THE FATE OF ADPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE DURING AMYLOLYTIC CORROSION OF STARCH GRANULES, AND ITS RELATION TO STARCH GRANULE STRUCTURE[1]

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ABSTRACT

ADPG-alpha-glucan glucose transferase is so strongly adsorbed to the starch granule that it is very difficult to extract. Because of the possibility that the enzyme might be released during amylolytic attack (corrosion) of starch granules, its activity was measured in the starch of germinating corn, barley, smooth peas, and wrinkled peas. In all cases the activity decreased. There was no increase of activity in corn juice during germination. High-amylose wrinkled-pea starch contained much more initial glucosyltransferase activity than smooth-pea starch with less amylose, but there was no direct proportionality. During a period of darkness, not only did green plants lose their starch, but the glucosyltransferase activity in the juice disappeared as well. New glucosyltransferase appeared after the plants had been re-exposed to light. The general conclusion is that glucosyltransferase protein is an integral part of starch granule structure. Corrosion in starch granules of wrinkled pea and potato was studied with the electron microscope. A correlation was shown between type of corrosion, granule structure, and changes in glucosyltransferase activity.

Starch can be synthesized through various metabolic pathways in the plant cell, but there is still great uncertainty about the mechanisms involved (1,2). Two enzymes have been mainly studied in this connection (3). Phosphorylase has wide distribution in plants, occurs in the plastids (1), and can synthesize starch *in vitro* containing glucose-1-phosphate and a primer of the maltose series. The second enzyme is called adenosine diphosphate D-glucose: alpha-1,4-glucan alpha-4 glucosyltransferase. It is able to incorporate the glucose moiety of adenosine diphosphate glucose (ADPG) into oligosaccharides or starch; it is unable to produce starch from sugars or oligosaccharides *in vitro*, but has nevertheless become accepted as *the* starch-synthesizing enzyme; it also has wide distribution (4). We have argued that the experimental evidence available favors the assumption that phosphorylase is an important enzyme involved in starch synthesis (5).

The glucosyltransferase enzyme is found in the juices of plants, even if these plants produce starches of widely differing chemical composition (4), and it can be adsorbed from those juices to starch or its fractions (4,6). However, when one measures transferase activity in starches, it is found only in those containing amylose, and therefore we have postulated (5) that the presence of linear molecules might be a consequence of the strong affinity of the enzyme protein for starch. If amylose molecules were formed first, presumably by phosphorylase, their association with glucosyltransferase protein might prevent them from becoming branched by Q-enzyme. It would therefore be of great interest to find out whether the glucosyltransferase is adsorbed

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to a particular fraction of the starch. The enzyme protein might further be the genetic carrier determining shape and composition of the starch granule (5).

As a first experimental approach to these problems, we attempted to extract the glucosyltransferase enzyme from starch granules, and we found activity in the watery extract after grinding the granules for short periods (6); this effect is presently under study in our laboratories.

Another method would be to break down starch granules by means of amylases (so-called "corrosion") in the hope that the enzyme would be released. The most careful and practical way of carrying out such an experiment would be to allow the plants to corrode their own starch granules. The obvious objects to study here are germinating seeds and potatoes; the investigation could also be done by starving plants during a period of darkness, a treatment that causes the starch to disappear from the leaves. Would transferase, released from the starch into the cells, be available for renewed starch synthesis, if such plants were re-exposed to light?

Since an eventual release might be dependent upon the mode of corrosion, it was also necessary to obtain a more detailed picture of corrosion than is presently available; to study this aspect an electron microscope was used.

Materials and Methods

Seeds of corn (cultivar Golden Rocket), smooth peas (cultivar Alaska), wrinkled peas (cultivar Little Marvel), and barley (cultivar York) were germinated in the greenhouse. Starch was isolated by the method previously described (5). Amylose percentages were determined colorimetrically (5). Assays of ADPG-alpha-glucan glucosyltransferase activity were carried out by measuring the amount of adenosine diphosphate (ADP) produced, or the amount of glucose-^{14}C incorporated into starch after incubation with ADP-glucose-^{14}C, as discussed elsewhere (6). Enzyme activity was expressed in mμM ADP released or glucose-^{14}C incorporated per 5 mg. starch in 15 min.

