Plant Physiol. (1978) 62, 687–689

Diurnal Oscillation of Amylolytic Activity in Spinach Chloroplasts

Received for publication April 18, 1978 and in revised form June 15, 1978

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ABSTRACT

Chloroplasts isolated from spinach (*Spinacia oleracea* L., cv. vital^R) plants grown under controlled light/dark and temperature regimes, contained the phosphorolytic and amylolytic pathways for starch breakdown. The latter consists at least of α- and β-amylase and maltase. Only low amylolytic activity was observed in chloroplasts isolated during the light phase. In chloroplasts prepared during the dark phase, this activity was almost twice as high. These diurnal oscillations of the amylolytic activity were maintained when the plants were kept in prolonged darkness or continuous light. The amylolytic system exhibited a sharp pH optimum between 5.8 and 6.0. Phosphorylase activity, when assayed with saturating concentrations of inorganic phosphate, did not show diurnal fluctuations.

The well known phenomenon of diurnal starch accumulation and disappearance in chloroplasts of assimilatory tissues can be explained by an alternating predominance of synthesis over degradation and *vice versa*. The regulatory mechanisms responsible for these oscillations still need more elucidation. A major regulatory role may be ascribed to Pi which, at higher concentrations, inhibits starch biosynthesis (6, 8, 13–15) and stimulates degradation by phosphorylase (8, 14, 17).

Phosphorolysis is apparently not the sole route of starch degradation in the chloroplast. Considerable contribution of starch hydrolysis to the total starch degradation was demonstrated by Levi and Gibbs (11), whereas other investigators ascribed only minor importance to amylolysis (8, 14). Amylolytic starch degradation could be regulated by means of pH shifts in the stroma (21) since amylases seem to exhibit rather acidic pH optima (5, 18). In addition, other regulatory features of these enzymes are also known, *e.g.* the conversion of latent to active β-amylase by the reversible reduction of its sulfhydryl groups (16) or the inhibition of α-amylase by its product maltose (20).

In the following, evidence for considerable amylolytic activity in isolated chloroplasts will be presented which, in contrast to that of phosphorylase, exhibits an endogenous diurnal rhythm.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L., cv. vital^R) was grown in the local jail and transferred 10 days prior to the experiments to a growth chamber adjusted to give a light period of 12 hr at 25 C during the light and 15 C during the dark phase. The light intensity was 14 klux. At intervals of 3 hr chloroplasts were isolated from 25 g of fresh leaves following the procedure described earlier (2). Buffers A and B described by Jensen and Bassham (9) were used without Pi. The intact plastids were washed twice in buffer B and then disrupted by the addition of 5 ml of bidistilled H_2O to the pellet. Aliquots of this suspension were used for Chl (1) and starch determination. The soluble enzymes were separated from the membranes by centrifugation at 45,000*g*.

Amylase activity was determined by the method of Vieweg and De Fekete (20); however, instead of Somogy's reagent, Bernfeld's dinitrosalicylic acid reagent (4) was used for the detection of reducing groups. The standard incubation mixture contained in a total volume of 1 ml: 60 mM sodium-citrate buffer (pH 6.1), 1.5 mM NaF, 2 mg of soluble starch or β-limit dextrin and 0.5 ml of chloroplast extract. After 30 min at 37 C, 1 ml of dinitrosalicylic acid reagent was added and the mixture was then kept for 5 min at 100 C. After cooling *A* was read at 540 nm. Limit dextrin was prepared from amylopectin (Sigma) by exhaustive degradation with β-amylase (Boehringer), heat inactivation of the enzyme, separation of the denaturated protein by centrifugation, precipitation of the glucans with methanol, and lyophilization of the material (De Fekete, personal communication). Analysis of the degradation products of soluble starch and of limit dextrin was performed by paper chromatography using the solvents 1-butanol-pyridine-water-acetic acid = 60:40:30:3, water-saturated phenol-water-acetic acid-1 M EDTA = 480:160:10:1, and propionic acid-water (352:448)-1-butanol-water (750:50) = 1:1. The spots were detected with alkaline silver nitrate (19).

