UDPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE AND AMYLOSE CONTENT OF SOME STARCHES DURING THEIR DEVELOPMENT AND UNDER VARIOUS EXTERNAL CONDITIONS[1]

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ABSTRACT

Low activity of UDPG-alpha-glucan glucosyltransferase in waxy corn varieties had suggested that this enzyme might be connected with the presence of amylose in most starches. Starches varying in amylose content were isolated for the determination of glucosyltransferase activity. Use was made of the natural increase in amylose content of starch during its development in corn endosperm or tobacco leaves; in other cases, changes in amylose percentage were induced by varying the environmental conditions (light, temperature) during growth. The results obtained so far do not provide evidence for a direct proportional relationship between glucosyltransferase activity and amylose content, although in some cases both decrease or increase simultaneously. The results suggest that plastid proteins may be important in determining the final composition of the starch granule.

All higher plants produce starch at one stage or another in the form of granules. It is a general rule that in higher plants granules are initiated inside small cell organelles, called plastids, of which there are various types (1). Those plastids which contain chlorophyll and represent the site of photosynthesis are called "chloroplasts." They possess a complicated lamellar structure, embedded in a proteinaceous ground substance or stroma. Starch granules formed during the day as the final product of photosynthesis appear in the stroma, in between the lamellae; they are always very small, may disappear again during the night, and constitute the so-called assimilatory starch. Obviously, leaves are the main organs in which assimilatory starch is produced.

In other plant organs, such as seeds, bulbs, tubers, roots, and stems, starch granules are likewise deposited inside plastids, but in these cases the plastids do not contain chlorophyll and show hardly any internal structure. Starch granules in such "amyloplasts" are larger and are stored for long periods; they represent the reserve type of starch. In exceptional cases, such as tobacco, the chloroplasts of the young leaf are transformed into amyloplasts as the leaf matures. The young tobacco leaf therefore contains assimilatory starch, whereas in the fully expanded leaf, reserve starch is found.

Critical reviews of the enzymatic reactions that may be involved

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UDPG is an abbreviation for uridine diphosphate glucose.

in starch synthesis have been written by Badenhuizen (2) and by Whelan (3).

In vitro, the following reactions have been established: 1) phosphorylase (P-enzyme) produces amylose (linear alpha-glucan chains) from D-glucose-1-phosphate (G-1-P) in the presence of malto-oligosaccharides as primers; 2) a branching enzyme (Q-enzyme) produces amylopectin from amylose; 3) a transferase is able to remove the glucose moiety from uridine diphosphate glucose (UDPG) and transfer it to both amylose and amylopectin molecules. This enzyme is therefore a (U)(A)DPG-alpha-glucan glucosyltransferase, also called "starch synthetase." There is much uncertainty about the functions of these enzymes *in vivo* and their relation to other enzymes in the living cell.

One can easily demonstrate that P-enzyme is localized in the plastids, and presumably this would also apply to Q-enzyme, since all starch granules contain amylopectin. However, when both enzymes act together *in vitro,* always a branched product is obtained, and the question arises: Why do we find linear molecules in natural starches?

The alpha-glucan glucosyltransferase is localized in the starch granules; attempts to extract it have failed so far. P-enzyme is also adsorbed to the starch, but can be removed with cold acetone (4). The transferase appears to elongate starch molecules; it has not been shown to initiate starch formation, but it can act upon starch whenever this has been formed (4,5,6,7). The name "starch synthetase" implies that this enzyme would be solely responsible for starch synthesis, but for this there is as yet no evidence. There is, unfortunately, no reason to believe with Leloir (8) that "we now know in general how polysaccharides are formed," and for the time being we prefer to avoid the name "starch synthetase." There are indications that ADPG is the natural substrate, rather than UDPG (5,6). Both P-enzyme (9,10) and alpha-glucan glucosyltransferase (6) occur in leaves, so that, whatever the differences between assimilatory and reserve starch may be, their mechanism of synthesis appears to be the same.