For the study of bean leaf juice, the fully developed primary leaves of *Phaseolus vulgaris L.* (cultivar Dwarf Horticultural) were used. Two leaves were homogenized with 3 ml. 0.001*M* citrate buffer pH 7.0 containing 0.001*M* EDTA. The homogenate was left in ice-cold water for 30 min. After being filtered through fine cheesecloth and centrifuged twice at 10,000 r.p.m. for 10 min. at 0°C., the supernatant was removed and used for the determination of ADPG-alpha-glucan glucosyltransferase activity. The standard incubation mixture contained: ADP-glucose, 0.3 μM (1,250 c.p.m.); EDTA, 0.1 μM; glycine buffer, pH 8.4, 4 μM; 5 mg. of soluble starch, and 20 μl. of juice in a total volume of 45 μl. A similar procedure was used for tobacco leaves.

To obtain juice from germinating corn kernels, 20 g. of endosperm tissue was ground in a mortar with 10 ml. glycine-EDTA buffer pH 8.4. The resulting brei was forced through cheesecloth; after two centrifugations about 5 ml. of juice was obtained, which was used for the assay of transferase

activity. Potato juice was isolated in a similar way.

Reaction mixtures containing glucosyltransferase were incubated for 15 min. at 37°. For estimating uptake of glucose-^{14}C by starch it was necessary to solubilize the starch. This was done by leaving the starch overnight in 0.2 ml. 1*N* NaOH and breaking up the lumps with a glass rod. After 5 ml. of water was added, the solution was neutralized with 1*N* acetic acid and the final volume was then adjusted to 10 ml. Three 1-ml. aliquots of this solution were plated on aluminum planchets and the activity was measured with a Nuclear-Chicago gas-flow counter.

Small pieces of wrinkled-pea cotyledons and potato tubers in a well-advanced stage of germination were treated with 1.2 or 5% $KMnO_4$ solution at room temperature, or fixed in a 1% OsO_4 solution adjusted to pH 7.2 with veronal buffer at 4°C. After the material was washed and dehydrated in an alcohol series, it was embedded in methacrylate or araldite. Sections were made with a Porter-Blum microtome and studied with a Phillips 100 electron microscope.

Experimental Results

Corrosion. Plastids in wrinkled-pea cotyledons produce only one starch granule (Fig. 1). At a later stage star-shaped fissures appear in the starch

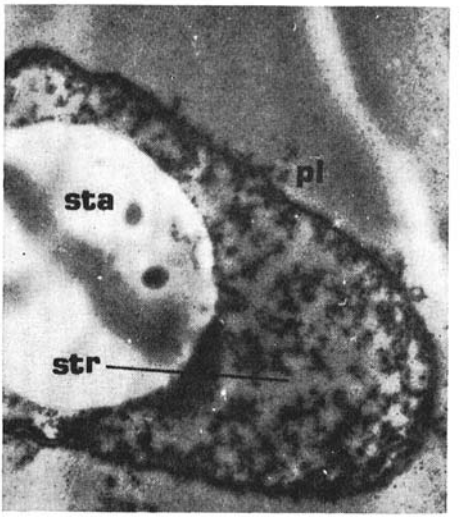

Fig. 1. Amyloplast containing one simple starch granule, in a cotyledon cell of wrinkled pea. Fixation: 1% OsO_4. About 8,000×. **sta,** starch; **str,** stroma; **pl,** plastid membrane.

granule (1), dividing the granule into a number of sectors. During the process of germination the sectors separate from each other and the starch granule fragments become subjected to rapid corrosion, starting at that part

which was near the center. Therefore we see an increasing number of starch granules falling apart during germination, while the fragments decrease in size. The electron micrographs showed how the fragments are corroded. At first the fragments were more or less kidney-shaped and they were lined by densely osmiophilic cytoplasmic particles which eventually penetrated into the depressed area (Fig. 2). The endoplasmic reticulum in the cytoplasm