Phosphorylase activity was measured with soluble starch according to the method described by Bergmeyer *et al.* (3) with 45 mM Pi. In a total volume of 1.7 ml 500 μl of chloroplast extract were assayed. One unit (U) of enzyme activity produced 1 μmol of reducing groups or 1 μmol of glucose 1-P/min. Starch content was determined after removal of Chl with acetone and ethanol and extraction of soluble compounds with hot water. The insoluble material was then hydrolyzed with 1.1% HCl for 1 hr at 100 C. The solubilized material (200 μl) was further incubated at pH 4.6 with α-amyloglucosidase (1.4 U, Boehringer Mannheim) for 14 hr and glucose was determined with hexokinase and glucose-6-P dehydrogenase.

RESULTS

Recently, data have been presented which suggest that isolated pea chloroplasts, in contrast to a cytoplasmic fraction, contain only low specific activities of β- and no α-amylase at all (14). Therefore, the amylolytic activity in the spinach chloroplast preparation had to be located carefully. However, more than 98% of the amylase activity of the chloroplast suspension was liberated only upon osmotic disruption of the plastids (Table I). Therefore the amylolytic activity should be ascribed to the chloroplasts.

The amylolytic activity of isolated chloroplasts proved to depend strongly on the growth conditions. High amylase and α-glucosidase activities could only be detected with chloroplasts from plants grown under a light/dark regime of 12/12 hr with the temperature program shown in Figure 1. No influence of these growth parameters on the activity of phosphorylase could be observed.

Together with the effect of growth conditions, amylolytic activity could vary depending of the time of measurement as has been described for *Kalanchoë* leaves (20).

To examine this suggestion intact chloroplasts were prepared at intervals of 3 hr during the day and both the amylolytic and phosphorolytic activities as well as the starch content were determined. Hydrolytic activity was measured as increase in reducing

Table I. Liberation of amylolytic activity by osmotic rupture of isolated intact chloroplasts

The chloroplasts were prepared as described; the incubation period was 5 min in order to avoid considerable disruption which took place at longer periods. The activity of the intact chloroplast preparation was corrected for 24 % of broken plastids, as measured with the ferricyanide method (7).

	Amylolytic activity mU/mg Chl
Intact chloroplasts	0.8
Osmotically disrupted chloroplasts	
chloroplast extract	66.8
membranes	2.9

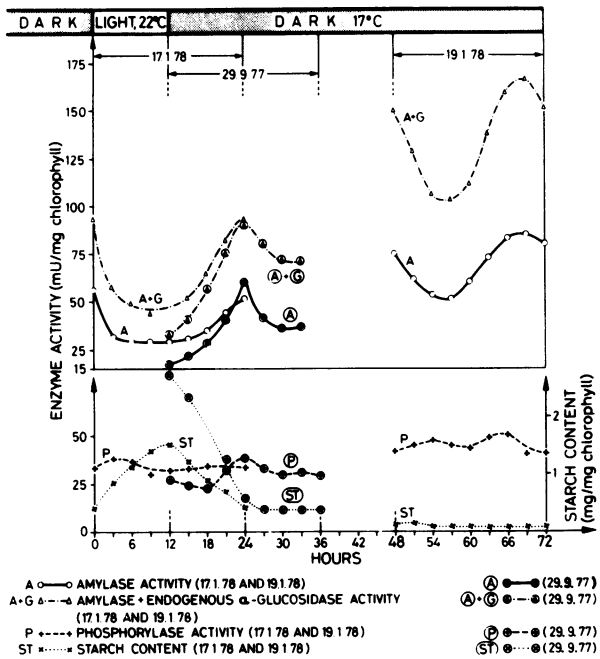

FIG. 1. Diurnal behavior of amylolytic (upper part) and phosphorolytic (below) activities of chloroplasts from spinach plants which were kept under a light/dark regime of 12/12 hr (17.1.78) or in continuous darkness immediately after a light period (29.9.77) or in darkness for 3 days (19.1.78). Hydrolytic activity was determined from the liberation of reducing groups from soluble starch. Amylase activity was calculated to produce 50% of the total reducing groups when purchased maltase was added to the assay. Phosphorylase activity was determined with saturating concentration of Pi. Dotted lines show starch content of chloroplasts on a Chl basis.

