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Interaction of Hydrolytic and Phosphorolytic Enzymes of Starch Metabolism in *Kalanchoë daigremontiana*

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Abstract. The degradation of starch by a protein fraction of *Kalanchoë daigremontiana* Hamet et Perrier, obtained by ammoniumsulfate precipitation (30–70%), was found to be catalyzed by α- and β-amylase (EC 3.2.1.1 and EC 3.2.1.2, respectively) and by starch phosphorylase (EC 2.4.1.1). The activity of these enzymes was determined by chromatographic analysis of the reaction products; separation and identification of α-amylase was accomplished by heat-inactivation of β-amylase and α-glucosidase. When the interaction of amylolytic and phosphorolytic enzymes was comparatively studied, it was found that without inorganic phosphorus in the reaction mixture, ^{14}C-starch was converted predominantly to maltose and glucose; supplementation with 1–10 mM orthophosphate (Pi) resulted in an increase in glucose-1-phosphate formation and a concomitant reduction of maltose production. Since the total volume of starch degradation remained approximately constant, Pi apparently inhibits β-amylase (Ki about 3 mM Pi). Thus, free Pi in the cell participates in the regulation of starch catabolism, serving as a substrate for starch phosphorylase while simultaneously reducing the production of maltose. With respect to glucan synthesis, adenosinediphosphoglucose-α-1,4-glucosyltransferase (EC 2.4.1.22), maltose phosphorylase and maltose-glucosyltransferase were also found to be active. The last-named enzyme catalyzes an exchange between dextrins and is considered to provide primer carbohydrates for the synthesis of polyglucans.

Abbreviations: ADPG = adenosinediphosphoglucose; G1P = glucose-1-phosphate; PEG = polyethylenglycol; PEP = phosphoenolpyruvate; Pi = orthophosphate

Key words: Amylases – Crassulacean acid metabolism – *Kalanchoë* – Maltose phosphorylase – Phosphorylase – Starch metabolism.

Introduction

The diurnal cycle of acidification and deacidification, which is a characteristic feature of Crassulacean acid metabolism (CAM), is intimately related to a concomitant rhythm of starch degradation and starch synthesis (see review by Kluge and Ting, 1978). Enzymes of starch metabolism have been investigated by Sutton (1975a, b), who demonstrated the presence of starch phosphorylase (EC 2.4.1.1) and β-amylase (EC 3.2.1.2) in the CAM plant *Kalanchoë daigremontiana;* the complementary α-amylase (EC 3.2.1.1) was tentatively identified in the same plant by Vieweg and Fekete (1977). Phosphorylase and both amylases serve the purpose of degrading starch and providing substrates for glycolysis (Sutton, 1975b). During the latter process, phosphoenolpyruvate, the precursor of malate during dark CO_2 fixation, is formed. Though we know that glycolytic intermediates participate in the regulation of CAM (Sutton 1975a, b; Ting and Osmond, 1973), it is still unclear which substrate enters the glycolytic pathway: whether the phosphorolytic enzyme provides glucose-1-phosphate or whether the hydrolytic enzymes-amylases in concert with α-glucosidase (EC 3.2.1.20) provide glucose, which must then be phosphorylated. Since both enzyme systems occur in the same cell, the question of mutual interaction and regulation also arises. In the course of our study we examined this question; furthermore we

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tested the activity of adenosinediphosphoglucose-α-1,4-glucosyltransferase (EC 2.4.1.22), of glucosyltransferase, as described by Linden et al. (1974), and gave particular attention to maltose phosphorylase (Schilling and Kandler, 1975) and its potential role in the synthesis of primers for glucan synthesis.

