The Biochemical Basis of Grain Hardness in Wheat

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ABSTRACT

The possibility was investigated that grain hardness in wheat is associated with differences in the composition of the protein matrix which holds the starch granules together. Such an explanation seems unlikely since hard and soft near-isogenic lines were found to have similar protein composition by protein mapping of gliadin proteins and by gel filtration and dodecyl sulfate electrophoresis of 4M urea extracts of gluten proteins. The degree of adhesion between starch and protein appears to be a more likely explanation of hardness. The amount of soluble material extractable from starch granules prepared from a range of wheats is shown to be a function of hardness. This material contains about 30% protein, and possesses protease, α-, and β-amylase activities. The nonprotein portion is mainly carbohydrate which, on hydrolysis, gives glucose with traces of xylose, arabinose, and mannose.

The importance of grain hardness as a quality parameter lies in its effects on the milling properties of wheat and other cereals, and in particular on the amount of damaged starch produced during this operation. For this reason various practical tests have been developed for the assessment of hardness (1–4), although the biochemical factors responsible for this character are poorly understood.

Several explanations of the biochemical basis of hardness have been proposed. First, Symes (5) has suggested, on the basis of an article by Greer et al. (6), that "the effect of changing this gene [for hardness] is to change the type of protein that is laid down within the grain." Thus, grain hardness may relate to the strength with which the protein matrix entraps the starch granules, and also possibly to the structure of the granules themselves. On the other hand, the tightness of adhesion between protein matrix and starch granules has been implicated in determining grain hardness (7).

This article describes an examination of this first suggestion by comparing protein composition for near-isogenic lines of wheat of different grain hardness (8). The second possibility is also studied, by examining the amount and composition of the material at the starch-protein interface. The results of these studies reinforce conclusions reported earlier (7,9) that grain hardness is related to the adhesion between starch and protein in the endosperm.

MATERIALS AND METHODS

Australian wheats of the varieties Timgalen, Falcon, Heron, and Olympic were obtained from variety trials conducted by the Agricultural Research Institute, Wagga Wagga, N.S. W., and the North West Wheat Research Institute, Narrabri, N.S.W. Hard and soft lines of the varieties Heron and Falcon (5,8) were also provided by the Agricultural Research Institute, Wagga. All wheats were milled to approximately 70% extraction on a Buhler experimental mill.

Preparation of Starch and Storage Protein

Starch and storage protein were separated from pin-milled flour by solvent flotation (10,11). The pin-milling step, performed on an Alpine Kolloplex Mill (160Z), was repeated if necessary to ensure that all flour aggregates were disrupted.

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This was checked microscopically and by ensuring that no more than 5% of particles by weight had a diameter of greater than 40 μ (as determined with the Shimadzu Sedimentation Balance).

For solvent separation, 300 g. flour was suspended in 800 ml. chloroform-benzene (density 1.45). The protein-rich material that rose to the surface after standing for 2 days was further purified by resuspension and flotation from chloroform-benzene mixtures of density 1.34 and finally 1.32. The yield of purified storage protein (90 to 95% protein) was generally about one-third of the protein in the flour taken. The starch-rich sediment from the original solvent suspension was purified by successive settling from chloroform-benzene mixtures with densities of 1.47 and 1.49. Finally, aleurone cell material was removed by two successive suspensions in chloroform-tetrachloroethylene (density 1.52). The yield of starch, which floated in this solvent, was generally about 30% of the original flour weight.

More recently, higher yields (about 60%) of equally pure starch have been obtained by suspending pin-milled flour in chloroform-tetrachloroethylene (density 1.49). The starch sedimented by centrifuging for 30 min. at 4,000 r.p.m. was purified by flotation from a mixture of the same solvents with density of 1.51.

Purified starch contained 1 to 3% protein, but was essentially free from particles of storage protein on the basis of microscopic examination after staining for protein with fast green. However, storage protein was observed attached to the surface of granules prepared from hard wheat types (7).

