.

It is of interest that hexokinase is detected since *C. reinhardtii* is unable to grow on or respire exogenous glucose in the dark (4). The block in utilization of exogenous glucose can be ascribed to lack of glucose permeability. On the other hand, the metabolic means of utilizing internally generated glucose is available.

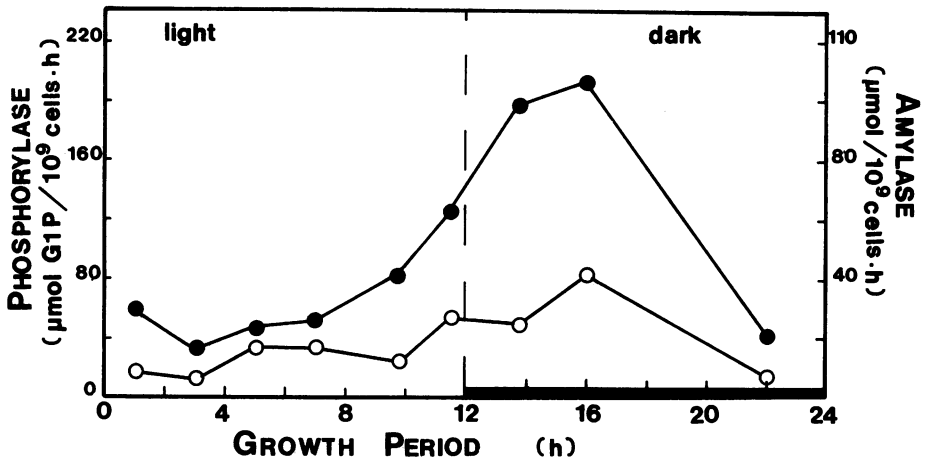

FIG. 1. Variation in the activities of phosphorylase (●) amylase (○) activities in synchronously grown *C. reinhardtii* 11-32/90 harvested at different times in the growth cycle.

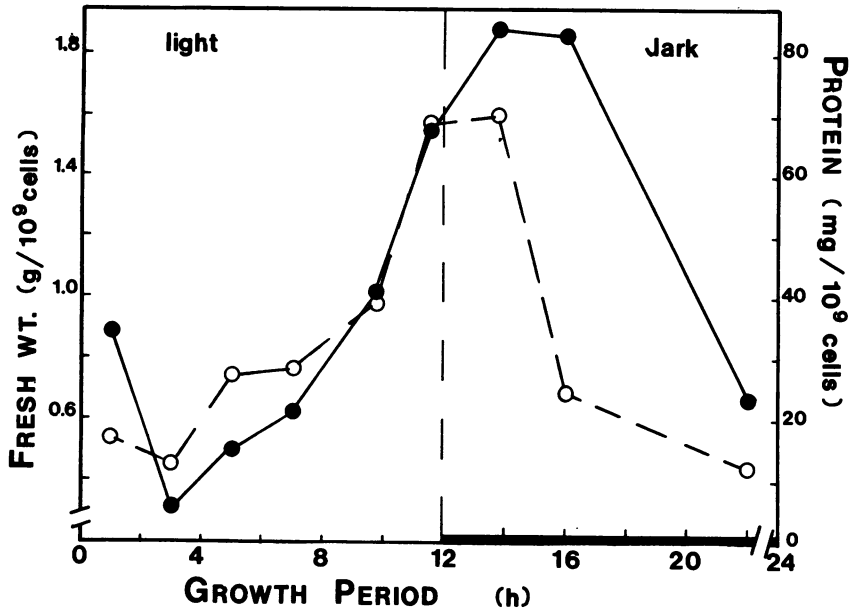

FIG. 2. Variation in fresh weight (●) and total soluble protein (○) of *C. reinhardtii* 11-32/90 harvested at different times in the growth cycle. Cell samples were the same as those in Figure 1.

