Endosperm Cell Wall Modification in Sorghum Grain During Germination

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ABSTRACT

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Both barley and sorghum are used as malting cereals, but sorghum endosperm cell walls, unlike those of barley, persist during germination. In spite of their persistence, however, the cell walls are subject to chemical and physical modification. This study was concerned with such changes. A procedure involving ball milling and wet sieving isolated the cell walls. They were isolated from sorghum grain as well as from kernels after a six-day germination period. The cell walls from both of these isolations were examined by both transmission and scanning electron microscopy. They were also fractionated into water- and alkali-soluble fractions as well as an insoluble fraction. Large amounts of protein were associated with the cell walls isolated from grain, but germination reduced this from 46 to 19%. During this period, the amount of cell wall was reduced, with the water- and alkali-soluble fractions having the biggest reductions. Electron micrographs revealed extensive pitting during germination. Diferulic and ferulic acids were the only polyphenols detected in the isolated cell walls. They were found associated with the alkali fraction, and germination did not cause any reduction in their content.

An understanding of modification during germination of cereal grains must depend in part on a knowledge of endosperm cell wall composition and ultrastructure. During germination, these cell walls must be modified in some manner to allow for the mobilization of the starch and protein reserves. The general pattern of modification in germinating barley was established as breakdown of cell walls, protein hydrolysis, and starch hydrolysis (Fretzdorff et al 1982). However, during germination in sorghum, the cell walls persisted even though the cell contents were degraded (Glennie et al 1983).

Although sorghum endosperm cell walls persisted during germination, their chemical composition was modified. After germination for six days, major changes were found in hemicellulose B, and the ratio of glucose to xylose in hemicellulose A changed from 15:1 to 3:1 (Woolard et al 1977b).

The cell walls of some plant species are less susceptible to degradation than others. One possible reason for this reduced susceptibility could be the esterification of some of the cell wall polysaccharides with diferulic and ferulic acids (Hartley and Jones 1976, 1977). Diferulic acid was identified in the cell walls of wheat endosperm (Markwalder and Neukom 1976), whereas ferulic acid was found in cell walls isolated from barley endosperm (Fincher 1976). In the present study, isolated cell walls from sorghum endosperm both before and after germination were examined for

the presence of diferulic and ferulic acids.

Structural modification and changes in cell wall polysaccharides during germination have been studied in barley (Morrall and Briggs 1978). Along with the chemistry, the enzymes responsible for this cell wall modification during barley germination have also been described (Bramforth 1982). Like barley, sorghum is also used as a malting grain, but very little information is available on cell wall modification of sorghum during germination. Since residual cell walls could play a detrimental role in the brewing process, from slower filtering rates to haze production, the fate of these cell walls during malting must be understood.

MATERIALS AND METHODS

Germination

Grain samples of *Sorghum bicolor* (L.) Moench cultivar Barnard Red were surface sterilized with a 0.2% Adcodyne (iodophore) solution and steeped for 16 hr, with a water change and 1-hr air rest every 3 hr. Germination was accomplished in nylon mesh bags in a Forma Scientific incubator at 28°C and at 99% relative humidity (rh). The grain was steeped twice a day (1 × 5 min, 1 × 10 min) in water containing 0.05% hypochlorite. Excess moisture was removed by centrifugation at approximately 270 × *g* in an AEG spin dryer for 3 min. After six days the kernels were dried at 50°C for 24 hr in a forced-air oven until the sample was approximately 6% moisture.

Isolation of Endosperm Cell Walls

Several established techniques were investigated for the preparation of sorghum cell walls (Mares and Stone 1973, Thompson and La Berge 1981), but they all proved to be inadequate. These methods did not break up the hard sorghum endosperm sufficiently to release the cell wall, so the following procedure was developed.

The ungerminated grain was pearled in a Miag rice pearler until approximately 33% of the weight of the original grain was removed. The pearled grain was milled in a Miag mill set at its finest setting. Endosperm cell walls were prepared from this flour by ball milling and wet sieving.

