Identification of Starch from Various Maize Endosperm Mutants via Ghost Structures

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ABSTRACT

Commercial maize and waxy maize starches and starches from six single-endosperm mutants, eight double mutant combinations, and one triple mutant were examined by a new, simple, rapid, inexpensive technique that requires only a small amount of starch. This technique can be applied in a way that is nondestructive of the germ and the majority of the endosperm of a kernel. The technique involves cooking without shear, staining with iodine, and examining by light microscopy. Each of the 17 starches examined exhibited repeatable unique cooking behaviors and dilute paste compositions that allowed each to be distinguished clearly from the others. Differences found were in the number and shapes of unswollen or only slightly swollen granules; the number, shapes, stained color intensities, and sizes (degrees of swelling) of swollen granules; the number, shapes, and stained colors and intensities of granule ghosts (remnants); and the number of “clusters” present. Clusters, which are reported for the first time, are groups of small units (4–12) with a dark blue center, sometimes surrounded by an envelope. It is suggested that this method has great potential as a screening tool for plant breeders in comparing the behaviors of genetically modified starches to those of chemically modified starches.

Several endosperm genes have been shown to modify the behavioral and functional properties of maize (Zea mays L.) starch biopolymers via structural modifications (Shannon and Garwood 1984, Asaoka et al 1993). Renewed interest in genetic modification of starch has been spurred by several factors, but progress has been slowed by the lack of a method to screen thousands of kernels for a proper genetic combination. Different methods and combinations of methods have been used to examine the effect of genetic modifications on starch properties. Differential scanning calorimetry (DSC) has been used most extensively for screening the characteristics of the starch of maize endosperm mutants (Krueger et al 1987, Brockett et al 1988, De Boer 1991, Inouchi et al 1991, Friedman et al 1993). However, this method may miss important behavioral characteristics and functional properties of starches. For example, the DSC curves obtained from waxy maize and normal maize starches are essentially identical in terms of onset, peak, and endpoint temperatures, and there is only a small difference in the enthalpies of gelatinization; hence, the very important functional properties of waxy maize starch might not have been discovered using this technique. Others (De Boer 1991, Friedman et al 1993) have used the Brabender Viscoamylograph to characterize cooking behaviors and paste characteristics of starches from maize endosperm mutants, but large quantities of starch are needed for this analysis. Gel-permeation (size-exclusion) chromatography, a time-consuming procedure, has also been used as a means of characterizing starches from maize endosperm mutants (Yeh et al 1981, Wang et al 1993). Scanning electron microscopy (SEM) has been used to relate granule morphology to starch genotype (Fannon et al 1992a, Katz et al 1993, Wang et al 1993), but this technique gives no indication of behavioral properties. SEM has also been used to relate paste structures to paste properties (Fannon et al 1992b,c). Sandstedt et al (1968) characterized the starch from five endosperm mutants known to affect the amylose-amylopectin ratio (amylose extender [ae], dull [du], sugary 1 [su1], sugary 2 [su2], and waxy [wx]) and various combinations of these mutants by the degree of water absorption. None of these techniques satisfy the plant breeders’ requirements for a screening method that is simple, rapid, cost effective, and nondestructive of kernels. Therefore, application of these techniques is limited, and with the exception of DSC, they have not been used as screening tools.

Here we describe a method that is simple, inexpensive, and rapid. It consists of undercooking a very dilute starch suspension in an autoclave, staining the paste with iodine, and viewing the results in a light microscope. It requires only very small amounts of starch and, hence, can be employed without kernel destruction, although that aspect is neither described nor used in this initial report. The application of this method to screen various endosperm mutants of maize is reported. We also report, for the first time, the observance of a clusterlike structure in normal maize starch and the starches of some maize endosperm mutants that have been cooked in this way.

MATERIALS AND METHODS

Starches

Commercial maize starch (A.E. Staley Mfg., Decatur, IL) from normal endosperm and its waxy (wx) mutant counterpart were used. Starch from single endosperm mutants (floury 2 [fl2], dull [du], brittle 1 [bt1], shrunken 1 [sh1], and sugary 2 [su2]) and from double mutants (waxy floury 1 [wxfl1], waxy shrunken 1 [wxsh1]) and a triple mutant (amylose extender dull shrunken 1 [su1fl2sh1]) was isolated by a modification of Watson’s method (1964). All genetic stocks were from Purdue University, all had an Oh43 background, and all were selfed at least seven times. For bulk isolation, maize kernels (50) from a single selfed ear were steeped overnight in 0.54% Na2SO4 in a water bath maintained at 45°C. The sodium sulfate solution was decanted, and the kernels were ground in 50 mM NaCl in a Waring blender. The slurry was passed through a 75-μm mesh sieve. The slurry was then agitated with tolune (1:10, v/v) for 10 min and centrifuged at 800 × g. The supernatant (tolune and interphase) was discarded. This process was repeated at least six times (until the tolune interphase was clear). The starch was then washed with distilled deionized water, 70% ethanol, and acetone, and then air-dried.

