Relationship Between Endosperm Texture and the Occurrence of Friabilin and Bound Polar Lipids on Wheat Starch[1]

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ABSTRACT

Endosperm texture affects the milling and end-use properties of wheat grain. A better understanding of the physical-chemical mechanism and the genetic control of endosperm texture in wheat would aid in breeding, marketing, and utilization of grain. Here, we report on the relationship between endosperm texture and the occurrence of friabilin, a family of 15-kDa proteins, and bound glyco- and phospho-lipids on water-washed wheat starch. These two classes of bound polar lipids follow the same pattern of occurrence as friabilin: approximately equal levels in soft and hard wheat flour, much reduced levels in water-washed soft wheat starch compared to flour, and much reduced levels in hard wheat starch compared to soft. The type and quantity of these bound polar lipids is highly conserved among both soft and hard wheat starches. Further, these lipids are implicated in the interaction of friabilin with soft wheat starch. Propan-2-ol and water (90:10), which is effective in removing bound polar lipids from starch, renders most friabilin components extractable with an aqueous salt solution. These results suggest that most friabilin components interact with starch through lipid-mediated hydrophobic interactions and ionic interactions. In addition, the results provide both an additional biochemical marker for grain softness and new insight into the possible physical-chemical mechanism and the genetic control of endosperm texture in wheat.

Endosperm texture is a primary determinant of the processing and end-use quality of wheat (*Triticum aestivum* L.) grain. Endosperm texture is controlled by one or two major genes (reviewed by Pomeranz and Williams 1990). Mattern et al (1973) and Law et al (1978) demonstrated that the major genetic element controlling endosperm texture resides on the short arm of chromosome 5D (5DS). Although extensively studied, no direct causal relationship between the genetic and the physical-chemical basis of endosperm texture has been established. Greenwell and Schofield (1986) reported the existence of friabilin, a 15-kDa protein on water-washed wheat starch that was perfectly correlated (no exceptions) with the qualitative level of endosperm hardness. Friabilin was abundant on soft, scarce on hard, and absent on durum (AABB tetraploid) water-washed wheat starch. This friabilin-endosperm softness relationship was confirmed in hundreds of genotypes (Greenwell and Schofield 1986, Morris et al 1994). Schofield and Greenwell (1987) also established that the occurrence of friabilin on starch is controlled by the short arm of chromosome 5D. Jolly et al (1993) extended these findings by demonstrating that the control of friabilin in whole grain (both hard and soft) resides on 5DS.

As compelling as these data may be, a causative role of friabilin in conferring endosperm softness remains speculative. However, the evidence does indicate that some underlying relationship be-

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tween friabilin occurrence and endosperm texture exists. Once considered to be a single, starch-granule-associated protein, more recent studies indicate that friabilin is a family of highly related proteins (Morris et al 1994) that occur in hard as well as soft wheat endosperm (Jolly et al 1993). Malouf et al (1992) and Jolly et al (1993) suggested that the occurrence of friabilin on soft wheat starch may be an artifact of the starch isolation procedure. Jolly et al (1993) reported that soft wheat starch possessed only ~5% of the friabilin originally present in the endosperm.

The physical interaction of friabilin with soft wheat starch is poorly understood. Friabilin is easily and quantitatively extracted from soft wheat starch with sodium dodecyl sulfate (SDS) (Greenwell and Schofield 1986, Jolly et al 1993, Morris et al 1994) or a combination of propan-2-ol, water, and NaCl (Morris et al 1994). Individually, aqueous alcohol and salt solutions are largely ineffectual (Morris et al 1994). These results suggest that friabilin may interact with starch through both hydrophobic and ionic interactions. Characterizing the association of friabilin with isolated starch may provide greater insight into the physical-chemical mechanism and the genetic control of endosperm texture in wheat. Here, we present our initial results describing the relationship between endosperm texture and the occurrence of friabilin and bound polar lipids on water-washed wheat starch.

MATERIALS AND METHODS

Prime starch was isolated from straight-grade flour of the soft wheat cultivars Lewjain, Hill 81, Penawawa, Stephens, Dirkwin, and Centennial and the hard wheat cultivars Butte 86, McKay, Wanser, Yecora Rojo, Klasic, and Westbred 906R using the dough-ball method of Wolf (1964). The starches were air-dried and ground using a Tecator Cemotec 1090 mill (setting 2) (Boulder, CO). Straight-grade flour from these cultivars was also used for protein and lipid extractions.