Flour (100 g) was placed in a 2.25-L ball mill container, and 1 L of 60% aqueous tert-butanol containing 0.05% dithiothreitol was added. This removes the contaminating prolamin proteins (Taylor 1983). The 60% aqueous tert-butanol dissolves prolamin, and the dithiothreitol breaks disulfide bridges. The sample was ball milled for 48 hr and then wet sieved on bolting cloth having a pore size of 74 μm. The residue on the bolting cloth was stirred repeatedly in 70% aqueous ethanol to remove free starch granules. The sample was ball milled for five 24-hr periods in 70% ethanol with wet sieving and washing with the same solvent after each 24-hr period. The sample was examined by light microscopy to determine the presence of free or embedded starch.

The final residue on the bolting cloth was suspended in 2 L of 70% ethanol, allowed to stand for 5 min, and the top half decanted. The residue in the bottom half was resuspended four times to yield a white suspension of endosperm cell walls, and the coarser fraction was left behind. The cell walls were dried by successive washing with 96% ethanol, absolute methanol, and *n*-pentane.

Since the germinated kernels were friable and could not be pearled, another technique was used to prepare endosperm cell walls from these germinated kernels. The kernels were dried and then crushed by hand with a metal roller. After being rolled for 5 min, the flour that passed through a 200-μm sieve was collected. To further improve this cell wall preparation, the sedimentation period at the end of this procedure was increased to 15 min.

Starch Determination

Endosperm cell walls (5 mg) were moistened with 1 ml of 96% ethanol, and 5 ml of H_2O was added. The sample was heated on a water bath at 95°C for 1 hr to gelatinize the starch. After cooling, 20 μl of amyloglycosidase suspension (*Aspergillus niger*, Boehringer Mannheim, 100 mg/10 ml) was added, and the preparation was incubated at 36°C for 18 hr. The digest was washed through a sintered glass funnel with H_2O and the glucose in the filtrate determined by the high-performance liquid chromatography (HPLC) method of Wight and van Niekerk (1983).

Fractionation of Isolated Endosperm Cell Walls

The isolated cell walls were fractionated according to the procedures of Thompson and La Berge (1981) with slight modification. Water was extracted at room temperature three times of 2 hr. The alkali extracts were dialyzed against several changes of water and then freeze dried. Aliquots of the alkali-soluble fractions were subsequently fractionated into hemicelluloses A and B by the methods of Woolard et al (1977b).

Protein Determination

Protein (N × 6.25) was determined by the method of Thomas et al (1967).

Electron Microscopy

For scanning electron microscopy, the cell walls were fixed in 5% gluteraldehyde, dehydrated through an ethanol series, and critical-point dried (amyl acetate-CO_2). The samples were attached to metal stubs with double-sided Sellotape and sputter coated with gold-palladium. Stubs were examined in a JEOL JSM35 scanning electron microscope at an accelerating voltage of 15 kV.

Material for transmission electron microscopy was prepared as previously described (Glennie et al 1983).

Diferulic and Ferulic Acid Determination

The diferulic and ferulic acids were released from the cell walls by hydrolysis overnight at 4°C in 2*M* NaOH under N_2. Acid hydrolysis greatly reduces the yield of cinnamic acid derivatives (Ibrahim and Towers 1960). After acidifying, the samples were extracted with ethyl acetate, and these extracts were used for polyphenol determination on thin-layer chromatography (TLC). The concentration of polyphenol was determined by a modified Folin-Ciocalteu method (McGrath et al 1982), using ferulic acid as a standard. Thin-layer chromatography was performed using solvents described by Hartley and Jones (1976) and Markwalder and Neukom (1976). The compounds were located by UV light (350 nm for nonfluorescent plates and 254 nm for fluorescent plates) and by spraying with Pauly's or Folin-Ciocalteu reagents.