The discovery that waxy corn is deficient in UDPG-alpha-glucan glucosyltransferase (11) was of great interest, since it provided for a link with the production of amylose, of which fraction, as is well known, very little is found in waxy varieties. The following questions could now be asked: Are amylose and amylopectin, as found in ordinary starch, the products of two separate reaction systems? What is the function of the glucosyltransferase in this respect? The fact that glucose is transferred to both amylose and amylopectin (7) does not support the existence of separate reaction systems for their synthesis. It is also of interest to note that after the incorporation of glucose-^{14}C from

UDPG-^{14}C the specific radioactivity of the amylose fraction was consistently higher than that of the amylopectin fraction (7).

In addition to the properties mentioned above, alpha-glucan glucosyltransferase is able to form malto-oligosaccharides of low degree of polymerization (DP). Such oligosaccharides can act as a primer for phosphorylase, but not for glucosyltransferase, action during the process of starch formation. These features were recently confirmed by Frydman (4), who also mentioned the possibility that there might be two related enzymes, one for the extension of starch polymer molecules and one for the extension of oligosaccharide molecules. Examples of pairs of related enzymes, which distinguish between substrates of high and substrates of low DP, are R-enzyme and limit dextrinase (12), and Q- and T-enzymes (13).

Elongation of amylose and amylopectin molecules presupposes, but does not explain, their presence. The glucosyltransferase has, however, another property which could be of importance in the production, or perhaps preservation, of linear molecules: the enzyme is very strongly adsorbed to the starch. All determinations of glucosyltransferase activity, when done with starch suspensions, are topochemical reactions, confirming the inclusion of ground substance from the plastid as observed with the electron microscope (14).

From the foregoing the following conclusion could be drawn: the formation of amylose and amylopectin is brought about by one enzyme system, presumably the P- and Q-enzymes; but some linear molecules are prevented from becoming branched, and this is somehow connected with the presence of alpha-glucan glucosyltransferase. One might further assume that protection against branching could be provided by the adsorption of the enzyme to a number of amylose molecules.

On the basis of such a working hypothesis a relationship between glucosyltransferase activity and amylose content could be expected. In order to investigate whether such a relationship exists, starches of varying amylose content are required, preferably isolated from one species of plant.

One possibility is to make use of the well-known increase in amylose content of endosperm starches during the development of the cereal grain. It is less well known that a similar process takes place in the maturing tobacco leaf (15), making this another interesting object of study. One should remember that the tobacco plant is an exception, in that its chloroplasts are gradually transformed into amyloplasts as the leaf ages; instead of the customary small assimilatory starch granules characteristic for chloroplasts, we find larger starch granules of the reserve type, which are often compound.

One can also try to influence amylose content by growing plants under various conditions of light and temperature. We have used both experimental approaches in relation to glucosyltransferase activity, and the results of our first experiments are reported in this paper.

Materials and Methods

The following diagram summarizes the plants studied and the methods used to obtain varying amylose contents in their starches.

A. During development in { corn endosperm; tobacco leaves }

B. Under different environmental conditions { constant illumination { tobacco; beans; *Pelargonium* }; different temperature { waxy corn; potato tubers on stem } }

The Plants. Development of corn endosperm was studied in high-amylose corn, where we might have expected a more dramatic increase in amylose content than in ordinary corn. Two varieties, numbered 1026 and 1027, were kindly provided by M. Zuber, Crops Research Division, University of Missouri. They were grown in the open and self-pollinated; endosperm starch was collected at various periods up to a month after pollination. Some pericarp starch was also collected.

Ears of waxy corn were harvested on the 10th day after pollination, just before starch was deposited in the endosperm. The ears were then grown in the dark on 4% sucrose solutions at various temperatures until development ceased.

All other plants were grown in the greenhouse under the experimental conditions indicated below. Tobacco leaves (var. Harrow Velvet) in various stages of development were harvested simultaneously and the starch was isolated from each leaf separately. The first fully developed leaf was taken from *Phaseolus vulgaris* (bean plant), but of *Pelargonium* sp. several leaves were harvested from one plant and their starches were mixed in one sample.