Preparation of Extracts

Starch (10 g.) as prepared above was extracted four times with 25 ml. sodium pyrophosphate (0.01M, pH 7.0). The extracts were dialyzed against water and freeze-dried.

Protein contents were determined by the method of Lowry et al. (12) while carbohydrate contents were assayed by the phenol-sulfuric acid procedure of Dubois et al. (13).

Estimation of Grain Hardness

Hardness was determined as particle size index (PSI) using a Labconco mill. Meal was sieved for 2 min. on a No. 15 nylon screen in a Simon laboratory sifter (14).

Determination of Proteolytic Activities

Proteolytic activities of the freeze-dried extracts were determined by the method of Anson (15) as modified by Kunitz (16). The extracts were dissolved in 1 ml. of acetate buffer (0.1M, pH 4.7) to give a solution containing 2.3% protein. The results were expressed as "hemoglobin units on tyrosine basis" (HUT) where 1 HUT is the amount of enzyme that produces, in 1 min. under the described conditions, a hydrolysate whose absorbance at 275 nm. is the same as that of a solution containing 1.10 g. per ml. tyrosine in 6×10^{-3}N HCl.

Determination of Amylase Activities

α-Amylase activity was determined by the method of Perten (17) using β-limit dextrin prepared by the β-amylolysis of soluble starch as substrate. Freeze-dried pyrophosphate-soluble material was dissolved in aqueous calcium chloride (0.2%) to

give a protein concentration of 4 mg. per ml. Portions of this working solution (3 ml.) were incubated with the substrate (1 ml.) at 30°C. Aliquots of 1 ml. were removed at 5-min. intervals and added to dilute iodine solution (17). Results were calculated as SKB units per ml. of protein solution.

β-Amylase activity was determined by the method of Bernfeld (18). Freeze-dried pyrophosphate-soluble material was dissolved in phosphate buffer (0.02M, pH 6.9) to a concentration of 23 mg. protein per ml. Enzyme activity is expressed as mg. maltose liberated in 3 min. after incubation at 20°C. with 1 ml. of the protein solution.

Assay for Starch Synthesizing Activity

The method of Yin and Sun (19) was employed using 75 mg. of freeze-dried extract in 1 ml. of acetate buffer (0.1M, pH 6.0). Incubation with glucose-1-phosphate (1% in the same buffer) was continued for periods of up to 16 hr.

Hydrolysis and Chromatography of Extracts

Freeze-dried extract (50 mg.) was hydrolyzed for 8 hr. in 0.5N HNO_3 at 105°C. The hydrolysate was neutralized by passage through Dowex 50W and evaporated to dryness. Unhydrolyzed and hydrolyzed extracts were chromatographed using ethyl acetate:pyridine:water (2.4:1.0:0.8) as solvent. Reducing sugars were revealed with the ammoniacal-silver nitrate spray (20).

Gel Electrophoresis

Following disc electrophoresis at a running pH of 9.5 (21), gels were stained as recommended by Silano and Pocchiari (22). Electrophoresis in sodium dodecyl sulfate was performed according to Weber and Osborn (23), with subsequent staining as described by Fairbanks et al. (24).

RESULTS AND DISCUSSION

Protein Compositions of Hard and Soft Isogenic Lines

To examine the possibility that grain hardness might be related to the composition of the protein binding the starch granules together, hard and soft isogenic lines were compared for gluten and gliadin composition. The size distribution of protein, extracted with 4M urea from freeze-dried gluten, was examined by gel filtration on Sephadex G-150 as described by Simmonds and Wrigley (25). Bushuk and Wrigley (26) demonstrated by a similar technique that there were differences in protein composition between hard red spring wheat and soft white winter wheat. However, virtually identical composition was consistently observed for protein from the hard and soft isogenic lines from Heron and also from Falcon, as judged by the proportions of glutenin, gliadin, and albumin, and by the proportions of large and medium glutenin (according to the definition of Simmonds and Wrigley, 25).