Flour proteins were extracted using sample buffer (62.5 m*M* Tris, pH 6.8, 20 g ml^{-1} SDS, 100 ml L^{-1} glycerol and 12.5 mg L^{-1} bromophenol blue) at room temperature for 0.5 hr. Flour-sample buffer ratio was 1:25. The extract was centrifuged (10,000 × *g*, 15 min) and the pellet discarded. The proteins in the supernatant were reduced by adding 50 ml L^{-1} β-mercaptoethanol and heating at 70°C for 15 min. The supernatant was centrifuged (10,000 × *g*, 15 min), and 10 μl was applied to an SDS polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Water-washed prime starch was extracted with propan-2-ol and 0.1*M* NaCl (1:1) (propanol-salt); 50 m*M* tris(hydroxymethyl)-amino methane, pH 6.8; 50 m*M* NaCl (Tris-salt); propan-2-ol and water (90:10) (propanol-water); or hexane at a starch-solvent ratio of 1:10 at room temperature for 0.5 hr (Morris et al 1994). Extracts were centrifuged (10,000 × *g*, 15 min), and the starches discarded or retained for sequential extractions, as appropriate. The proteins in the supernatants were either: 1) reduced and loaded onto SDS-PAGE as described for the flour proteins (Fig. 1); or 2) precipitated with 3–5 volumes of acetone overnight at −20°C, pelleted (10,000 × *g*, 15 min), washed with acetone, air-dried, solubilized at the rate of 1 mg starch equivalent per microliter in sample buffer containing 50 ml L^{-1} β-mercaptoethanol, heated at 70°C for 15 min, and centrifuged as before. A sample volume of 5 μl was loaded on the SDS-PAGE gels.

Proteins were separated using a 0.75-mm thick 13.5% T/2.6% C SDS-PAGE resolving gel (Laemmli 1970) in a mini-gel format (Bio-Rad Laboratories, Hercules, CA). Glycerol was substituted for water in the resolving gel at 100 ml L^{-1} to reduce protein diffusion. The stacking gel was 4% T/2.6% C. Both gels were crosslinked with piperazine diacrylamide (Bio-Rad). Molecular weight markers were obtained from the same source. Electrophoresis was conducted at a constant 200 V for 55 min. The gels were fixed and silver stained (Morris et al 1994).

Free lipids were extracted from flour and starch with hexane (sample-to-hexane ratio of 1:10) for 0.5 hr at room temperature and discarded. Flour and starch were air-dried. Bound lipids were extracted from 2 g of flour and 12 g of starch with propan-2-ol and water (90:10) at a sample-to-solvent ratio of 1:6 for flour and 1:3 for starch, for 15 min at room temperature. The extracts were centrifuged (10,000 × *g*, 15 min), the pellets discarded, and the supernatants were taken to dryness at 50°C under N_2 gas.

Group separation of bound lipids was accomplished using a modified procedure of Prieto et al (1992). The bound lipids were dissolved in 4 ml of hexane, ethyl acetate, and acetic acid (95:5:0.2) (initial solvent) and applied to a silica Sep-Pac cartridge (Millipore Corp., Milford, MA) prewashed with hexane and the initial solvent. Neutral lipids were eluted from the cartridge with 5 ml of the initial solvent. The cartridge was washed with 5 ml of hexane and ethyl acetate (95:5) and partially dried. Glycolipids were eluted with 5 ml of tetrahydrofuran, acetonitrile, and propan-2-ol (35:35:30). The cartridge was again partially dried, and phospholipids were eluted with 5 ml of acetonitrile and methanol (35:65). All three eluted lipid fractions were taken to dryness at 50°C under N_2 gas and redissolved in chloroform and methanol (2:1) to a final concentration of 5-mg flour equivalents, or 60-mg starch equivalents per microliter.

