JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Why Do Gelatinized Starch Granules Not Dissolve Completely? Roles for Amylose, Protein, and Lipid in Granule "Ghost" Integrity

MARTINE R. DEBET[†] AND MICHAEL J. GIDLEY*[,†,‡]

Unilever R&D Colworth, Sharnbrook, Bedford MK44 1LQ, United Kingdom, and Centre for Nutrition and Food Sciences, Hartley Teakle Building, University of Queensland, St. Lucia, Brisbane, Queensland 4072, Australia

After gelatinization in water, starch granules persist in swollen hydrated forms known as ghosts. Three potential mechanisms for ghost formation are tested. Proteins and lipids on the granule surface are found to be a determinant of ghost robustness, but not ghost formation. Proteins inside pre-made maize or wheat starch ghosts are degraded extensively by proteases without any apparent change in ghost properties, making an internal protein cross-linking mechanism unlikely. Waxy maize mutants with a range of amylose contents have ghost integrities that correlate with (low) apparent amylose levels. It is hypothesized that ghost formation is due to cross-linking of polysaccharide chains within swollen granules, most likely involving double helices formed from polymer chains that become free to move following heat-induced granule swelling. The size and robustness of granule ghosts is proposed to be determined by the relative rates of swelling and cross-linking, modulated by surface non-polysaccharide components.

KEYWORDS: Starch; amylose; amylopectin; granule; granule ghosts; gelatinization

INTRODUCTION

Starch granules represent nature's solution to the problem of long-term storage of carbohydrate energy in plants by packaging hydrophilic glucose polymers (amylose, amylopectin) into a stable condensed form that is capable of controlled degradation by enzymes to release glucose at a later stage. Much progress has been made in identifying the genetic and biochemical basis for the production of amylose and amylopectin molecules, including subtleties of branch length distributions and molecular weight (*1*, *2*). Similarly, numerous studies of starch granules have revealed a hierarchical structure with a logical connection between the nature of branched amylopectin molecular architecture and longer distance scale features (*3*, *4*). In contrast to the natural functions of starch, which take place under ambient conditions, most food and industrial uses of starch involve a heating step that is designed to disrupt the ordered arrangement of polymers within granules ("gelatinization"). Indices of granular order such as crystallinity (X-ray diffraction), helical order (^{13}C CPMAS NMR), and regularity of amylopectin clusters (small-angle X-ray scattering) show that all detectable order is lost following completion of the gelatinization process.

The characteristic physicochemical and rheological properties of gelatinized starches in excess water can be attributed to a combination of solubilized polymers (amylose and/or amylopectin) and residual granular structure (*5*, *6*). Although the solution and gelation properties of amylose and amylopectin have been studied extensively (*6*−*8*), less attention has been paid to the nonsolubilized portion of gelatinized granules. These granular remnants are frequently termed "ghosts" because of their retention of particulate character following loss of significant granule contents (*9*). Granule ghosts have characteristic microscopic features depending on botanical and genetic origins (*10*,*11*), but show no evidence for the structural organization of polymers characteristic of native granules (*12*).

The presence of granule ghosts in gelatinized starch systems leads to a characteristic "short" texture that differs from the "long" viscous texture characteristic of dissolved polysaccharides. One consequence of the industrial chemical modification of starches can be to emphasize the importance of granule ghosts in functional performance. For example, chemical cross-linking can result in cooked microstructures dominated by swollen granules with the rheology of a particulate dispersion (*13*) that has more in common with other cross-linked particles such as chromatography beads than with solubilized amylose and/or amylopectin. Elements of this rheology are also found for cooked dispersions of native starch granules, but other factors such as swollen granule morphology and/or leached components must also be important as viscoelastic properties are not fully explicable in terms of a homogeneous particulate dispersion (*14*).

When native starch granules are heated in water, the sequence of gelatinization events occurs at temperatures typical for the

* Address correspondence to this author at the Centre for Nutrition and Food Sciences, Hartley Teakle Building, University of Queensland, St. Lucia, Queensland 4072, Australia (telephone +61733652145; fax +61733651177; e-mail mike.gidley@uq.edu.au).
[†] Unilever R&D Colworth.
[‡] University of Queensland.

10.1021/jf070004o CCC: $37.00
Published on Web 05/16/2007

botanical and genetic origin of the starch. Once the major long- and short-range polysaccharide order has been lost (as monitored by, e.g., differential scanning calorimetry, X-ray diffraction/scattering, and NMR), swelling of granules occurs to various extents depending on starch type. In most cases, provided shearing is not excessive, it is at this point that granule ghosts become evident. For a few starches, for example, potato, swelling is so extensive that it is difficult to recover granule ghosts, but for most other starches, ghosts can be isolated for further study (*9*−*12*).

The structural factors that contribute to ghost integrity are not well understood. It seems likely that specific stabilizing factors exist in starches, as other biopolymer particles held together by non-covalent stabilization mechanisms do not form ghosts. Examples include the typical commercial forms of agar and gelatin that contain extensive double- and triple-helical structures, respectively, that prevent dissolution at cold temperatures. However, heating to temperatures above that required for melting of helices results in dissolution to a true molecular solution with no evidence for any residual ghost structures. By analogy, once granular double helices have been melted, typically at 60−80 °C, it might be expected that granules would dissolve. As this does not happen, additional structural features seem to be responsible for the persistence of ghosts in gelatinized starches. At least five structuring hypotheses can be proposed:

(a) Amylopectin molecules are of such high molecular weight that nonspecific hydrodynamic entanglements are sufficiently extensive that an essentially permanent physically entangled structure is produced. This hypothesis seems unlikely to be dominant as amylopectin-rich starches typically give lower yields of granule ghosts with reduced stability compared with amylose-containing starches (*9*−*12*).

(b) As yet unidentified covalent cross-links between starch polymers are present. This is a credible hypothesis in that chemical cross-linking results in increased ghost integrity. However, no evidence for this mechanism has been found for native starches to date.

(c) A surface film or coat around granules limits expansion and prevents dissolution. Evidence for this comes from microscopic observations of apparently hollow cores in ghosts coupled with a well-defined outer surface (*10*, *11*, *15*). The surface regions of many granules contain proteins and lipids (*16*, *17*), providing the potential for film formation. It has been shown that surface proteins and lipids play a major role in restricting the postgelatinization swelling of starches such as wheat and maize (*18*).

(d) Effective cross-linking of starch polymers by proteins present inside the granule takes place. Potential cross-links could be either covalent or non-covalent and may be formed during biosynthesis or as a consequence of thermal unfolding during gelatinization. The major protein in most granules is the so-called waxy protein or granule-bound starch synthase (*1*, *17*). Proteins have been visualized within ghosts from some starches (*19*), and there is evidence that they are involved in granule swelling and ghost integrity in mung bean starch (*20*). Disulfide cross-linking of proteins within rice granules has been linked with altered pasting properties (*21*) and waxy maize starches with elevated internal protein content, but similar amylose contents have been shown to have altered rheological properties (*22*, *23*).