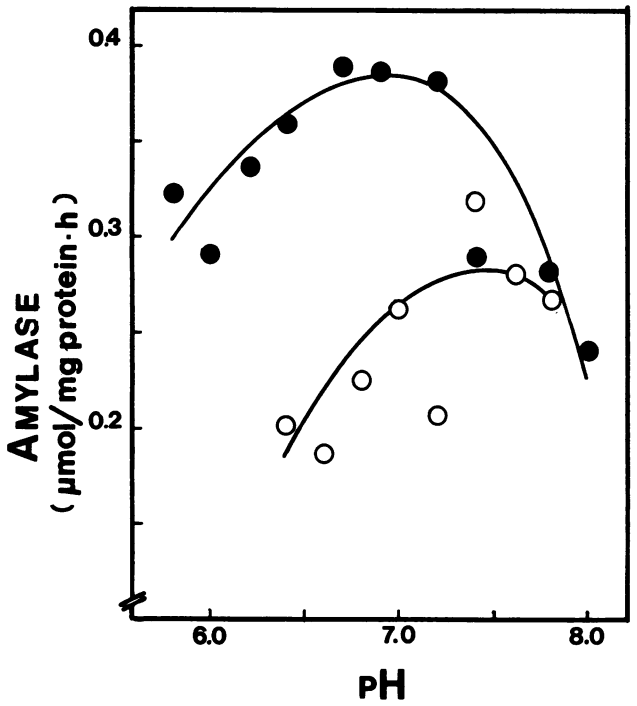

FIG. 3. Effect of pH on the activity of *C. reinhardtii* Dangeard 137c amylase assayed in potassium phosphate (●) and Hepes-NaOH (○) buffers. Activity is expressed as μmol reducing equivalents/mg protein·h.

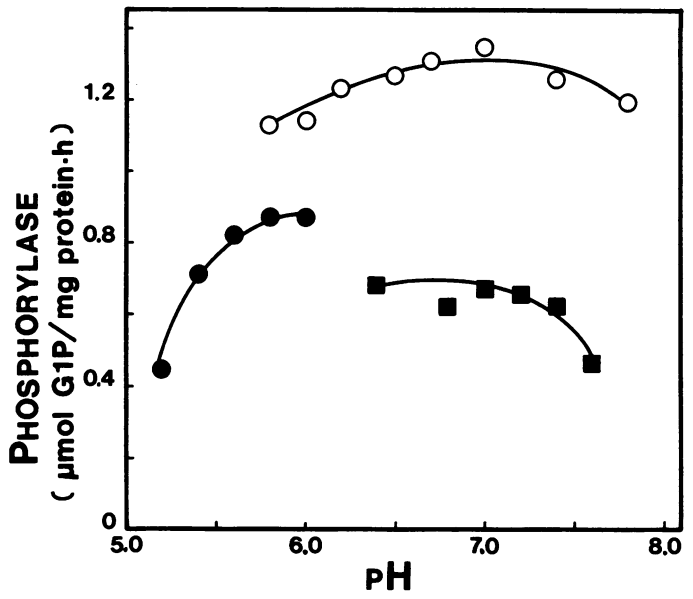

FIG. 4. Effect of pH on the activity of *C. reinhardtii* Dangeard 137c phosphorylase in potassium phosphate (○), Hepes-NaOH (■), and sodium acetate (●) buffers.

Intracellular Localization. Table I shows the intracellular distribution of amylase and phosphorylase in *C. reinhardtii* 11-32/90. Isocitrate dehydrogenase and NADP-glyceraldehyde-3 P dehydrogenase are included in the table as extra- and intrachloroplast markers, respectively. Since the enzyme activity, on a Chl basis, is the same in the protoplast as in the intact chloroplast, it is clear that amylase and phosphorylase are restricted to the chloroplast. This enzymic distribution is like that found in the green alga *D. marina* (6), but is very unlike that of plant leaves where amylases and phosphorylases of spinach and pea leaves were found both inside and outside of the chloroplast (14).

