Wheat Hardness. II. Effect of Starch Granule Protein on Endosperm Tensile Strength[1]

R. B. MALOUF,[2,3] W. D. A. LIN,[2] and R. C. HOSENEY[2]

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ABSTRACT

A hard and a soft wheat flour were fractionated into starch, gluten, and water solubles. These fractions were interchanged and reconstituted into doughs, which were dried and ground. Tablets made from these reconstituted flours had low tensile strength when the starch fraction was from the soft wheat, regardless of the source of the other two fractions. Tablets had intermediate tensile strength when made with soft wheat gluten and the hard wheat starch fraction. Proteins, including the 15-kDa sodium dodecyl sulfate-polyacrylamide gel electrophoresis band, were removed from the soft wheat starch fraction by using either pronase or sodium dodecyl sulfate. These treatments did not gelatinize the starch. The starch fraction from soft wheat with the surface protein removed gave tablets with high tensile strength. These results support the hypothesis that the 15-kDa protein associated with soft wheat starch granules has a dominant influence on wheat endosperm texture. Pronase treatment removed essentially all protein from the starch fractions, suggesting that nearly all proteins associated with starch granules are on the surface.

Endosperm texture (hardness) in common wheat (*Triticum aestivum* L.) is mainly under genetic control (Symes 1965, 1969) and is probably expressed by adhesion between starch granules and the surrounding matrix of storage protein (Barlow et al 1973). However, the actual mechanism by which endosperm texture is expressed is unknown.

Literature reviews on possible mechanisms for phenotypic expression of wheat hardness are discussed in the companion paper (Malouf and Hoseney 1992). Several factors have been shown not to influence endosperm hardness. For example, within a cultivar, total protein content usually does not correlate with hardness (Yamazaki and Donelson 1983, Miller et al 1984, Pomeranz et al 1985). Also, gliadins do not appear to influence hardness, because no qualitative differences were seen among gliadins separated by two-dimensional electrophoresis from near-isogenic hard and soft wheats (Simmonds et al 1973).

Another factor to consider is continuity of the starch-protein interface, indicated by endosperm translucence. Stenvert and Kingswood (1977) proposed that this continuity bestows endosperm hardness. However, this proposal is shown to be incorrect by the existence of opaque hard wheats and translucent soft wheats (Hoseney 1987).

Starch granules isolated from hard or soft wheats were equal in their resistance to being pierced by a micropenetrometer (Barlow et al 1973). The protein strength in endosperm fragments of hard and soft wheats was also equal when tested with this technique. The matrix protein was about 20% harder than the starch granules in both HRW and SRW. Therefore, differences in endosperm hardness are not caused by starch granules alone or by the protein matrix alone. The most logical remaining explanation is that in hard wheat the matrix protein adheres more strongly to starch granules than in soft wheat (Barlow et al 1973).

There is considerable evidence in the literature to support the above hypothesis. First, after milling, large amounts of protein remain attached to starch granules in hard wheat flour. In soft wheat flour very little protein remains attached to starch granules (Hoseney and Seib 1973, Simmonds et al 1973). Second, starch damage during milling is typically greater in hard wheat than in soft wheat (Evers and Stevens 1985). If the starch granules are of equal strength in soft and hard wheat, as indicated by Barlow et al (1973), then hard wheat starch granules and protein bind more tightly, resulting in more starch damage during milling. Another consequence of starch granules and protein binding tightly is that when kernels are fractured, some hard wheat starch granules are broken, but almost no soft wheat starch granules are broken (Hoseney and Seib 1973). Third, as hard wheat kernels are fractured, the initial breaks follow cell walls, showing that the cell wall is weaker than the starch-protein interface in the cell interior. In soft wheat, initial breaks do not follow cell walls (Hoseney 1986). If cell walls are equally strong in hard wheat and soft wheat, then the starch-protein interface is stronger in hard wheat. Fourth, in hard wheat the surrounding protein wets the starch granule surface better in than in soft wheat (Hoseney and Seib 1973). Most surfaces are microscopically rough, and a good adhesive must wet the surface to provide strong contact (Kettleborough 1990).