The lipid fractions were chromatographed on precoated thin-layer silica gel G plates (20 × 20 cm) (Aldrich Chemical Co., Milwaukee, WI) following the procedure of Morrison et al (1980). The plates were conditioned by vertical development for 19 cm with chloroform and methanol (2:1) and heat-activated at 125°C for 1 hr. The cooled plates were spotted with 8 μl of the appropriate lipid solution per lane. The neutral lipids were separated in a sequential two-solvent system: 1) ethyl ether, toluene, ethanol, acetic acid (10:50:2:0.2) to 10 cm from the origin, and 2) ethyl ether and hexane (6:94) to 16 cm from the origin. The glycolipids were separated in a two-solvent system: 1) chloroform, acetone, acetic acid, and water (10:90:2:3) to 13 cm from the origin, and 2) ethyl ether and acetic acid (99:1) to 16 cm from the origin. The phospholipids were separated in chloroform, methanol, ammonium hydroxide, and water (65:35:5:2.5). After development, the plates were air-dried, and the lipids were visualized

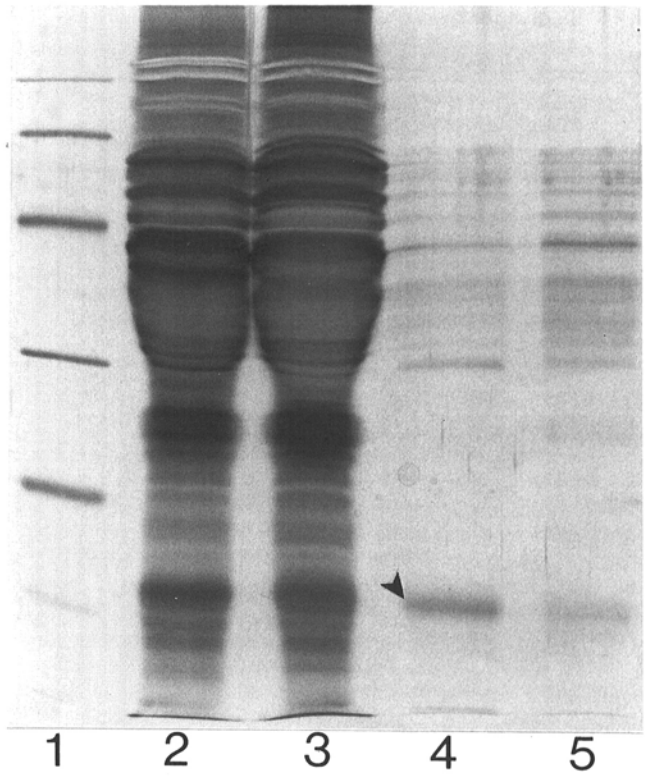

Fig. 1. Silver-stained electropherogram of proteins from flour (lanes 2 and 3) and prime starch (lanes 4 and 5) from soft (Lewjain, lanes 2 and 4) and hard (Butte 86, lanes 3 and 5) wheats. The extracts from either 0.4 mg of flour or 5.0 mg of starch were applied per lane. Lane 1: molecular weight markers of M_r 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 k. Friabilin is indicated by the arrow.

by spraying with sulfuric acid and methanol (1:1) and charring on a 500° C hot plate. All ratios used in this article refer to weight for solids and volume for liquids.

RESULTS

Friabilin occurs in soft and hard wheat endosperm at approximately equal amounts (Fig. 1, lanes 2 and 3). Western blots confirm that the heavy band at 15 kDa, in both soft and hard, is predominately friabilin (data not shown). During the aqueous isolation of soft wheat starch, as little as one per cent of the original friabilin partitions with the starch, apparently associating with the granule surface (lane 4 and western blot data not shown). In hard wheat, the proportion is relatively much less, so that isolated hard wheat starch has little friabilin associated with it (lane 5).

As shown earlier (Morris et al 1994), a combination of propan-2-ol and NaCl is effective in the quantitative extraction of friabilin from starch (Fig. 2, lane 2). Tris-salt was largely ineffectual at breaking the interaction of friabilin with the surface of the starch granule (Fig. 2, lane 3). Some protein at 15 kDa is extracted, though, suggesting either low extraction efficiency or selective extraction of one or more components of the friabilin protein family. Tris-salt is effective at removing the major protein of M_r 30 kDa and additional, larger proteins above 30 kDa. Following the extraction with Tris-salt, propanol-salt removes the remaining friabilin (lane 4).