Standard ferulic acid was obtained from Sigma Chemical Co. while a standard of diferulic acid was extracted from wheat endosperm (after alkali hydrolysis) and purified by preparative TLC.

RESULTS AND DISCUSSION

Isolation of Endosperm Cell Walls

The technique of ball milling and wet sieving proved successful in the preparation of sorghum endosperm cell walls. Although the technique is not quantitative, a large number of cell wall isolations gave an average of 0.25 g of endosperm cell walls from 100 g of pearled sorghum grain. Thompson and La Berge (1981) obtained 1.25 g of endosperm cell walls from 100 g of pearled barley. Expressed on a 1,000-kernel basis, the sorghum grain gave 43.7 mg of cell wall per 1,000 kernels, and the germinated grain gave 15.7 mg of cell wall per 1,000 kernels. The starch content of the isolated sorghum grain cell walls was 5.7% compared to 11.7% starch for the barley cell walls. The starch content of the isolated sorghum cell walls did not decline after germination.

Cell Wall Extraction

The yields of fractions after successive water and alkali extractions both before and after germination are presented in Table I. Very little of the isolated sorghum endosperm cell wall is water soluble—9% compared to 17% for barley (Fincher 1976) or 43% hot water-soluble for barley (Thompson and La Berge 1981). The cold water-soluble gum from sorghum gave a D-glucan, and the hot water-soluble gum gave another glucan. The two glucans differed in their ratio of (1 → 4)- to (1 → 6)-linkages (Woolard et al 1977a, 1977c). The alkali-soluble fraction is the largest fraction of

sorghum endosperm cell walls. The isolated cell walls have 46% protein associated with them, and since the bulk of this protein is alkali soluble, this could account for the size of this fraction. Although this protein is associated with the isolated cell walls, it is not part of them. No attempt was made to remove this protein, as it would involve an enzyme degradation, and the aqueous medium of the enzyme could alter the composition of the cell walls. Also, the enzyme preparations could contain enzymes that degrade cell walls.

The insoluble fraction of sorghum grain cell walls was 23%; by comparison, the insoluble fractions of barley endosperm cell walls was 6–7% (Fincher 1976, Thompson and La Berge 1981). If the protein associated with the alkali-soluble fraction of sorghum cell walls was ignored and only the carbohydrate taken into consideration, then the proportion of insoluble carbohydrate increases. This is probably the clue to the stability of the sorghum cell walls during germination. This insoluble cell wall fraction of sorghum may be more resistant to enzyme attack than the more soluble cell walls of barley; hence, sorghum endosperm cell walls are not readily degraded during germination.

Germination caused a marked change in the amount of cell wall isolated and in the ratios of the fractions of different solubilities (Table I). All three fractions declined during germination, with the water- and alkali-soluble fractions suffering the biggest decline. During germination, the protein content of the cell wall preparations declined from 46 to 19%. Since the alkali-soluble fraction contained most of the protein associated with the cell walls, this decline in protein might account for much of the loss of the alkali solubles.

Morphological Changes in Endosperm Cell Walls

A typical fragment of endosperm cell wall isolated from the grain is shown in Fig. 1A. Only intracellular cell wall surfaces as shown were found; apparently the cell walls from adjoining cells did not separate easily to allow intercellular surfaces to be exposed. The cell wall appears to be very concrete and has what is probably protein associated with it. This material adhering to the cell walls was probably protein, since the cell walls had 46% protein associated with them. Also, this material was very similar to the matrix protein identified in other micrographs (Hoseney et al 1974, Taylor et al 1984). Matrix protein, which surrounds the starch granules, contains small gaps that were probably occupied by

TABLE I
Fractionation of Endosperm Cell Walls from Sorghum Grain Before and After Germination (average of 40 determinations)

Sample	Water Soluble (mg/g cell wall)	KOH Soluble (mg/g cell wall)	Insoluble (mg/g cell wall)
Before germination	0.93[a] (4.00)[b]	6.75 ± 1.24 (29.45)	2.33 ± 0.28 (10.18)
After germination	0.62 ± 0.26 (0.97)	4.17 ± 0.35 (6.45)	4.16 ± 0.68 (6.53)

[a] This fraction was calculated as the difference between the sum of the other two fractions and 100.

