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MECHANICAL PROPERTIES OF STARCH, PROTEIN AND ENDOSPERM AND THEIR RELATIONSHIP TO HARDNESS IN WHEAT

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Abstract

Various mechanical properties of whole endosperm, starch granules, and storage protein deposits were compared to determine whether the findings were consistent with the current theories on wheat hardness. All mechanical measurements were performed \textit{in situ} without the use of solvents, resins, or polishing compounds that could alter the properties of the specimens. The results showed that the starch and protein components had elastic and creep properties. There were no significant differences in any of the mechanical properties of the starch and protein components either within a variety or among soft, hard, and durum wheat types. Tensile strength ($S_u$), compressive strength ($S_{\text{max}}$), toughness ($W_{\text{max}}$), modulus of elasticity ($E$) and strain to fracture ($\epsilon_{\text{max}}$) were measured in samples of whole endosperm from various soft, hard, and durum wheats. The mean values for each parameter were highest for durum caryopses and lowest for non-vitreous caryopses of soft wheats. The hard wheat samples were intermediate. The mechanical properties of some vitreous caryopses of two soft wheat varieties were comparable to those of durum and hard wheat caryopses. These results do not support theories that suggest endosperm hardness is directly attributable to a cell product made exclusively in either hard or soft wheat varieties.

Key Words: Nanoindentation, indentation hardness, micropenetrometer, ultramicrohardness, single-grain hardness, microstructure, SEM.

Introduction

The characterization of the mechanical properties of materials is important in gaining a better understanding of their functional properties (Benham and Crawford, 1987). One commonly measured mechanical property is hardness. Hardness is defined as a measure of resistance to deformation by abrasion, indentation, scratching, machining, etc. and is typically quantified on an arbitrarily defined hardness scale (ASTM, 1991, Benham and Crawford, 1987). Different hardness tests performed on the same materials may give different hardness ratings because each test measures somewhat different characteristics (ASTM, 1991). Hardness tests are often chosen for a specimen, especially small specimens, because other mechanical tests may be difficult or too involved to perform.

Wheat caryopses are small and possess a complex geometry which makes the characterization of their mechanical properties difficult. Consequently, a number of simple, rapid, bulk hardness methods such as near-infrared analysis (NIR hardness) have become widely used in the industry (Williams and Sobering, 1986, Norris et al., 1989). Wheat hardness is a parameter of interest in the industry because it correlates well with various functional properties of commercial importance. For instance, tempering conditions and flour yield, flour particle size and shape, and flour density correlate with wheat hardness (Pomeranz and Williams, 1990).

The inheritance of wheat hardness is controlled by one or at the most two major genes and some modifying genes (Symes, 1969). However, the direct cause of mechanical hardness is not fully understood. Barlow \textit{et al.} (1973) ruled out the possibility that differences in wheat hardness could be due to differences in the hardness of the endosperm components (starch and protein). They used a conventional indentation hardness test (CIHT) to demonstrate that indentation hardness of starch and protein among hard and soft wheat types was similar. One limitation of CIHTs is that they may not provide accurate data for viscoelastic materials due to the effects of elastic recovery (Oliver \textit{et al.}, 1985, Tabor, 1985, Doerner and Nix, 1986). Depth-sensing indentation instruments (DSII) have been developed which correct for
elastic recovery and measure various mechanical properties including indentation hardness. One of the objectives of this study was to use a DSII to determine whether any mechanical properties of starch and protein correlate with differences in endosperm hardness.

Based on their earlier conclusion that the hardness of the endosperm components was similar for hard and soft wheats, Barlow et al. (1973) suggested that hardness could be dependent on the degree of starch-protein adhesion. Greenwell and Schofield (1986) isolated a 15 kDa polypeptide from starch preparations which they suggested could impair starch-protein adhesion and induce a soft texture. The gene controlling synthesis of this protein is located on chromosome 5D, the same chromosome that contains the major gene for hardness. However, it has not been conclusively shown whether the 15 kDa polypeptide directly determines mechanical hardness or is simply a genetic marker for soft wheat varieties.

One way to study the importance of a polypeptide in determining hardness is to correlate the intensity of its electrophoretic band with actual hardness scores of wheats ranging from very soft to very hard. Although bulk hardness scores for hard and soft wheats seldom overlap (Pomeranz and Williams, 1990), there is considerable variation in hardness among varieties of a given class (Williams and Sobering, 1986, Norris et al., 1989, Glenn et al., 1991). The hardness scores of single caryopses vary more than bulk hardness scores (Gaines, 1986, Pomeranz et al., 1988, Eckhoff et al., 1988) and may even overlap for some hard and soft wheats (Gaines, 1986, Glenn and Saunders, 1990). Documenting the precise amount of overlapping in hardness scores among single caryopses is difficult because hardness values for most single-caryopsis hardness tests are affected by variation in size, shape, and density among caryopses (Eckhoff et al., 1988, Gaines, 1986, Pomeranz et al., 1988). However, in an earlier study (Glenn and Saunders, 1990), a bulk hardness test was used to show that the hardness values of vitreous caryopses sorted from two soft wheat samples fell in the lower hardness range of hard wheats. This finding was unexpected since the vitreous soft wheat samples contained the 15 kDa polypeptide thought to induce a soft texture.