Propan-2-ol and water (90:10) extracted no detectable friabilin from starch (data not shown). Friabilin was quantitatively extracted using propanol-salt following a preextraction with propanol-water (Fig. 2, lane 5). However, extracting starch with propanol-water conferred Tris-salt extractability on a major portion of friabilin (or components) (Fig. 2, lane 6). Again, differences in recovery could result from either efficiency or selectivity of extraction. Sequential extraction of starch with propanol-water and Tris-salt, followed by propanol-salt produced a faint band at 15 kDa (Fig. 2, lane 7), suggesting that a small portion of friabilin may require the combined, synergistic action of propanol, water, and salt.

In contrast to propanol-water, extracting starch with hexane did not affect friabilin extractability (Fig. 2). Extracting starch with hexane followed by propanol-salt indicated that hexane did not extract friabilin (Fig. 2, lane 8), nor did hexane confer Tris-salt extractability; a faint band, similar to that obtained with Tris-salt alone (lane 3), was observed (lane 9).

Two classes of bound polar lipids follow the same pattern of occurrence as friabilin. The amount of bound glycolipids is roughly equivalent in soft and hard wheat (Fig. 3A, lanes 1 and 2). The level of bound glycolipids associated with water-washed starch from soft wheat was about one-tenth that found in soft flour (Fig. 3A, lane 3 [note loadings]). Bound glycolipids were essentially absent in water-washed starch from hard wheat (Fig. 3A, lane 4).

Bound phospholipids exhibited a pattern similar to that of glycolipid occurrence: similar between soft and hard wheat flours (Fig. 3B, lanes 1 and 2), much reduced in water-washed soft wheat starch compared to flour (Fig. 3B, lane 3 vs. lane 1 [note loadings]), and much reduced in hard wheat starch compared to soft (Fig. 3B, lanes 3 and 4). In addition, the low level of phospholipids found associated with hard wheat starch generally exhibited different mobilities and therefore would be considered to be different species.

The pattern of bound lipid occurrence in soft and hard wheat starches is highly conserved among hexaploid wheats. A survey of six hard and six soft wheat cultivars indicated that the level of bound glycolipids was consistently high in soft and nearly absent in hard (Fig. 4). Likewise, bound phospholipids were prevalent in soft and present at much reduced levels in hard (Fig. 5). Further, the level of the various bound polar lipids among the soft and the hard wheat starches was similar both in terms of types of lipids and relative abundance.

Visualization of neutral lipids, using the thin-layer chromatography system described above, indicated that there were no salient differences between hard and soft wheat flours or starches (data

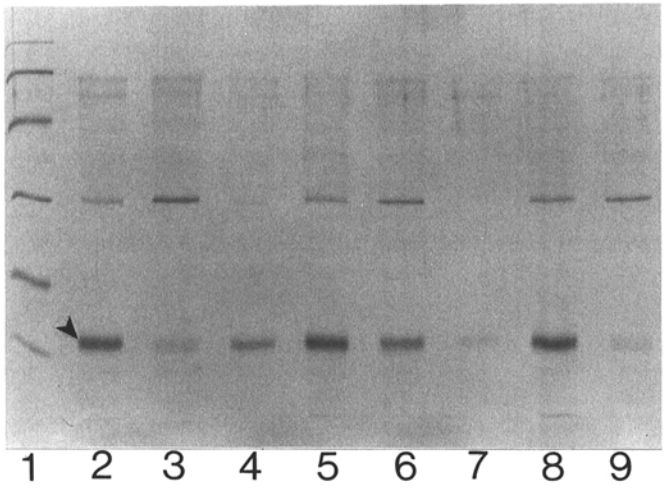

Fig. 2. Silver-stained electropherogram of proteins from prime starch from soft (Lewjain) wheat (lanes 2–9) extracted as follows: lane 2, propanol-salt; lane 3, Tris-salt; lane 4, propanol-salt following a pre-extraction with Tris-salt; lane 5, propanol-salt following a pre-extraction with propanol-water; lane 6, Tris-salt following a pre-extraction with propanol-water; lane 7, propanol-salt following a sequential, pre-extraction with propanol-water and Tris-salt; lane 8, propanol-salt following a pre-extraction with hexane; and lane 9, Tris-salt following a pre-extraction with hexane. Lane 1: molecular weight markers of M_r 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 k. Friabilin is indicated by the arrow.