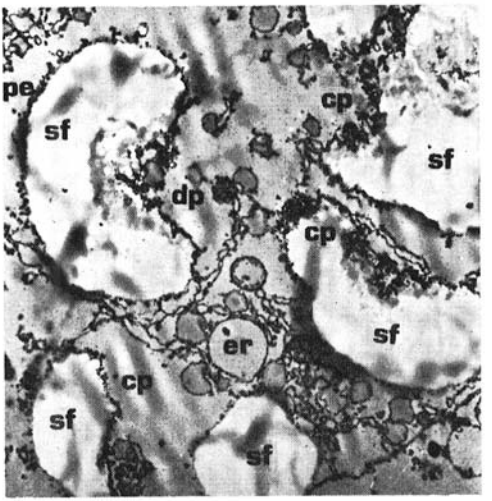

Fig. 2. Group of corroding starch granule fragments in a cotyledon cell of germinating wrinkled pea. Fixation: 1% OsO_4. About 3,300×. **sf,** starch granule fragments; **cp,** cytoplasmic particles; **dp,** depressed area of fragment; **pe,** periphery of fragment; **er,** swollen endoplasmic reticulum.

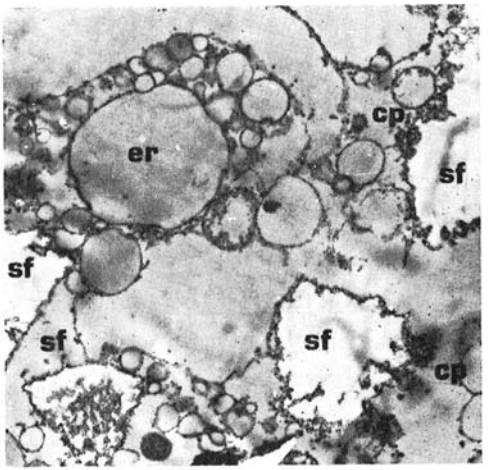

Fig. 3. Advanced stages of corrosion of starch granule fragments in a cotyledon cell of germinating wrinkled pea. Fixation: 1% OsO_4. About 6,000×. **sf, cp,** and **er** as in Fig. 2.

had the vacuolated structure, usually seen after OsO_4-fixation. At a later stage the fragments became smaller and irregularly shaped; the penetration of protoplasmic particles into the interior was stronger in the smallest residues (Fig. 3).

Eventually very small residues were left in the last stages before total disintegration (Fig. 4). One may assume that amylase had penetrated into

Fig. 4. Starch residue in a cotyledon cell of wrinkled pea after 13 days of germination. Fixation: 5% $KMnO_4$. About 17,300×. A reticulate structure has become visible in some places. Note residue of plastid membrane. Inset: reticulate structure from another residue (same magnification).

all parts of these small residues. Only where this was the case did permanganate treatment reveal a reticulate structure in the residues, reminiscent of similar structures observed during the growth of a starch granule (1). Finally, the residues assumed a granular aspect, and these little bodies were eventually liberated.

The fragments shown in Figs. 2 and 3 did not have a trace left of the plastid in which they were formed and which is so clearly visible in Fig. 1. These fragments were therefore in direct contact with the cytoplasm. It is the rule in germinating seeds that the plastid membrane degenerates, exposing the starch granules to enzymatic action in the cytoplasm. In smooth peas the plastid membranes were even found to disappear during the last stages of maturation, and none were seen after the first day of germination (7). However, Fig. 5 shows clearly that corrosion can also take place while the plastid is still intact. We can see in this picture that grana and lamellae had been pressed against the peripheral plastid membrane, as de-

scribed by several authors (8,9). It is, however, unusual that such structures are still present during germination. Corrosion is evident from the fact that the stroma can be seen penetrating into the depressed area of the largest