Materials and Methods

Plant Material and Preparation of the Protein Fraction

Plants of *Kalanchoë daigremontiana* Hamet et Perrier were cultivated in a growth chamber with controlled environment at 28° C and 30% relative humidity during a 9-h light period, and at 17° C and 80% relative humidity during a 15-h dark period. Illumination was provided by high-flux fluorescent lamps (Philips TLM 115 Watt/33 RS) supplemented by Fluora red-light fluorescent lamps (40 Watt). The light intensity at the plant level was 40 Klux. *K. daigremontiana* leaves (50 g) were harvested at the end of the light period and homogenized together with 10 g insoluble polyvinylpolypyrrolidone (PVP) in 125 ml 0.1 M tris(hydroxymethyl) aminomethane (Tris), pH 8.0, containing 10 mM mercaptoethanol, 10 mM $MgCl_2$ and 0.1% polyethylenglycol (PEG) 6000. The slurry was squeezed through nylon cloth of 10 µm pore diameter and the filtrate subjected to $(NH_4)_2SO_4$ precipitation. The fraction between 30 and 70% saturation was dissolved in 3 ml of an appropriate buffer, and was filtered to remove substrates and other low molecular weight compounds through Sephadex G25 (Pharmacia, Uppsala, Sweden). This protein fraction was used in the assay of the different enzymes.

Heat Inactivation of β-Amylase

The protein fraction (0.5 ml, 1 mg protein) in acetate buffer, 10 mM, pH 4.8, containing 5 mM dithioerythritol (DTE) and 20 mM $CaCl_2$, was incubated for 10 min with 1 mg ^{14}C-starch (39 µCi), (Amersham Buchler, Braunschweig, West Germany) at 30° C. The reaction was stopped by 2 min boiling and the reaction products were analyzed. Three ml of the same protein fraction were brought to pH 8.0 by buffer-exchange on Sephadex G25 (Tris buffer 50 mM, 5 mM DTE, 20 mM $CaCl_2$) and incubated for 10 min at 70° C. After cooling in ice the precipitated protein was centrifuged and the clear supernatant readjusted to pH 4.8 by filtration over Sephadex G25 equilibrated with the original 10 mM acetate buffer. The protein solution diluted by two filtration steps was concentrated to 2 ml with an Amicon concentrator (B 15; Amicon Corporation, Lexington, Mass., USA). The heat-treated protein solution (0.5 ml containing 0.27 mg protein) was incubated with 1 mg ^{14}C-starch (39 µCi) for 10 min at 30° C as the untreated solution had been before. The reaction products were separated chromatographically.

In order to test for the reaction products of starch breakdown by authentic amylases, α-amylase from *Bacillus subtilis* and β-amylase from *Ipomoea batatas* were subjected to a comparative test (enzymes purchased from Boehringer, Mannheim, West Germany). One unit of each enzyme was separately incubated with 1 mg ^{14}C-starch (39 µCi) in 0.5 ml 10 mM acetate buffer, pH 4.8, containing 5 mM DTE and 20 mM $CaCl_2$ for 10 min at 30° C and the compounds formed were identified by paper chromatography.

Competitive Assay of Amylase and Phosphorylase

The reaction mixture contained in a total volume of 1 ml 50 mM N-2-hydroxyethylpiperazin-N′-2-ethane-sulphonic acid (HEPES), pH 6.8, with 10 mM Mg^{2+} and 5 mM DTE, 1 mg *K. daigremontiana* protein, 1 mg ^{14}C-starch (39 µCi) and 0–10 mM orthophosphate. Samples of 0.1 ml were taken during a 30-min incubation at 30° C, boiled for 2 min, and analyzed.

Enzyme Assays

For assaying maltose-phosphorylase activity, the *Kalanchoë* protein fraction (0.5 ml) was incubated at pH 6.8 (50 mM HEPES, 10 mM Mg^{2+}, 5 mM DTE) with 1 µmol [^{14}C]glucose-1-phosphate ([^{14}C]G1P) (0.9 mCi/mmol) and 1 µmol glucose in a total volume of 1 ml. The assay was also carried out in the reverse direction using labeled glucose and unlabeled G1P.