(e) Cross-linking of amylose or long branches of amylopectin occurs within swollen granules. In native granules, amylose is believed to be present predominantly in a single-chain state, as evidenced by an ability to leach at least partially from gelatinized granules. Once amylopectin double helices have been dissociated by heat and granules have begun to swell, amylose molecules are expected to be prone to double-helix formation with other amylose molecules or possibly long branches of amylopectin. If enough of these events occur within the swollen granule, then a network of cross-links could be formed that prevents the dissolution of ghosts. There is a strong thermodynamic driving force for this mechanism as amylose double helices melt at ca. 140−160 °C (*7*, *24*); that is, there is a 50−100 °C undercooling of ordered structures.

It is known that lipid complexes are present within many starch granules and that levels correlate with the rate and extent of granule swelling (*17*). However, lipids complex with single molecules of amylose (or long chains of amylopectin), and therefore complexation itself cannot lead to the effective cross-linking of polymer molecules that is necessary for a stable ghost structure.

As indicated above, hypothesis a seems unlikely, and there is no supporting evidence for hypothesis b. In this paper, tests of hypotheses c−e are described. Approaches employed include selective extraction of granule surfaces, treatment of ghosts with protein-degrading enzymes, and the study of waxy maize mutants (*25*) that have significant protein contents within granules and variable (low) amylose contents.

MATERIALS AND METHODS

Materials. Starches were obtained from commercial sources: wheat starch from A.B.R., Corby, U.K.; waxy and regular maize starches from National Starch, Bridgewater, NJ. Alkaline proteinase K, EC 3.4.21.14, from the fungus *Tritirachium album*, and acidic bromelain, EC 3.4.22.4, from pineapple *Ananas comosus* stem (B5144, batch 103 H4052), were obtained in purified form from Sigma. Proteinase K (P2308) batch 123H6885 was used with maize ghosts, and batch 124H6850 was used with wheat ghosts. Pronase E (BDH, 39052 2P; and Sigma, P6911) and crude bromelain (Sigma, B2252) were found to contain traces of amylase activity and were not used further.

Protein molecular weight (M_w) markers for electrophoresis were obtained from Sigma, except for the gel shown in **Figure 3**, which used the Rainbow protein molecular weight markers from Amersham International, Little Chalfont, U.K.

Isolation of Starch Ghosts. Ghosts were isolated on a small scale from commercial maize and wheat starches following an adaptation of a method reported previously (*12*). Starch (100 mg) was suspended in a small amount of cold water and then poured into hot water (95 °C; 20 mL). The dilute suspension (0.5% starch) was kept at 95 °C for 30 min under static conditions (without shear) and then centrifuged hot inside a preheated centrifuge to minimize the risk of retrogradation of amylose leached from gelatinized granules (45 °C, 2000*g* for 15 min). The supernatant was removed, and the spun ghosts were washed twice by resuspension in hot water (90 °C) with gentle manual stirring or whirl-mixing followed by centrifugation. The washed ghosts were finally resuspended in excess water and either examined directly or freeze-dried. Ghost yield was defined as the weight ratio of freeze-dried pellet to initial starch. For the larger preparation scale (2.5 g in 500 mL), the centrifuge was not preheated as the larger sample volume allowed better heat retention during centrifugation, and ghosts were washed using cold water.

Characterization of Starch or Granule Ghosts. Amylose/amylopectin ratios were determined using the method of Morrison and Laignelet (*26*). Protein extracts from starches were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (*27*), using the Phastsystem from Pharmacia, Uppsala, Sweden. Laemmli buffer [2% SDS + 10% v/v glycerol + 1% dithiothreitol (DTT) + 0.01% bromophenol blue + 0.3125 M Tris-HCl adjusted to pH 6.8] was added to starch (1 mL to 150 mg), ghosts (900 μL to 50 mg), or extracts (material leached from starch on preparation of ghosts) (900 μL to 100 mg). After mixing, the

mixtures were placed in a boiling water bath for 5 min (2 min for standards), cooled to room temperature, and then centrifuged at 11600*g* for 10 min. The supernatants (typically 1 μL) were loaded onto the gels. Proteases were dissolved in Laemmli buffer (1 mg/1 mL), immediately boiled for 10 min, and then typically diluted 10 times for loading. The gels were gradient (8−25%) SDS polyacrylamide precast gels from Pharmacia. The gels were run automatically in the control unit at a constant voltage of 250 V. Proteins were visualized by silver staining as described by Morrissey (*28*).

For assessment of ghost integrity by microscopy, freeze-dried ghosts were suspended in water (1.25% w/v) by whirl-mixing and left to hydrate at 4 °C for at least 2 h without shear. Freshly prepared or freeze-dried rehydrated ghosts were stained with I_2/KI solution (0.2/2.0%), viewed under interference contrast using a Leitz Ortholux 2 microscope and photographed using a JVC KY30 video camera.

Depletion of Proteins and Lipids from Granule Surfaces. Treatment of starch granule slurries (40% w/v) with sodium dodecyl sulfate (SDS, 2% w/v) at 20 °C was used to extract surface proteins and lipids. Extracted granules were isolated by centrifugation and washed extensively with cold water as described (*18*).

Protein-Rich Waxy Maize Mutants. Four unusual waxy maize mutants reported to display a low amylose phenotype but with high levels of integral protein have been identified (*25*). Three of the mutants (wx C31, wx R, and wx S5) were grown at Custom Farm Seeds and then in glasshouses at Unilever R&D Colworth, alongside a normal waxy maize starch (wx P6). Cobs were harvested 50 days after anthesis and stored at −20 °C until required.

Kernels were ground in buffer (Tris-HCl, 50 mM, pH 7.5, EDTA 10 mM, and DTT 2.5 mM). The paste was then filtered through four layers of muslin and the residue extracted further until apparently free of starch. The extract was then washed by repeated (at least three times) centrifugation and resuspension in fresh extraction buffer. The recovered pellet was finally washed in acetone and dried by evaporation at room temperature.

Protease Treatment of Wheat and Maize Granule Ghosts. Protease activity of proteinase K was assayed as 10.8 tyrosine units/mg in phosphate buffer (0.067 M, pH 7.4) and that of bromelain as 0.4 tyrosine unit/mg in acetate buffer (0.2 M, pH 4.5). One tyrosine unit hydrolyzes casein at pH 7.4 or hemeoglobin at pH 4.5 to produce color equivalent to 1.0 μmol (181 μg) of tyrosine per minute at 40 °C. The absence of starch depolymerizing activity was demonstrated by measuring the viscosity of a solution of waxy maize starch in the relevant buffer incubated with a range of levels of protease over 24 h. The time required for the solution to flow between two graduations on a 0.1 mL pipet was measured. The deliberate addition of very low levels of α-amylase reduced flow times very quickly. Only those batches of protease that showed no difference in flow times from controls after 24 h of incubation were used for subsequent experiments.

For proteolysis trials, freeze-dried granule ghosts (100 mg) were hydrated in 5 mL of relevant buffer at 4 °C for 16 h and then treated at 40 °C for 24 h with 5 mL of protease dissolved in buffer (proteinase K, 5.4 tyrosine units/mg of ghost, 0.067 M phosphate buffer, pH 7.4; or bromelain, 0.025 tyrosine unit/mg of ghost, 0.2 M acetate buffer, pH 4.5). The 1% w/w suspension of granule ghosts was then centrifuged (1612*g*, 10 min) and the supernatants assayed for soluble carbohydrate and residual proteolytic activity. The pellet of granule ghosts was washed three times in water and then freeze-dried. Controls were also prepared by adding boiled/deactivated enzyme solution or buffer to suspensions of granule ghosts.