[b] Numbers in parentheses = milligrams per 1,000 kernels.

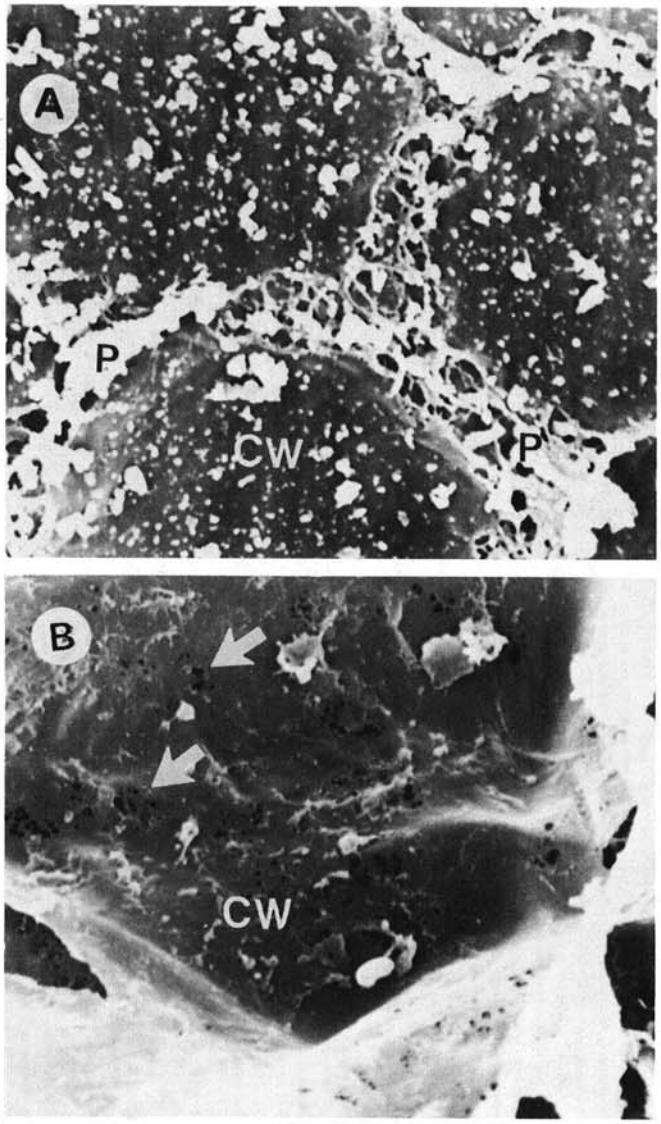

Fig. 1. Scanning electron micrographs of isolated sorghum endosperm cell walls. **A,** cell walls from ungerminated grain showing protein matrix adhering to inner surface (P). CW = cell wall. **B,** cell walls from kernels germinated for six days. Very little protein is present, and the cell wall is extensively pitted (arrows). CW = cell wall.