groups upon addition of chloroplast extract to soluble starch. In order to determine whether amylase or α-glucosidase were rate-limiting in starch hydrolysis, purchased α-glucosidase was added to the assay in parallel samples. Except at the stages of maximal hydrolytic activity no increase of reducing groups was obtained upon addition of α-glucosidase (maltase). Therefore amylase rather than α-glucosidase activity was usually rate-limiting. If β-amylase activity exceeds that of α-amylase, maltose will be the predominating saccharide in the hydrolysate. Since maltose was the only product of amylolytic activity detectable besides glucose (see Fig. 3) and because maltase is known to hydrolyze maltose preferentially, amylolytic activity was estimated to produce at least approximately 50% of the reducing groups found in the combined assay of chloroplast extract plus exogenous maltase. Typical results obtained during 24 hr with plants of a standard day/night rhythm are shown by the curves from January 17, 1978 in Figure 1. Amylase activity varied between 30 mU during the light period and 55 at the middle of the dark phase whereas phosphorylase activity remained nearly constant at about 35 mU. As was to be expected starch content of the chloroplasts and amylase activity behaved in an opposite manner and the enzyme activity at each interval of the dark phase was high enough to account for the total starch degradation observed.

In a second series of experiments (Fig. 1, curves dated September 29, 1977) the behavior of amylase activity was studied during a prolonged dark phase of 24 instead of 12 hr in order to examine whether the amylolytic activity is correlated to the starch content. The same fluctuations were found as had been observed during a normal light/dark change. The relative high starch content at the beginning of these experiments was due to the high rate of starch synthesis usually observed with plants grown in summer. Phosphorylase activity exhibited a small increase between hr 6 and 9, however, no rhythmic oscillation was observed. Finally, a similar oscillation of amylase activity was found from hr 36 to 60 of continuous darkness at which interval the starch content was constant and virtually zero (Fig. 1, curves data January 19, 1978). The level of amylolytic activity increased significantly with prolonged darkness whereas phosphorolytic activity was enhanced only slightly.

Measurement of the amylolytic activity of chloroplasts from plants which were kept in continuous light was rather troublesome since heavy starch accumulation prevented the isolation of intact plastids. Therefore the light intensity was reduced to 0.7 klux during the induction period and maintained during the experiment. This treatment resulted in a low amylolytic activity which again exhibited the diurnal oscillations, but now during the prolonged light phase (Fig. 2). The second maximum was less pronounced than the first indicating an inhibitory effect of continuous light on amylase formation or activation. This is in agreement with the finding that prolonged darkness increased the level of amylase activity.

In order to characterize the amylolytic activity of spinach chloroplasts the degradation velocities of soluble starch and β-limit dextrin were determined at the stage of maximum activity and compared with those of purchased α-amylase (hog pancreas). Table II shows a significant lower rate of β-limit dextrin hydrolysis than of soluble starch cleavage. On the contrary purchased α-amylase hydrolyzed β-limit dextrin more readily. These findings indicate that the chloroplast extract contained both α- and β-amylase and that the latter was predominant.

Paper chromatography of the hydrolysates corroborated this interpretation. At a stage where the endogenous α-glucosidase was not limiting starch hydrolysis, only glucose could be detected in the hydrolysate, whereas at the points of highest amylase activity, some maltose but no other oligosaccharides were found besides glucose (Fig. 3).

With respect to the regulation of the amylolytic activity the pH optima are of great importance. One sharp optimum was found at pH 5.8 to 6.0 (Fig. 4).

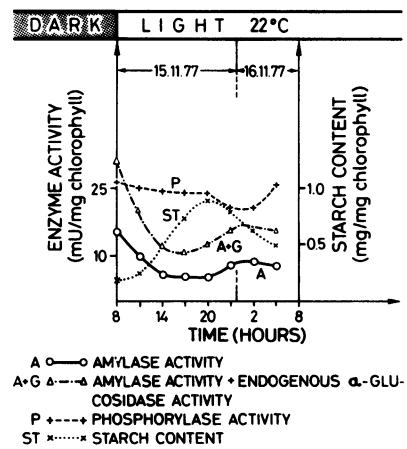

FIG. 2. Behavior of amylolytic and phosphorolytic activities of chloroplasts from spinach plants which were kept under continuous light for 24 hr following a series of normal light/dark periods. Light intensity was reduced to 0.7 klux instead of 14 klux in order to keep starch synthesis low. For more details see legend of Figure 1.

