Electron Microscopy of Starch Granules Modified by Bacterial α-Amylase[1]

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ABSTRACT

Starch granules of various types (potato, corn, amylomaizes, waxy-maize, and wheat) were studied by light and electron microscopy before and after attack by α-amylase. Differences in the digestion processes of these starches were revealed. By combining the enzymatic treatment with a lintnerization or a cautious periodic oxidation of the granules, it was possible to define more accurately some characteristics of the granular structure. It was shown in particular that the organization of the peripheral and mid areas and of the central part of potato starch granule may be different. The results are discussed in relation to previous studies.

During the past 15 years, the study of starch by electron microscopy has advanced our knowledge of the ultrastructure of the granule.

Most workers are agreed that, except for wheat starch, the surface of native starch granule is smooth and uniform (1-4). In wheat starch, the first-formed and larger granules frequently possess an equatorial, circular groove (5,6), and their surface presents small depressions, which correspond to localizations of small granules that are formed later (6).

Regarding the submicroscopic structure of starch, different workers have presented different views of micellar organization (7-12). Moreover, the distribution of crystalline and amorphous areas for different types of starch is not yet known.

It was thought that evidence from enzymatic digestion might aid in resolving these problems. Only a few studies by electron microscopy have been carried out to explore the manner of attack of α-amylase on various starch granules (11,13,14,15). In the present paper, the granular structures of the more characteristic types of starch were studied by light and electron microscopy before and after attack by α-amylase from *Bacillus subtilis*. Bacterial degradation of lintnerized potato starch was also made, to have better information on the granule backbone.

MATERIAL AND METHODS

Both tuber (potato) and cereal (wheat, corn, waxy-maize, and amylomaizes) starches were used. The characteristics of these samples are given in Table I.

The samples of wheat and corn starches were prepared by a laboratory according to Mercier (16); and the two amylomaizes, by a process described by Saint-Lebe et al. (17). Waxy-maize, corn, potato, and wheat starches were also obtained commercially.

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TABLE I. CHARACTERISTICS OF THE STARCHES STUDIED

Starch Type	Extraction Process	X-ray Spectrum	Amylose Content %	Damaged Granules in the Starting Samples %	Hydrolysis after 24 hr. %
Potato	Commercial	B	22	<0.1	2
Amylomaize	Laboratory (17)	B	38	<5	3.8
Amylomaize	Laboratory (17)	B	72	<10	4.1
Corn	Laboratory (16)	A	24	<5	22
Corn	Commercial	A	22	<20	24
Wheat	Laboratory (16)	A	25	<5	24
Wheat	Commercial	A	25	<40	41
Waxy-maize	Commercial	A	1	>95 (see the text)	39

These starches vary in their X-ray spectra—type-A for wheat, corn, and waxy-maize; and type-B for potato and amylomaizes (16)—and in their amylose contents as determined by iodine absorption at 20°C. (18).

The *"in vitro"* attack by bacterial α-amylase (Mann Research Laboratories) was effected according to Mercier (16). A 2.5% stirred suspension of starch in 5 mmoles phosphate buffer (pH, 6.9) with 0.01% sodium merthiolate as an antiseptic was degraded by 5 ml. of bacterial α-amylase preparation, computed to contain 3% enzyme on a substrate basis. After 24 hr. (37°C.), the digestion was stopped by adding a sufficient amount of 95% ethanol with 1.5% acetic acid to obtain a final 80% ethanol concentration. The residual starch was washed with distilled water and separated by centrifugation. Percentage hydrolysis was measured by spectrophotometry after colorimetric dosage with anthrone (16).

In addition, a sample of potato starch was acid-treated for 8 months at room temperature, according to Lintner (19), before being submitted to bacterial α-amylolysis.

Observations with the light microscope were made on the Wild model M20. To estimate the percentage of damaged granules of the starting samples, the method of Sandstedt and Mattern (20), with dilute iodine solution, or the method of Baker and Hobson (21), with a double stain, was employed (Table I). Examination of the starch before and after attack by bacterial amylase was performed directly in a drop of water.