Several methods have been developed through which various mechanical properties can be characterized in small, irregularly shaped samples. Compression and tension tests have been used in conjunction with scanning electron microscopy (SEM) to characterize qualitative and quantitative properties of wheat endosperm (Stenvert and Kingswood, 1977, Spanoudakis and Young, 1984, Glenn et al., 1991). The second objective of this investigation was to determine both the qualitative and quantitative range in the mechanical properties of caryopses from two soft wheats with mechanically hard caryopses that contain the 15 kDa polypeptide. The mechanical properties of the soft wheat endosperm are compared with those of endosperm from various hard and durum wheats.

Materials and Methods

Indentation Tests

**Variety Selection** Wheat samples that were harvested in 1987 were obtained from three USDA regional wheat quality laboratories and stored until used. The soft wheat varieties used in the study were Crew and Arthur. Crew is a soft-white-winter club wheat (*Triticum aestivum* L.) multiline cultivar made up of a composite of 10 closely related soft wheat lines (Allan et al., 1983). Arthur (*Triticum aestivum* L. em Thell.) is a soft red winter wheat cultivar (Patterson et al., 1974). Crew and Arthur were selected because, similar to hard wheats, they contained vitreous caryopses with the capacity to form a smooth-planar surface when sectioned (1 μm) on a microtome.

Additionally, hard (Arizona and Len) and durum (breeder selections 127 and 134) wheat classes were selected for indentation tests. Near-infrared (NIR) hardness scores (AACC, 1983) were performed on each of the varieties selected. NIR hardness was 32 for bulk samples of Crew and Arthur. However, vitreous caryopses that were hand sorted rendered NIR hardness scores of 53 and 46 for Crew and Arthur, respectively. The NIR hardness readings ranged from 46 for vitreous caryopses of Arthur to 125 for breeder selection 134.

**Sample Preparation** The caryopses were prepared for indentation tests by forming a smooth, planar surface in the caryopsis' mid-section using a microtome. This was accomplished by removing the germ end of the caryopsis with a razor blade. The cut end of the caryopsis was sanded and adhered to an aluminum SEM stub using a cyanoacrylate adhesive. Approximately 2 mm of the brush end of the caryopsis was removed by sanding before mounting the sample in the chuck of a microtome (Sorvall MT-2). Serial sections (1 μm) were removed from the caryopsis until intact sections were produced. Previous observations (Glenn and Saunders, 1990) indicated that the sample surface was relatively smooth and planar when sections made on the microtome remained intact. The samples were equilibrated at room conditions (25 °C, 74% relative humidity (h_r)) for 24 hour prior to performing indentation tests. The moisture content of the endosperm under such conditions is approximately 15% (Glenn et al., 1991).

**Light Microscopy** The distribution of starch and storage protein deposits was investigated in both vitreous and non-vitreous wheat caryopses using light microscopy. A 1 mm slice of a wheat caryopsis was cut with a surgical blade and fixed overnight at 4 °C in a solution containing 6% glutaraldehyde, 0.05 M cacodylate buffer and 0.16% CaCl_2. The sample was then fixed at 4 °C for 24 hours each in solutions containing 25% and 50% glutaraldehyde. The sample was infiltrated 72 hours at 4 °C with JB4 resin containing a catalyst and placed in flat plastic molds which then were filled with resin and covered with paraffin. The resin was allowed to polymerize for 4 days in an oven set at 55 °C. Dry sections

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(2 μm) were made on a Sorvall MT-2 microtome using glass knives. The sections were mounted on glass slides and stained with 0.3% fast green for 4 minutes. Stained sections were photographed on a Nikon Fluophot light microscope.

**SEM.** Various samples of wheat caryopses that had been prepared on the microtome were studied before or after performing indentation tests using SEM. All samples observed using SEM were viewed without chemical fixation. The samples were first gold sputter coated (200 nm) then viewed in an Hitachi S-530 SEM operated at 10 kV.

**Test Procedure** The SEM stubs containing the microtome prepared samples were affixed (cyanoacrylate) to a precision X-Y-Z sample manipulation table of a DSII (Nano Indenter II, Knoxville, TN). The table first positioned the samples under the objective lens of a reflective light microscope to specify the location of the indentations. The microscope was equipped with Nomarski Interference Contrast infinity-corrected objectives which provided high resolution, high contrast images. The samples were observed at 600X (80X objective with a numerical aperture of 0.90). Efforts were made to identify the starch and protein components of the endosperm without the aid of aqueous stains which cause hydration and swelling of the starch and could alter the mechanical properties of the starch and protein. Fortunately, the starch component of the endosperm was readily discerned without stains. Storage protein deposits were also discerned without stains and were identified as amorphous structures in the subaleurone region encircled by granules of intermediate size. Protein deposits and starch granules approximately 5 μm or larger were targeted for indentation since objects of that size were well within the limits of indentation placement (± 200 nm) and indentation diameter (< 1 μm).

A normal test involved automatically positioning the sample with the precision X-Y-Z sample manipulation table below the diamond indenter and adjacent to the target. In the pretest mode, the surface of the caryopsis was located by lowering the indenter at a relatively high, constant rate and determining the point where a change in indenter velocity occurred. The sample manipulation table then positioned the first target area just below the tip of the indenter. In the test mode, a constant deformation rate of 10 nm/sec was maintained by increasing the load as needed. When a total displacement of 100 nm was reached, the load was held constant for 10 seconds to observe the creep behavior of the starch and protein. The probe was not retracted from the sample at a constant rate but rather by reducing the load incrementally which allowed more data points to be gathered along the unloading curve.

**Data Analysis.** The parameters measured were indentation hardness, Young’s modulus (E), percent creep, percent elastic displacement (De) and percent plastic displacement (Dp) (Fig. 1). Indentation hardness was calculated based on the plastic hardness point (Hp).