High-amylose starch (ae V) and starch from double mutant combinations (aedu, aesu2, aexw, dull horned [duh], dusu2, and duwx) were gifts from American Maize-Products (Hammond, IN).

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Sample Preparation for Light Microscopy

Starch (500 mg) was cooked in 250 ml of distilled deionized water under static conditions in an autoclave (121°C, 20 psi) for 45 min, unless otherwise specified. (Much smaller amounts can be used, e.g., 2 mg in 1 ml of water in a microcentrifuge tube. The procedure described here is for isolated starch as used in development of the method.) Pastes were centrifuged at 1,000 × g for 2 min. The supernatant was decanted, and the pellet was washed with 200 ml of boiling water and centrifuged as above for a total of four washes. A 100-μl suspension of the pellet was then stained with 10 μl of an iodine solution (5 g of I₂ and 5 g of KI dissolved in 100 ml of water) that had been filtered through Whatman No. 3 paper. The samples were observed under a bright field light microscope (Vanox S, Olympus Optical, Tokyo, Japan) and photographed (Kodak Ektachrome 160T film). All starches were examined several times with identical results.

Scanning Electron Microscopy

Dry starch samples were observed as described by Fannon et al. (1992a) using scanning electron microscopy (JEOL JSM-840).

Defatting Normal Maize Starch

Corn starch (5 g) was defatted in a Soxhlet extractor with 500 ml of 3:1 (v/v) 1-propanol and water as described by Hoover et al. (1993).

RESULTS AND DISCUSSION

Discussions of the biochemical changes effected by various mutations have been presented previously; refer to Shannon and Garwood (1984) and Preiss (1988) for details.

Normal Maize Starch

The granule envelopes (ghosts) of normal maize starch stained reddish brown, suggesting that they are made up of amyllopectin (Fig. 1A), which is in agreement with previously reported results (Langton and Hermansson 1989, Derek et al. 1992). However, some ghosts had a purplish tint, which may indicate either an incomplete leaching of amyllose, or the presence of longer chains in their amyllopectin molecules. Svegmark and Hermansson (1993) observed that some potato starch ghosts gelatinized in a 2% amyllose solution had stained a light violet color, indicating a partial amyllose content. They further suggested that the amyllose in these ghosts is intragranular and did not originate from dissolved amyllose. It is apparent from Figure 1A that ghosts of normal maize starch granules were the envelopes of highly swollen granules. Some were collapsed; others had disintegrated to some extent, but the majority still maintained their integrity. The most striking feature of normal maize starch pasted under the conditions of this study was the appearance of clusters made up of four to 12 individual units, each of which had a dark blue center. These clusters probably originated from granules rather than being formed from a molecular dispersion, because some clusters still had a membranelike structure surrounding them. The clusters could also be seen in normal maize starch cooked at atmospheric pressure with slight shear (M. R. Jacobson, M. Obanni, and J. N. BeMiller, unpublished data). The number of these clusters was greatly reduced when corn starch was defatted (not shown), but was not affected when starch was treated with a protein-reducing agent such as dithiothreitol (data not shown). These observations confirm the generally held belief that individual normal maize starch granules exhibit a range of cooking behaviors.

bt₁ Mutant Maize Starch

The ghosts of bt₁ starch resembled those of normal corn starch; they might have been smaller. The cook contained more fully swollen granules that stained a purplish color. It also contained clusters, and it was clear that some clusters were enveloped by a membranelike structure (Fig. 1B).

du Mutant Maize Starch

Starch from this endosperm mutant can be classified as a high-amylose starch (Yeh et al. 1981). The cook of this starch contained a more elongated, gelatinized (no longer birefringent), but not highly swollen granules that stained the deep blue color characteristic of high-amylose starch (Fig. 1C). Fully swollen granules and granule remnants that stained like amyllopectin could also be seen, making the cook appear as if it had been produced by a mixture of two granule types. The ghosts of this starch were generally smaller than those of normal maize starch (compare Fig. 1C to Fig. 1A). Although this paste contained significantly fewer clusters than did pastes of normal and bt₁ mutant endosperm maize starches, a few were seen (Fig. 1C), but they are not clearly evident in the figure.

fb₂ Mutant Maize Starch

Starch from the fb₂ endosperm mutant of maize, when cooked under the conditions described, produced significantly more granule debris (Fig. 1D) than did normal maize starch or the bt₁ or du endosperm mutant starches. This cook also contained more fully swollen granules that stained more densely with iodine. The ghosts also appeared to be smaller than those of the previously described starches. No clusters were observed in the fb₂ starch paste (Fig. 1D), which indicated that this characteristic was inherent to the type of starch rather than being an artifact of cooking. (The same equipment and procedures were used throughout this study.) This starch has the same relative amyllose content as does normal maize starch. Therefore, it would be expected to have approximately the same amount of bound lipids, for Morrison and Milligan (1982) found a positive correlation between amyllose content and lipid content of starches from several maize endosperm mutants. The cluster formation could, therefore, be used as a means of distinguishing between genetically modified starches.