The samples were embedded in methacrylate after a preparative treatment previously described (10,11), and cut into sections about 0.1 μ thick. These were studied in a Siemens electron microscope, Elmiskop 1, operated at 80 kv.

This preparative treatment before embedding consists of fixation with silver after a mild periodic oxidation and action of sodium thiosemicarbazide (11). The basis of the method is to achieve local oxidation of anhydroglucose units which are in the accessible regions of the starch granule without destroying the structure of macromolecular associations. However, the oxidation rate varies from one starch to another according to its type, amorphous content, and the extent of damage. Therefore, the method must be adapted for every particular sample (10,11).

In certain cases, to achieve stability of the preparation in the electron beam, sections were shadowed with gold in the Micro-BA 3 Balzers apparatus.

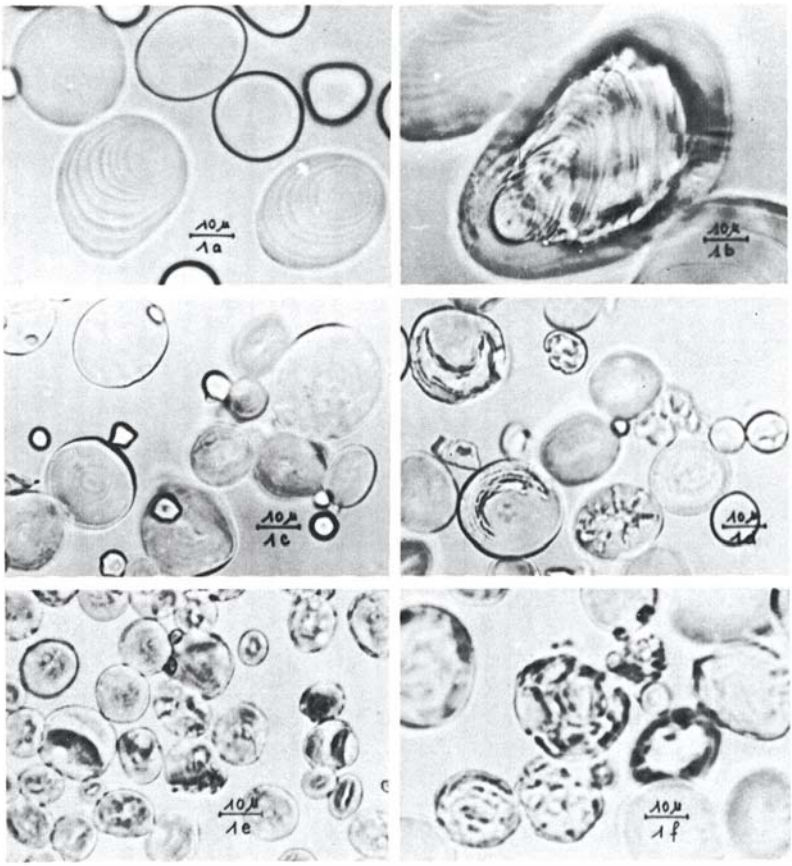

Fig. 1. Photomicrographs of starch granules. Top line, potato (commercial preparation): a) native, b) α-amylase modified. Middle line, wheat (laboratory-extracted): c) native, α-amylase modified. Bottom line, wheat (commercial preparation): e) native, f) α-amylase modified.

RESULTS AND DISCUSSION

Light Microscopy

Figure 1 shows potato and wheat starches before and after attack by bacterial α-amylase. Potato starch is difficult to hydrolyze, as was shown by Leach and Schoch (22), who observed "no evidence of potato starch granule erosion even after 56% hydrolysis." Nevertheless, in our experiments, although the extent of damaged grains in the starting sample was less than 0.1% [by the method of Baker and Hobson (21)], there were at least five times more starch grains damaged after 24 hr. of hydrolysis, especially among the largest grains. In these, the region of the hilum and the surrounding area were strongly attacked, the external part being more resistant (Fig. 1, b). With wheat starch, the observations confirm those described by Sandstedt (23): The attack on most granules progresses both tangentially, forming concentric patterns, and radially, forming radial channels. It is of interest to notice

the important difference between commercial and laboratory-separated wheat-starch samples. The amount of damaged granules is much greater in the commercial (Fig. 1, e and f) than in the laboratory sample (Fig. 1, c and d). Furthermore, after bacterial α-amylase attack, all the granules of the commercial starch are damaged (41% hydrolysis), whereas in the laboratory sample (24% hydrolysis), intact granules can be observed.