The Hp value was recorded as the X-intercept of a straight line ("Derived" line in Fig. 1) that passed through the initial slope of the unloading curve. Indentation hardness was measured as the applied load divided by the projected area of contact between the indenter and the sample. The modulus of elasticity was calculated using a Poisson’s ratio of 0.32. The modulus was calculated from the slope of the linear portion of the unloading curve ("Derived" line in Fig. 1). Elastic displacement (De) was calculated as the difference in displacement at Hp and the point where the unloading curve reached zero force. Plastic displacement was calculated as the difference in displacement between initial and final displacements at zero load.

Indentation tests were made on one caryopsis of each of the six wheat varieties. Seven measurements each were made on starch granules and protein deposits for each variety. An F-test (Steel and Torrie, 1980) was performed on the data to determine whether there were significant varietal differences in the mean values for the various mechanical properties measured. Tests were also performed on two polished reference materials (SiO2 and Si) to demonstrate the precision of the DSII on more typical samples.

**Endosperm Compression Tests**

**Variety Selection** In addition to the six varieties used in the indentation tests, a third soft wheat variety, Hillsdale, was included because in contrast to the samples of Crew and Arthur, it contained vitreous caryopses with relatively low NIR hardness scores. Bulk samples of vitreous and non-vitreous caryopses were hand sorted from samples of Crew, Arthur, and Hillsdale. The NIR hardness scores for each of the samples are given in Table 1.

**Sample Preparation** Cylinders of wheat endosperm were prepared for uniaxial compression tests by turning individual caryopses on a lathe as previously described (Glenn et al., 1991). Briefly, 2 mm of the germ end of each caryopsis was removed by sanding. The caryopsis was centered and affixed in an erect position on an aluminum SEM stub using a cyanoacrylate adhesive. Cylinders of endosperm approximately 2.5 mm in height and 1 mm in diameter were formed by cutting the sample on a lathe (Unimat-SL Model DB 200, American Edelstaal Inc., New York). The cylinders were equilibrated to approximately 15% moisture content by incubating them 3 days in a glass jar held at 71% hr, with a diluted acid solution (36.9% H2SO4).

**Test Procedure** A compression load was applied to the samples by crushing them at a constant rate (0.5 mm/min) between two flat plates using an Instron (model 4502). Force-displacement curves were recorded to the point of breakage for each sample. The compression tests were performed on at least ten samples for each variety.

**SEM** Failure analysis of samples broken under
compressive stress was performed by SEM. The samples typically failed in shear. Efforts were made to compare regions where a similar mode of failure occurred by photographing surfaces that had fractured at approximately a 45° angle. The samples were gold coated and viewed in the SEM as described earlier.

Data Analysis Data from force-displacement curves were used to determine compressive strength ($S_{\text{max}}$), which is the maximum compressive stress that a sample can sustain, stiffness ($E$), which is the ratio of stress to corresponding strain below the proportional limit of the force-deformation curve, toughness ($W_{\text{max}}$), which is proportional to the area under the force-deformation curve, and $e_{\text{max}}$, which is the percent strain required to cause the sample to fail (Glenn et al., 1991). Significant differences in mean values of different varieties were tested for using Fisher's Protected LSD.

Tension Tests

Variety Selection Samples that were tested in tension were prepared from the same varieties tested in compression with the exception of the durum breeder selection 127.

Sample Preparation Individual caryopses were centered on and attached to aluminum SEM stubs in an erect position as described previously. Approximately 2 mm of the brush-end was cut away using a lathe. The lathe was then used to form a dumbbell shaped sample that had a diameter of approximately 1 mm at the center. All samples were incubated for 48 hours at 71% h, to attain a sample moisture content of approximately 15%.

Test Procedure Tension tests were performed by individually mounting the aluminum SEM stubs containing the sample in an inverted position on the bottom of the Instron cross-head. The cross-head was lowered to where the sample just contacted a flat plate coated with cyanoacrylate adhesive. The glue was allowed to dry 1 hour before proceeding. The samples were tested under tension until breakage occurred.

SEM Failure analysis of samples broken under tension stress was performed by SEM. The samples were gold coated and viewed in the SEM as described earlier.

Data Analysis Tensile strength ($S_u$), the maximum tension force a sample can sustain, was calculated from force-deformation curves. Significant differences in mean values of different varieties were tested for using Fisher's Protected LSD.

Results

Indentation Tests

Light Microscopy The stained sections of wheat endosperm clearly showed starch granules and storage protein deposits. Storage protein deposits were largest in the subaleurone region of vitreous caryopses regardless of variety (Fig. 2).
Mechanical Properties of Wheat Endosperm