Enzyme Levels throughout the Cell Cycle. Since all of the amylase and phosphorylase of *C. reinhardtii* 11-32/90 is within the chloroplast, as is the starch, measurements of their activities in extracts of whole cells are equivalent to measurements of activity present at the site of their substrate, starch. Therefore, variations in enzymic activity of whole cell extracts should represent variations in the cellular capacity for starch degradation.

The variation in the activities of phosphorylase and amylase in *C. reinhardtii* 11-32/90 over a 24-h growth cycle are illustrated in Figure 1. Phosphorolytic activity increases 6-fold over the last 4 h of the light period and the first 4 h of the dark period, to a maximum activity of 214 μmol glucose 1-P/10^9 cells·h. Activity then declines to one-sixth of the maximum activity by 3 h of illumination (43 μmol glucose 1-P/10^9 cells·h).

The increase in amylolytic activity in the light begins slightly later than the increase of phosphorylase, but also reaches maximum activity after 4 h darkness (42 μmol reducing equivalents/

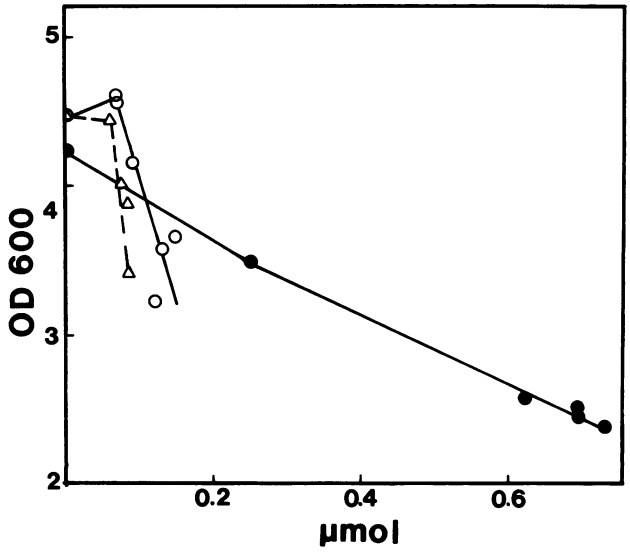

FIG. 5. Effect of hydrolysis of potato amylopectin by partially purified *C. reinhardtii* Dangeard 137c amylase (Δ) and (○) (two experiments) and by sweet potato β-amylase (●) on the loss of iodine binding. Each β-amylase sample contained 0.24 unit of sweet potato β-amylase. The initial concentration of amylopectin was 0.6 mg/ml.

10^9 cells·h), declining to one-eighth of the maximum value by 3 h illumination (7.3 μmol reducing equivalents/10^9 cells·h).

Starch, the substrate for these enzymes, reaches a maximum at the end of the light phase (110 μmol C/10^8 cells) and minimum at the end of the dark phase (3 μmol C/10^8 cells) (R. Gfeller, private communication). Therefore, enzyme activity varies with substrate levels.

Although amylase and phosphorylase are most active in the early dark period, it can be seen in Figure 1 that both are active over the entire cell cycle, indicating that the capacity to degrade starch by both hydrolytic and phosphorolytic means is retained even at low substrate levels. In contrast are the results of Wanka *et al.* (18), where amylase activity could not be detected during the middle of the light period in synchronously grown *Chlorella*. Diurnal fluctuation in amylase levels, but not phosphorylase levels, has been demonstrated in spinach leaves (13).