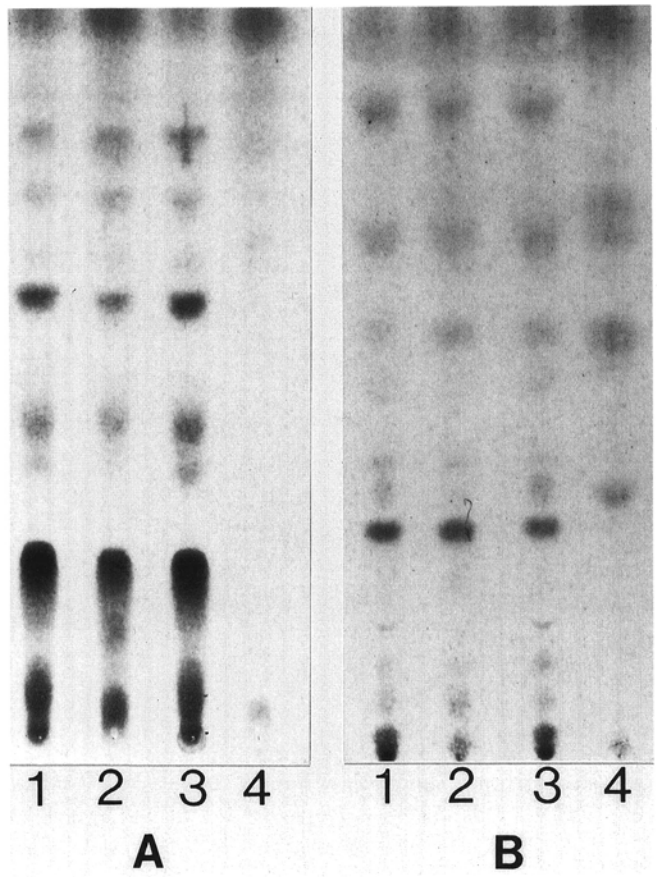

Fig. 3. Thin-layer chromatogram of bound glycolipid **(A)** and bound phospholipid **(B)** from flour (lanes 1 and 2) and prime starch (lanes 3 and 4) from soft (Lewjain, lanes 1 and 3) and hard (Butte 86, lanes 2 and 4) wheat. Extracts from either 40 mg of flour or 480 mg of starch were applied per lane and chromatographed.

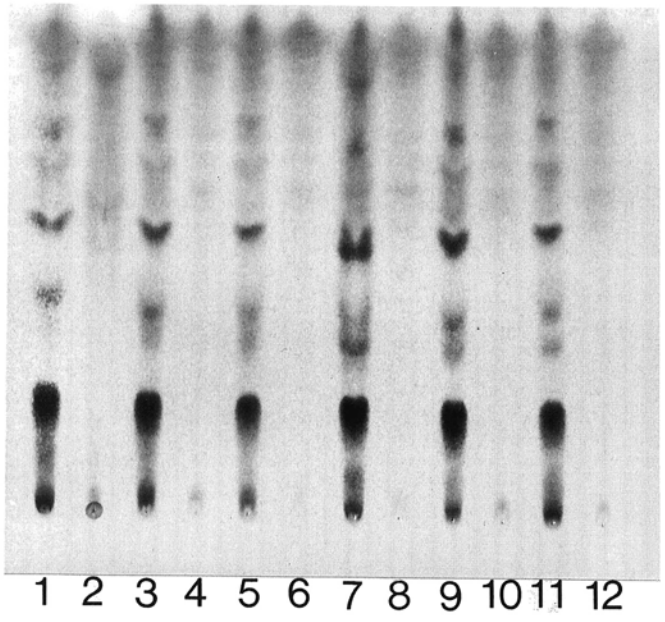

Fig. 4. Thin-layer chromatogram of bound glycolipid from prime starch from six soft (lanes 1, 3, 5, 7, 9, and 11) and six hard (lanes 2, 4, 6, 8, 10, and 12) wheat. Extracts from 480 mg of starch were applied per lane and chromatographed.