Barlow et al (1973) and Simmonds et al (1973) proposed that water-soluble protein acts as an adhesive between starch and protein. They found that water-soluble protein was concentrated around starch granules and suggested that hard wheat has greater amounts of water-soluble protein at this location than does soft wheat. However, no further evidence in support of this view has been published. Alternative explanations for their data include two possibilities: 1) equal amounts of water-soluble protein surrounded starch granules in the kernel, but sample preparation removed more of this protein from the soft wheat starch granules, and 2) rather than contributing to starch-protein adhesion, water-soluble protein might be bound to hard wheat starch granules by some other adhesive substance.

Starch-Granule Protein

Greenwell and Schofield (1986), Schofield (*personal communication*), and Schofield and Greenwell (1987) have consistently found a strong 15-kilodalton (kDa) electrophoretic band associated with starch granules from soft wheat. In this procedure hard wheats give a weak 15-kDa band, and durum wheats (being extremely hard) give no 15-kDa band. From this, Greenwell and Schofield suggested that a 15-kDa protein decreases adhesion at the starch-protein interface. Starch granule proteins (SGPs) from several hundred wheats from around the world follow this electrophoretic pattern, and essentially no exceptions have been found. To obtain these bands, flour-water doughs were formed, starch granules were washed out, and SGPs were extracted with 1% (w/v) sodium dodecyl sulfate (SDS), typically at 50°C, and precipitated with acetone. Proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a linear gradient of 7.5–25% acrylamide. This procedure consistently yielded 10 SDS-PAGE bands (5, 8, 15, 19, 30, 59, 77, 86, 96, and 149 kDa) that differed from gluten proteins when compared using two-dimensional electrophoresis (isoelectric focusing in the first dimension and SDS-PAGE in the second).

From these 10 consistent electrophoretic bands isolated from starch, Schofield and Greenwell (1987) have designated five bands (59, 77, 86, 96, and 149 kDa) as coming from the interior of the starch granule. Extraction at 20°C with 1% SDS did not remove the interior proteins, whereas extraction and gelatinization at 50°C with 1% SDS removed four of the five interior bands.

[1]Contribution 91-271-J of the Kansas Agricultural Experiment Station.

[2]Graduate research assistant, graduate research assistant, and professor, respectively, Department of Grain Science and Industry, Kansas State University, Manhattan 66506-2201.

[3]Present address: American Institute of Baking, 1213 Bakers Way, Manhattan, KS 66502-4576.

Extraction of the 149-kDa band with 1% SDS required a temperature of at least 90°C (Gough et al 1985, Schofield and Greenwell 1987).

SGPs of 30 kDa and 59 kDa have been purified. A monoclonal antibody developed against the 59-kDa band apparently bound to the interior of immature starch granules in a pattern similar to the concentric rings of starch. The antibody also bound to the surface of starch granules (Greenwell and Schofield 1987, Schofield and Greenwell 1987). The 59-kDa band was found in SGP from normal triticale, rye, barley, oats, maize, rice, pea, potato, and tapioca but not in SGPs from the waxy cultivars of barley, maize, and rice, which were essentially without amylose (Schofield and Greenwell 1987). This suggests that the 59-kDa band from maize SGPs is the enzyme that synthesizes amylose (uridinediphosphoglucose starch transferase) (Shure et al 1983).

Schofield and Greenwell (1987) also reported a genetic study in which genes bestowing endosperm softness and the strong 15-kDa band segregate together in 53 out of 56 wheat lines. These lines were from a cross between the soft wheat Chinese Spring and a line of Chinese Spring in which the 5D chromosome had been replaced by the 5D chromosome of the hard wheat Hope. Because of uncertainty about these data, Schofield and Greenwell concluded that genetic control of endosperm softness and the strong 15-kDa band is either by one gene or by two genes that are physically very close on the chromosome. Study of whole-chromosome-substitution lines confirmed that the gene controlling the strong 15-kDa band is on the 5D chromosome (Schofield and Greenwell 1987). Study of near-isogenic lines of hard and soft wheats confirmed that the gene controlling the strong 15-kDa band segregated with the gene controlling endosperm hardness (Greenwell and Schofield 1987, Schofield *personal communication*).