Figure 2 shows the starch of waxy-maize, corn, and amylomaize before and after attack by bacterial α-amylase. The grains of amylomaize starches (Fig. 2, e and g) show a heterogeneity of form and size already mentioned (23-26). Estimation of damage by the iodine method (Table I) shows that the extent of damage in commercial starch is greater than in the laboratory-prepared sample. Only a few of the nonfilamentous grains appear to be damaged. All the grains of waxy-maize are colored red, with intensely blue centers, as already indicated by Badenhuizen (27). With Baker and Hobson's method (21), 95% of the waxy-maize granules are colored an intense blue (Table I).

After 24 hr. of hydrolysis, waxy-maize and corn starch granules (Fig. 2, f and d) are nearly all fragmented; the enzyme seems to act both near the hilum area and at the periphery of the granule. With amylomaize starches (Fig. 2, f and h), the degradation is not very noticeable on the micrograph, but in general an attack similar to that on potato starch was observed.

Electron Microscopy

In native starches, the presence of successive layers, alternatively dark and light, is observed.

We must note the great difference between our results and those of Mussulman and Wagoner (26). According to them, the native starch granules observed without staining have alternatively dense and clear concentric layers, the first supposedly being more crystalline and the second more amorphous. In the present case where starch granules are stained by silver, the alternation of dark and light layers, on the contrary, indicates differences in susceptibility to oxidation. A more extensive periodic oxidation occurs in the dark layers, which are presumably less organized and more reactive than the light ones (10,11).

Potato Starch. In the native granule (Fig. 3, a), it may be observed that the layers differ in number and depth, as pointed out by Buttrose (14). Figure 3, b, shows a picture after amylase attack. The split seen is attributable to a rupture in the ultrathin section. The sections were so fragile that it was necessary to reinforce them with a light shadow of gold. The external border of the grain is generally intact. A successive detachment of the different tangential layers may be noticed (Fig. 3, b and c).

Wheat Starch. Unmodified wheat starch (Fig. 4, a) also has a lamellar structure, with an alternation of dark and light layers, the layers being thinner and more numerous toward the periphery. Under our experimental conditions, the commercial sample reacts more strongly with silver than the extracted laboratory starch, some granules in the latter having no reaction at all. Laboratory wheat starch is hydrolyzed by means of radial canals which start at the periphery of the granule, particularly along the equatorial groove. The hydrolysis (Fig. 4, b), which shows a "saw-teeth" pattern, is more extensive in the intermediate part of the granule. Hydrolysis seems to be greater for the dark layers (Fig. 4, c).