The reaction mixture for assaying adenosinediphosphoglucose-α-1,4-glucosyltransferase (EC 2.4.1.22) activity contained 0.5 ml *Kalanchoë* protein fraction, 50 mM N-tris(hydroxymethyl)-methylglycine Tricine), pH 8.0, with 10 mM Mg^{2+}, 5 mM DTE, 1 mg soluble starch, and 1 µmol ADP-[^{14}C]glucose (0.9 mCi/mmol) in a final volume of 1 ml.

The incubation mixture for maltose-glucosyltransferase assays consisted of 50 mM HEPES, pH 6.8, containing 10 mM Mg^{2+}, 5 mM DTE, 0.5 ml protein fraction, 1 µmol [^{14}C]glucose (0.9 mCi/mmol), and 1 µmol maltose in a total volume of 1 ml.

Incubation time for all assays was 30 min, incubation temperature 30° C; samples were taken at intervals. Protein was determined with Coomassie brilliant G 250 Blue according to Sedmark and Grossberg (1977). The ^{14}C-labeled radiochemicals were purchased from Amersham Buchler, Braunschweig, West Germany. Stock solutions of ^{14}C-labeled radiochemicals were regularly checked for purity by paper chromatography in the solvent described by Linden et al. (1974).

Product Analysis

For the analysis of the products of the enzyme reactions, the proteins in the samples taken during the incubation period were precipitated by 2 min boiling and removed by centrifugation. The carbohydrates were then separated by paper chromatography (Whatman Nr. 1), descending in one direction, using butanol-pyridine-acetic acid-water 60:40:3:3 (v/v) (Aspinall et al., 1958), or ethylacetate-pyridine-water 100:40:30 (v/v) (Linden et al., 1974). The radioactive areas were located by autoradiography and counted with a Gasflow-G-M-tube (Berthold, Wildbad, West Germany). Reference carbohydrates were detected by spraying with alkaline $AgNO_3$ (Trevelyan et al., 1950).

Results

Separation of α-amylase and β-amylase was achieved by heating the protein fraction (30–70% ammonium-sulfate precipitation) of *K. daigremontiana* to 70° C for 10 min in the presence of Ca^{2+}; the latter protects α-amylase from destruction (Clutterbuck and Briggs, 1973; Rodaway and Kende, 1978). About 73% of the incubated protein was precipitated and thus eliminated. The test carried out before and after heat treatment showed the characteristic reaction products of both enzymes (see Table 1). The predominant degradation product of starch by the untreated enzyme mixture was maltose; the dextrins, maltotriose to maltopentaose, were found only at a low level. The appearance of glucose as shown in Table 1 is a consequence of the action of α-glucosidase which leads

Table 1. Comparative action of α- and β-amylase from *Kalanchoë daigremontiana* and from commercial preparations on ^{14}C-starch (1 mg, 39 μCi) with regard to reaction products.
The reaction mixture was incubated at pH 4.8 and 30° C for 10 min

Enzyme	Starch conversion (%)	Radioactivity of reaction products (%)					
		Glucose	Maltose	Isomaltose	Malto-triose	Malto-tetraose	Malto-pentaose
Kalanchoë protein preparation	46.1	59.4	24.3	1.6	9.0	4.1	1.6
Kalanchoë protein preparation, heat-inactivated	10.0	7.8	12.6	3.9	24.0	25.6	24.7
β-Amylase *(Ipomoea batatas)*	45.6	2.6	87.0	2.9	3.7	3.7	0
α-Amylase *(Bacillus subtilis)*	62.4	6.2	16.4	2.4	24.1	18.1	32.4

to the final degradation of maltodextrins formed. Heat treatment of the protein fraction reduced the activity of α-glucosidase markedly; nervertheless only a low percentage of the ^{14}C of the reaction products was detected in maltose whereas maltotriose, maltotetraose and the maltopentaose comprised about 75% of the starch-degradation products. This result is a clear indication of the action of α-amylase. Support of this contention was obtained by comparing the degradation products of authentic α- and β-amylase. With the exception of the presence of α-glucosidase, the reaction products of heat-treated and untreated *Kalanchoë* protein with ^{14}C-labeled starch matched, those of α- and β-amylase.