Table 1. Mechanical properties of wheat endosperm for vitreous caryopses of two durum and hard wheat varieties and three soft wheats that include non-vitreous samples as determined by compression and tension tests.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Class</th>
<th>NIR</th>
<th>$S_{\text{max}}$</th>
<th>$S_{\text{max}}$ Range</th>
<th>$E^\prime$</th>
<th>$E$ Range</th>
<th>$W_{\text{max}}$</th>
<th>$W_{\text{max}}$ Range</th>
<th>$e_{\text{max}}^w$ (%)</th>
<th>$S_u^v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>134u</td>
<td>DURUM</td>
<td>125</td>
<td>54.6a</td>
<td>47.3-65.4</td>
<td>1.75a</td>
<td>1.25-2.17</td>
<td>5.35a</td>
<td>2.2-10.8</td>
<td>11.7a</td>
<td>12.69a</td>
</tr>
<tr>
<td>127u</td>
<td>DURUM</td>
<td>124</td>
<td>55.5a</td>
<td>50.6-60.6</td>
<td>1.78a</td>
<td>1.36-2.11</td>
<td>5.24a</td>
<td>1.6-9.7</td>
<td>11.3ab</td>
<td>-.-</td>
</tr>
<tr>
<td>Arizona</td>
<td>HW</td>
<td>92</td>
<td>53.4ab</td>
<td>49.0-57.0</td>
<td>1.75a</td>
<td>1.39-2.19</td>
<td>3.74b</td>
<td>2.7-5.5</td>
<td>8.8b</td>
<td>13.67a</td>
</tr>
<tr>
<td>Len</td>
<td>HRS</td>
<td>86</td>
<td>47.7b</td>
<td>40.4-54.4</td>
<td>1.56ab</td>
<td>1.27-1.81</td>
<td>2.24bc</td>
<td>1.1-3.5</td>
<td>6.3c</td>
<td>13.41a</td>
</tr>
<tr>
<td>Crew V</td>
<td>CLUB</td>
<td>53</td>
<td>46.7b</td>
<td>38.9-58.0</td>
<td>1.56ab</td>
<td>1.35-2.17</td>
<td>2.10cd</td>
<td>1.0-3.8</td>
<td>6.1c</td>
<td>12.00a</td>
</tr>
<tr>
<td>Crew NV</td>
<td>CLUB</td>
<td>12</td>
<td>16.5d</td>
<td>12.7-21.4</td>
<td>0.65d</td>
<td>0.43-0.84</td>
<td>0.36f</td>
<td>0.24-0.51</td>
<td>3.4d</td>
<td>3.53c</td>
</tr>
<tr>
<td>Arthur V</td>
<td>SRW</td>
<td>46</td>
<td>42.9b</td>
<td>24.1-61.7</td>
<td>1.36bc</td>
<td>0.89-1.84</td>
<td>1.93cde</td>
<td>0.52-5.1</td>
<td>5.8cd</td>
<td>7.92b</td>
</tr>
<tr>
<td>Arthur NV</td>
<td>SRW</td>
<td>23</td>
<td>18.2d</td>
<td>13.5-21.4</td>
<td>0.71d</td>
<td>0.47-0.91</td>
<td>0.41f</td>
<td>0.24-0.77</td>
<td>3.4d</td>
<td>3.19c</td>
</tr>
<tr>
<td>Hillsdale V</td>
<td>SRW</td>
<td>32</td>
<td>32.0c</td>
<td>25.7-38.1</td>
<td>1.24c</td>
<td>0.96-1.48</td>
<td>0.70def</td>
<td>0.49-1.04</td>
<td>3.4d</td>
<td>3.82c</td>
</tr>
<tr>
<td>Hillsdale NV</td>
<td>SRW</td>
<td>23</td>
<td>22.3d</td>
<td>15.3-27.9</td>
<td>0.76d</td>
<td>0.35-0.98</td>
<td>0.51ef</td>
<td>0.33-0.65</td>
<td>3.8cd</td>
<td>3.21c</td>
</tr>
</tbody>
</table>

$S_{\text{max}}$: Stress required to cause compressive failure (compressive strength).

$E^\prime$: Initial tangent modulus of elasticity (stiffness).

$W_{\text{max}}$: Calculated from sample volume and area under force-displacement curve up to compressive failure.

$e_{\text{max}}^w$: Strain required to cause compressive failure.

$S_u^v$: Tensile stress required to cause sample failure (tensile strength).

Breeder selection.

Mean values within columns followed by a different letter are significantly different at the 1% level (Fisher’s protected lsd).

Starch granules were easily located on the surface of unstained samples using the optical system provided on the DSII. The storage protein deposits in the subaleurone region were harder to identify than the starch because of occasional cavities left by starch granules that had been dislodged during the sectioning process. The starch cavities were largely beyond the focal plane of the microscope which gave them an appearance similar to the amorphous deposits of storage protein. However, in cases where the indenter was positioned over a cavity the tests were automatically aborted as the depth limit setting of the DSII was exceeded.

SEM The endosperm of non-vitreous durum and hard wheat varieties was not suited to in situ indentation hardness measurements due to a dense distribution of intracellular spaces throughout its matrix (Fig. 3a). It was noteworthy that few starch granules became dislodged while sectioning non-vitreous hard and durum wheat caryopses. Non-vitreous soft wheat caryopses were also poorly suited for in situ indentation tests (Fig. 3b). The cut surface of the non-vitreous soft wheat caryopses was not smooth due to intracellular spaces and because of disruption of the starch and protein matrix during the sectioning process.

The sectioned surface of vitreous hard wheat caryopses appeared reasonably smooth (Fig. 3c). The endosperm had little or no intracellular space and few starch granules became dislodged when the caryopsis was sectioned. The surface of the starch granules, however, were not quite planar. The starch surfaces angled slightly toward the hilum of the granule. In addition, cracks and fissures were visible in SEM micrographs of the surface of microtome prepared samples. Some cracks were observed forming when the sample was first viewed in the SEM as reported earlier (Glenn and Saunders, 1990). Consequently, most of the cracks that occurred around the starch granules and between cells were assumed to be artifacts attributable to differential shrinkage in the evacuated chamber of the SEM.