Greenwell and Schofield (1986, 1987) have attempted to produce an antibody to a protein in the 15-kDa band but have had only limited success. Skerritt et al (1988) have also attempted to produce antibodies to the 15-kDa band, which was referred to as "a group of proteins of molecular weight 15,000" that "seem to be immunologically distinct" because three antibodies with very broad gluten specificity bound only weakly to it. Despite these difficulties, Greenwell and Schofield (1987), Schofield (*personal communication*), and Spencer (1988) did report developing a fluorescent-labeled monoclonal antibody to 15-kDa protein. Immunofluorescence was seen only on the surface of immature starch granules and not in the granule interior. The specific protein that hypothetically causes endosperm softness has been given the name *friabilin* (Spencer 1988).

Amounts of free polar lipids in wheats are influenced by at least two genes (Morrison et al 1989). One of these genes is very close on the chromosome to the softness gene, perhaps being allelic with it. Increased levels of free polar lipids are associated with increased endosperm softness (Morrison et al 1989).

Tensile Strength

Procedures dealing with the use of tablets to measure tensile strength are well established. Measurement of tablet strength is standardized in pharmaceutical research (Rudnick et al 1963, Shotton et al 1976, Ejiofor et al 1986). A round tablet is placed on its edge, then compressed between parallel, flat surfaces until fracture occurs. The maximum load sustained is a measure of tensile strength. Also, concrete or rock cylinders are similarly fractured to measure their strength properties (ASTM 1984a,b).

Tablets were prepared for this study as artificial wheat endosperm with a constant shape, size, and controlled composition, using the procedures of Malouf and Hoseney (1992). These tablets were fractured to show tensile strength, indicating the adhesion between components.

The objective of this study was to identify the flour fraction from *Triticum aestivum* L. that has a dominant influence on the tensile strength of tablets made from flour. A second objective was to determine whether the SGPs were on the surface or within the starch granules.

TABLE I
Flour Fractions Used to Make Tablets[a]

Fractions	Logan SRW[b]	Composite HRW[c]	Newton HRW[c]
Starch	88.9	85.7	87.9
Gluten	7.0	10.0	7.4
Water-solubles	4.1	4.3	4.7
Totals	100.0	100.0	100.0

[a] Analytical values are reported as percent dry basis.
[b] Soft red wheat.
[c] Hard red wheat.

MATERIALS AND METHODS

Flour

Three flours were used in this study (Malouf and Hoseney 1992). Logan soft red wheat (SRW) was milled in the Kansas State University (KSU) pilot mill to straight-grade flour. A composite of hard red wheat (HRW) was milled to straight-grade flour on Ross experimental mills. Newton HRW was milled to a patent flour on Ross experimental mills.

The Logan SRW and composite HRW flours were fractionated into gluten, starch, and water solubles as described by Malouf and Hoseney (1992). The proportions obtained for all fractions and flours are shown in Table I. A second quantity of Logan SRW flour was fractionated when the Newton HRW flour was fractionated. Resulting proportions from the second fractionation of Logan flour on a dry basis were 88.4% starch fraction, 7.1% gluten, and 4.6% water solubles. These proportions were similar to those of the first fractionation. This second set of Logan fractions was used in the same experiments in which the Newton fractions were used.

Extraction of Starch Fractions with NaCl

At 7°C, the Logan SRW starch fraction and the Newton HRW starch fraction were extracted twice with 0.1*M* NaCl, using a procedure similar to that of Lowy et al (1981). The first extraction was for 2 hr, the second for 1 hr. After each extraction, the starch was centrifuged at 2,000 × *g* for 15 min. The water control was treated the same way except that water was used instead of 0.1*M* NaCl. The other controls were not extracted (Fig. 1).

At 27°C the soft wheat starch fraction was extracted twice for 10 min with 0.1*M* NaCl. After each extraction the starch was centrifuged at 2,000 × *g* for 15 min.

Extracted starch fractions were washed four times by being slurried with 13 volumes of water for 5 min and centrifuged at 2,000 × *g* for 20 min. Washed starch fractions were slurried with a minimum of water, shell-frozen, and lyophilized at ≈60 mtorr.