As can be seen by comparison with the development of fresh weight and total protein (Fig. 2), amylolytic and phosphorolytic activity increase concomitantly with total protein, but the loss of activity in these starch utilizing enzymes is delayed relative to the loss of total cell protein.

pH Optima. The effect of pH on the enzymic activity of *C. reinhardtii* Dangeard 137c amylase and phosphorylase was determined over a range from approximately 5.5 to 8.0 and in the presence of different buffers. Maximal amylolytic activity is between pH 6.7 and 7.2 in K-phosphate and pH 7.2 and 7.6 in Hepes-NaOH buffer (Fig. 3) while phosphorylase possesses a broad pH optimum from 6.0 to 7.4 and is buffer independent (Fig. 4). The *Chlorella* enzymes exhibit similar pH response curves (not shown). Our results are consistent with those observed with spinach leaf amylase (12) and phosphorylase (16). The pH curve for *Chlamydomonas* maltase is quite broad from pH 6.0 to 8.0 (not shown). The dependence of amylase (Fig. 3 and 12) and the relative independence of phosphorylase (Fig. 4 and 16) and maltase on pH suggests that amylase, rather than the latter two enzymes, may regulate starch degradation through sensitivity to cellular pH. Consistent with this suggestion is the documentation of a downward shift in chloroplastic stromal pH from approximately 8 to 7 during a transition from light to dark (19), indicating that amylolytic activity in response to pH would function maximally in the stroma of the darkened chloroplast.

Extrachloroplastic amylases in contrast to their counterparts in the chloroplast tend to exhibit lower pH optima. Thus, amylases of plant storage tissue have maximum activity from pH 4.8 to 6 (17) and the cytoplasmic spinach leaf enzyme has a pH optimum between 6.0 and 6.5, which contrasts with pH 6.0 to 7.5 for the chloroplastic enzyme (12). There is one report (18), however, of pH optima of 6.0 for *Chlorella* amylase and phosphorylase.

Properties of Partially Purified Amylase. The *C. reinhardtii* amylase was partially purified to remove limit dextrinase which would interfere with differentiation between α- and β-amylolytic modes of hydrolysis. The preparation was also free of phosphorylase. Inasmuch as sweet potato β-amylase catalyzes a slow reduction in iodine staining per reducing equivalent formed by amylopectin hydrolysis, whereas loss of iodine staining is rapid relative to amylopectin hydrolysis catalyzed by our partially purified preparation, we concluded that the *C. reinhardtii* Dangeard 137c amylase is an α-amylase (Fig. 5). Kombrink and Wöber (6) suggested that both α- and β-amylase are present in the green alga *D. marina*, and our results do not rule out the possibility that both amylases are present in *C. reinhardtii*.

The *C. reinhardtii* amylase is inhibited 30 to 40% by 2 mM NEM[3] and 95% by 10 mM NEM. Neither 10 mM EDTA nor 10 mM $CaCl_2$ affect the enzyme activity. The amylase is heat labile; all activity is lost by heating at 55°C for 5 min in the presence of 10 mM $CaCl_2$.

Heat lability, sensitivity to NEM, and insensitivity to EDTA are properties shared with both spinach leaf amylases (12), whereas α-amylase from storage tissue are heat-stable in the presence of Ca^{2+} and are inactivated by EDTA.

CONCLUSION

The enzymes necessary for starch degradation and glucose utilization (amylase, phosphorylase, limit dextrinase, maltase, and hexokinase) are present in *C. reinhardtii*. Both amylase and phosphorylase are restricted to the algal chloroplast and the pH dependence of the amylase is consistent with activity in the darkened chloroplast. It is a 'leaf-type' α-amylase: heat labile, insensitive to EDTA, and requiring near neutral pH for activity.

Amylase and phosphorylase activities were detected throughout the cell cycle of the synchronously grown *C. reinhardtii* 11-32/90, so if this alga regulates its starch degradation at the initial enzyme reactions, it may be providing these enzymes with a suboptimal environment, such as elevated pH, rather than by regulating enzyme turnover. Thus, there seem to be two classes of amylases in green algae as well as in higher plants. Those, like *Chlorella* (18) and seed (15) amylases, which have low pH optima and are developmentally regulated, and those like the *C. reinhardtii* amylase and leaf amylases (12), which have higher pH optima and are only incompletely subject to developmental control.

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[3] Abbreviations: NEM, *N*-ethylmaleimide.

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