The surface structure of sectioned vitreous soft wheat caryopses varied among the varieties tested. As with the hard wheats, intact sections were obtained while sectioning vitreous caryopses of Crew and Arthur and a relatively smooth, planar surface was achieved. In contrast, no intact sections were obtained while sectioning vitreous Hillsdale caryopses. The surface of the
Figure 3. Scanning electron micrographs of the microtome prepared surfaces of wheat caryopses. (a) Non-vitreous durum wheat caryopsis. Non-vitreous durum and hard wheat caryopses typically had an extensive network of intracellular spaces. Note that very few starch granules were dislodged by the microtome preparation procedure which illustrates the degree of starch-protein adhesion. (b) Structure typical of non-vitreous soft wheats. The protein matrix and starch granules were severely disrupted during the sectioning process. (c) Structure typical of vitreous, mechanically hard endosperm (including vitreous caryopses of Crew and Arthur). The surface is relatively planar and smooth. The fissures around the starch granules were considered artifacts of SEM since fissure formation could be observed. Note that the starch granules were not perfectly planar but angled slightly toward the center. (d) Vitreous Hillsdale caryopses were mechanically soft and had many starch granules disrupted by the sectioning process which indicated poor starch-protein adhesion. Scale bar = 10 μm.

Hillsdale caryopses was not smooth and planar due to the numerous starch granules that became dislodged while sectioning (Fig. 3d).

Even though the precision of indentation placement was ± 200 nm, an effort was still made to use SEM to locate the indentations made in the six samples tested. The depth-finding indentation formed when the indenter was plunged into the sample (~10 μm deep) was visible using SEM (Fig. 4a). Each of the three corners of the three-sided pyramidal indenter was apparent (see arrows). However, the edges of the indentation that typically form a triangular image on the specimen (see dotted lines, Fig. 4b) were not visible. Efforts to verify the placement of the shallow (100 nm) indentations used in actual measurements were unsuccessful. The difficulty in locating the test indentations stemmed
Mechanical Properties of Wheat Endosperm

Figure 4. (a) Scanning electron micrograph of a depth-finding indentation made by the DSII. Note that the triangular impression (see dashed lines in figure b) that is typical of plastic materials is not visible. The 100 nm indentations made when testing the sample could not be located due to their small size. Scale bar = 10 μm.

Table 2. Mean values for various mechanical properties of starch and protein components of wheat endosperm for three soft and three hard wheat varieties as determined by indentation tests.

<table>
<thead>
<tr>
<th></th>
<th>Hardness (GPa)</th>
<th>E(MPa)</th>
<th>Creep (%)</th>
<th>De (%)</th>
<th>Dp(%)</th>
<th>Hp (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hard wheats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.41±0.22</td>
<td>5.8±1.7</td>
<td>7.7±0.92</td>
<td>34.8±10.1</td>
<td>65.2±10.1</td>
<td>103±7.5</td>
</tr>
<tr>
<td>Starch</td>
<td>0.39±0.14</td>
<td>5.9±1.2</td>
<td>8.7±0.95</td>
<td>35.0±5.8</td>
<td>65.0±5.8</td>
<td>104±4.2</td>
</tr>
<tr>
<td>Soft wheats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.42±0.18</td>
<td>6.1±1.6</td>
<td>8.4±1.3</td>
<td>35.4±8.2</td>
<td>64.6±8.2</td>
<td>104±6.9</td>
</tr>
<tr>
<td>Starch</td>
<td>0.48±0.16</td>
<td>6.6±1.6</td>
<td>8.4±1.3</td>
<td>38.2±5.6</td>
<td>61.8±5.6</td>
<td>101±4.0</td>
</tr>
<tr>
<td>SiO₂</td>
<td>9.31±0.47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Si</td>
<td>11.72±0.39</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

from their small size (< 1 μm in diameter), the proliferation of fissures produced in the SEM chamber, and the slightly angled surfaces of the starch granules (Fig. 3c).

Testing Procedure Force/displacement curves generated during indentation tests were similar for both the starch and protein components of the endosperm. The curves revealed that the starch and protein undergo considerable elastic and plastic displacement during deformation (Fig. 1). The displacement at Hp was an average of approximately 17% smaller than the total displacement as reflected in the slope of the unloading curve.

Data Analysis The data obtained from the in situ indentation tests were quite variable in comparison to data obtained for SiO₂ and Si (Table 2). Data from two of the 84 tests were excluded because they were completely beyond the range of the rest of the data. It is possible that in the case of the two samples, the indenter tip was positioned over a small cavity so that one or more of the three faces of the indenter contacted the sample surface before the tip. Since the data are based on the assumption that the indenter tip makes first contact with the sample surface, such values would not be accurate and should be excluded from the results.

The variability in the data was highest for indentation hardness. The coefficients of variation (cv) for indentation hardness of starch and protein were 36% and 48%, respectively. In contrast, the cv for SiO₂ (5%) and Si (3.3%) were much lower.

There were no significant differences (F test, 5% probability level) in values obtained for any of the measured parameters, including indentation hardness, due to variety or to the endosperm component (starch or protein). The DSII data revealed that both the starch and protein had creep and elastic behavior. Just under two-
Figure 5. Scanning electron micrographs of the failed regions of endosperm fractured under compressive stress. The cell contents of non-vitreous caryopses of Crew (a) and Arthur (b) typically were disrupted after failure. The region of failure had loosely held fragments of protein and starch granules. There was little or no protein adherent to the starch or any form in the protein matrix that resembled starch granule cavities. The cell contents of vitreous caryopses of Crew (c), Arthur (d), and a durum variety (e) remained relatively intact. Starch granule cavities were visible as well as partially embedded starch. There were some fractured starch granules (figure c - arrow). Scale bar = 10 µm.
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Figure 6 (at left). Scanning electron micrographs of the fractured surface of wheat caryopses that failed under tensile stress. The endosperm tissue remained intact during failure except where the fracture plane occurred. Endosperm tissue of a non-vitreous soft wheat (Crew) failed by separating at the starch-protein interface (a). Endosperm tissue of vitreous endosperm of Crew (b) and Arizona (c) failed by fracturing through starch granules. Scale bar = 10 μm.

thirds of the displacement was attributable to plastic displacement (Table 2). There was less variation in the data for $E$, % creep, $D_c$, $D_p$, and $H_p$ than for indentation hardness.