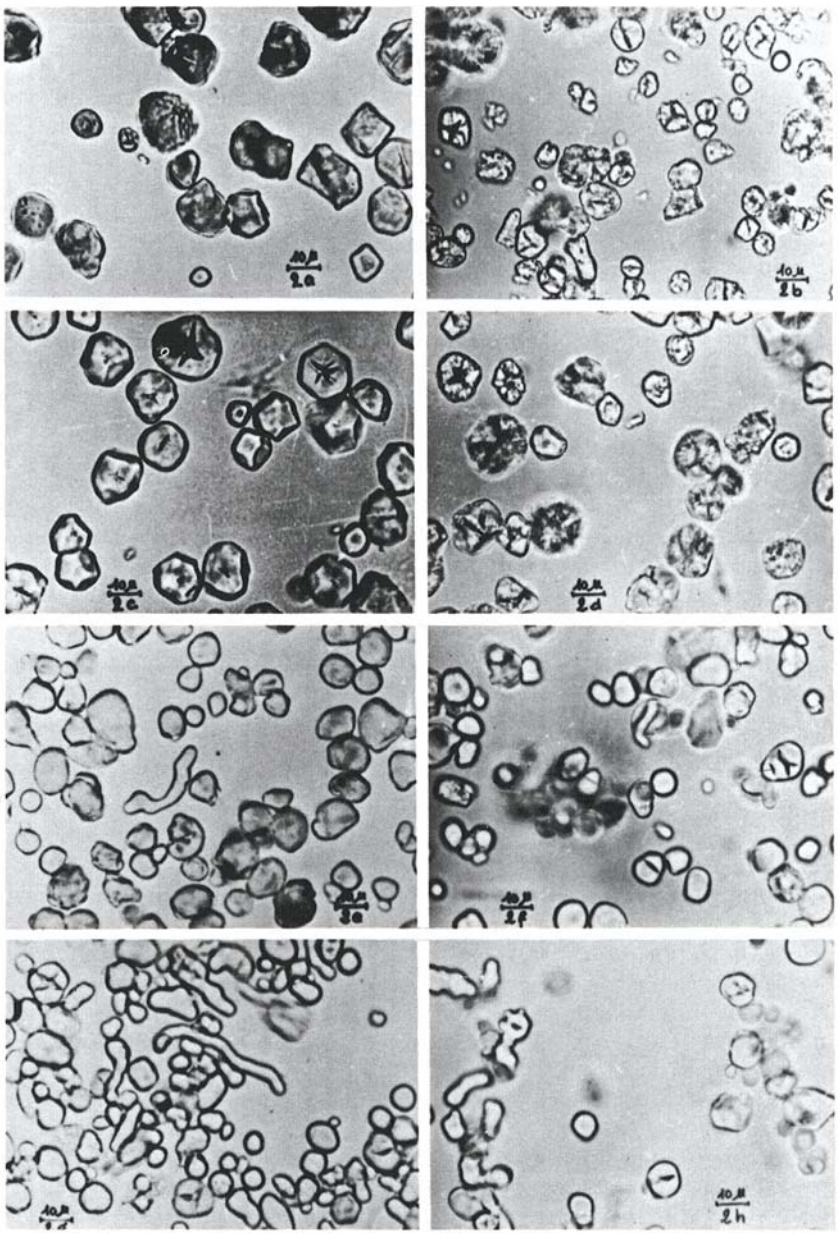

Fig. 2. Photomicrographs of starch granules. Commercial preparation - native: a) waxy-maize, c) corn. Commercial preparation - α-Amylase modified: b) waxy-maize, d) corn. Laboratory-extracted - native: e) 38% amylomaize, g) 72% amylomaize. Laboratory-extracted - α-Amylase modified: f) 38% amylomaize, h) 72% amylomaize.