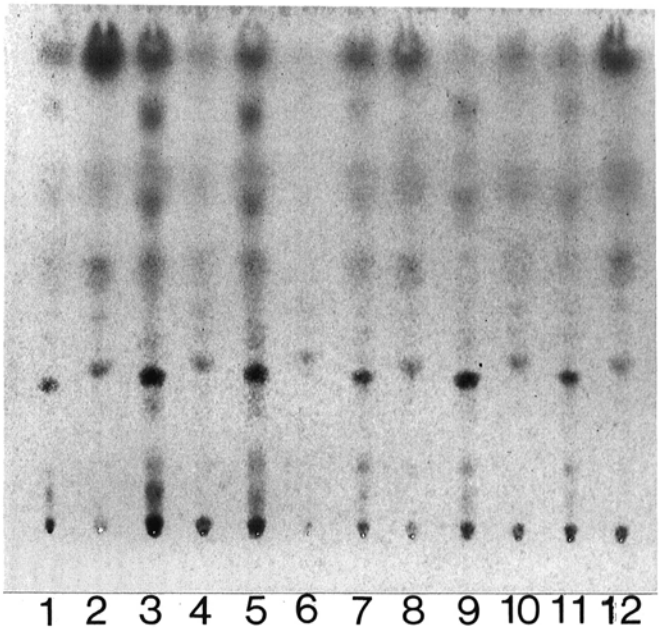

Fig. 5. Thin-layer chromatogram of bound phospholipid from prime starch from six soft (lanes 1, 3, 5, 7, 9, and 11) and six hard (lanes 2, 4, 6, 8, 10, and 12) wheat. The extracts from 480 mg of starch were applied per lane and chromatographed.

not shown). Neutral lipids showed the same 10-fold reduction in the amount of lipids bound to starch relative to the amount bound in the endosperm as was seen for polar lipids.

DISCUSSION

Circumstantial evidence indicates that friabilin is involved or related in some way to endosperm texture in wheat. The unbroken correlation of friabilin occurrence on water-washed starch with endosperm softness indicates that either friabilin plays a direct causative role or friabilin occurrence is a pleitropic effect of the *Ha* locus. Regardless, the occurrence of friabilin has: 1) provided a biochemical marker with which to characterize the genetic predisposition of individual wheat kernels for endosperm texture (Bettge et al 1992); and 2) provided a promising approach to elucidating, at the molecular level, the biochemical-genetic regulation of endosperm texture. To date, a direct causative role of friabilin has not been established. Rather, the occurrence of friabilin on water-washed soft wheat starch appears to be a partitioning phenomenon that occurs during dough development and subsequent starch isolation and recovery (Malouf et al 1992, Jolly et al 1993). Although Jolly et al (1993) found a significant correlation between the level of friabilin occurrence in whole-grain meal and grain hardness (particle size index of flours), no such relationship was found in the limited number of genotypes used here (Fig. 1).

The association of friabilin with soft wheat starch appears to involve bound polar lipids. Solvents capable of disrupting hydrophobic interactions, in addition to ionic bonds (e.g., SDS and propanol-salt) are required to quantitatively extract friabilin from water-washed starch. Apparently, this solvent requirement is not due to direct hydrophobic interactions of friabilin with the granule per se, because pre-extracting starch with propanol-water renders most of the friabilin (or components) extractable with Tris-salt. Some friabilin components, however, appear to be extractable with Tris-salt alone (no lipid removal pretreatment), while others appear to have an absolute requirement for solvents that can disrupt both hydrophobic and ionic interactions in concert. As friabilin is a family of highly related proteins (Jolly et al 1993, Morris et al 1994), these results may suggest variations in the way some friabilin components interact with starch.

The possibility that bound polar lipids are not involved in friabilin-starch interactions should be considered. In this case, the hydrophobic interactions would occur directly between friabilin and the starch granule. As such, treatment with propanol-water would disrupt interactions which would subsequently not be energetically favorable to re-form; Tris-salt would then disrupt the remaining, ionic interactions, effecting friabilin removal. With this model, lipids would merely occur coincidentally with friabilin on soft wheat starch.