Removal of Protein from Starch Fractions

Subsamples of Logan SRW and Newton HRW starch fractions were extracted with 1% SDS (w/v) at 25°C for 40 min (Gough et al 1985) or subjected to pronase activity at 37°C and pH ≈8.3 for 4 hr (Russell et al 1987). Lyophilized pronase (0.2800 g) was added to 200 ml of 0.01*M* $NaHCO_3$. Pronase, nonspecific proteases from *Streptomyces griseus,* was obtained from

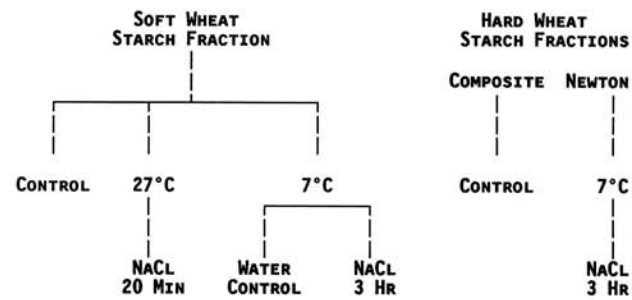

Fig. 1. Extraction of starch fractions with 0.1*M* NaCl.

Boehringer Mannheim, West Germany, specified by the supplier to have an activity of ≈2.5 × 10^4 PUK per grams of lyophilized pronase (1987/88 catalog 165 921, lot 11088320-30, Feb. 89).

After treatment with SDS or pronase, the starch was centrifuged at 1,200 × *g* for 10 min, then washed 10 times by being slurried with 16 volumes of water for 5 min and centrifuged at 1,200 × *g* for 10 min. Washed starch fractions were slurried with a minimum of water, shell-frozen, and lyophilized at ≈70 mtorr (Fig. 2).

Making Tablets

In three experiments flour fractions were combined and made into tablets, using methods of Malouf and Hoseney (1992). The fractions from the composite HRW flour and the Logan SRW flour were interchanged in all possible combinations. The starch fractions depicted in Fig. 1 were combined with water solubles and gluten from hard wheat. The starch fractions depicted in Fig. 2 were also combined with water solubles and gluten from hard wheat.

Electrophoresis

Samples were prepared for SDS-PAGE by incubating 0.250 g of the starch fraction (≈10.8% moisture) with 2 ml of 1% (w/v) SDS at 50°C for 20 min, centrifuging the mixture at 2,000 × *g* for 20 min, and discarding the centrifugate. The supernatant was added to 6 ml of acetone, which precipitated the protein; the mixture was centrifuged at 2,000 × *g* for 20 min; and the supernatant was discarded. The centrifugate was added to 1 ml of deionized water, lyophilized, and added to 0.250 ml of the treatment buffer. The treatment buffer, preparation, and gel use were performed according to the description in HSI (1983), except that the separating gel had a linear gradient of acrylamide from 7.5 to 25% (w/v).

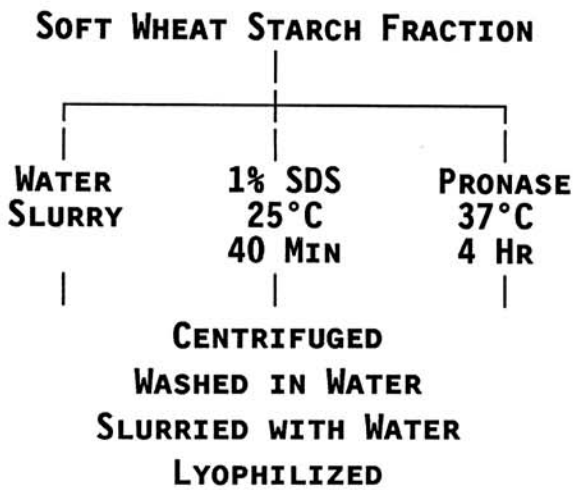

Fig. 2. Removal of protein from a starch fraction. SDS = sodium dodecyl sulfate.

