NOTE

Size-Distribution of Starch Granules Isolated From Hard Red Winter and Soft Red Winter Wheats

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Grain hardness (in particular, wheat hardness) is an important characteristic that plays a significant role in marketing wheat. A variety of methods exist for the determination of the degree of hardness; however, the methods involve some sort of mechanical disruption of the wheat grain and the measurement of this disruption. Little work has been conducted to determine whether hardness affects various grain storage components such as starch and proteins. It is generally thought that hardness results from the strength of starch-protein interactions (Barlow et al 1973). Softness may be caused by the presence of the 15-kDa protein called friabilin, which is isolated from the starch of soft wheats (Greenwell and Schofield 1986, Schofield and Greenwell 1987). A close spacial relationship exists between starch and protein at maturity. This relationship was formed during grain development and drying and may influence starch granule morphometry. Starch granule mean area, standard deviation of granule area, and coefficient of variation were shown by Pitts et al (1989) to be indicators of differences between hard and soft wheat endosperm cell geometry. We previously developed quantitative image analysis techniques for studying wheat starch isolated from maturing caryopses (Bechtel et al 1990, 1991).

This article investigates differences in starch granule size distributions among a group of hard and soft red winter wheats to determine whether isolated starch can be used as a discriminator between hard and soft wheats.

MATERIALS AND METHODS

Samples and Starch Isolation

Samples used in this study were selected from a large group of wheats grown in 1988 as part of the Kansas winter wheat performance test. The wheats were selected because they varied widely in near-infrared reflectance (NIR) hardness values, protein content, growing location, and milling quality. In addition, some had hardness values that caused them to be misclassified by NIR (Table I). This selection process, although arbitrary, resulted in a number of samples that were difficult to classify as hard or soft based on NIR hardness values.

Starch was isolated from the wheat as previously described (Bechtel et al 1990). The embryo was removed from individual caryopses by cutting off the lower level of the caryopsis with a razor blade. The remainder of the caryopsis was then minced with a new razor blade. The caryopsis-endosperm pieces were placed in 4°C buffer (25 m*M* Tricine, 5 m*M* magnesium acetate, and 50 m*M* potassium acetate, pH 7.5). A 2:1 volume ratio of buffer to endosperm was used. The mixture was homogenized in a Tekmar Tissumizer (model SDT, Cincinnati, OH) with high-torque speed control (model TR-5T) at a moderate setting for 30–60 sec. The resulting slurry was squeezed through a single layer of sterile gauze (0.5 mm mesh) into 1.5-ml microfuge tubes, rinsed with 0.5 ml of buffer, and spun for 60 sec in an Eppendorf 5412 centrifuge. The supernatant was discarded, and the pellet was resuspended in water and centrifuged again. This washing process was conducted twice, and care was taken to retain starch tailings at the starch-buffer interface.

Samples of starch were lyophilized and stored at −20°C until used. Analysis of starch was conducted by rehydrating a portion of the starch in water and mixing it with glycerol to make a 20% solution (to decrease Brownian movement). The starch slurry was thoroughly mixed, a small drop was placed on a microscope slide, and a cover glass was placed over the suspension. Isolated starch was viewed as previously described (Bechtel et al 1990) with dark-field microscopy, and the images were recorded with a Cohu solid-state black-and-white camera (model 4815-2000) and a Panasonic AG-2510 videocassette recorder. Forty separate fields of view from at least three separate slide preparations were recorded on video. The number of starch granules counted per sample varied from 3,238 to 14,671. Recorded images were captured from videotape with a Panasonic AG-2510 videotape recorder interfaced to a Kontron Image Analysis system (Roche Image Analysis Systems, Ellon College, NC).