Identical patterns were obtained for hard and soft isogenic lines when 4M urea extracts of gluten were compared by dodecyl sulfate electrophoresis (9,23) in 8% polyacrylamide containing 4M urea (Fig. 1). Patterns were also similar for pairs of isogenic lines if disulfide linkages were broken, before electrophoresis, by treatment with mercaptoethanol.

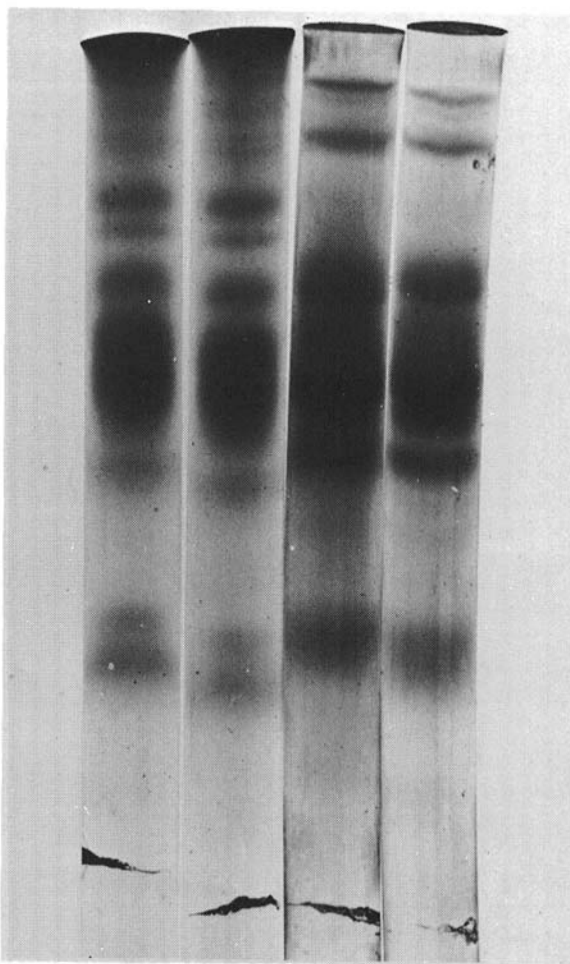

Fig. 1. Dodecyl sulfate electrophoresis of gluten proteins from soft (gels 1 and 3) and hard (gels 2 and 4) isogenic lines of cv. Heron (numbering gels from the left). For gels 3 and 4, extracts were treated with mercaptoethanol before electrophoresis (9,23). The position of the tracking dye is marked at the bottom of each gel.

Another aspect of protein composition was compared in the hard and soft isogenic lines by extracting gliadin proteins from the flour and fractionating them by combined gel electrofocusing and gel electrophoresis (27), a procedure known to show differences in protein composition between many wheat varieties, including those of differing hardness. The use of near-isogenic lines for this comparison offered the advantage that only biochemical differences directly related to hardness were likely to be detected. Figure 2 shows the gliadin patterns obtained for hard and soft lines of Heron. Gliadin composition, as revealed by this discerning method, was considered to be qualitatively identical, and no quantitative differences were