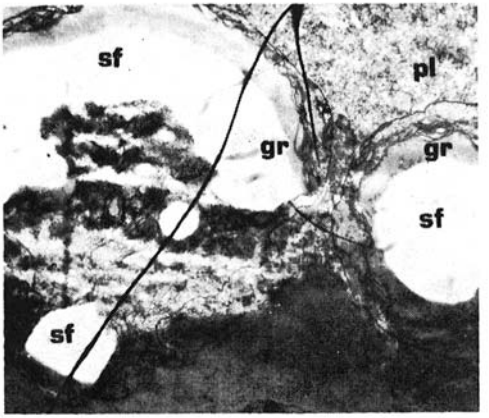

Fig. 5. Two intact plastids containing lamellar structures and corroding starch granules in a cotyledon cell of germinating wrinkled pea. Fixation: 1% OsO_4. 4,000×. **pl**, plastid membrane; **gr**, granum; **sf**, starch granule fragment. (Dark lines are caused by a flaw in the negative.)

fragment in one of the two plastids (Fig. 5). In addition, there are two very small residues of fragments in the same plastid. The stroma still contains grana, but they were degenerating. Dense cytoplasmic particles, as we see them in Figs. 2 and 3, are not visible.

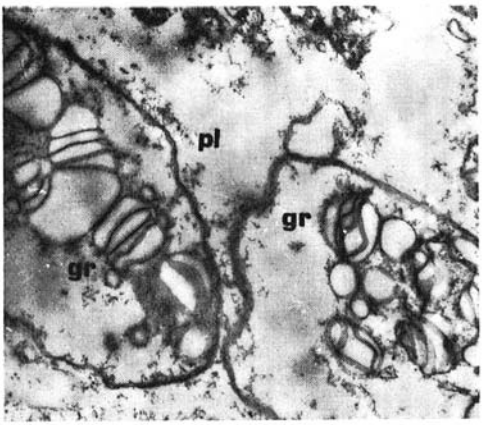

Fig. 6. Two plastids with swollen grana and no starch, in a cotyledon cell of germinating wrinkled pea. Fixation: 1% OsO_4. 8,000×. **gr** and **pl** as in Fig. 5.

Bain and Mercer (9) detected, in pea cotyledons, plastids that had never formed starch. We found such plastids also in germinating wrinkled peas (Fig. 6). Remnants of plastid membranes were often seen near the smallest

starch residues (Fig. 4), and sometimes an uninterrupted membrane enclosed a conglomerate of such residues.

In contrast, the plastids in germinating potatoes always remained intact, and only in very rare cases did the stroma penetrate into the residue of the corroded starch granule. The starch granules were degraded from the periphery and became spindle-shaped (Fig. 7). Even the smallest residues

Fig. 7. Spindle-shaped starch granule residue in a plastid of a parenchyma cell of germinating potato tuber. Fixation: 1.2% $KMnO_4$. 20,000×. The knife has cut tangentially through the plastid, so that the plastid membrane is not well defined.

showed no fissures through which enzymes could enter. Throughout the process of corrosion, the starch residues remained coated with a thin pseudomembrane. In addition there was often a second, more osmiophilic, pseudomembrane, which was either fused with the one bordering the starch residue or located at a certain distance away from it (Fig. 7). It is not known whether these structures have any significance.

TABLE I

ADPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE ACTIVITY IN STARCH AND JUICE OF THE ENDOSPERM OF CORN KERNELS AT VARIOUS STAGES OF GERMINATION

Germination Time	Transferase Activity			Amylose Content
	Starch		Juice	
	ADP	Glucose-^{14}C	Glucose-^{14}C	
days	*mμM/5 mg./15 min.*	*mμM/5 mg./15 min.*	*mμM/10 μl./15 min.*	*%*
0	46	48	15.5	16.4
3	41	38	15.5	16.2
6	28	23	12.5	16.8
9	16	15	10.0	16.6

Activity of the ADPG-Alpha-Glucan Glucosyltransferase during Corrosion. The values obtained for the starch and the juice from corn kernel endosperms are summarized in Table I. There is good agreement between the

two methods of enzyme determination. Glucosyltransferase activity of the starch decreased to about one-third of the original activity during a period of 9 days, whereas the amylose content remained unaltered. The little glucosyltransferase activity found in the juice soon started to decline. Evidently there was no release of the transferase enzyme during starch granule corrosion.