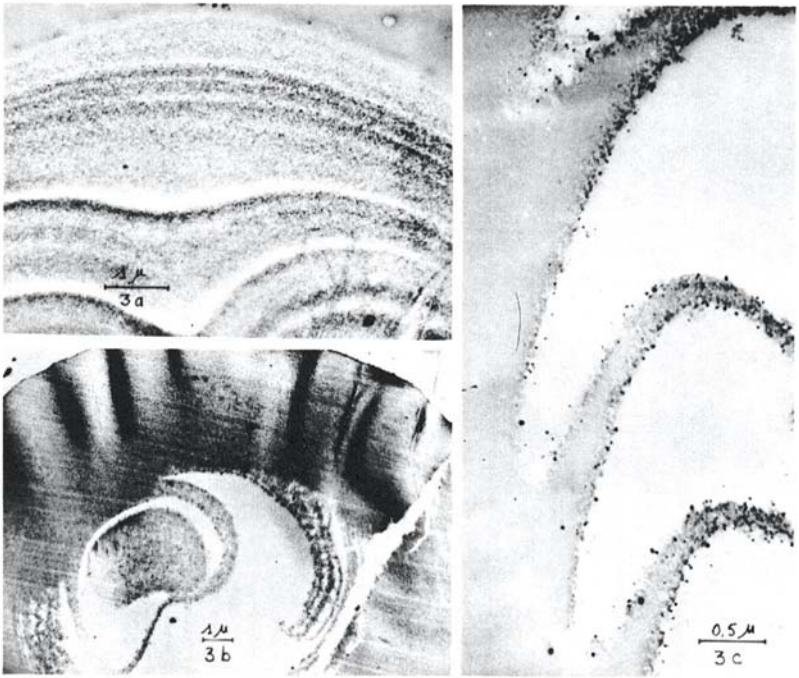

Fig. 3. Electron micrographs of potato starch. Commercial preparation: a) unmodified; b and c), α-amylase modified. To achieve stability of the preparation in the electron beam, section b was lightly shadowed with gold.

Waxy-Maize Starch. In this type of starch, there is a little difference in thickness between the inner and outer layers (Fig. 5, a). The granule is attacked by the enzyme (Fig. 5, b) through corrosion canals and probably through some fissures existing in the native granule, hydrolysis being more effective in the darker, more silver-reacting layers (Fig. 5, c).

Corn Starch. Figure 6, a and b, shows the same type of structure and enzymatic degradation as for waxy-maize starch. Here it is quite apparent that the layers more extensively digested correspond to the dark, more reactive layers (Fig. 6, c). The differences in the thickness of the dark layers, particularly as between Figs. 6a and 6b, are due to different conditions of the staining treatment. With the α-amylase-attacked starch (Fig. 6, b) the periodic acid treatment was necessarily shorter than for native starch (Fig. 6, a), and resulted in less extensive darkening (11).

Amylomaize Starches. Figure 7 shows the appearance of amylomaize starches containing 38 (Fig. 7, a) and 72% amylose (Fig. 7, c, d, and g) and their respective appearances after digestion (Fig. 7, b, e, f, and h). As the amylose content increases, the number of alternate dark and clear layers decreases. In contrast with the observations of Mussulman and Wagoner (26), who suggested an even distribution of the crystalline regions in amylomaize, we usually observed a more reactive area