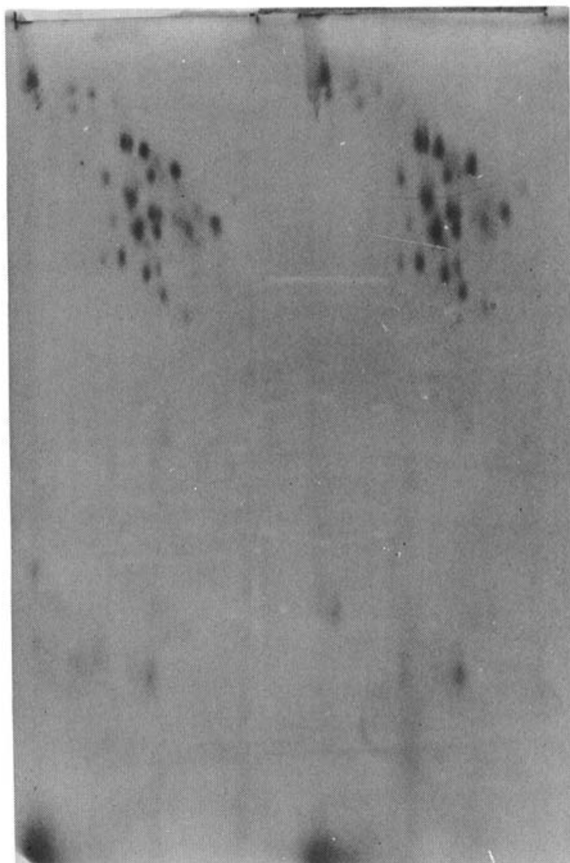

Fig. 2. Protein mapping of proteins for hard (left) and soft isogenic lines of cv. Heron. Gel electrofocusing was performed in the first dimension in the range pH 5 (left) to pH 9, followed by starch-gel electrophoresis (second dimension) in aluminum lactate buffer, pH 3.2 (27).

evident by visual inspection. Thus gliadin composition appears to be independent of grain hardness.

These observations modify the suggestion (5,6) that differences in hardness may be due to differences in the protein matrix which binds the starch granules together. At least gluten protein, as examined above, does not appear to be responsible for these differences.

Barlow et al. (7) also concluded that the properties of the protein matrix were not related to hardness as a result of micropenetrometer tests. They found that the resistance to piercing of endosperm fragments from hard and soft wheats was similar not only for storage protein, but also for starch granules. All these results thus focus attention on the possibility that grain hardness is a result of tighter adhesion between starch granules and storage protein in hard wheats than in soft. This explanation implies that specific compounds responsible for this adhesion may

be present in the zone between the surface of the starch granule and the protein matrix surrounding it (28–30). If this is true, the production of these specific cementing substances should, like grain hardness itself (5,8), be genetically controlled.

Extraction of Soluble Material from Starch Granules

Previous studies (7) stressed the importance of the material extractable with neutral pyrophosphate buffer from the region between the starch granules and the storage protein. Figure 3 shows that two to three times more of this material could be extracted from starch granules prepared by solvent flotation from hard wheats (of lower PSI) compared with soft wheats. These results suggested the possibility that one or more of the components in these extracts may be functioning as a cementing substance between the surface of the starch granule and the surrounding storage protein matrix. Thus the composition of the material was examined further.

Composition of Soluble Material from Starch Granules

The material extracted from starch granules contained carbohydrate and protein in the ratio of about 2:1. For the wheats examined (see Fig. 3) there were minor variations in this proportion, but these were not related to PSI.

The carbohydrates present in unhydrolyzed extracts of all samples consisted mainly of glucose and maltose. In addition, maltotriose was identified. Higher