A computer program was written to acquire images from the running videotape, which kept track of incoming images by measuring image gray levels. Image acquisition was started for a sample from a white image frame, which was used to separate sample sets from one another on the videotape. The incoming image was used as a reference image and stored for comparison

TABLE I
Wheat Samples of Isolated Starch

Class	Variety	NIR[a] Hardness	Protein (%)
Group 1			
Soft	Caldwell 1	44.0	13.4
Soft	Caldwell 2	33.2	9.4
Soft	Compton 1	54.8	14.8
Soft	Caldwell 3	23.9	11.6
Soft	Becker 1	38.7	14.0
Hard	TAM 107 2	114.8	14.3
Group 2			
Hard	TAM 108 1	48.1	15.5
Hard	TAM 108 2	80.3	14.0
Hard	Arkan 1	53.9	16.7
Hard	Newton 6	59.1	14.9
Group 3			
Soft	Caldwell 4	33.4	10.2[b]
Soft	Compton 3	26.3	13.4[b]
Soft	Becker 2	16.2	12.7[b]
Soft	Compton 2	39.8	10.8[c]
Soft	Mo 9965	20.8	10.9[c]
Hard	Newton 3	73.4	12.3[d]
Hard	TAM 107 1	72.5	13.7[d]
Hard	Newton 1	47.9	15.3[d]
Hard	Newton 2	96.8	14.5[d]
Hard	Newton 4	56.5	10.2[d]
Hard	Newton 5	79.5	13.0[d]
Hard	Triumph 64 1	60.0	15.5[d]
Hard	Triumph 64 2	96.4	15.6[d]
Hard	Arkan 2	102.2	15.2[d]
Hard	Arkan 3	74.9	13.5[d]

[a] Near-infrared reflectance.
[b] Mean of type B starch granules greater than 10 μm.
[c] Mean of type B starch granules equal to 10 μm.
[d] Mean of type B starch granules less than 10 μm.

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to the next acquired image. Acquired frames were subtracted from the previously recorded frame, and when the difference of the subtracted image exceeded a preset threshold value, the program was triggered to acquire the next image. A command that turned the videotape recorder to pause allowed time for the appropriate calculations to be conducted before the next frame was acquired. Data was printed in the form of histograms plotting the number of granules versus equivalent diameter.

RESULTS

Histograms of equivalent diameter—$2(\text{area}/\pi)^{1/2}$—for the 25 hard red winter (HRW) and soft red winter (SRW) wheat samples were coded in such a manner that they could not be identified by variety and/or class. The histograms then were visually sorted into groups based on histogram shape. Three groups were identified. Group 1, represented by Caldwell 1, had an equivalent diameter histogram shape in which the small type C granules (0–5 μm equivalent diameter) were present in large numbers. The remainder of the starch in the form of type A (> 16 μm equivalent diameter) and type B (5–16 μm equivalent diameter) granules occurred at a much lower frequency (Fig. 1A). Type B granules occurred about twice as often as did type A granules. Group 1 consisted of six wheats—five SRW and one HRW (Table I). Samples that were placed in group 2 had a size distribution that was approximately triangular (Fig. 1B) and consisted of four HRW wheats (Table I). Type C granules were the most numerous, followed by a nearly linear decrease in relative numbers of type B and A granules. The third group of wheats were depicted by a histogram that was a distinctly trimodal distribution for the starch (Fig. 1C and D) and contained the remaining five SRW and 10 HRW wheats (Table I). Further visual comparison of group 3 histograms revealed that HRW wheat type B granules had mean equivalent diameters of less than 10 μm (Fig. 1C), whereas SRW samples had mean diameters greater than 10 μm (Fig. 1D). Using these three groupings based on histogram shape, only one sample (TAM 107 2) was not correctly classified out of 25 wheats.