The data for glucosyltransferase activity in the starch of germinating wrinkled peas, smooth peas, and barley are collected in Table II. In these

TABLE II

ADPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE ACTIVITY IN STARCH FROM GERMINATING WRINKLED PEAS, SMOOTH PEAS, AND BARLEY

Germination Time	Wrinkled Pea		Smooth Pea		Barley	
	Transferase	Amylose	Transferase	Amylose	Transferase	Amylose
days		%		%		%
0	83	68.7	30	35.7	29	20.0
3	43	66.8	21.5	37.3	0	20.3
6	31	68.8	19	36.8	0	20.1
9	9	65.2	12.5	35.1	0	19.8
12	0	60.9				
15	...	50.0				

cases no activity determinations were done in the juices. Activity was measured in ADP-units.

In general, glucosyltransferase activity was considerably reduced after 9 days of germination, and at about that time the amylose content of wrinkled-pea starch started on a downward trend, after it had remained constant during the first week (smooth peas and barley did not have sufficient starch left after 9 days for further investigation). It is extremely interesting that wrinkled-pea starch, which contains much more amylose than the starch of smooth peas, also had a much higher initial transferase activity. However, although the wrinkled-pea starch contained 33% more amylose than that of smooth peas, its transferase activity was 53% higher, so that there was no direct proportionality.

Nevertheless, some indication of a relation between amylose and glucosyltransferase may have been obtained. In both cases the glucosyltransferase activity dropped to about the same level after 9 days of germination, and the decrease in activity was therefore much more rapid in wrinkled-pea starch (90%) than in smooth-pea starch (60%). Sometimes starch from mature barley endosperms showed some glucosyltransferase activity, as in the experiment recorded in Table II; sometimes it had none; but in all cases no trace of the enzyme was found in barley starch as soon as germination had started. ADPG-alpha-glucan glucosyltransferase activity was measured in juice from bean and tobacco plant leaves 1) after the plants had been growing in the greenhouse, 2) after a dark period to remove the starch, and 3) after re-exposure to light in the greenhouse. In addition, we tested the juice of etiolated plants that had germinated in the dark and had never been exposed

to light. Figure 8 is a histogram of the results. Both etiolated and green starch-containing bean leaves contained a fair amount of glucosyltransferase activity. During the dark period the activity disappeared from the green leaves; after return to normal conditions the enzyme reappeared in the juice, with larger recovery in bean leaves than in tobacco leaves. In this

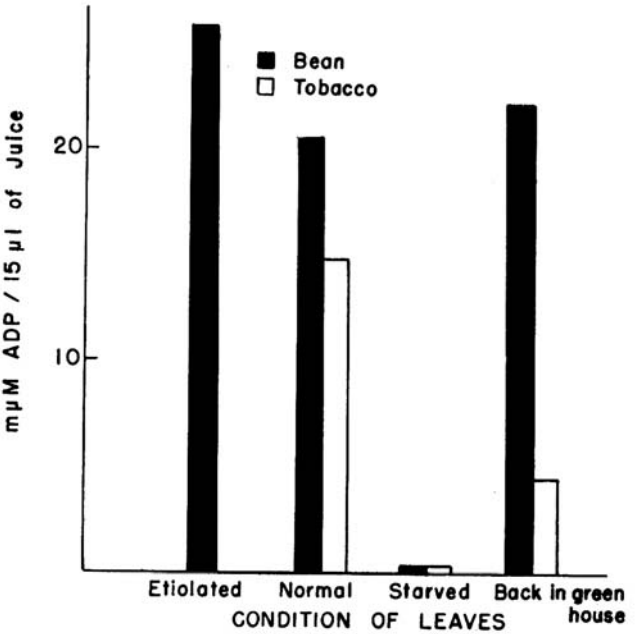

Fig. 8. ADPG-alpha-glucan glucosyltransferase activity in bean and tobacco leaf juice under various external conditions. Etiolated—germinated in the dark; the leaves are small, the stems very elongated, and there is no chlorophyll. Starved—a green plant kept in darkness until starch has disappeared; the leaves are still green.

first experiment, glucosyltransferase activity was measured in ADP-units. The results were the same when the experiment was repeated during incubation of the juice with ADP-glucose-^{14}C for the measurement of glucosyltransferase activity. We intend to investigate in more detail the effect of external conditions, such as light intensity and photosynthetic activity, on enzyme recovery.