Endosperm Compression Test

SEM The SEM images of the endosperm cylinders that failed during the compression tests revealed two distinct fracture patterns; one characteristic of non-vitreous soft wheats (Figs. 5a,b) and the other characteristic of all the vitreous (Figs. 5c,d) wheat samples tested excluding Hillsdale. The non-vitreous samples failed in a manner that largely disrupted the endosperm structure (Figs. 5a,b). For the most part, there were very few starch-protein aggregates or even intact starch granule cavities (Figs. 5a,b). The protein matrix structure generally appeared discontinuous.

The fracture behavior of the vitreous caryopses of Crew and Arthur (Figs. 5c,d) were similar to that of the hard and durum wheats (Fig. 5e). The caryopses typically failed along a fracture pathway that left the remaining cellular structure relatively intact. Failure between whole cells was common. Very few fractured starch granules were found in the failed region (Figs. 5c,d). Typically, starch granule cavities and partially embedded starch exposed at the failure zone were visible (Figs. 5c-e).

Data Analysis Differences in the mechanical properties of the endosperm as a whole were evident among the different wheat varieties tested in compression (Table 1). Arizona had $S_{max}$ and $E$ values similar to those of durum wheats but had lower $W_{max}$ and $e_{max}$ values. The vitreous endosperm of Crew and Arthur had properties similar to the hard wheat variety, Len. However, as evidenced by the range in values, there were individual vitreous caryopses of both Crew and Arthur that exhibited mechanical properties similar to the durum caryopses (Table 1). The vitreous caryopses of Hillsdale had higher $S_{max}$ and $E$ values than non-vitreous Hillsdale caryopses and significantly lower values for $S_{max}$, $E$, $W_{max}$ and $e_{max}$ than the hard and durum wheats. The non-vitreous caryopses of Crew, Arthur and Hillsdale all had similar values for each of the parameters measured. The $S_{max}$ values obtained from the single caryopses strongly correlated ($r^2 = 0.83$) with the NIR hardness scores from bulk samples.
**Tension Tests**

*SEM* The SEM images of endosperm tissue that failed under tensile stress revealed two dissimilar modes of failure. In both cases, the endosperm failed along a plane perpendicular to the direction of the force. The endosperm was not disrupted except along the fracture zone. The endosperm tissue of non-vitreous caryopses of Crew and Arthur failed around the starch granules. No fractured starch granules could be seen (Fig. 6a). In contrast, vitreous endosperm of Crew and Arthur and of the hard and durum wheat varieties contained many fractured starch granules (Figs. 6b,c). The fractured starch was evident by the angled starch surfaces that sloped toward the hilum.

**Data Analysis** The tensile strength ($S_u$) of the endosperm tissue was much weaker under tension than compression (Table 1). The mean $S_u$ of vitreous caryopses of Crew was similar to that of the durum and hard wheat varieties tested. The $S_u$ of the non-vitreous caryopses of Arthur and Crew was much lower than that of the vitreous endosperm tested. The $S_u$ of vitreous caryopses of Arthur was intermediate (Table 1). There was a good correlation ($r^2 = 0.74$) between $S_u$ of single caryopses and NIR hardness scores of bulk samples.

**Discussion**

Accurate values for indentation hardness may be difficult to obtain in viscoelastic materials using a CIHT because creep and elastic recovery can affect the final indentation size (Tabor, 1985, Doerner and Nix, 1986). DSII data are valuable because DSII can detect differences in creep behavior and correct for elastic recovery. In addition, DSII can measure various other parameters that characterize material properties (Oliver et al., 1985, Pollock et al., 1985). The data from the DSII confirmed the findings of Barlow et al. (1973) of no differences in indentation hardness of starch and protein among hard and soft wheat classes. The indentation hardness values were reasonably close for both studies. Barlow et al. (1973) reported slight differences in hardness between the starch and protein components although no statistical information was provided. Results of the present study indicated there were no differences between starch and protein in any of the mechanical properties measured. The apparent discrepancy in the DSII and CIHT results could be due to a greater precision in the CIHT tests or to differences in sample preparation or test conditions.

The variability of the DSII data was high for the starch and protein when compared with the data for SiO$_2$ and Si. The greater precision obtained for SiO$_2$ and Si may be attributed to their extremely smooth surface. Surface roughness is a known cause for variable readings (Tabor, 1985, Pollock et al., 1985) and is commonly alleviated by polishing the sample surface as was done with the SiO$_2$ and Si samples. The microtome technique used in the current study circumvented problems associated with the use of polishing pastes but may have resulted in more variability. In spite of the variability, some trend in the data was expected if substantial differences in the mechanical properties of the starch or protein existed among hard and soft wheat types. Since no such trends could be detected, the work was not pursued any further.