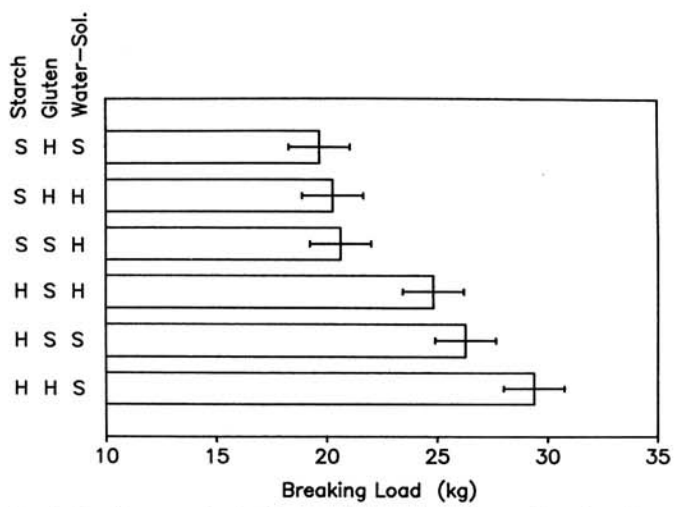

Fig. 3. Tensile strength of tablets made from interchanged fractions from hard wheat flour (H) and soft wheat flour (S). Each horizontal bar shows the mean from four tablets in one experimental unit. Error bars show the 95% confidence interval for replicate experimental units.

RESULTS AND DISCUSSION

Interchanged Fractions

Tablets made with interchanged flour fractions had low tensile strength when the starch fraction was from the soft wheat, even when the gluten, water solubles, or both were from hard wheat (Fig. 3). The starch fraction from the hard wheat together with gluten from the soft wheat produced tablets with intermediate tensile strength, showing that gluten has a minor effect on tablet tensile strength. The source of water solubles did not influence tablet tensile strength. When both the gluten and the starch fractions were from the hard wheat, tablets had high tensile strength. These results showed that the starch fraction had the dominant influence on tablet tensile strength.

NaCl-Extracted Wheat Starch Fractions

Further investigation of the soft wheat starch fraction was also suggested because Greenwell and Schofield (1986) had reported a correlation between soft wheat and a 15-kDa protein extracted from starch granules. They proposed that the 15-kDa protein decreases adhesion between starch granules and the surrounding matrix of storage protein. Extraction with NaCl removed much of the 30-kDa protein, but the 15-kDa protein remained with the starch fractions (Fig. 4).

In this study, a strong 15-kDa band was obtained from both hard and soft wheat starch. The apparent cause for this was the different procedure used for isolating starches. Greenwell and Schofield initially formed a dough, but in this study, a slurry was initially formed. When Greenwell and Schofield's procedure was followed, the 15-kDa band was strong from soft wheats and

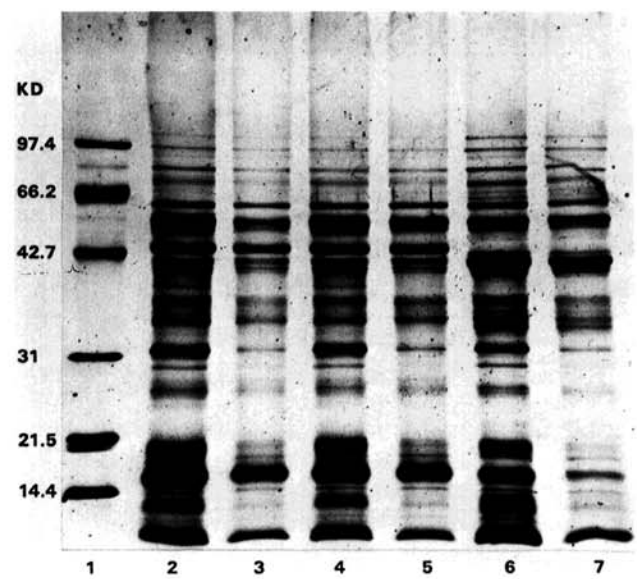

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of starch fractions extracted with 0.1*M* NaCl. Lane 1: M_r standards. Lane 2: soft endosperm, no extraction. Lane 3: soft endosperm, extraction with NaCl at 27°C. Lane 4: soft endosperm, extraction with water at 7°C. Lane 5: soft endosperm, extracted with NaCl at 7°C. Lane 6: hard endosperm, no extraction. Lane 7: hard endosperm, extraction with NaCl at 7°C. Protein applied to the gel was extracted with 1% sodium dodecyl sulfate at 50°C for 20 min.

weak from hard wheats (Fig. 5). However, Glenn and Saunders (1990) reported that "the intensity of the 15-kDa polypeptide did not reflect the textural hardness of wheat endosperm" from hard and soft wheat varieties.