Soluble Carbohydrate Assay. Supernatants from suspensions of protease-treated ghosts were assayed for soluble carbohydrate using the phenol−sulfuric acid colorimetric assay (*29*). To 200 μL of diluted sample was added 0.2 mL of 5% w/v phenol solution in water (in triplicate). The samples were then incubated at 70 °C for 5 min, after which 1.0 mL of concentrated sulfuric acid was added. The hydrolysates were left to cool, and absorbance was read at 492 nm. A standard curve (typically from 0 to 100 μg) was constructed for each batch of carbohydrate determination by diluting a stock solution of D-glucose (anhydrous) in water (or relevant buffer). The stock was kept at 0−4 °C.

Table 1. Yields of Ghosts (Percent w/w) with Standard Deviations (σ_{n-1}) and Numbers of Replicates

	from untreated starch	from 1× SDS washed starch
maize	57.6 ± 2.3,[a] $n = 3$ 68.4 ± 2.6,[b] $n = 6$	49.4 ± 2.1,[a] $n = 2$ 62.4,[b] $n = 1$
wheat	43.1 ± 0.6,[a] $n = 2$ 59.7 ± 1.5,[b] $n = 3$	48.6 ± 3.6,[a] $n = 2$ 63.0,[b] $n = 1$

[a] Small-scale preparation method (100 mg in 20 mL). [b] Large-scale preparation method (2.5 g in 500 mL).

RESULTS AND DISCUSSION

Characteristics of Ghosts. Granule ghost yields for maize and wheat starches were 57.6 and 43.1%, respectively, for the small-scale method or 68.4 and 59.7%, respectively, for the larger scale method of preparation (**Table 1**). These values are higher than those reported previously (18 and 32% for maize and wheat starches, respectively, large-scale method) (*12*). This difference may be due to use of more concentrated starch suspensions for preparing ghosts and/or use of centrifugation instead of settling for harvesting of ghosts. Incomplete settling might lead to loss of ghost material upon removal of supernatants. Reproducibility of yields between preparation batches was generally good (**Table 1**). Yields from other starches (waxy maize, pea, and waxy pea) were similar to those of wheat and maize starches, ranging between 40 and 71% by the small-scale method (data not shown).

Extraction of wheat and maize starches with sodium dodecyl sulfate (SDS) prior to gelatinization results in greatly enhanced swelling (*18*). This effect seems to be linked to the extraction of surface protein and lipid components of isolated granules, mainly representative of the matrix in which starch granules are embedded in their botanical source. It might be expected that removal of swelling-restricting surface components would result in a decreased recovery of granule ghosts. However, yields of ghosts following SDS treatment are similar (**Table 1**), being slightly reduced for maize and slightly raised for wheat.

Yields of ghosts should be interpreted in combination with an assessment of ghost integrity. Integrity was assessed qualitatively by microscopy of both fresh and freeze-dried resuspended ghosts. For example, ghost yield from waxy maize was found to be high (71%), but ghosts were found to be larger/more swollen than maize starch ghosts and showed poor integrity, especially after freeze-drying and resuspension. Ghost integrity was qualitatively highest for maize (**Figure 2a**), intermediate for wheat, and lowest for waxy maize. Maize ghost integrity was sufficient to survive freeze-drying, although some damage to the ghosts was apparent (**Figure 2b**).

The partitioning of amylose and amylopectin in preparations of ghosts and extracted (solubilized) material from maize and wheat starches is shown in **Table 2**. Ghosts contain less amylose than starch, with only 12 and 6% (w/w) amylose for maize and wheat ghosts, respectively (**Table 2**). Depletion of amylose in ghosts was reported previously (*12*), consistent with partial leaching of amylose from granules during heating. The low residual amylose content in ghosts does not rule out the possibility of amylose-based cross-linking, as amylose can form a permanent network from a concentration as low as 1% w/v (*7*, *8*).

Gel electrophoresis (SDS-PAGE) was used to characterize proteins associated with starches and ghosts. Wheat and maize starches show protein profiles characteristic of their botanical origin (**Figure 1**). The profile of maize starch granule proteins shows the storage protein zein as a low M_w protein doublet (22−

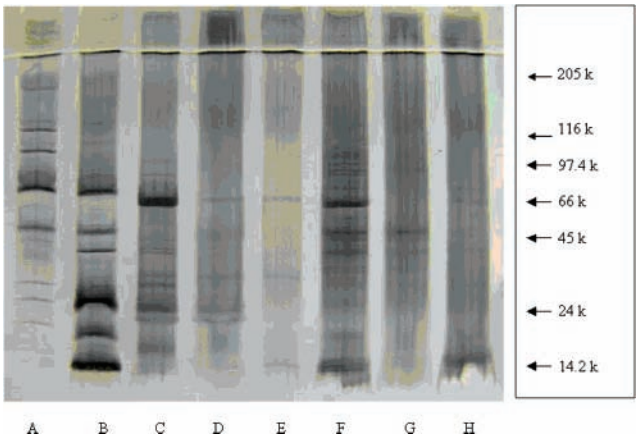

Figure 1. SDS-PAGE analysis of proteins associated with starch, ghosts, and extracts (material leached during preparation of ghosts): protein standards of high (lane A) and low molecular weight (lane B) (0.03 μg of each protein), maize starch (lane C), maize ghosts (lane D), maize extracts (lane E), wheat starch (lane F), wheat ghosts (lane G), and wheat extracts (lane H). Extracts were from 150 mg of starch, 50 mg of ghosts, and 100 mg of leached material.

24 kD), the "waxy" (granule-bound starch synthase I) protein at about 60 kDa, and a few higher M_w proteins. Zein is a characteristic granule surface protein, originating from the endosperm in which the granules are embedded in nature. Proteins associated with maize starch have been described by Goldner and Boyer (*30*), Echt and Schwartz (*25*), Mu-Forster et al. (*31*), Mu et al. (*32*), and Han and Hamaker (*33*). Wheat starch shows a more complex protein profile, ranging in M_w from a few kilodaltons to over 100 kDa as described previously by Greenwell and Schofield (*34*), Schofield and Greenwell (*35*), Rahman et al. (*36*), and Zhao and Sharp (*37*). A 15 kDa protein is characteristic of starch granule surfaces (*35*). For both maize and wheat starches, proteins of 60 kDa and higher are considered to be internal to the granule and to be biosynthetic proteins trapped during the deposition of granules.

Protein profiles for granule ghosts are fainter than those of the original granular starch for both wheat and maize starches (**Figure 1**). Maize ghosts do not appear to be enriched in a specific protein compared with the starting starch (**Figure 1**, lanes C and D). Solubilized material from the preparation of maize granule ghosts does not show a zein doublet. As there is no indication of enrichment of zein in ghosts, we suggest that zein was solubilized away from the granule and aggregated under the heating conditions of ghost preparation, leading to segregation as protein particles with the ghost fraction. Support for this suggestion comes from the observation that a proportion of the proteins extracted from granule ghosts do not enter the polyacrylamide gel (top of lane D, **Figure 1**) and the detection of protein-rich particles in ghost preparations by fluorescence microscopy (not shown). The protein profile of wheat ghosts appears depleted in the ca. 15 kDa protein (**Figure 1**, lanes G and F), but a band of similar size is present in the ghost extracts (**Figure 1**, lane H). This suggests that this wheat granule surface protein is solubilized during ghost preparation. Thus, for both wheat and maize starch, proteins characteristic of granule surfaces are affected significantly by the production of granule ghosts.