Small tubers were produced in sterile culture on stem pieces of potato plants inserted into agar, following the method of Mes and Menge (16). They were allowed to grow in the dark at various temperatures; the agar contained 10% sucrose.

Isolation of Starch. For the isolation of leaf starches the following method proved successful. The leaf parenchyma was homogenized in a Waring Blendor with sufficient ice-cold water for 5 min. The suspension was filtered through three layers of muslin under reduced pressure and the residue was extracted once more. The filtrates were combined and quickly centrifuged at 35,000 r.p.m. for 10 min. The clear

supernatant was discarded and the residue triturated with ice-cold 1*M* NaCl solution, after which the suspension was allowed to stand for 5–10 min. at 0°C. before centrifugation; this process was repeated four to five times. Finally the residue was suspended in 1*M* NaCl, peroxide-free ether was added, and the mixture was shaken in a separatory funnel. After a few minutes' standing, a layer containing tissue debris settled in between the NaCl solution and the ether. This layer was shaken once more with NaCl solution, and the starch was removed from the combined NaCl solutions by centrifugation, washed several times with ice-cold water, then with acetone at −15°C., and dried *in vacuo*.

Starch was isolated from endosperm as follows: Endosperms were removed from the kernels and homogenized in cold water in a Waring Blendor. After filtration through fine cloth and centrifugation of the filtrate at 4°C., the supernatant was discarded. Generally the starch was covered with a layer containing various impurities. This layer was carefully removed with a spatula and the remaining starch was resuspended in cold 1*M* NaCl solution. After the suspension was shaken with peroxide-free ether, the remaining impurities were removed and the starch was further purified as described for leaf starch.

For the isolation of starch from small tubers the latter were treated with 0.004*M* KCN after slicing and then ground in a mortar. Further treatment was as described above.

Amylose Percentage. We adopted Augustat's colorimetric method (17) with slight modifications. A series of starches, of which the amylose content had been estimated by means of potentiometric iodine titration, were used as test objects. The results compared well and could be easily reproduced with an accuracy ± 1%. We found the method to be a very convenient one.

Starch (10 mg.) was moistened with 2 drops of 80% ethanol and the lumps were broken up with a glass rod. After addition of 0.7 ml. 1*N* NaOH and occasional stirring, the mixture was allowed to stand for 24 hr. at room temperature. It was then quantitatively transferred to a beaker with about 200 ml. of water. The pH was adjusted to 5 with 0.5*N* HCl and the solution transferred to a 500-ml. flask with another 200 ml. of water. Vigorous shaking was important at this stage. After addition of 5 ml. of a solution containing 0.2% iodine and 2% potassium iodide, the solution was diluted further to 500 ml., shaken well, and allowed to stand for 15 min. before an aliquot was read in a Beckman photocolorimeter at a wave length of 660 mμ. Amylose content was calculated from a calibration curve, which had been prepared

from the readings given by mixtures of amylose and amylopectin of varied composition.

Alpha-Glucan Glucosyltransferase Activity. UDPG was used as substrate and the amount of UDP liberated by enzyme action determined in the usual way (4,11,19). Activity is expressed as mμM UPD/5 mg. starch/15 min. Reproducibility was good for starch suspensions, considering the topochemical nature of the reaction. Occasionally large deviations were encountered in the replications; much more has to be learned about the properties of the enzyme before such changes are understood. A similar situation has been found in relation to UDPG-glycogen transglucosylase (18).