Another difference is that Greenwell and Schofield's procedures dealt only with prime starch. In this study the complete starch fraction, which included the tailings starch, was always used.

Tablets made from NaCl-extracted starch fractions showed that the extraction did not change tablet tensile strength (Fig. 6). This experiment did not show whether the 15-kDa protein is directly involved in tablet tensile strength. Therefore, other means were sought to remove the 15-kDa protein.

Protein-Depleted Soft Wheat Starch Fractions

To remove the 15-kDa protein, the soft wheat starch fraction was treated with SDS or with pronase. SDS removed the smaller proteins but left proteins of ≈59 kDa and larger (Fig. 7). Pronase removed all of the protein to the extent that no electrophoretic bands stained with Coomassie blue R-250 (Fig. 7).

This pronase treatment was replicated, using another subsample from the same soft wheat starch fraction and a subsample from the Newton hard wheat starch fraction. Results were nearly the same. Pronase removed all SGP, except a very faint band of ≈64 kDa.

Neither of these treatments destroyed birefringence of the starch granules, indicating that gelatinization had not occurred. Enzymes are too large to enter intact, ungelatinized, starch granules (Brown and French 1977); therefore the results of the test show that nearly all proteins associated with wheat starch granules are on the surface. This conclusion is in conflict with Schofield and Greenwell's conclusion (1987) that the five electrophoretic bands of the larger proteins came from the interior of the granule.

Additional evidence that SGPs are located only on the exterior of wheat starch granules comes from experiments in which dyes were used to stain SGPs (Seguchi and Yamada 1989). Amido black 10B (naphthol blue black, $M_r = 616$) (Venkataraman 1952) is assumed to penetrate to the interior of starch granules (Brown and French 1977), but this dye did not stain apparently ungelatinized starch granules that had been extracted previously with 1% SDS containing 1% 2-mercaptoethanol. Furthermore, the extracted material contained protein larger than 50 kDa, contrary to what would be expected from the work of Schofield and Greenwell (1987).

Tablets made with the pronase-treated soft wheat starch fraction had essentially the same tensile strength as tablets with a hard wheat starch fraction (Fig. 8). Tablets with the SDS-treated soft wheat starch fraction had almost as much tensile strength as tablets with the pronase-treated soft wheat starch fraction (Fig. 8). These results suggested that removal of the smaller proteins from the soft wheat starch fraction allowed strong adhesion between starch granules and the surrounding matrix of storage protein. Increased tablet tensile strength cannot be attributed solely to removal of the 15-kDa protein, according to these results, because other proteins were also removed. However, taken together with the work of Schofield and Greenwell (1987), these results strongly suggest that the 15-kDa protein does cause endosperm softness.

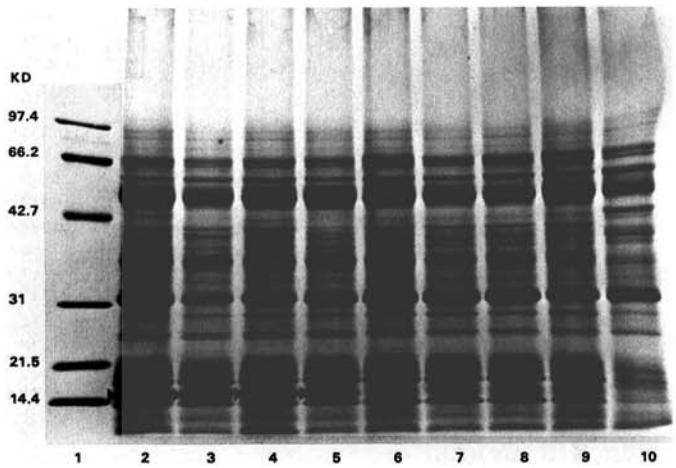

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of starches isolated according to Greenwell and Schofield's procedure. Arrows (▶) indicate the strong 15-kDa bands from the four soft wheats. Lane 1: M_r standards. Lanes 2–5: soft endosperm. Lanes 6–10: hard endosperm. Protein applied to the gel was extracted with 1% sodium dodecyl sulfate at 50°C for 20 min.