Effects of Surface Proteins and Lipids on Ghost Formation and Integrity. For the range of starches examined here and previously (*12*), starch protein (and lipid) content appears to be linked with ghost integrity, as ghosts of high integrity

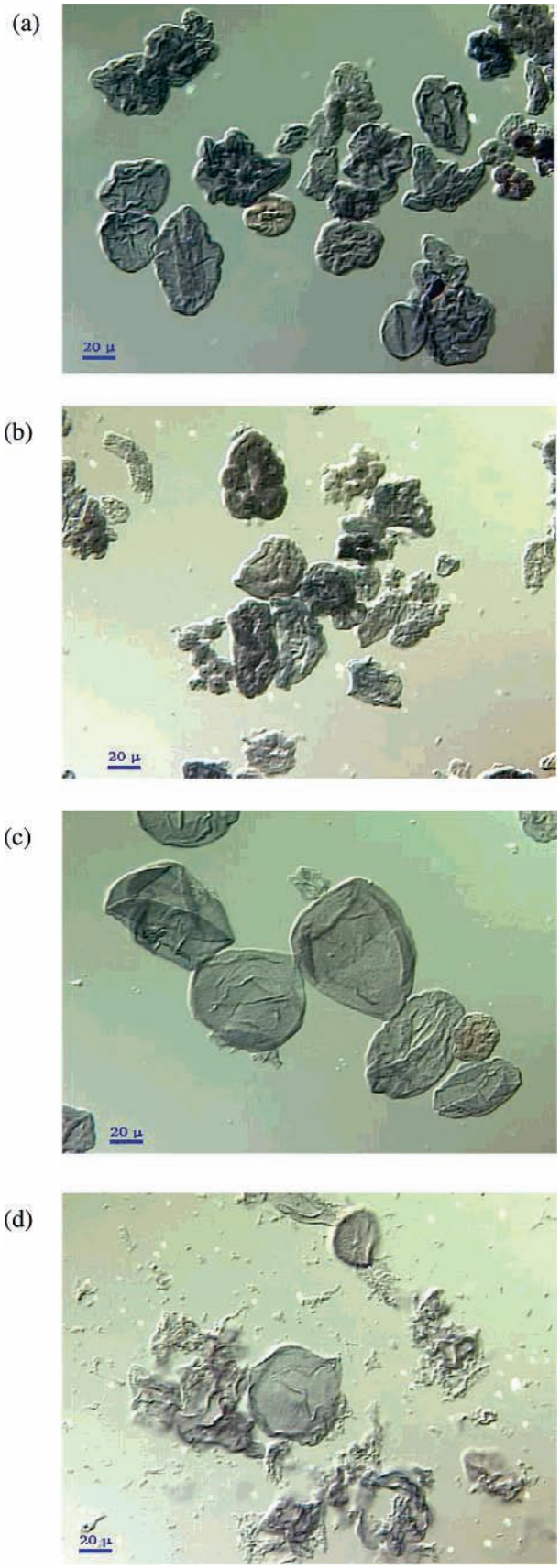

Figure 2. Micrographs of ghosts prepared from maize starch before and after extraction once with 2% w/v SDS at room temperature and subsequent extensive water washing. Ghosts were prepared from untreated maize starch [freshly prepared (**a**) or after freeze-drying and resuspension (**b**)] and from SDS washed maize starch [freshly prepared (**c**) or after freeze-drying and resuspension (**d**)].

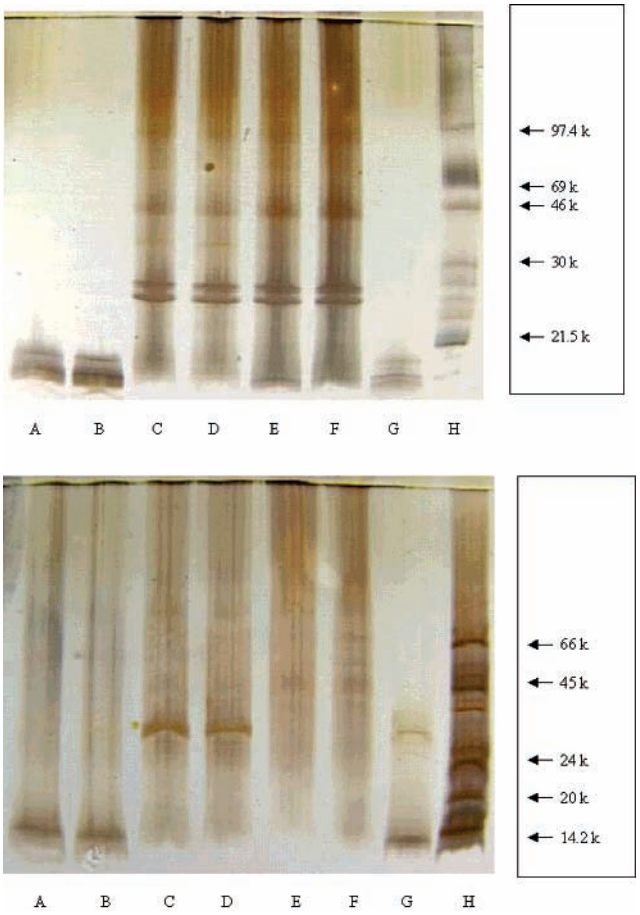

Figure 3. SDS-PAGE analysis of proteins associated with starch ghosts after treatment with buffer containing active, deactivated, or no protease. (Top) Maize ghosts after treatment with active proteinase K (lanes A, B), with deactivated proteinase K (lanes C, D), or with buffer containing no proteinase (lanes E, F). Controls shown are proteinase K (lane G) (0.1 μg) and molecular weight standards (lane H) (0.03 μg of each protein). (Bottom) Wheat ghosts after treatment with active proteinase K (lanes A, B), with deactivated proteinase K (lanes C, D), or with buffer containing no proteinase (lanes E, F). Controls shown are proteinase K (lane G) (0.1 μg) and molecular weight standards (lane H) (0.03 μg of each protein).

Table 2. Composition of Wheat/Maize Ghosts and Solubilized Material

	total amylose (% w/w)	ghost yield[a] (% w/w)	amylose content of granule ghosts (% w/w)	amylose content of soluble extract (% w/w)
maize	25.2	63.0	11.8	71.5
wheat	27.3	61.2	6.2	70.1

[a] Yield relative to original starch weight. Yield relative to total material recovered (ghosts + extract) was higher at 72.8% (maize) or 66.5% (wheat), due to some material loss during extraction.

can be prepared from protein-rich (and lipid-rich) barley, wheat, and maize starches, but not from protein-poor (and lipid-poor) waxy maize or potato starch, which yielded a gelatinous mass rather than particulate ghosts. The role of starch granule surface proteins and lipids in ghost integrity (hypothesis c) was tested by extracting starch using SDS at room temperature (*18*) before ghosts were prepared. Yields of ghosts from gelatinized maize and wheat starches were not significantly affected by prior SDS extraction (**Table 1**). However, maize ghosts from SDS-extracted granules were more swollen (**Figure 2c**) and suffered more damage during freeze-drying and resuspension (**Figure 2d**). SDS extraction of wheat starch also resulted in increased swelling of ghosts and reduced stability to freeze-drying. Pellet volumes of ghost preparations were typically twice as great for ghosts prepared from SDS-treated starch compared with untreated starch, for both preparation scales. These results are consistent with a general role for granule-surface proteins and lipids in affecting the stability of ghosts by restraining expansion during heating (*18*). However, whereas the properties of ghosts are affected, the ability to form them does not seem to require granule-surface proteins and lipids (**Table 1**). Extraction of granule-surface proteins and lipids results in more expanded and therefore more fragile ghosts. This is consistent with effects on starch paste rheology: SDS-treated wheat and maize starches show a reduction of paste viscosity on heating (*18*), most likely due to shear-induced breakdown of highly expanded ghost structures. These results are also consistent with the study of Han et al. (*22*, *23*), who found that toluene/water-extractable material, that is, surface protein (and lipid), appeared to be influencing the rheology, and therefore the swelling and rigidity, of protein-rich waxy maize mutants.