Experimental Results

The values obtained for high-amylose corn are summarized in Table I. Variety 1027 grew more rapidly than variety 1026. In both

TABLE I
STARCH IN DEVELOPING KERNELS OF HIGH-AMYLOSE CORN

Tissue	Post-Pollination Time	Amylose		UDPG-Alpha-Glucan Glucosyltransferase Activity	
		Var. 1026	Var. 1027	Var. 1026	Var. 1027
	days	%	%		
Endosperm	14	..	9.0	..	..
	16	17.0	25.0	5.0	18.0
	18	20.0	35.0	10.0	40.0
	20	24.0	43.0	38.0	30.0
	26	49.0	47.5	50.0	65.0
	30	54.6	52.0	70.0	80.0
Pericarp	12	7.0	11.0	36.5	50.0
	26	20.0	..	52.5	..

there was a regular increase in amylose content of *endosperm* starch, but the initial amylose percentages were higher in var. 1027 than in var. 1026. In both varieties glucosyltransferase activity increased during development, and var. 1027 had a better start than var. 1026. Therefore, in this experiment, amylose content and glucosyltransferase activity in developing endosperm starch showed some proportionality. Amylose percentage of *pericarp* starch likewise appeared to increase, but, in contrast to the high level eventually reached in the endosperm starch, it stopped at 20%. Although there was an increase in glucosyltransferase activity, its value was much higher than one would have expected on the basis of the endosperm data, so that there is no correlation in this case.

Tobacco plants grown in the greenhouse and subjected to the

normal day-night periodicity showed the expected increase of amylose content in their leaf starch when harvested from top to bottom (up to 30%). Many determinations of glucosyltransferase activity were made in these starches, but the results were irregular and showed no relationship to amylose content.

More revealing were the results obtained from tobacco plants grown in the greenhouse under constant illumination. Amylose content had a constant value of 20% for all leaves from all plants, but glucosyltransferase activity showed a wide variation. A few of the data have been collected in Table II. The corresponding leaves, numbered 1 to 4, were not necessarily of exactly the same physiological age, since not all plants had grown to the same height. We see that there are differences in activity level between plants, and within one plant there is a tendency for glucosyltransferase activity to increase as the leaf develops, followed by a decrease. With constant illumination leaves produced more (reserve) starch per unit of weight than under normal conditions; the leaves were yellowish of color and the starch was easier to isolate.

TABLE II

UDPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE ACTIVITY IN STARCH OF TOBACCO GROWN UNDER CONSTANT ILLUMINATION
(Amylose % of all starch constant at 20)

LEAF	PLANT I	PLANT II	PLANT III
1	38.3	10.0	36.5
2	23.0	25.0	38.5
3	6.0	7.5	45.0
4	6.0	. .	10.0

Assimilatory starch in the leaves of *Phaseolus* or *Pelargonium* did not show a decrease in amylose content when the plants were subjected to constant illumination; on the contrary, there was a little improvement.

However, when *Pelargonium* plants were first destarched by leaving them in the dark for 3 to 4 days, then subjecting them to constant illumination for several days, there was a sharp drop in both amylose content and glucosyltransferase activity of the leaf starch (Table III). Some varieties of *Pelargonium* were more responsive to the treatments applied than others.

Perhaps this was a consequence of the starvation process. Madison (9) had found that destarching was damaging to tobacco leaves and that it caused loss of P-enzyme activity. When we destarched *Pelargonium* and then returned the plant to normal day-night conditions, the starch produced contained normal, or even increased, amylose con-

TABLE III

AMYLOSE PERCENTAGE AND UDPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE ACTIVITY IN PELARGONIUM LEAF STARCH UNDER VARIOUS CONDITIONS

EXPERIMENT No.	CONSTANT ILLUMINATION, DESTARCHED		DAY-NIGHT			
			Not Destarched		Destarched	
	Amylose	Transferase	Amylose	Transferase	Amylose	Transferase
	%				%	
1	11.0	12.5[a]	20.8	37.0[a]	26.5	63.0
2	11.2	25.0	21.2	48.0	20.0	43.0

[a] Sample had been kept at room temperature for 8 months.

tent and glucosyltransferase activity (Table III). As in tobacco, constant light had the effect of reducing amylose content.