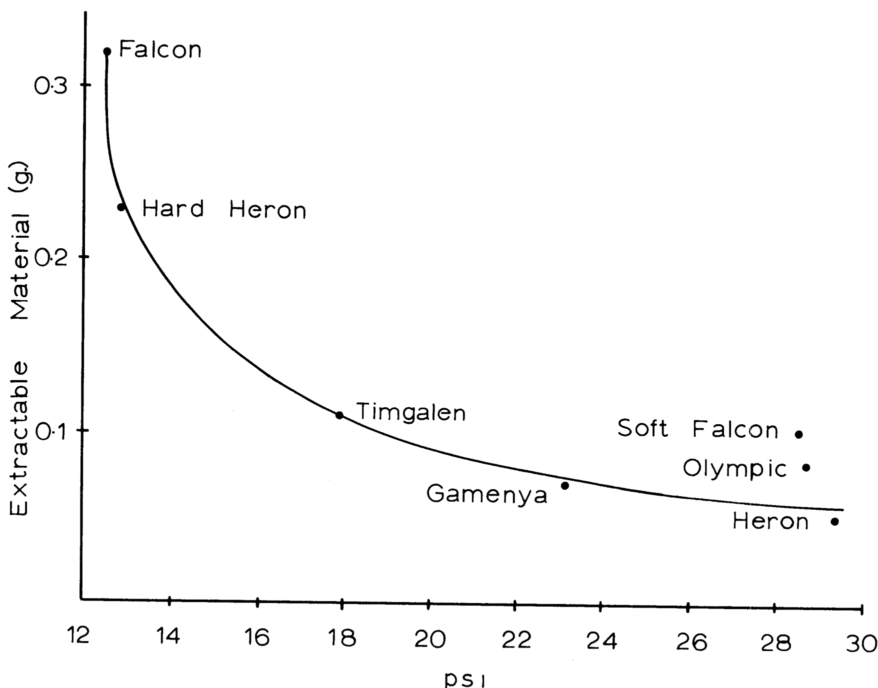

Fig. 3. Relationship between PSI and the weight of material extractable with pyrophosphate buffer from solvent-sedimented starch granules (10 g.) for a range of varieties. Each point represents a single determination. The same trend was observed in parallel experiments.

oligosaccharides were also evident, but specific components could not be definitely identified because of streaking. On hydrolysis, the major product obtained was glucose as reported previously (7), but further work has revealed the presence of trace amounts of xylose, arabinose, and mannose for all varieties. The presence of xylose and arabinose might indicate trace contamination of the starch with endosperm cell-wall material.

Protein Composition of Starch Granule Extract

Electrophoretic analyses showed that most of the proteins extractable with pyrophosphate from whole flour were also present in extracts of starch granules for the range of wheats examined, but minor differences in protein composition could be detected (Fig. 4). By using differential staining (22) to distinguish gliadin and albumin proteins, it was evident that some gliadin protein was dissolved by the fourfold extraction with pyrophosphate. In general, there was more gliadin and less albumin protein in extracts from flour compared with extracts of the corresponding starch. However, starch extracts for hard wheat (e.g., Timgalen) contained more gliadin than starch extracts for soft wheat (e.g., Summit).

These results presumably reflect the different mode of release of starch from hard and soft endosperm during milling, as suggested already by morphological studies (7). Starch is released from a soft wheat reasonably free of adhering storage protein; thus, the extracted protein is largely albumin. On the other hand, a significant proportion of storage protein (indicated by the presence of gliadin) adheres to starch granules from hard wheats so that extracts contain rather more gliadin protein, though not as much as extracts of whole flour.

Enzymatic Activities of Starch Granule Extracts

Preliminary tests on the pyrophosphate extracts of starch granules showed the presence of protease and amylase activities. There were no significant differences in activity between several varieties examined. A set of typical results is shown in Table I. Although lower by a factor of 10^4 than the figures given by Tipples and Tkachuk (31), nearly all of the β-amylase activity present in the endosperm of hard wheats could be accounted for in the starch granule fraction. A lower proportion of the endosperm enzyme activity was detected in association with the isolated starch granules from soft wheats, since in this case the starch fraction contained less endosperm protein. The low enzyme recoveries observed may have been caused by the solvent treatment and freeze-drying associated with the isolation process.

Demonstration of the presence of these hydrolytic enzymes in extracts of starch granules might provide an explanation for the discrepancy between our results and those of Rohrlich et al. (32), who suggested that only substances of low molecular weight are associated with the surface of the starch granule. Extensive proteolysis and amylolysis during extraction of starch and dialysis of extracts might be expected to reduce proteins and carbohydrates below the maximum molecular weight of 700 that they observed.

The results of our carbohydrate analyses of starch granule extracts suggest that some of the extracted material might be partially completed starch chains. It thus seems possible that some of the material surrounding the starch granules is a residue of the starch-synthesizing mechanism. However, it was not possible to demonstrate starch phosphorylase activity by the assay method used (19).

The presence has been demonstrated, in unripe grain, of α-amylase activity

which decreases as maturity is reached (33,34). It seems likely that some of this activity is specifically localized in the area surrounding the starch granules. Olered and Jönsson (33) suggest that this amylase activity makes only a minor contribution to the total hydrolysis of starch on germination. It may, however, be partly responsible for more subtle changes in endosperm properties during some processing procedures.