Table II. Amylolytic breakdown of soluble starch and β-limit dextrin with a chloroplast extract and purchased α-amylase

The standard assay was used with 500 µl chloroplast extract or 1 unit of α-amylase (hog pancreas), citrate buffer (60 mM, pH 6.1), NaF (1.5 mM) soluble starch or β-limit dextrin (2 mg) in a total volume of 1 ml.

	substrate (2 mg/assay)	incubation period	reducing groups produced by chloroplast extract	chloroplast extract + exogenous maltase	α-amylase
		min		µmol	
Experiment 1	soluble starch	10	1.2	1.2	-
	soluble starch	30	2.6	2.7	0.5
	β-limit dextrin	10	1.0	1.1	-
	β-limit dextrin	30	2.3	2.5	1.0
Experiment 2	soluble starch	30	3.4	-	-
	β-limit dextrin	30	2.2	2.3	-

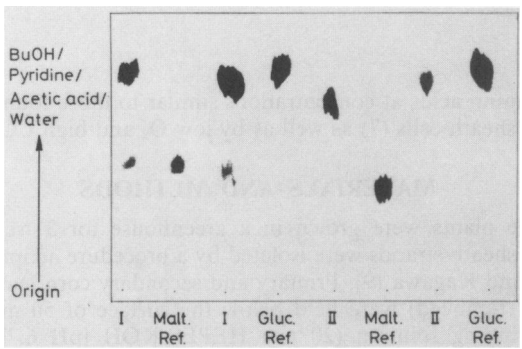

FIG. 3. Paper chromatograms showing products of soluble starch breakdown with chloroplastic amylolytic system. Standard degradation assay was employed. Prior to paper chromatography, the lysate was desalted by passing small columns of Dowex 1-X8 and Dowex 50-W. I: stage of maximal activity; II: stage of minimal amylase activity.

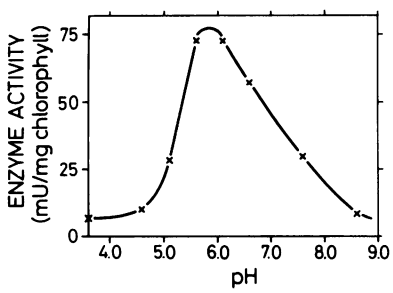

FIG. 4. Effect of pH on amylolytic activity of spinach chloroplasts. Standard assay was employed. pH 3.6 to 6.6: 60 mM citrate buffers. pH 7.6 to 8.6: 40 mM Tris-HCl buffers.

DISCUSSION

The data presented in this communication indicate that hydrolytic breakdown of starch may play an important role for the regulation of the starch content of the chloroplast. Production of maltose from starch by intact chloroplasts has already been shown (11, 13). However, as the activity of amylase depends on the time of day and conditions of growth, the extent of amylolytic breakdown could have been underestimated even by those workers that have found such activity.

The diurnal oscillations of amylase activity which were unchanged under conditions of continuous darkness and, at least, during 24 hr of continuous light, are probably caused by an endogenous rhythm. A regulation of the amylolytic activity by chloroplast metabolites seems unlikely since the chloroplast content was diluted upon osmotic rupture by a factor of 50 and in the assay by an additional factor of 2. Further dilution of the chloroplast extract in the assay again by a factor of 2 did not change the specific activity. Thus, an allosteric influence of metabolites of the chloroplast on the amylase activity can be ruled out. However, amylolytic activity could be doubled by addition of DTT (data not shown). Therefore, some activation of amylase could be brought about in the light by formation of a complex with an endogenous dithiol (22). However, this activation of the amylolytic system might be superimposed by a pH effect on its activity. At pH 8, which is reached in the stroma upon illumination (21), the amylases should be inhibited by roughly 80%. At pH 7 which was found in the stroma of darkened chloroplasts (21) the inhibition would be only by 50%. Taking into account that the suggested light activation is amply compensated by the strong inhibition at pH 8, a diurnal oscillation between approximately 12 mU/mg Chl in the light and 30 mU/mg Chl in the dark (calculated from a 50% inhibition at pH 7) should result. The often cited inhibition of α-amylase by higher maltose concentrations seems to play a minor role since the endogenous α-glucosidase (maltase) exhibited activities at least in the same order of magnitude as the amylases.

Our results do not suggest that phosphorolytic starch breakdown is of minor importance. In this work saturating Pi concentrations were used; however, *in vivo* the Pi concentration in the chloroplast might oscillate (10, 12) leading to fluctuations of phosphorylase activity which parallel those of the amylolytic system described here.

Acknowledgments—The authors thank O. Kandler (Botanisches Institut, Universität München) and E. -D. Schulze (Lehrstuhl Pflanzenökologie, Universität Bayreuth) for the use of growth chambers and D. Thomas for linguistic help with the manuscript.

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