The compression and tension data of the whole endosperm were much more discriminating for hardness than the DSII data. These data in conjunction with qualitative results based on SEM micrographs were useful in evaluating different theories on wheat hardness. Barlow et al. (1973) suggested that mechanical hardness in wheat was attributable to a difference in the strength of starch-protein adhesion. There is clear evidence that particle-matrix adhesion affects the strength of materials. Spanoudakis and Young (1984) showed that fracture strength of epoxy resin reinforced with spherical glass particles was markedly increased when the glass particles were pre-coated with a coupling agent. Fracture strength decreased when the glass particles were pre-coated with a release agent.

Qualitative data support a major role for starch-protein adhesion in determining mechanical hardness in wheat (Glenn and Saunders, 1990). In the current study, starch-protein adhesion was apparent when observing caryopses that had been sectioned on the microtome or had been fractured under tension. In the microtome-prepared samples, the starch granules that became entrapped during the sectioning process remained in place in caryopses with high $S_{max}$ values but were dislodged in caryopses with low $S_{max}$ values. In the caryopses fractured under tensile stress, the protein matrix pulled away from the starch in the mechanically soft samples with low $S_u$ values. However, in samples with high $S_u$ values the starch granules adhered to the protein matrix and fractured under the tensile stress.

In spite of the evidence in support of Barlow’s starch-protein adhesion theory, the mechanism governing starch-protein adhesion is still not fully known. One possibility is that a cell product that influences starch-protein adhesion is deposited at the starch-protein interface. Simmonds et al. (1973) suggested that an "adhesive" protein could be responsible for starch-protein adhesion. However, they were unable to specifically identify such a protein. Greenwell and Schofield (1986) isolated a 15 kDa polypeptide from washed starch preparations that was most abundant in soft wheat varieties. They suggested that the polypeptide was localized on the starch granule surface and could act as a "release" protein that impairs starch-protein adhesion in soft wheat varieties. Their work has stimulated wide interest and is consistent with the genetic basis for hardness.

An innovative effort to demonstrate a direct role for the 15 kDa polypeptide in determining hardness was reported by Malouf et al. (1992). They tested the strength of tablets formed of starch and gluten from soft and hard wheats. Tablets made with soft wheat starch had low strength regardless of the source of gluten. Tablet strength from soft wheat starch pre-treated with...
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SDS or pronase was nearly equal to that of tablets made with hard wheat starch. Their results showed that differences in tablet strength were directly attributable to SDS soluble and pronase digestible material associated with the soft wheat starch. The 15 kDa polypeptide was among several other polypeptides removed by the SDS and pronase treatment. Although the data do not conclusively demonstrate that the 15 kDa polypeptide was directly responsible for the differences in tablet strength, the results are consistent with the theory of Greenwell and Schofield.

The results of the current study are not consistent with the theory of Greenwell and Schofield (1986). Glenn and Saunders (1990) demonstrated that samples of vitreous caryopses of Crew and Arthur contained the 15 kDa polypeptide and were mechanically hard based on NIR hardness data. The data from the present study further show that Crew and Arthur have some caryopses with $S_{max}$, $E_{max}$, $e_{max}$ and $S_h$ values similar to those of some hard wheat samples. These results indicate that at least some soft wheat varieties have the inherent ability to form endosperm with mechanical properties similar to hard and durum wheats, even in the presence of the 15 kDa polypeptide. What direct role the 15 kDa polypeptide plays only a role of minor importance.

In conclusion, the cause of mechanical hardness in wheat endosperm appears related to the degree of starch-protein adhesion. However, the direct cause of starch-protein adhesion remains unclear. This investigation showed that the range in hardness of individual caryopses from different wheat classes can overlap even though their bulk hardness scores do not. Further studies that attempt to establish a direct relationship between a cell product and mechanical hardness would do well to correlate the quantity of the cell product with actual hardness data based on both single caryopsis and bulk hardness tests.

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References


Discussion with Reviewers

R.C. Hoseney: Do you have evidence that the vitreous kernels selected from the soft wheat cultivars actually contain the 15 kDa protein?

Authors: In an earlier paper (Glenn and Saunders, 1990) we separated SDS-extracted polypeptides from starch preparations of various soft and hard wheats using polyacrylamide gel electrophoresis. Twenty gram quantities of both vitreous and non-vitreous samples of Crew and Arthur were tested and found to contain the 15 kDa polypeptide. The ideal approach would be to quantify the 15 kDa polypeptide in single caryopses after measuring their mechanical properties. Unfortunately, single caryopses assays for the 15 kDa polypeptide are beyond our present capabilities.

J. Lawton: What was the percent of the vitreous and non-vitreous kernels for the different wheat varieties used?

Authors: The soft wheat varieties used in this study had a range in vitreousness. The majority of the caryopses had both vitreous and non-vitreous regions with the greater portion being non-vitreous. It was our intent to test caryopses at both ends of the range. Accordingly, caryopses that were completely vitreous or completely non-vitreous (2%-4%) were selected. It should be noted that it was very unlikely that the vitreous soft wheat caryopses were actually hard wheat caryopses intermixed with soft wheat samples. The size, shape and color of the vitreous caryopses were comparable with the rest of the sample. In addition, all of the vitreous soft wheat samples contained the 15 kDa polypeptide.

J. Lawton: What did the SEM's of the vitreous Hillsdale look like after the compression test? Could you provide a SEM of the crushed Hillsdale?

Authors: The SEM's looked very similar to those of the non-vitreous samples of Crew and Arthur. It seems redundant to show an SEM of the crushed Hillsdale.

J. Lawton: Did vitreous Hillsdale fail like the vitreous or the non-vitreous kernels of the other soft wheats?

Authors: Vitreous Hillsdale failed like the non-vitreous samples of the other soft wheats.