Discussion

The corrosion of starch granules in higher plants was first extensively studied by Krabbe (10) and later by Meyer (11), Badenhuizen (12) and Sandstedt (13). It appears that most starches sooner or later develop cracks through which the amylolytic enzymes can penetrate (*endo*corrosion). In contrast, relatively few starches, among them potato starch and assimilatory

starch in the leaves (14), are attacked at the periphery of the granules only and therefore show what may be termed *exo*corrosion.

Our electron pictures (Figs. 2 to 5) demonstrate that the fragments of wrinkled-pea starch are subject to both endo- and exocorrosion. Since only one starch granule is formed per plastid in the cotyledons of wrinkled peas (Fig. 1), the mature granules with their radial cracks are simple, and not compound. The cracks will have dislocated the molecular pattern, with the result that the fragments become more accessible to amylase action. One therefore expects endocorrosion to proceed initially at the inner parts of the fragments and exocorrosion at the periphery, and this is what we see. Starch from smooth peas also shows endocorrosion (10,12), but there is no fragmentation.

In most cases the fragmented starch granules of wrinkled pea become exposed to the cytoplasm because the plastid has degenerated. Corrosion seems to be connected with the action of cytoplasmic particles, which are mainly visible after OsO_4-fixation, and may carry amylolytic activity. However, some plastids in wrinkled-pea cotyledons did not produce starch (Fig. 6); in other cases we found intraplastidal corrosion (Fig. 5) and even a persistent plastid membrane around fragments in the last stages of corrosion. In corn endosperm, too, some plastids may not have formed starch, although they contain phosphorylase (15). Evidently the availability of substrates will determine whether starch is formed or not, and to what extent. If the starch granule remains small it does not need to destroy all stroma lamellae. In principle there is no difference between the structure of the plastids in Figs. 5 and 6, and therefore we do not think that there are two different types of plastids, as suggested by Bain and Mercer (9).

Electron pictures of growing starch granules have been interpreted to show incorporation of particles from the stroma into the periphery of the granules, resulting in a reticulate structure (16). A similar structure becomes visible in the last stages of corrosion (Fig. 4) and may well represent a protein skeleton pervading the starch granule after the incorporation of stroma during the process of apposition (1). The glucosyltransferase protein should be part of this network.

There is evidence that during the process of starch isolation the granules may become coated with proteins adsorbed from the juice; these proteins have glucosyltransferase activity (6). Differences in adsorption of enzyme and differences in the porosity of the starch granules may be contributing factors to the lack of proportionality found between amylose content and glucosyltransferase activity in such cases as smooth-pea and wrinkled-pea starches (Table II). The important fact remains that high-amylose starch of wrinkled peas had much higher glucosyltransferase activity than the smooth-pea starch with less amylose. Enzymes may be present in larger quantities than needed and so mask their exact relationships to certain molecules.

The experiments reported in this paper demonstrate that the glucosyltransferase loses its activity as the starch is broken down during corrosion. In

germinating seeds the starch-containing tissues are disintegrating (7), and therefore the loss of glucosyltransferase activity may be due to the hydrolytic conditions generally prevailing in such tissues.

However, leaves which had been starved in the dark contained tissues which remained vigorous and capable of reverting to normal metabolism, and in such leaves the glucosyltransferase activity also disappeared with the starch. One may conclude that the enzyme became an integral part of the granule structure. When the latter is disrupted by amylolytic degradation of the starch molecules, the glucosyltransferase protein is changed simultaneously so that it loses its enzyme activity. Its reconstitution is a subject for further research.

Potato-starch granules remain closed systems during their exocorrosion in the plastid. Consequently, these residues retain high glucosyltransferase activity throughout the corrosion (17). Further investigations on potato starch are in progress.

It has been shown before that amylolytic corrosion is a process which both in the way and the rate it proceeds, is dependent upon the structure of the starch granule (11,12,13). It may be suggested that proteins, such as that of the glucosyltransferase, could play an important role in determining that structure, and perhaps even the shape of the granule.

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