Particularly interesting were the small potato tubers raised on stem pieces at various temperatures. As Table IV shows, the starch from these tubers had an unusually high amylose content, constant for all temperatures. More surprising, however, was the rise and decline of glucosyltransferase activity as temperature increased. At 10°C. less glucosyltransferase had been formed than at 18°C., but at 30°C. all activity had disappeared. It is therefore possible that amylose is present in the starch granule without there being any glucosyltransferase activity.

TABLE IV

AMYLOSE PERCENTAGE AND UDPG-ALPHA-GLUCAN GLUCOSYLTRANSFERASE ACTIVITY IN STARCH FROM POTATO TUBERS GROWN IN STERILE CULTURE AT VARIOUS TEMPERATURES

TEMPERATURE	AMYLOSE	TRANSFERASE
°C.	%	
10	25	15
18	25	28
27	25	11
30	25	0

Finally, we wanted to make sure that there was no detectable glucosyltransferase activity in waxy corn, even at various temperatures. Fredrick (20) had found that the blue-green alga *Oscillatoria*, which at room temperature contains glycogen, produced a quantity of linear molecules at 5°–10°C. Waxy corn cobs grown at 10°C. were too retarded in development to be useful; they had not proceeded beyond the stage normally reached on the 10th day after pollination. At 18°, 24°, and 30°C., development had been normal; in none of the starches isolated could glucosyltransferase activity be detected. They were all of the waxy type and showed increasing molecular association at higher

temperature (increase in birefringence end point) and a tendency to irregular granule shape at 30°C.

Discussion

In two experiments a proportional relationship was found between amylose content and UDPG-glucosyltransferase activity; they both increased in endosperm starch of high-amylose corn, and both decreased in *Pelargonium* leaf starch formed after starvation followed by constant illumination. In all other experiments glucosyltransferase activity bore no relationship at all to amylose content and it is therefore impossible to predict one from the other. Comparing starches from different plant species and varieties of varying amylose content, Frydman failed, as we did, to find a correlation between amylose content and glucosyltransferase activity (4).

Nevertheless, there is no measurable activity of UDPG-glucosyltransferase in waxy corn, whereas all amylose-containing starches possess the enzyme. According to Frydman (4), waxy corn does contain some ADPG-glucosyltransferase (its activity being demonstrable with ADPG as substrate), but most of the activity goes into the production of oligosaccharides and very little into the formation of starch. We suggest that this small amount of glucosyltransferase, after inclusion in the granule, may be responsible for the development of "blue cores" in waxy starch granules (1).

Glucosyltransferase activity in the living cell appears to be dependent upon environmental conditions, especially light and temperature, and also on the stage of development or the physiological age of the tissue. The interference of these factors brings about discrepancies between amylose percentage and glucosyltransferase activity. Our figures, for instance those of Table IV, show that the enzymatic activity of the glucosyltransferase has no importance for amylose formation. Its protein could, however, form a complex with some amylose molecules, whether it is active or not, and this possibility will be further investigated.

Protein-polysaccharide complexes have been studied in systems containing glycogen on the one hand, and alpha-amylase (21), phosphorylase (22), or concanavalin (23) as the other reactant. The structure and concentration of the participating substances, and the size of the carbohydrate molecule, were found to be important factors in the formation of the complexes. Such factors undoubtedly influence starch formation in the plastid; in turn they are genetically controlled.

In the past the formation of amylose and amylopectin has often been seen as two separate processes. This concept carried the difficulty

that no mechanism was available for spatial separation of the soluble enzymes involved. The formation of protein-starch complexes would provide for a spatial separation between glucosyltransferase and the phosphorylase system, the latter being the one system responsible for starch formation. We visualize that the whole process takes place within the limits of the coacervation droplets formed in the stroma of the plastid (14).

We are still far from being able to explain why amylose content is depressed in starch formed under constant illumination (cf. Tables II and III), or why an amylose content of about 20% is so common in most starches. It may be that a wide variation of the protein-carbohydrate ratio in the reaction mixture has very little influence on the composition of the complex, as was found for glycogen and alpha-amylase (21). We are hopeful that this new experimental approach has possibilities for the elucidation of those processes which control starch formation in the plant cell.

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