Table 3. Carbohydrate Solubilized (Percent w/w) after Incubation of Maize and Wheat Ghosts with Proteinase K and Bromelain[a]

	maize ghosts		wheat ghosts	
	proteinase K	bromelain	proteinase K	bromelain
controls (buffer free of protease)	3.2	3.0	7.5	3.2
	3.8	2.7	7.4	4.2
digests (active enzyme)	3.7	3.0	7.4	3.3
	3.7	2.8	9.3	2.8

[a] Data shown are for two independent treatments of each type.

Protease Digestion of Wheat and Maize Starch Ghosts. Although surface proteins may not be crucial in the maintenance of ghost integrity, proteins integral to the granule may be more relevant (hypothesis d). Mung bean and other legume starch granules tend to have high thermal stability and are more resistant to shear than other starches, behaving similarly to some cross-linked starches (*20*, *38*). It has been proposed that mung bean starch granules are stabilized by protein cross-links, as bromelain treatment has been reported to decrease granular integrity and increase susceptibility of mung bean starch granules to a starch hydrolytic enzyme (*20*, *39*).

Two wide-spectrum proteases, proteinase K and bromelain, were chosen to attempt exhaustive hydrolysis of proteins within maize and wheat granule ghosts. Proteinase K is an alkaline protease (pH range 7−12), whereas bromelain is acidic (pH range 4−6). Proteinase K or bromelain treatment of maize and wheat ghosts did not lead to any significant change in the amount of carbohydrate solubilized compared with buffer controls (**Table 3**). To determine whether protease treatment resulted in digestion of proteins within granule ghosts, SDS-PAGE analysis of treated ghosts (after water washing three times and freeze-drying) was carried out. Results for proteinase K are shown in **Figure 3**. Lanes A and B (**Figure 3a**) showing the protein profile of treated maize ghosts were clear of high molecular weight proteins, unlike control incubations (lanes C−F). Proteinase K preparations contained two main bands, one at about 14 kDa and a broad band below 14 kDa (lane G). Loading of protease is higher for ghost lanes A−D (if all enzyme added to ghosts in buffer had precipitated onto ghosts, then it would be 25 μg) than in lane G (0.1 μg). At least some of the low molecular weight protein in enzyme-treated ghosts (lanes A and B) is likely to be from the proteinase preparation (cf.

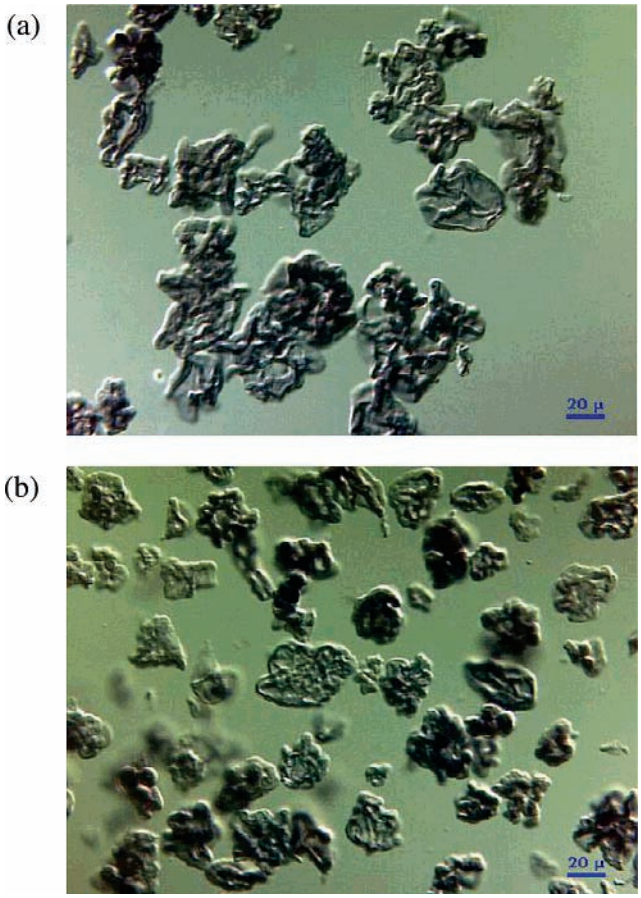

Figure 4. Micrographs of maize starch ghosts after treatment with buffer containing (**a**) no protease or (**b**) active proteinase K. Ghosts were washed three times with water, freeze-dried, and then resuspended.

lane G), but the presence of some degraded starch-derived proteins/peptides is also likely. Nevertheless, whatever protein material remains in maize ghosts after proteinase K treatment is no longer of native molecular weight and is therefore the product of proteolytic action. As the protein profile of untreated wheat ghosts is faint (**Figure 1**, lane G), the difference in ghost proteins before and after protease treatment is more difficult to observe (**Figure 3b**). It appears, however, that proteinase K has also digested most wheat ghost proteins. For both digests, protease activity was still substantial at the end of the reaction (at least 50% of starting levels).

Despite the extensive degradation of granular proteins, the properties of ghosts were very similar after proteinase K treatment, as found previously for barley ghosts (*12*). Particle size distribution was not changed significantly (results not shown), and the appearance of maize ghosts by microscopy was similar (**Figure 4**). This suggests that neither integral nor surface proteins are crucial for the maintenance of ghost integrity. Although considered to be unlikely, it is not possible from the present results to discount the existence of a limited number of small peptide cross-links that survive proteolysis. The results reported make it unlikely that protein cross-links alone are responsible for the integrity of either maize or wheat granule ghosts.

After bromelain action, the protein profiles of both maize and wheat ghosts were slightly less stained, but the difference was minimal (not shown). Zein, whether at the maize granule surface or more likely solubilized away from the granule, did not appear to be hydrolyzed. The limited action of bromelain

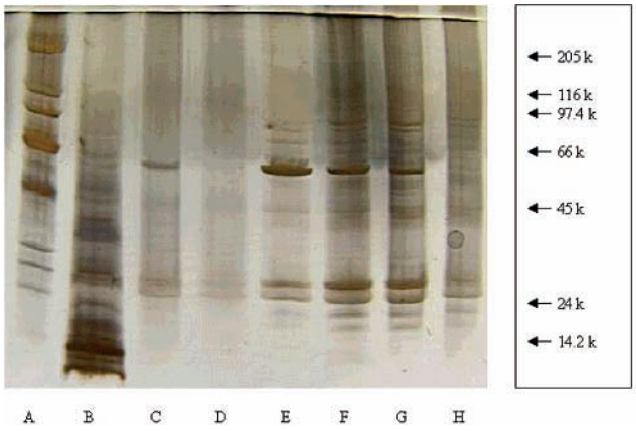

Figure 5. SDS-PAGE analysis of proteins associated with commercial and mutant maize starches. Standards were of high (lane A) and low (lane B) molecular weight (0.08 μg of each protein). Data are shown for commercial maize (lane C) and waxy maize (lane D), mutants wx S5 (lane E), wx R (lane F), wx C31 (lane G), and control waxy maize (lane H).

may be due to the inherently low proteolytic activity of the (amylase-free) preparation used.