In a comparative experiment both starch degrading enzyme systems (α- and β-amylase and starch phosphorylase) were investigated simultaneously and competitively by incubating the protein fraction with ^{14}C-starch and various concentrations of Pi (Fig. 1). Despite the lower pH optima of amylases, between 4.75 and 6 (Thoma et al., 1971), a pH of 6.8 was chosen, similar to the pH in the cytoplasm at the onset of the dark-acidification cycle in CAM. Without Pi in the incubation medium, the amylases catalyze predominantly the formation of maltose; indicative of the action of α-amylase, an elevated percentage of label in maltotriose was observed. Supplementation of the assay mixture with 1–10 mM Pi, the substrate of starch phosphorylase, results in increasing formation of G1P and, as indicated in Fig. 1, in a concomitant reduction of maltose synthesis. The level of maltotriose and maltotetraose showed a minor decline as a response to higher Pi concentrations. These changes, however, were not a consequence of starch degradation accelerated by the additional action of phosphorylase, since starch consumption remained almost constant between 63 and 65% in all 5 assays (Fig. 1), but were apparently a consequence of the inhibition of amylase, particularly β-amylase, by Pi.

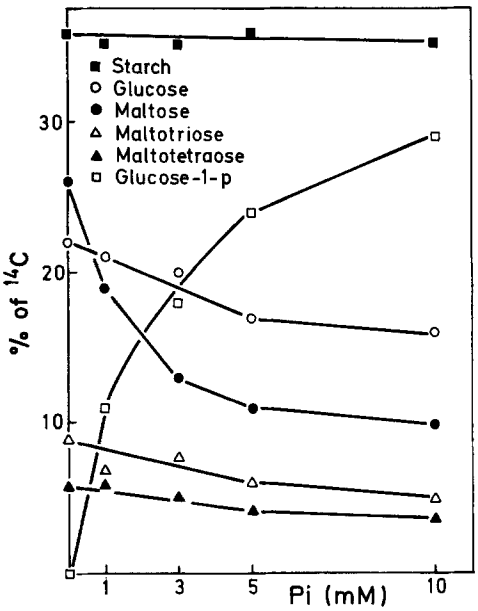

Fig. 1. Effect of various concentrations of Pi upon starch degradation and reaction products by a 30–70% $(NH_4)_2SO_4$ fraction from leaves of *Kalanchoë daigremontiana* after incubation for 10 min at pH 6.8

Maltose phosphorylase, which is the alternative enzyme for the synthesis of maltose and catalyzes a reaction between G1P and glucose (Schilling and Kandler, 1975) was found to be active in the crude extract of *K. daigremontiana* (Table 2). The chromatographic analysis of reaction products in the assay showed 15.3% of the total label to be in maltose when [^{14}C]G1P was used as substrate. A small fraction of the label was also recovered in maltotriose. Phosphatase present in the crude protein fraction poses a problem in as much as labeled G1P added as substrate is subject to a large extent to dephosphorylation. This fact tends to complicate the evaluation of kinetic parameters since the concentration of the one sub-

Table 2. Formation of products from a mixture of glucose (1 μmol) and glucose-1-phosphate (G1P) (1 μmol) as glucosyl donors by the 30–70% $(NH_4)_2SO_4$ protein fraction from *Kalanchoë daigremontiana* incubated at pH 6.8 for 10 min

Donor	Acceptor	% of total radioactivity				
		G1P	Glucose	Fructose	Maltose	Maltotriose
[^{14}C]G1P	Glucose	11.2	64.0	6.7	15.3	2.8
G1P	[^{14}C]Glucose	0	90.2	0	6.7	0.5

Table 3. Enzyme activities of the 30–70% $(NH_4)_2SO_4$ protein fraction of *Kalanchoë daigremontiana*