Two classes of polar lipids, bound glycolipids and bound phospholipids, are associated with water-washed soft, but not hard wheat starch. These lipids are found at much higher levels (more than 10-fold) in flour compared to water-washed starch, and also in approximately equal amounts in hard and soft wheat (determined by visual evaluation of chromatograms and gravimetric measurements, data not shown). In this way, the occurrence of these lipids parallels the occurrence and approximate stoichiometry of friabilin. The relationship between bound lipids reported here and the free lipids reported by Morrison et al (1989) and assigned to 5DS is at present unknown. Although the interaction of friabilin with neutral bound lipids cannot be ruled out, the occurrence of similar levels of neutral lipids between soft and hard wheat starches suggest a less likely involvement compared to the bound polar groups.

In parallel to friabilin occurrence, the various constituent glyco- and phospho-lipids appear to be highly conserved in soft wheat. As such, these lipids, like friabilin, could serve as biochemical markers for endosperm texture (Bettge et al 1992).

At present, it is premature to advance a new hypothesis from these data regarding the physical-chemical mechanism or genetic control of endosperm texture in wheat. These data do, however, provide a better understanding of how friabilins interact with starch, and a previously unknown aspect of wheat grain hardness.

LITERATURE CITED

BETTGE, A. D., MALKAWI, H. I., GREENBLATT, G. A., and MORRIS, C. F. 1992. Single kernel analysis of wheat hardness using a biochemical marker, friabilin. Cereal Foods World 37:570.

GREENWELL, P., and SCHOFIELD, J. D. 1986. A starch granule protein associated with endosperm softness in wheat. Cereal Chem. 63:379-380.

JOLLY, C. J., RAHMAN, S., KORTT, A. A., and HIGGINS, T. J. V. 1993. Characterisation of the wheat M_r 15,000 "grain-softness protein" and analysis of the relationship between its accumulation in the whole seed and grain softness. Theor. Appl. Genet. 86:589-597.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

LAW, C. N., YOUNG, C. F., BROWN, J. W. S., SNAPE, J. W., and WORLAND, J. W. 1978. The study of grain protein control in wheat using whole chromosome substitution lines. Pages 483-502 in: Seed Protein Improvement by Nuclear Techniques. International Atomic Energy Agency: Vienna, Austria.

MALOUF, R. B., LIN, W. D. A., and HOSENEY, R. C. 1992. Wheat hardness. II. Effect of starch granule protein on endosperm tensile strength. Cereal Chem. 69:169-173.

MATTERN, P. J., MORRIS, R., SCHMIDT, J. W., and JOHNSON, V. A. 1973. Locations of genes for kernel properties in the wheat variety 'Cheyenne' using chromosome substitution lines. Pages 703-707. 4th Int. Wheat Genetics Symp.: Columbia, MO.

MORRIS, C. F., GREENBLATT, G. A., BETTGE, A. D., and MALKAWI, H. I. 1994. Isolation and characterization of multiple forms of friabilin. J. Cereal Sci. 21:167-174.

MORRISON, W. R., TAN, S. L., and HARGIN, K. D. 1980. Methods for the quantitative analysis of lipids in cereal grains and similar tissues. J. Sci. Food Agric. 31:329-340.

MORRISON, W. R., LAW, C. N., WYLIE, L. J., COVENTRY, A. M., and SEEKINGS, J. 1989. The effect of group 5 chromosomes on the free polar lipids and breadmaking quality of wheat. J. Cereal Sci. 9:41-51.

POMERANZ, Y., and WILLIAMS, P. C. 1990. Wheat hardness: Its genetic, structural, and biochemical background, measurement, and significance. Pages 471-548 in: Advances in Cereal Science and Technology. Vol. 10. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.

PRIETO, J. A., EBRI, A., and COLLAR, C. 1992. Optimized separation of nonpolar and polar lipid classes from wheat flour by solid-phase extraction. J. Am. Oil Chem. Soc. 69:387-391.

SCHOFIELD, J. D., and GREENWELL, P. 1987. Wheat starch granule proteins and their technological significance. Pages 407-420 in: Cereals in a European Context. I. D. Morton, ed. Ellis Horwood: Chichester, U.K.

WOLF, M. J. 1964. Wheat starch. Pages 6-9 in: Methods in Carbohydrate Chemistry, Vol. 4. R. L. Whistler, ed. Academic Press: New York.

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