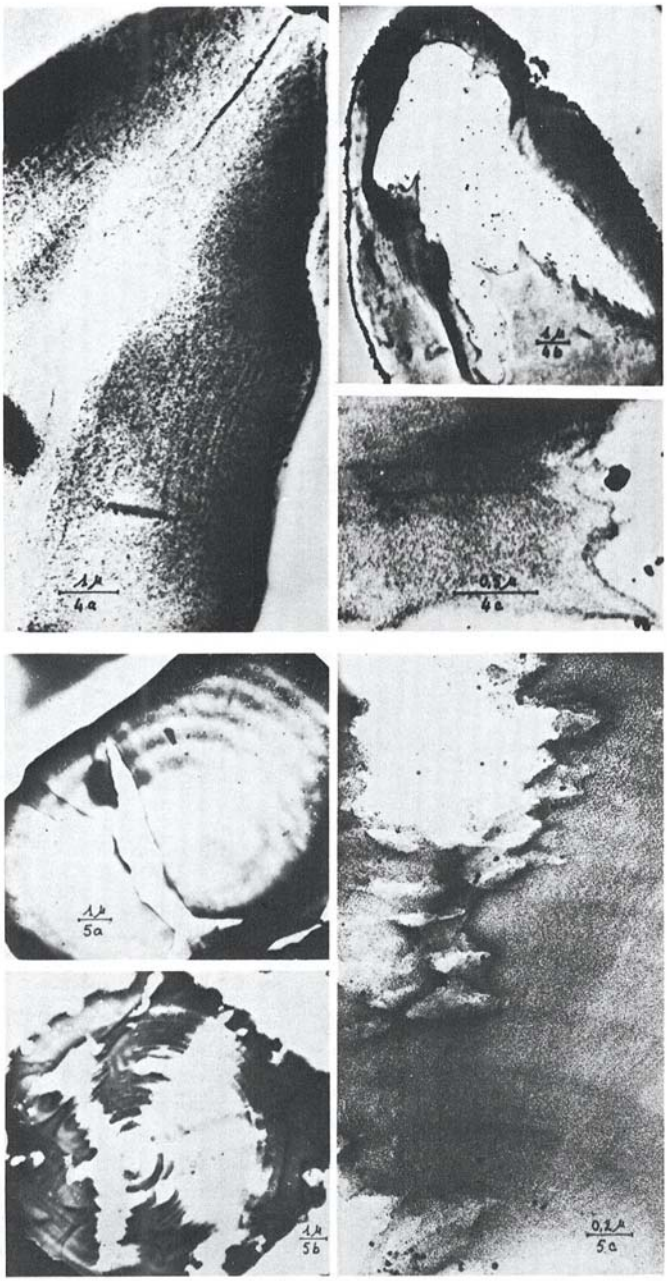

Fig. 4. Electron micrographs of laboratory-separated wheat starch: a) native; b) and c), α-amylase modified.

Fig. 5. Electron micrographs of commercial waxy-maize starch: a) native; b) and c), α-amylase modified.

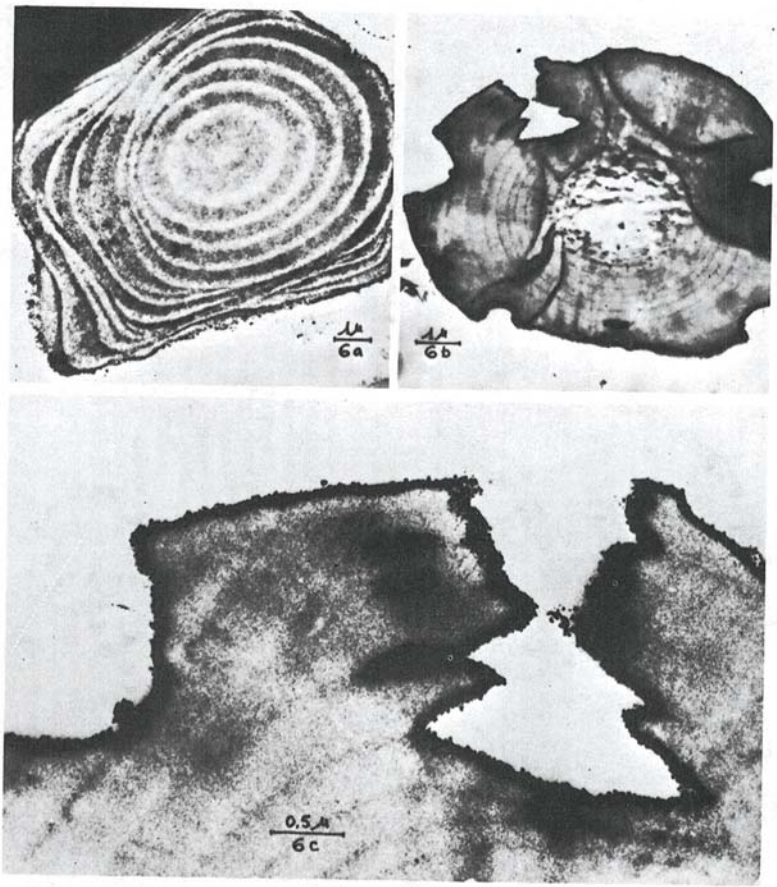

Fig. 6. Electron micrographs of laboratory-separated corn starch: a) native; b) and c), α-amylase modified.

in the central part of the nonfilamentous granules (Fig. 7, c and d). This would indicate that the amorphous region is especially localized there. However, in certain filamentous granules (Fig. 7, g) there was a more even distribution of the reactive material. All the ultrathin sections (Fig. 7, a, c, d, and g) showed a particularly deeply staining border which probably should be ascribed to the presence of lipoproteic impurities on the granule surface. These may persist in spite of the process of purification (16). The presence of these impurities was directly verified with osmium tetraoxide and fast-green staining under the light microscope and by lipid and protein determination (16).

The enzyme attacks the granule either at the central part or along corrosion canals, starting from the periphery of the granule. Again, and particularly with the 72% amylose-content starch (Fig. 7, f, g, and h), the parts of the granule which react more rapidly with periodic acid are those which are degraded more easily by bacterial α-amylase.