DISCUSSION

The theory that endosperm hardness resides in the strength of the starch-protein interface (Barlow et al 1973) has led to several investigations regarding this interaction. That the interaction between starch and protein results in the phenomenon called hardness is supported by the fact that proteins from hard and soft wheats have similar mechanical properties, as do the starches from hard and soft wheats (Barlow et al 1973). The finding of the 15-kDa protein friabilin, consistently associated with soft wheat starch, also supports the view that starch-protein interactions influence grain hardness (Greenwell and Schofield 1986, Schofield and Greenwell 1987). A difference in the interaction between endosperm components during development might influence the size and shape of starch granules. Consequently, the interaction between starch and protein during growth and development may result in a different morphology for starch from hard or soft wheats. Hard wheats were found to have a larger mean starch granule area and fewer small starch granules than did soft wheats (Pitts et al 1989), whereas another study using the same samples reported that hard wheats had a smaller mean starch granule area and a greater number of small granules than did the soft wheats (Glenn et al 1992). Both of these studies used sectioned material and had sampling problems associated with viewing only portions of granules (Glenn et al 1992). The large amount of variation that occurs within and among grains makes it imperative that large sample sizes be used when sectioned material is analyzed (Gaines et al 1985).

Digital image analysis of isolated starch granules allows for a highly accurate measurement of size distribution of starch granules (Bechtel et al 1990) but does not allow for in situ comparisons within the grain. Quantitative image analysis of isolated starch does allow for measurement of various shape