Although this study has not found any evidence for bromelain affecting maize or wheat ghosts, the amount of amylose leached out of mung bean starch granules has been reported to increase if the gelatinized granule is further incubated with bromelain (*20*, *39*). It remains to be determined if the difference in behavior in our study is due to the batches of bromelain used in the two studies or a difference between mung bean and wheat/maize starches.

Low-Amylose Maize Mutants with Variable Waxy Protein Contents. The most abundant protein in amylose-containing starches is the so-called waxy protein, or granule-bound starch synthase I, the enzyme primarily responsible for amylose synthesis. In most starches, there is a correlation between the amount of amylose and the amount of waxy protein. There is also a general correlation between amylose content, protein content, and ghost integrity, at least for the same botanical source. This makes it difficult to separate the relative effects of protein and amylose on ghost integrity.

Waxy mutants lack the waxy protein and therefore have correspondingly reduced amylose contents. The waxy maize mutants selected for this study are unusual, in that they display the waxy starch phenotype, by containing little or no amylose yet high levels of waxy protein (*25*). These plants synthesize a defective waxy protein (*25*). The commercial and the control waxy maize (wx P6) starches have no detectable waxy protein (**Figure 5**, lanes D and H), whereas wx C31, wx R, and wx S5 have increasing amounts of the ca. 60 kDa waxy protein (**Figure 5**, lanes G, F, and E, respectively). Line wx S5 has a waxy protein level at least comparable with regular maize that has 6 times the amylose content (**Table 4**). All mutants appear to contain detectable amounts of surface protein (**Figure 5**, lanes E−G), somewhat higher than the levels in commercial starches (**Figure 5**, lanes C and D), presumably due to the mild starch isolation method used for the mutant lines.

Two of the waxy mutants contain small amounts of amylose, as determined by an iodine-based method (*26*) (**Table 4**). The amount of amylose increases with the amount of waxy protein (**Table 4**). Gelatinization behavior (endotherm onset temperature and enthalpy assessed by DSC) is similar for all waxy mutants (not shown). Mutant starches display a typical waxy maize starch viscosity profile, as assessed by Rapid Visco analysis, with a

Table 4. Composition and Properties of Waxy Mutants and Comparison Starches[a]

	starch		ghosts	
	waxy protein	amylose content (% w/w)	yield (% w/w)	integrity
wx C31	+	–0.2	52	low
wx R	++	2.0	68	medium
wx S5	+++	3.5	78	high
control wx P6	–	0.0	49	low
commercial maize	+++	21.6	58	very high

[a] Waxy protein levels are ranked according to **Figure 5**. Yields of ghosts are from the small-scale method. Ghost integrity is categorized on the basis of light microscopy observation of never-dried samples.

high swelling rate and extent (not shown). The higher amylose containing starches wxR and S5 show slightly elevated viscosity, as expected. However, shear breakdown, estimated by the decrease in viscosity between peak and paste viscosities relative to peak viscosity, is similarly extensive for all starches.

Ghosts freshly prepared from the mutants display very different degrees of integrity. As expected, integrity is low for the control line wx P6 containing neither waxy protein nor amylose: very few intact ghosts are visible, and the particulate material consists mostly of small fragments (**Figure 6a**). Integrity is also low for the mutant wx C31, with no amylose and some waxy protein (**Figure 6b**). Integrity then increases with waxy protein and amylose contents in wx R (numerous ghosts together with many small fragments) (**Figure 6c**) and wx S5 (ghosts without fragments) (**Figure 6d**). The same trend was seen after freeze-drying and resuspension of the ghosts (data not shown).

Ghost yield and integrity (fresh and after freeze-drying) generally seemed to increase with amylose and waxy protein contents under low shear conditions (**Figure 6**). However, wx C31, which contains the waxy protein, and wx P6, which does not, showed a similar behavior. The waxy protein does not appear to confer (additional) integrity to the wxC31 ghost. Together with the finding (**Figure 3**) that extensive proteolytic degradation of the waxy protein does not affect significantly ghost integrity, the present results suggest that ghost integrity is related more to apparent amylose levels than to protein content.

Removal of starch granule surface proteins from a different set of protein-rich waxy maize mutants by toluene/water treatment led to more swollen and fragile granules (*22*). It is possible that for those mutants, surface proteins could be modulators of swelling, as seen for wheat and maize starches in this study. However, some waxy protein was also extracted by the toluene treatment alongside the surface proteins (not observed with SDS extraction), so it is difficult to compare results with those reported here.

Factors Contributing to Granule Ghost Integrity. Three hypotheses for the structural origins of ghosts formed after thermal gelatinization of starch granules in excess water have been tested. These are based on the following: a surface film around granules rich in protein and lipid that limits expansion and prevents dissolution; cross-linking involving proteins inside the granule; and cross-linking (presumably by double helices) of amylose and/or long amylopectin branches (measured as "apparent amylose" by iodine titration).

The results described are not consistent with either of the first two mechanisms being the sole origin for ghosts. Effective removal of surface lipids and proteins does not prevent ghost