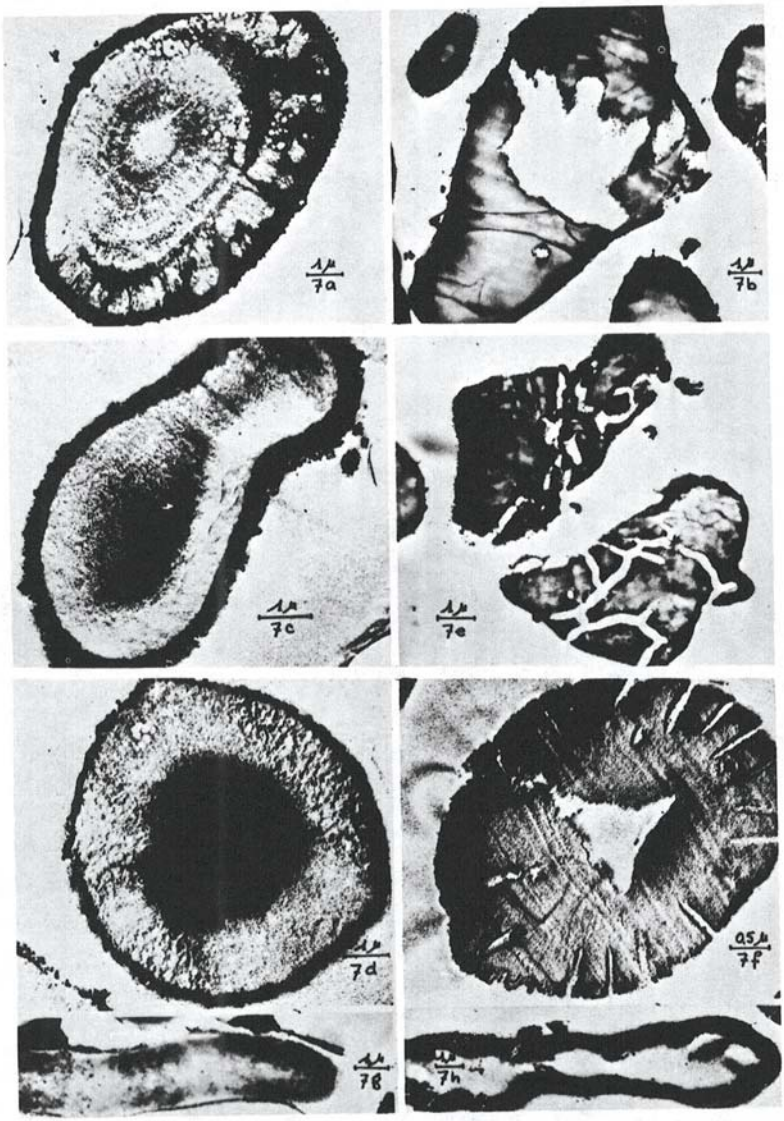

Fig. 7. Electron micrographs of laboratory-extracted amylomaize starches. Native: a) 38% amylose content; c), d), and g), 72% amylose content. α-Amylase modified: b) 38% amylose content; e), f), and h), 72% amylose content. To achieve stability of the preparation in the electron beam, sections a, c, d, f, and h were shadowed with gold.

Lintnerized Potato Starch. After lintnerization, α-amylase attack, and staining with silver (11), we have been able to observe that a radial organization (Fig. 8, b), with strands made of helical microfibrils, exists near the hilum of potato starch. In the middle part of the granule, these helical microfibrils are tangentially oriented (Fig. 8, c and d).

Comparison Between the Various Types of Starch-Granule Structures and Their Behaviors Toward α-Amylase

Alpha-amylolysis of isolated starch *in vitro* can occur by penetration of the enzyme into the granule, either by pitting or by fissures formed on the surface during the processing of starch. It appears that the starches which are rapidly digested with bacterial α-amylase are those whose surfaces are readily attacked, with the formation of canals (wheat, corn, and waxy-maize). This is true even for the laboratory samples, which do not have a large number of initially damaged grains (wheat and corn). In the case of potato starch, as soon as the attack reaches the internal regions, hydrolysis proceeds very rapidly, so that no fragments remain such as can be seen in the cereal starches, according to the results of Leach and Schoch (22) and our own observations (28). However, the surface of the grain in potato starch shows a much greater resistance to enzymatic attack than that of wheat and corn.