J. Lawton: The NIR test placed both vitreous Arthur and Crew much lower on the hardness scale than the hard wheats tested. However, vitreous Arthur and Crew showed no difference compared to the hard wheats for \( S_{\text{max}} \) and vitreous Crew showed no difference for \( S_w \). Do you think the NIR test is a more sensitive test for hardness than the compression or tensile tests?

Authors: If you compare one parameter, such as \( S_{\text{max}} \), with NIR hardness, you will find it is less sensitive. Earlier studies (Glenn et al., 1991) indicate that NIR hardness is a measure of strength (\( S_{\text{max}} \)) and ductility (a function of \( e_{\text{max}} \)). If you consider all of the parameters measured (\( S_{\text{max}}, e_{\text{max}}, E, W_{\text{max}}, S_w \)) on single caryopses, you will find they are very sensitive and they can be obtained on a single caryopsis. If that is true, the obvious question is "why are the five single-caryopsis parameters similar for Crew and Len while their NIR scores differ?"

The NIR tests required about 1500 hand-sorted vitreous Crew caryopses. These caryopses were mostly vitreous but contained some caryopses with non-vitreous regions. Caryopses with non-vitreous regions tend to lower the NIR scores.

J. Lawton: Could the NIR test due to its grinding be influenced more by the adhesion of the protein to the starch and the compression and tensile tests more influenced by matrix continuity?

Authors: The grinding process used in the NIR test breaks down the caryopses using a combination of shear, compression and tension stress. Even with compression tests there are tension and shear forces generated within the sample. The tension test was the most likely to differentiate specific structural features. Tension-tested samples did not fail in shear as did the compression tested samples. Tension tests appeared to be a very good indicator of starch-protein adhesion.

J. Lawton: The tensile test that was done would seem to be influenced by the continuity of the protein matrix. A test specimen that was solid all the way across would seem to be stronger than a specimen that had an non-continuous matrix. Were any non-vitreous hard wheats tensile strength tested, and if so how did their tensile strength compare with vitreous hard wheats? Also did they fail like vitreous hard wheats?

Authors: The authors main interest was to document that some soft wheats have the inherent ability to form endosperm that is mechanically hard. The authors only looked at a few non-vitreous hard wheat samples. These
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samples had a lower tensile strength than the vitreous samples. Failure analysis was not performed on the non-vitreous hard wheats.

C.R. Martin: Do you believe the data?  
Authors: The authors believe the data and encourage others to include single-caryopses tests in their studies on wheat hardness.

C.R. Martin: Do the micrographs show any evidence to support any conclusions about differences between the way hard and soft varieties break apart?  
Authors: While most soft wheat varieties are mechanically soft, there are some varieties with the capability of forming caryopses with mechanically hard endosperm. The micrographs provide evidence for distinguishing between caryopses that are mechanically hard or soft. The evidence is especially convincing when micrographs of tension, compression and microtome prepared samples are combined. However, a mechanically hard caryopsis from a soft wheat variety will not be distinguished from a caryopsis with similar mechanical properties from a hard wheat variety.

C.R. Martin: Can the micrographs be analyzed in a manner that describes the distribution or continuity of the material surrounding starch granules?  
Authors: The micrographs of the microtome prepared samples (Fig. 3) are ideal for analyzing the matrix surrounding the starch. The microtome technique gives a cross-sectional view of the starch and matrix material.

C.R. Martin: Was the starch-protein interface strength tested by the indentation measurements?  
Authors: No. The authors contemplated such a feat but quickly disregarded efforts to attempt it after considering the complexity involved in obtaining meaningful data.

C.R. Martin: Were the indentation tests measuring the same thing (large protein deposits) or were the things measured (vitreous material) always the same?  
Authors: The indentation tests were performed separately on both the protein deposits and the starch granules.

C.R. Martin: Did any portion of the Intron test sections contain bran or vascular bundle material? Was it all endosperm?  
Authors: The sample cylinders were all endosperm.

C.R. Martin: What effect would the adhesive that penetrated the vascular bundle capillaries have on Intron tests?  
Authors: The sample cylinders were all endosperm. The adhesive used to attached the samples to the stub was applied as a small drop (approximately 10 μm) with a pipet. The excess was immediately blotted with a tissue. There appeared to be very little capillary movement of the glue beyond the base of the sample. The authors feel that capillary movement of the glue was not an important factor.

C.R. Martin: How are the results of mechanical properties tests related to the distribution or continuity of protein within the kernel?  
Authors: The authors assessed whether the protein matrix was continuous or not based on their interpretation of SEM micrographs of unfixed tissue that had been sectioned on a microtome. Both the vitreous and non-vitreous samples of Hillsdale had a discontinuous protein matrix. The vitreous Hillsdale samples had higher protein content and a more densely packed protein matrix. These samples had greater strength than the non-vitreous samples. The vitreous caryopses of Crew had a continuous protein matrix that was also densely packed. The vitreous Crew samples also exhibited starch-protein adhesion. These samples had greater $S_{\text{max}}$, $E$, $W_{\text{max}}$, $S_u$ than vitreous Hillsdale caryopses.

C.R. Martin: What is the difference between adhesion and cell product theory in wheat hardness?  
Authors: The adhesion theory offered by Barlow et al. (1973) attributes wheat hardness to the degree of starch-protein adhesion. The data in the current study concur with the adhesion theory. The cell product theory attempts to account for the mechanism of starch-protein adhesion. That is where there is uncertainty. The theory of Greenwell and Schofield is very interesting but in this instance, it was not consistent with the data.