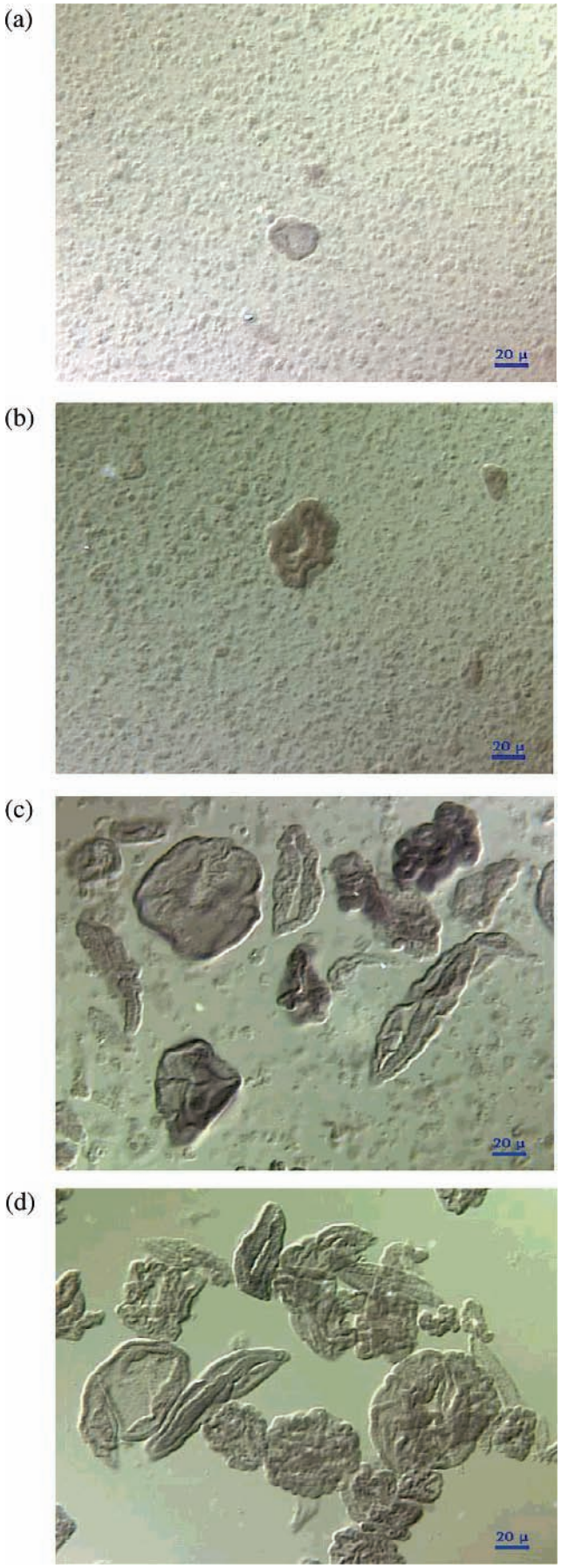

Figure 6. Micrographs of ghosts freshly prepared from mutant waxy maize starches. Data are shown for control waxy maize (wx P6) (**a**) and mutants wx C31 (**b**), wx R (**c**), and wx S5 (**d**).

formation, and proteolytic treatment of ghosts resulted in extensive breakdown of all granular proteins without any apparent change in ghost properties. We hypothesize that double-helical cross-linking of glucan segments is a necessary component of granule ghost stability. This is supported by the correlation between ghost formation and amylose content at low amylose levels (**Table 4** and **Figure 6**). Ghosts are fragile and ill-defined at <1% apparent amylose, but are robust and well-defined at 2% amylose and higher. The fact that (fragile) ghosts can be isolated from waxy starches with zero apparent amylose content requires that at least a small portion of amylopectin be involved in cross-linking. The fact that no detectable carbohydrate order is found for ghosts by X-ray diffraction, ^{13}C NMR, or DSC (*12*) suggests that, if double-helical order is present within ghosts, it is at a level of <5% (estimated detection limit by NMR or DSC). Comparison with chemically cross-linked starches (*40*) shows that a very low level of cross-linking (equivalent to much less than 1% of glucose residues) is needed to prevent dissolution during ghost formation. Such a level of helix formation is below the detection limits of current methodologies. Comparison with glycogen is also instructive: despite being a high molecular weight α (1 → 4)-based glucan like starch polymers, there is no evidence for ghost formation as dissolution in water is complete, especially at elevated temperatures. We propose that this difference is due to the branch lengths of glycogen being too short to form double helices, compared with the longer branch lengths of amylopectin.

The proposed primary mechanism for ghost formation involves a competition between swelling and cross-linking in starch granules heated to above gelatinization temperatures in excess water. When swelling is very rapid (and extensive), as for potato starch (possibly due to the relatively high level of phosphate substituents compared with other starches), cross-link formation is proposed to be too slow (and/or polymer concentration too low) to result in coherent ghost structures. On the other hand, the slower swelling of wheat and maize starches is proposed to give sufficient time and polymer concentration for glucan cross-linking to take place, leading to the formation of robust ghosts. Native starch granules are stabilized by double helices based on amylopectin branches; the amylose fraction is considered to be in a single-chain state, prevented from forming double helices by segregation from other amylose molecules. The biological rationale for this is that any amylose-based double helices are likely to be resistant to α-amylase digestion ("resistant" starch) and therefore lead to both a reduction in available energy and a nondigestible residue with no obvious clearing mechanism in the plant. Above the temperatures required for melting of amylopectin double helices ("gelatinization"; typically 60−75 °C), the hydrophilic nature of all starch polymers will drive expansion of the granule. As this expansion takes place, the amylose molecules previously prevented from forming double helices are hypothesized to be able to find partner molecules and form double helices. The melting temperature for amylose-based double helices is above 130 °C, so there is a significant undercooling that can drive double-helix formation. The melting temperature of amylose/amylopectin double helices (one chain from each type of polymer) has been reported (*41*) to be similar to that of amylopectin alone (typically 50−80 °C) and therefore does not provide a mechanism for ghost stability at 90−95 °C. Provided enough restraining double helices are formed (<1% of glucose residues based on analogy with chemically cross-linked starches) before molecular dissolution takes place, a stable granule ghost will be formed. The microstructure of gelatinized starch granules is reported to consist of a fine fiber network (presumably retrograded amylopectin and amylose), with fiber frequency appearing to increase from the inside toward the outside of the granule ghost (*15*). This suggests that the effective proposed glucan cross-links are located in the outer regions of granule ghosts. This is consistent with a mechanically effective "skin" in granule ghosts as inferred from micromechanical measurements (*42*). Although starch polymers are proposed to be the primary structuring feature in ghosts, surface proteins/lipids can play a role in determining final ghost properties. For those starches (e.g., wheat and maize) that have restricted swelling due to surface proteins/lipids (*18*), ghosts prepared from surface-extracted granules show greater expansion due to the removal of constraining proteins and lipids, leading to a greater extent of swelling before sufficient double-helix formation occurs to prevent dissolution.

The degree of expansion and the presence or absence of surface proteins and lipids can have a major influence on the stability of ghosts to, for example, centrifugation or freeze-drying and resuspension. It should also be noted that a significant minority of starch polymers are not present in recovered granule ghosts, suggesting that some polymers are able to migrate away from the granule before being trapped by cross-linking (e.g., leaching of amylose from wheat and maize starch) or that swelling of some granules is so extensive that the resultant ghosts are too fragile to be examined.

ACKNOWLEDGMENT

We thank Alison Smith (John Innes Institute, Norwich, U.K.) for advice on selecting the waxy maize mutants, Virgil Ferguson (Custom Farm Seeds, U.S.A.) for locating and growing the waxy maize mutants, and Sue Robinson and Sue Webb (Unilever R&D Colworth) for growing the increased seed.

LITERATURE CITED

(1) Smith, A. The biosynthesis of starch granules. *Biomacromolecules* **2001**, *2*, 335−341.
(2) Buleon, A.; Colonna, P.; Planchot, V.; Ball, S. Starch granules: structure and biosynthesis. *Int. J. Biol. Macromol.* **1998**, *23*, 85−112.
(3) Waigh, T. A.; Kato, K. L.; Donald, A. M.; Gidley, M. J.; Clarke, C. J.; Riekel, C. Side-chain liquid-crystalline model for starch. *Starch/Staerke* **2000**, *52*, 450−460.
(4) Jane, J.-L.; Shen, J. J. Internal structure of the potato starch granule revealed by chemical gelatinization. *Carbohydr. Res.* **1993**, *247*, 279−290.
(5) Evans, I. D.; Haisman, D. R Rheology of gelatinized starch suspensions. *J. Texture Stud.* **1979**, *10*, 347−370.
(6) Miles, M. J.; Morris, V. J.; Orford, P. D.; Ring, S. G. The roles of amylose and amylopectin in the gelation and retrogradation of starch. *Carbohydr. Res.* **1985**, *135*, 271−281.
(7) Gidley, M. J.; Bulpin, P. V. Aggregation of amylose in aqueous systems: the effect of chain length on phase behaviour and aggregation kinetics. *Macromolecules* **1989**, *22*, 341−346.
(8) Clark, A. H.; Gidley, M. J.; Richardson, R. K.; Ross-Murphy, S. B. Rheological studies of aqueous amylose gels: the effect of chain length and concentration on gel modulus. *Macromolecules* **1989**, *22*, 346−351.
(9) Stark, J. R.; Aisien, A. O.; Palmer, G. H. Studies on starches from Nigerian sorghum. *Starch/Staerke* **1983**, *35*, 73−76.
(10) Obanni, M.; BeMiller, J. N. Identification of starch from various maize endosperm mutants via ghost structures. *Cereal Chem.* **1996**, *72*, 436−442.
(11) Obanni, M.; BeMiller, J. N. Ghost microstructures of starch from different botanical sources. *Cereal Chem.* **1996**, *73*, 333−337.