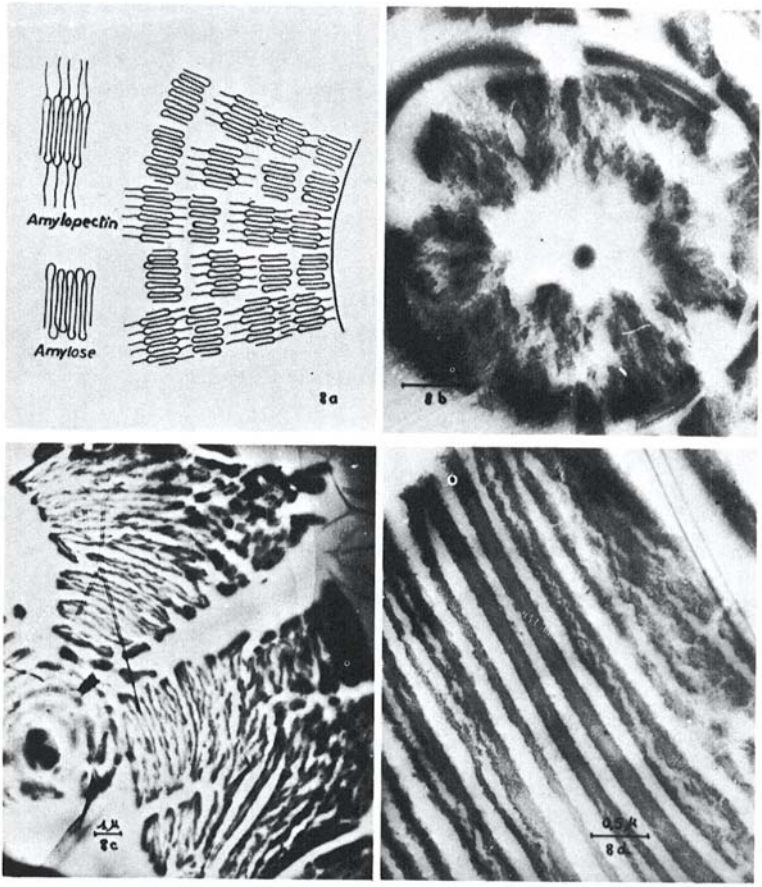

Fig. 8. a) Mühlethaler (9) scheme of starch organization: b), c), and d), are electron micrographs of lintnerized potato starch after α-amylase modification.

Apart from certain amylomaize granules, the starch grain shows the alternation of successive tangential layers. However, in our experiments with potato starch, after lintnerization, α-amylase attack, and staining with silver, a radial organization can be seen in the center of the grain and a tangential organization in the median part.

A radial organization of starch micelles has been observed by different authors (3,7,11,12). Mühlethaler (9), after Buttrose (14), has suggested a scheme in which amylose and amylopectin chains are folded in accordion-like pleats (Fig. 8, a) and arranged side by side in tangential layers. This explanation could reconcile the seemingly contradictory views of tangential and radial organizations in the starch grain.

With cereal starches of the type-A X-ray-diffraction spectrum, the different layers have a resistance to α-amylolysis which probably varies, as their periodic acid reactivity, with their crystallinity.

The fact that, after the enzyme has penetrated into the grain, the attack on the internal region is more rapid in potato than in wheat and corn starches, is in accord with the suggestion that in the crystalline regions of type-B starch the polymer molecules are more loosely bound than in those of type-A starch (16,29). It would be interesting to compare this with the type of radial organization which exists in the central region of the grain of potato starch.

In the case of amylomaize starches, in spite of the B-type X-ray-diffraction spectrum, the interchain associations were shown to be stronger than in the potato starch granule (16,30), which can be related to the mode of attack of the grain.

Acknowledgments

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