Enzyme	Activity ($\mu mol \cdot mg^{-1} protein \cdot h^{-1}$)
α-Glucan phosphorylase	4.0
β-Amylase	4.3 mg[a]
α-Amylase	1.2 mg[a]
Maltose phosphorylase	2.0
ADP-glucose-starch glucosyltransferase	3.5
Maltose-glucosyltransferase	2.1

[a] mg starch converted into dextrins of 2–5 glucosyl units

strate (G1P) is reduced while that of the second (glucose) is increased. However, label lost from G1P is fed into glucose and thus utilized via this compound for the labeling of maltose. The reverse experiment, in which [^{14}C]glucose and unlabeled G1P were employed, yielded a lower incorporation of ^{14}C into maltose. This is once more a consequence of the action of phosphatases present in the assay mixture; in this case, they reduce the concentration of G1P, but simultaneously dilute the specific activity of the labeled glucose and hence lower the incorporation of radioactivity into maltose. As a side result of this assay, the formation of fructose from ^{14}C-G1P demonstrates that at least some of the enzymes of the glycolytic pathway are active in the protein fraction of *K. daigremontiana*.

The only enzyme catalyzing starch synthesis tested was ADPG-starch glucosyltransferase (Recondo and Leloir, 1961). In this assay, which gave an activity of this enzyme of 3.5 $\mu mol \cdot mg^{-1}$ protein $\cdot h^{-1}$, the complexity of the reaction sequence going on in crude extracts became apparant. Long incubation times such as 30 min have turned out not to be representative if enzymes at the crossroads of metabolic pathways are investigated. After 30 min of incubation, the starch formed from ADPG had already lost about 25% of its radioactivity compared to the sample taken after 15 min. Amylases and α-glucosidase apparently degrade the newly formed starch; thus, the radioactivity in glucose showed a considerable increase, from 26.6 to 42.6% of total ^{14}C, which might also be partly the result of ADPG degradation by which labeled glucose was liberated.

As shown in Table 3 the extract of *K. daigremontiana* also contains a maltose-glucosyltransferase, confirming the report by Linden et al., (1974); this enzyme transfers the nonreducing glucose unit of maltose onto free glucose. In this manner an exchange of label between maltose and glucose is accomplished: thus, the incubation of labeled glucose and unlabeled maltose together with the *Kalanchoë* protein yielded ^{14}C-maltose at a rate of 2.1 $\mu mol \cdot mg^{-1}$ protein $\cdot h^{-1}$.

A small amount of ^{14}C (1.5% of total label) was detected in maltotriose, which is probably a result of the action of another enzyme from among the multitude of glucose and glucan transferases which are known to occur in plants (Jones and Whelan, 1969; Linden et al., 1974). The alternative explanation, an exchange between glucose and a maltotriose contamination in the maltose employed can be dismissed because the purity of substrates used was analyzed prior to the experiment and no maltotriose was detected.

Discussion

Degradation of starch can be accomplished by two enzyme systems, α- and β-amylase in sequence with α-glucosidase, or starch phosphorylase. The latter enzyme provides G1P which can enter the glycolytic pathway without further activation and consumption of ATP. Though phosphorylase is also being discussed with regard to starch synthesis and some assays of this enzyme are run in the direction of starch formation, the low concentration of G1P in starch-synthesizing tissues (Kaiser and Bassham, 1979) and the low affinity of phosphorylase for G1P point to a predominance of phosphorylase during starch mobilization (Burr and Nelson, 1975; Steup and Latzko, 1979).

Vieweg and Fekete reported in 1977 that α- and β-amylase were active in tissues of *Kalanchoë daigremontiana;* however, the identification of both enzymes was carried out only by measurement of reducing groups in the enzyme assay after incubation with either soluble starch or β-limitdextrin. In our experiments the starch degradation products by the protein fraction of *K. daigremontiana* were subjected to paper chromatography; thus, the activities of α- and β-amylase could be clearly identified and distinguished. Heat treatment of the protein fraction led to the inac-

tivation of β-amylase and α-glucosidase, and consequently the products of the α-amylase action were detected without being superimposed by large amounts of maltose and glucose (Table 1). The results of the product analysis were supported by the analysis of reaction products of authentic amylases; hence the presence of α-amylase in *K. daigremontiana* can be considered as confirmed.