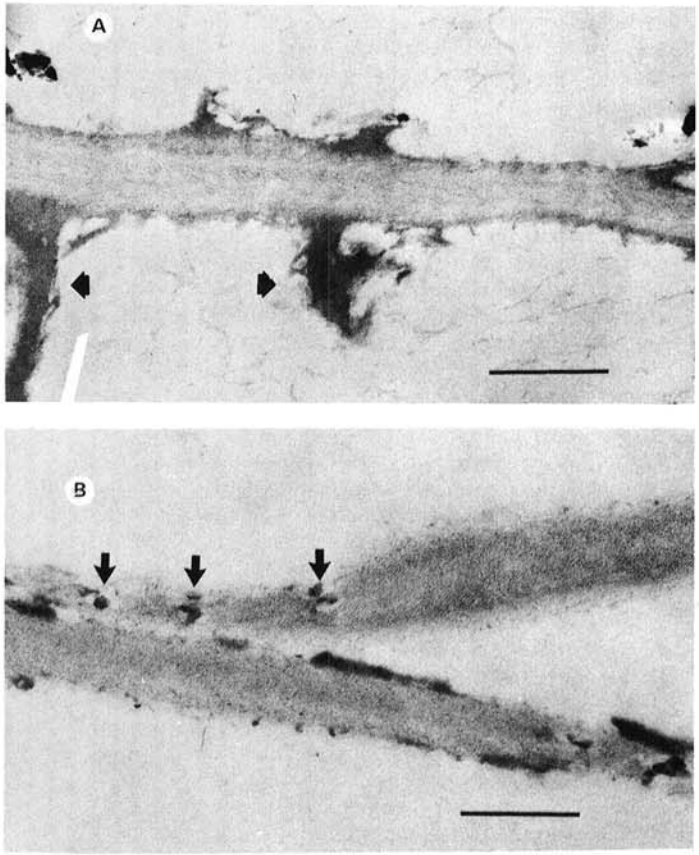

Fig. 2. Transmission electron micrographs of isolated sorghum endosperm cell walls. **A,** cell walls from ungerminated grain. Arrow indicates adhering protein. Bar = 0.5 μm. **B,** cell walls from grain germinated for six days. Little protein is associated with the cell walls, which are pitted (arrow). Bar = 0.5 μm.

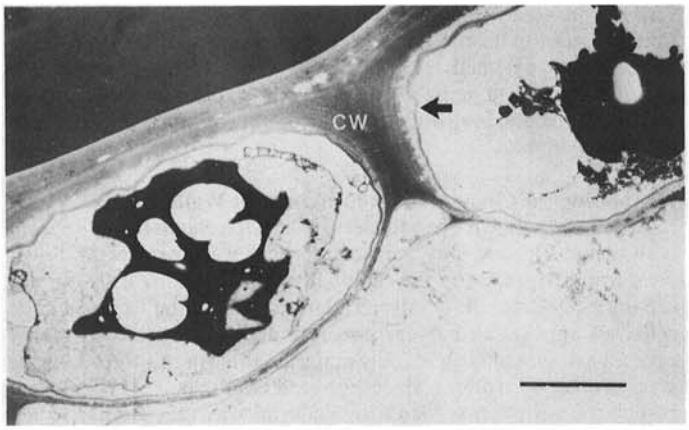

Fig. 3. Transmission electron micrograph of aleurone cell walls after germination for 12 days. The aleurone cell walls are much thicker than the other cell walls, and all cell walls persist even though cell contents are much diminished. CW = cell wall. Arrow indicates area of cell wall degradation. Bar = 5 μm.

protein bodies before they were extracted with aqueous tert-butanol. The apparently loose debris associated with the cell walls is characteristic of cell walls isolated from ungerminated grain.

Because the cell walls of sorghum persist after germination, they could be isolated and examined by electron microscopy. Although the cell walls persisted, they were physically changed after the grain was germinated for six days (Fig. 1B): All the loose debris had gone, very little protein was associated with it, and the cell wall was extensively pitted. Extensive pitting is characteristic of cell walls from germinated grain. This pitting probably facilitates the translocation of mobilized endosperm reserves to the growing embryo. In barley, entire endosperm cells adjacent to degraded cells were identical to cells in ungerminated grains. This suggests that the intact cell wall is an effective barrier of starch- and protein-hydrolyzing enzymes (Gram 1982). Therefore, this cell wall must be breached, either by complete degradation, as in barley or by pitting, as in sorghum.