sh₁ Mutant Maize Starch

The cook of this maize endosperm mutant contained a mixture of two types of ghosts: some that stained purplish with iodine and others that stained like amyllopectin (Fig. 1E). It is unlikely that the mixture represents a genetic segregation because the starch of this mutant was from the Oh43 inbred line which was selfed for several generations; thus all the loci in this inbred line are expected to be fixed or homozygous. Only a few clusters could be identified in this cook.

su₂ Mutant Maize Starch

Some starch granules from this endosperm mutant were oddly shaped (Fig. 2) and contained segments with distinct divisions between them. Segmented granules were previously described for the su₂, mutant by Sandstedt et al. (1968); similar granules can be seen in the su₂ micrograph presented by Sandstedt et al. (1968), although they did not mention them in the report. Polarized light revealed that the segmented granules of su₂ were birefringent, but did not show the typical Maltese cross of a starch granule (Sandstedt et al. 1968). We found that some of the segments remained together (Fig. 1F) when su₂ starch was cooked under the conditions described. The oddly shaped ghosts formed from the su₂ starch granules were, however, different from the clustered structures formed from starches from normal maize and bt₁ and du endosperm mutants.

ae Mutant Maize Starch

Granules from this mutant exhibited little swelling because of their high amyllose content. Elongated granules and granules with protuberances, which are characteristic of this starch, were observed after the cook (not shown). Some purple color in the background was evident.

duh Mutant Maize Starch

Starch from this mutant combination had the same apparent amyllose content as that of the single mutant du (31%; Katz et al.
Fig. 1. Micrographs of starch pastes prepared under static cooking conditions (121°C, 45 min) at a concentration of 0.02% and stained with an iodine solution. A, normal maize; B, bt1; C, dtu; D, βt2; E, sh1; F, s12; G, d1h; H, aedu; I, d1su2; J, aescu; K, aedashi; L, wx; M, wxfl1; N, duwx (15-min cook); O, duwx; P, aewx. All micrographs magnified 50×. Bar = 50 μm.
Granules from the duh endosperm mutant produced larger ghosts than did those of the du mutant (compare Fig. 1G to Fig. 1C). Gelatinized, but only slightly swollen, granules that stained a deep blue with iodine, indicating their high amylose content, also were present. Again the cook appeared as if it had been produced from a mixture of two granule types. Katz et al (1993) found that starch granules from duh were larger than those of the single mutant du.

**ae du Mutant Maize Starch**

Although this starch was produced by a plant homozygous for the double mutation at the ae and du loci, its cooked paste appeared as if it were a mechanical mixture of two types of granules: normal maize starch granules and granules of a high-amylose starch (Fig. 1H). In addition, small granules tended to aggregate around fully swollen granules or ghosts.

An interesting observation was that du, duh, and particularly ae du starches appeared to be a mixture of two different types of granules: those that appeared to be high-amylose granules and those resembling normal maize starch granules. While DSC analysis might result in double peaks indicative of two granule populations, it would seem that measurements such as apparent amylose content, average chain length, and iodine affinity, to name a few, that give averages of the entire population of granules, would miss the fact that two different types of granules with rather different behaviors are present rather than a starch of hybrid properties. However, ae starch granules were more or less homogeneous, and no highly swollen granules were observed. It, thus, might be inferred that corn plants carrying the ae gene have one type of amyloplast, whereas those carrying the du gene contain two types of amyloplasts. It can be deduced from this study that the mechanism by which a gene or a combination of genes increases the apparent amylose content of a starch granule is unique to that particular genetic combination.

**dusu2 Mutant Maize Starch**

Cooked granules of this double endosperm mutant starch contained segmented granules (Fig. 1I), characteristic of the su2 gene. The ability of the du gene to produce granules that formed ghosts upon cooking was somewhat diminished by its association with the su2 gene, and there were more granules with segments than granules that formed ghosts. This would suggest that the su2 gene is epistatic to du, a result which is in agreement with previously reported data for gelatinization temperature by Inouchi et al (1991).

**aesu2 Mutant Maize Starch**

The combination of these two genes resulted in a starch with granules that swelled little and did not form ghosts, in addition to elongated granules and granules with long protuberances (Fig. 1J). Cooked aesu2 granules were less swollen than were ae granules (not shown). No segmented granules or clusters, nor any background purple color, could be seen in aesu2 starch.