Various workers (35,36) have reported a drop in starch damage on milling and an improvement in protein shift (37) after cereal grits or endosperm fragments have been moistened with water or buffer and redried. Attack (possibly enzymatic) by

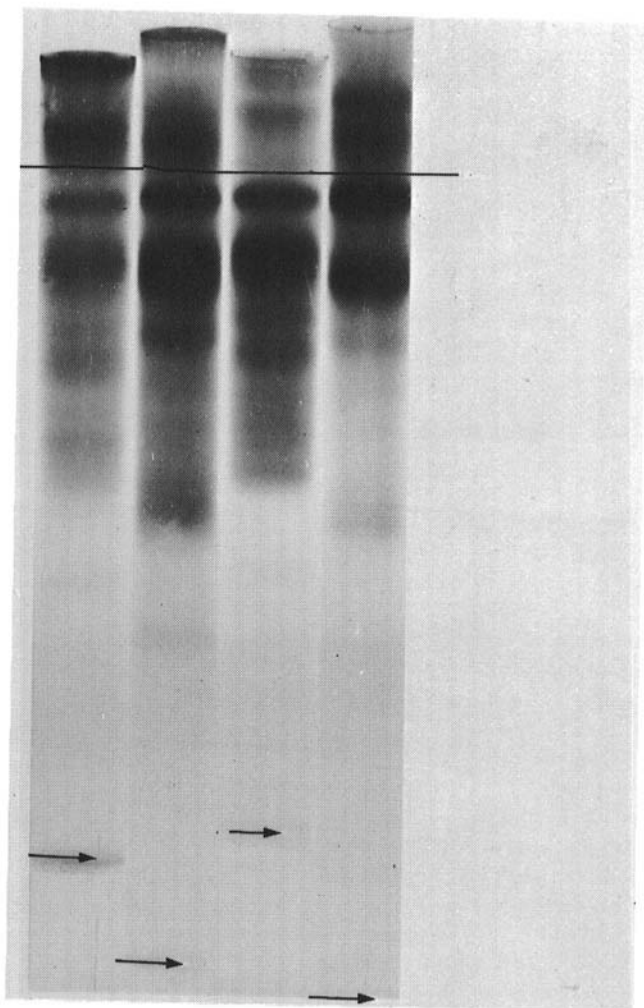

Fig. 4. Disc electrophoresis (21, running pH of 9.5) of proteins extracted with pyrophosphate buffer from Timgalen flour (gel 1, extreme left) and starch (gel 2) and from Summit flour (gel 4) and starch (gel 3). Differential staining (22) indicated that components above the horizontal line were gliadins, and below, albumins. Arrows show the position of the tracking dye.

TABLE I. ENZYMATIC ACTIVITIES OF PYROPHOSPHATE EXTRACTS OF FLOUR AND STARCH (CV. TIMGALEN)

Sample	Protease Activity HUT/mg. protein	α-Amylase Activity SKB units/ml.	β-Amylase Activity mg. maltose/ml.
Whole flour extract	0.285	0.15	0.06
Starch	0.166	0.07	0.05

aqueous solutions on a water-soluble cementing zone between starch and storage protein may provide an explanation for this phenomenon.

CONCLUSION

Evidence has been presented to suggest that adhesion between starch and storage protein is more important in determining grain hardness than is the composition of the protein matrix. Examination of the pyrophosphate-soluble material surrounding the starch granules from endosperm of a range of wheats did not implicate any specific compounds as adhesives at the starch-protein interface. However, the finding that starch granules of hard wheats have a larger amount of water-soluble material of uniform composition associated with them might in itself provide an explanation for greater adhesion in hard than soft wheats. Although it seems unlikely that any single factor will provide a complete explanation of grain hardness, adhesion between starch and protein is offered as one important aspect of this phenomenon.

Acknowledgment

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