Transmission electron micrographs also show the morphological changes in endosperm cell walls after germination. Figure 2A shows a fragment of cell wall isolated from grain. The protein associated with the cell walls does not actually line the cell wall but rather protrudes from it and probably occupies the space between the starch granules. After malting, the amount of visible protein is reduced, which supports the results from the protein determinations performed on the cell walls (Fig. 2B). Pits are frequently observed in the cell walls isolated from germinated grain, but they were not observed in the cell walls isolated from the ungerminated grain.

Diferulic and Ferulic Acids in Endosperm Cell Walls

Both diferulic and ferulic acids were isolated from the cell walls of the endosperm after alkali hydrolysis. No other polyphenols could be detected. The ferulic acid was identical to an authentic sample, and the diferulic acid was identical with that isolated from wheat with respect to R_f values, UV spectra (± NaOH), appearance under UV light (± ammonia), and reaction with Pauly's and Folin-Ciocalteu reagents. After a large number of TLC plates were examined, we estimated that the cell walls contained four to five times as much ferulic as diferulic acids.

The cell walls were fractionated to establish the location of the diferulic and ferulic acids. These acids could be found only in the alkali-soluble fraction that contained the hemicelluloses. They could not be detected in either the water-soluble gums or the alkali-insoluble residue. These results agree with those of Markwalder and Neukom (1976), who found these acids in the hemicelluloses of wheat. They suggested that the linking of polysaccharides by diferulic acid could reduce the solubility of such polysaccharides.

When the alkali-soluble hemicelluloses from sorghum were acidified to pH 5 with acetic acid, hemicellulose A precipitated while hemicellulose B remained in solution. These two fractions were examined for diferulic and ferulic acids, and although both fractions contained ferulic acid, diferulic acid could be found only in the more insoluble hemicellulose A fraction. Hemicellulose A from sorghum endosperm is composed of a glucan and an arabinoglucoxylan, and these polysaccharides were recovered in the ratio of 7.8:1 (w/w) (Woolard et al 1976). An arabinoxylan isolated from wheat bran cell walls contained ferulic acid (Smith et al 1981). In sorghum endosperm cell walls, both diferulic and ferulic acids were associated with the arabinoglucoxylan as well as with the glucan.

Just as germination caused a change in the ratio of cell wall fractions, it also brought about an apparent change in the ferulic acid content of the cell walls. The ferulic acid content of the cell walls increased from 0.08 ± 0.02% for ungerminated grain to 0.15 ± 0.03% for the germinated kernels. This probably did not occur because the cell walls acquired more ferulic acid but because the carbohydrate content of the cell walls decreased. This would account for the apparent increase in ferulic acid content. Similarly, the carbohydrate and protein content of the alkali-soluble fraction decreased. Hence, the relative amounts of diferulic and ferulic acids increased.

When examined with fluorescence microscopy, the cell walls of sorghum endosperms showed a blue autofluorescence (Earp et al 1983), which suggests that the diferulic and ferulic acids were distributed throughout the cell walls of the sorghum endosperm. In this respect, sorghum resembles barley, in which the endosperm cell walls were also found to be fluorescent (Fincher 1976).

Grain was germinated for 12 days to exaggerate cell wall modification; even after this period the cell walls persisted. The endosperm cells were empty and the aleurone cells contained very little, but very thin endosperm cell walls were present (Fig. 3). The cell walls between aleurone cells (approximately 4 μm thick) were much thicker than the starchy endosperm cell walls (approximately 1 μm thick). In the ungerminated grain the aleurone cell walls contained plasmodesmata interconnecting the aleurone cells. During germination these cell walls were modified by degradation around their inner surface rather than by extensive pitting.

CONCLUSIONS

Although the endosperm cell walls of sorghum persisted during germination, their chemical composition and solubility patterns changed. The ratio of water- and alkali-soluble fractions versus the insoluble residue of the cell walls decreased during germination. The major physical change to occur during this period was the abundant development of pits. Diferulic and ferulic acids were associated with the alkali-soluble fraction of the cell walls, and they did not decrease during germination.

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