While the ae du combination has approximately the same apparent amylose content as does dusu2 (Katz et al 1993), the distribution and packing of amylose into their respective granules could be different. It can be further concluded that certain genes complement each other in an additive manner (ae and su2) and result in granules with higher apparent amylose contents than affected by either of the genes independently. Because these mutant genes control the enzymes in the biosynthetic pathway of starch, the du gene might control at least two distinct steps. Starch biosynthesis is complex (Shannon and Garwood 1984, Preiss 1988) and deserves further attention.

**ae su2 Mutant Maize Starch**

This starch, like the ae du starch, contained at least two types of granules. However, the number of granules with intermediate swelling was greater than that found in ae du starch (Fig. 1K). Although this starch was classified as a high-amylose starch (51% amylose; Katz et al 1993), a comparison of Figures 1K and 1J indicates that it should behave differently than ae du starch, which appeared as a mixture of ghosts and uncooked granules, or ae starch (50% apparent amylose content). Addition of the sh1 gene to ae du substantially changed the starch by increasing the number of granules with the ability to form ghosts. This raises the question
of whether the sh₁ gene controls the branching enzymes and the formation of long chains in the amylpectin molecules. Results of interaction of these three genes suggests that sh₁ may be epistatic to aed₁u for the ability of granules to form ghosts; yet the aed₁u genes were epistatic to sh₁ for amyllose content. Complex interactions among the three genes is obvious, as is our lack of understanding of starch biosynthesis. Shannon and Garwood (1984) hypothesized that the sh₁ gene affects mainly sucrose synthase and, hence, decreases the total starch content of a maize kernel harboring this gene.

**Waxy and Waxylike Maize Starches**

**wx Maize starch.** Ghosts of waxy maize starch looked more filled, were wrinkled, and had the typical color of amylpectin from iodine staining (Fig. 1L). However, some ghosts stained purple, which indicated that these granule remnants probably were formed from contaminating normal maize starch. This contamination could result either from a lack of complete segregation during storage, transport, or the commercial milling process or from a natural outcrop in the production field of waxy maize seed. No clusters were found in the cooks of this starch. Although waxy maize contains significantly less lipid than does normal maize starch, again, this by itself does not constitute evidence of a linkage between lipid content and the ability of starch to form clusters. In fact, starches from ae, f₁, and su₁ endosperm mutants, which either have about the same or higher amyllose contents than does normal maize starch, and therefore would be expected to have about the same or higher lipid content than does normal maize starch (Morrison and Milligan 1982), did not form clusters, and _du_ mutant maize starch, which has a greater amyllose content, produces only a very few clusters. This is additional strong evidence that clusters are genetically controlled.

**duwx Mutant maize starch.** Although this starch, like wx maize starch, essentially contains only amylpectin, the abilities of their respective granules to form ghosts were not the same. When _duwx_ starch was cooked under the conditions described in this study, there were almost no complete ghosts left in the cook, which essentially contained only granule fragments in a solution of solubilized amylpectin (Fig. 1O). Again the ghosts with integrity most likely arose from contaminating normal maize starch. (The starch used was a sample of a factory-processed starch, but it is not known where or how the contamination occurred.) _Duwx_ starch had some unusual features that looked like ghost remnants surrounding a dark large staining center (Fig. 1O). When it was cooked for only 15 min, the starch from the _duwx_ mutant endosperm formed ghosts (Fig. 1N), but the majority of these granule envelopes were collapsed and appeared to be disintegrating. This starch has been described as behaving like a chemically cross-linked starch (Sandsdot et al. 1968, De Boer 1991, Friedman et al. 1993). This characterization is not supported by our observations.

**wxsh₁ and _wxs_ Mutant maize starches.** Starch from these _duwx_ endosperm mutants contain only amylpectin, like waxy maize starch, but possess distinct physical and functional properties (Friedman et al. 1993). Their uniqueness is supported by our analysis. The _wxsh₁_ mutant maize starch had no ghosts left in its cook (not shown). The _wxsh₁_ mutant starch had a few highly swollen granules that could be classified as ghosts (Fig. 1M). Its cooks were completely different in appearance from those of _wx_ maize (Fig. 1L) or _duwx_ (Fig. 1N) starch cooks, so it could be inferred that the ability of a native starch granule to form a ghost is also under genetic control.

**aewx Mutant maize starch.** Although two mutant alleles controlling high-amyllose content and no amyllose, respectively, made up this gene combination, the _wx_ allele is epistatic to _ae_ and the result is an amylpectin-only starch. Starch from this genetic combination had the ability to form ghosts (Fig. 1P), but their color was purplish, which suggests that these granule envelopes contained amylpectin with long chains, since their amyllose content is nil (Yeh et al. 1981). The _ae_ gene has been shown to increase both the number of long B chains and the average chain length of amylpectin (Ikawa et al. 1981, Wang et al. 1993).


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