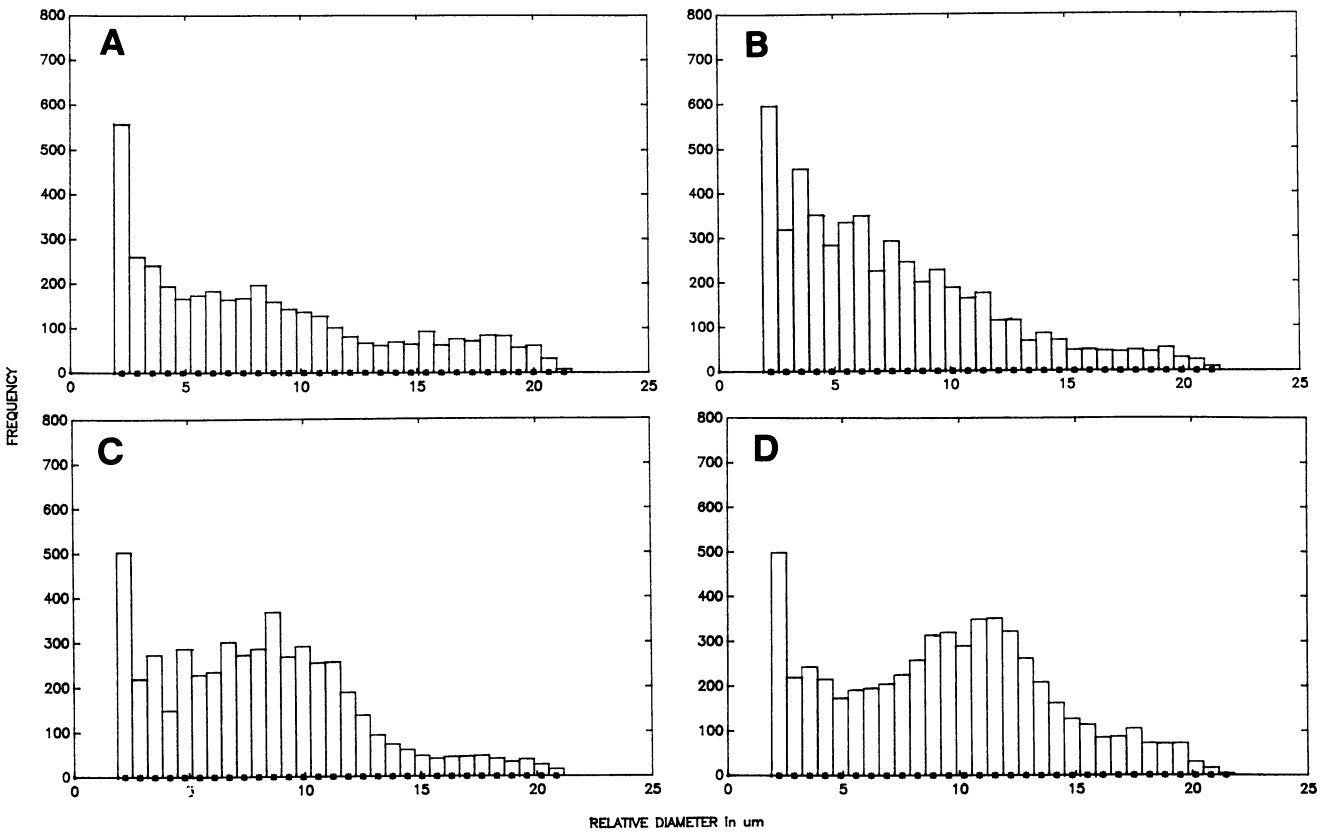

Fig. 1. Histogram of size distribution in starches. **A,** Caldwell 1 (representing group 1); **B,** Arkan 1 (representing group 2); **C,** Newton 3; and **D,** Compton 3 (representing hard and soft wheats in group 3).

factors that help reduce certain measurement errors. Perimeter-convex perimeter measurements are necessary internal software controls that allow the identification and rejection of starch granules that may be damaged and, more importantly, allow for the elimination of granules that may be touching each other or confused with nonstarch objects.

Image analysis of isolated starch can help identify problems with techniques. For example, image analysis of isolated starch using microscopy showed that starch was nearly circular but only about 5 μm thick (Bechtel et al 1990). Several other methods of starch quantification have typically assumed that the starch granule is spherical (Coulter counter: Morrison and Scott 1986, Morrison and Gadan 1987; sectioned material: Glenn et al 1992) rather than a prolate spheroid. This assumption leads to a larger volume (mass) for the type A granules being studied and skews that data toward the presence of a larger mass of type A granules than is actually present.

The samples used in this study were purposely selected to give a wide variation in NIR hardness values, protein content, growing location, and milling quality. The samples would, therefore, challenge the most discriminating method of hardness determination, NIR. Based on cursory comparison of the image analysis data, we were able to correctly class 24 of the 25 samples (only TAM 107 2 was not properly classed). The separation into hard and soft winter wheat classes was based on equivalent diameter histogram shape and on the mean diameter of type B granules estimated from the histogram. We found no significant difference in the relative quantity of type A granules between the two wheat classes, as previously reported for sectioned tissue (Pitts et al 1989, Glenn et al 1992). All 25 samples exhibited a type C class of starch granules (Bechtel et al 1990), and 15 of the samples showed a distinct trimodal distribution of starch.

The type C granules were actually underestimated in our data. At the microscope magnification we used, many type C granules were less than one pixel in size (but still visible in the microscope) and could not be detected with this methodology. In addition, some of the tiny granules are undoubtedly lost during the isolation procedure, and we have yet to quantify the number lost. In contrast to our method, other methods of analysis have shown only a bimodal distribution for wheat (Evers and Lindley 1977; Baruch et al 1979, 1983; Soulaka and Morrison 1985). Image analysis of sectioned endosperm tissue also showed a bimodal distribution for both hard and soft wheats (Glenn et al 1992). This may be because of the starch size classes that were chosen; the smallest class was 0–50 μm^2 in measured area, which corresponds to about 0–8 μm equivalent diameter and covers all of the type C as well as part of the type B classes.

In summary, we have been able to correctly identify 24 of 25 wheat samples as hard or soft by visually comparing histograms of size distribution of starch. The results of this preliminary investigation suggests that starch morphometrical parameters may be useful to separate hard red winter from soft red winter wheats. Further quantitative and statistical analysis currently in progress may identify these parameters more specifically.

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ERRATUM

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On page 27, the literature citation given in the first paragraph under Results and Discussion should read:

(Chan and Wasserman 1993).

On page 28, the fifth entry in Literature Cited should read:

CHAN, K. Y., and WASSERMAN, B. P. 1993. Direct colorimetric assay of free thiol groups and disulfide bonds in suspensions of solubilized and particulate cereal proteins. Cereal Chem. 70:22-26.