(12) Prentice, R. D. M.; Stark, J. R; Gidley, M. J. Granule residues and "ghosts" remaining after heating A-type barley starch granules in water. *Carbohydr. Res.* **1992**, *227*, 121−130.
(13) Evans, I. D.; Lips, A. Concentration dependence of the linear elastic behaviour of model microgel dispersions. *J. Chem. Soc., Faraday Trans.* **1990**, *86*, 3413−3417.
(14) Evans, I. D.; Lips, A. Viscoelasticity of gelatinized starch suspensions. *J. Texture Stud.* **1992**, *23*, 69−86.
(15) Atkin, N. J.; Abeysekera, R. M.; Robards, A. W. The events leading to the formation of ghost remnants from the starch granule surface and the contribution of the granule surface to the gelatinization endotherm. *Carbohydr. Polym.* **1998**, *36*, 193−204.
(16) Morrison, W. R. Starch lipids and how they relate to starch granule structure and functionality. *Cereal Foods World* **1995**, *40*, 437−446.
(17) Baldwin, P. M. Starch granule-associated proteins and polypeptides: a review *Starch/Staerke* **2001**, *53*, 475−503.
(18) Debet, M. R.; Gidley, M. J. Three classes of starch granule swelling: influence of surface proteins and lipids. *Carbohydr. Polym.* **2006**, *64*, 452−465.
(19) Han, X. Z; Hamaker, B. R. Association of starch granule proteins with starch ghosts and remnants revealed by confocal laser scanning microscopy. *Cereal Chem.* **2002**, *79*, 892−896.
(20) Oates, C. G. Studies on mung bean starch: granule stability. *Food Hydrocolloids* **1991**, *4*, 365−377.
(21) Hamaker, B. R.; Griffin, V. K. Changing the viscoelastic properties of cooked rice through protein disruption. *Cereal Chem.* **1993**, *70*, 377−380.
(22) Han, X. Z.; Campanella, O. H.; Guan, H. P.; Keeling, P. L.; Hamaker, B. R. Influence of maize starch granule-associated protein on the rheological properties of starch pastes. Part I. Large deformation measurements of paste properties. *Carbohydr. Polym.* **2002**, *49*, 315−321.
(23) Han, X. Z.; Campanella, O. H.; Guan, H. P.; Keeling, P. L.; Hamaker, B. R. Influence of maize starch granule-associated protein on the rheological properties of starch pastes. Part II. Dynamic measurements of viscoelastic properties of starch pastes. *Carbohydr. Polym.* **2002**, *49*, 323−330.
(24) Moates, G. K.; Noel, T. R.; Parker, R.; Ring, S. G. The effect of chain length and solvent interactions on the dissolution of the B-type crystalline polymorph of amylose in water. *Carbohydr. Res.* **1997**, *298*, 327−333.
(25) Echt, C. E.; Schwartz, D. Evidence for the inclusion of controlling elements within the structural gene at the waxy locus in maize. *Genetics* **1981**, *99*, 275−284.
(26) Morrison, W. R.; Laignelet, B. An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. *J. Cereal Sci.* **1983**, *1*, 9−20.
(27) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(28) Morrissey, J. H. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **1981**, *117*, 307−310.
(29) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.
(30) Goldner, W. R.; Boyer, C. D. Starch granule-bound proteins and polypeptides: the influence of the waxy mutation. *Starch/Staerke* **1989**, *41*, 250−254.
(31) Mu-Forster, C.; Huang, R.; Powers, J. R.; Harriman, R. W.; Knight, M.; Singletary, G. W.; Keeling, P. L.; Wasserman, B. P. Physical association of starch biosynthetic enzymes with starch granules of maize endosperm−granule-associated forms of starch synthase I and starch branching enzyme II. *Plant Physiol.* **1996**, *111*, 821−829.
(32) Mu, H. H.; Mu-Forster, C.; Bohonko, M.; Wasserman, B. P. Heat-induced fragmentation of the maize waxy protein during protein extraction from starch granules. *Cereal Chem.* **1998**, *75*, 480−483.
(33) Han, X. Z.; Hamaker, B. R. Location of starch granule-associated proteins revealed by confocal laser scanning microscopy. *J. Cereal Sci.* **2002**, *35*, 109−116.
(34) Greenwell, P.; Schofield, J. D. A starch granule protein associated with endosperm softness in wheat. *Cereal Chem.* **1986**, *63*, 379−380.
(35) Schofield J. D.; Greenwell P. Wheat starch granule proteins and their technological significance. In *Cereals in a European Context*; Morton, I. D., Ed.; Ellis Horwood: Chichester, U.K., 1987; pp 407−420.
(36) Rahman, S.; Kosar-Hashemi, B.; Samuel, M. S.; Hill, A.; Abbott, D.; Skerritt, J. H.; Preiss, J.; Appels, R.; Morell, M. K. The major proteins of wheat endosperm starch granules. *Aust. J. Plant Physiol.* **1995**, *22*, 793−803.
(37) Zhao, X. C.; Sharp, P. J. An improved 1-D SDS-PAGE method for the identification of 3 bread wheat "waxy" proteins. *J. Cereal Sci.* **1996**, *23*, 191−193.
(38) Hoover, R.; Sosulski, F. W. Composition, structure, functionality, and chemical modification of legume starches: a review. *Can. J. Physiol. Pharmacol.* **1991**, *69*, 79−92.
(39) Oates, C. G. Fine structure of mung bean starch: an improved method of fractionation. *Starch/Staerke* **1990**, *42*, 464−467.
(40) Wurzburg, O. B. Modified starches. In *Food Polysaccharides and Their Applications*; Stephen, A. M., Ed.; Dekker: New York, 1995; pp 67−97.
(41) Klucinec, J. D.; Thompson, D. B. Amylose and amylopectin interact in retrogradation of dispersed high-amylose starches. *Cereal Chem.* **1999**, *76*, 282−291.
(42) Evans, I. D.; Haisman, D. R.; Elson, E. L.; Pasternak, C.; McConnaughey, W. B. The effect of salivary amylase on the viscosity behaviour of gelatinized starch suspensions and the mechanical properties of gelatinized starch granules. *J. Sci. Food Agric.* **1986**, *37*, 573−590.

Received for review January 1, 2007. Revised manuscript received March 29, 2007. Accepted April 1, 2007.

JF070004O