In starch metabolism, Pi plays a major regulatory role, inhibiting starch synthesis at higher concentrations via ADPG-pyrophosphorylase (Chen-She et al., 1975; Peavey et al., 1977; Preiss and Levi, 1978) and accelerating starch degradation via phosphorylase (Heldt et al., 1977; Steup et al., 1977). In connection with phosphorylase, Pi serves as a substrate, and regulatory effects are based upon the mass action of Pi in the equilibrium of the reaction. However, Pi does not only act at the substrate level, but appears also to have a regulating function in the activity of amylases, in particular β-amylase, as demonstrated by our data in Fig. 1. By increasing the Pi concentration in a competitive enzyme assay the activity of phosphorylase was increased while the production of maltose was decreased (Ki about 3 mM Pi) in a reciprocal manner. This decrease cannot however be a consequence of the additional action of maltose phosphorylase which would convert maltose to G1P + glucose since Fig. 1 shows that the amount of glucose produced decreases with the increase of the Pi concentration. This argument receives further support from the fact that the total turnover of starch was not affected. Thus, the Pi concentration in the cell appears to direct the catabolism of starch. In the presence of sufficient Pi, the energy-saving phosphorylase reaction is turned on while the less efficient production of glucose via amylases and glucosidases is diminished. This finding would help explain the diurnal variations in amylase activity reported by Pongratz and Beck (1978) for spinach. Here, the availability of free Pi would regulate the activity of the dominating β-amylase. An additional factor of amylase regulation is constituted by the reaction product maltose, which according to Vieweg and Fekete (1977) acts in a feed-back type inhibition, the Ki however being 20 mM. Our present concept of the regulation of starch catabolism is shown in Fig. 2.

Maltose can arise through the catabolic action of amylases and in a synthetic manner via maltose phosphorylase (Schilling and Kandler, 1975). This enzyme, whose activity was assayed in the protein fraction of *K. daigremontiana,* is responsible for the rapid labeling of maltose in short-time CO_2 fixation experiments (Linden et al., 1975). Maltose phosphorylase serves two purposes: one, it catalyzes the formation of G1P from maltose, by-passing starch phosphorylase during starch breakdown; two, in synthesizing maltose from G1P it initiates a pathway leading to the formation of primer carbohydrates if it acts in concert with glucosyl- and glucantransferase. These transferases, which were described by Linden et al. (1974) for spinach, are also active in *K. daigremontiana.* The maltose-glucosyltransferase, which transfers the nonreducing moiety of maltose to free glucose, and the related enzyme, which converts two maltose molecules to maltotriose and glucose, could also be shown to operate in *K. daigremontiana.* These transfer reactions start with maltose phosphorylase and lead to the synthesis of glucans, which serve as primer carbohydrates for the net formation of starch.

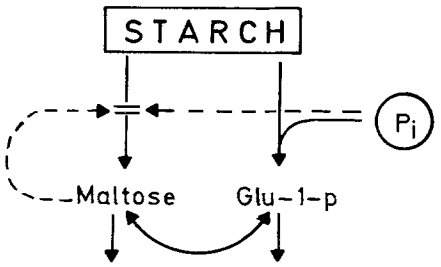

Fig. 2. Diagram of starch catabolism. The concentration of Pi in the cells appears to regulate the pathway of starch degradation. Pi serves as a substrate for starch phosphorylase and concomitantly inhibits β-amylase at a Ki of about 3 mM. Maltose, according to Vieweg and Fekete (1977), inhibits β-amylase in a feed-back type of inhibition (Ki 20 mM). The connection between the hydrolytic and the phosphorolytic pathway of starch breakdown is maintained by maltose phosphorylase which catalyzes the exchange between G1P and maltose

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