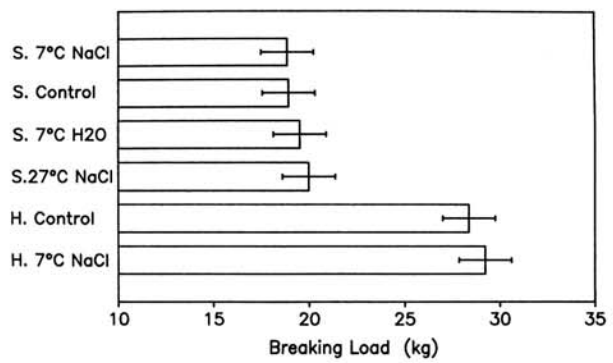

Fig. 6. Tensile strength of tablets made with starch fractions extracted with 0.1*M* NaCl. Starch fractions were from hard wheat flour (H) or soft wheat flour (S). Each horizontal bar shows the mean from four tablets in one experimental unit. Error bars show the 95% confidence interval for replicate experimental units.

CONCLUSIONS

Tablets made with interchanged fractions of starch, gluten, and water solubles had relatively low tensile strength when the starch fraction was from a soft wheat, regardless of the source of the

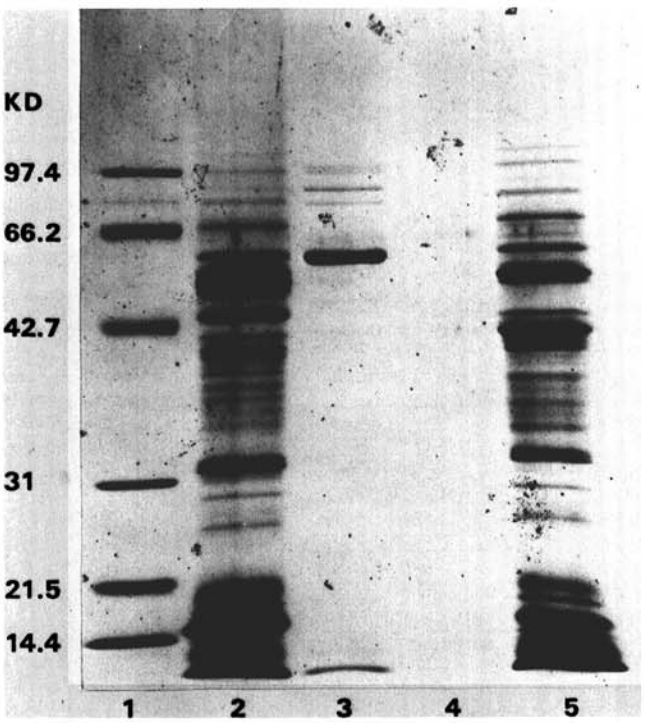

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soft wheat starch fractions treated with SDS or with pronase. Lane 1: M_r standards. Lane 2: soft endosperm, no treatment. Lane 3: soft endosperm, sodium dodecyl sulfate at 25°C. Lane 4: soft endosperm, pronase at 37°C. Lane 5: hard endosperm, no treatment. Protein applied to the gel was extracted with 1% sodium dodecly sulfate at 50°C for 20 min.

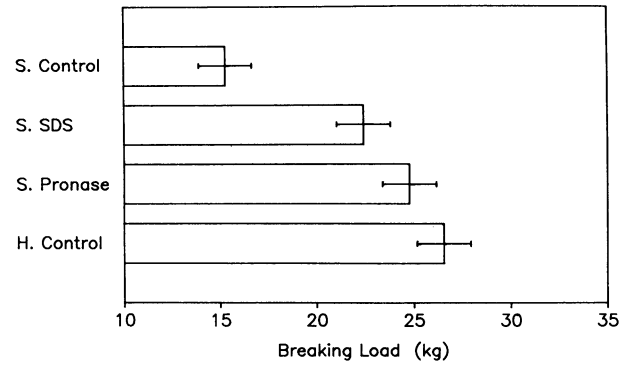

Fig. 8. Tensile strength of tablets made with soft wheat starch fractions treated with sodium dodecyl sulfate or with pronase. Starch fractions were from hard wheat flour (H) or soft wheat flour (S). Each horizontal bar shows the mean from four tablets in one experimental unit. Error bars show the 95% confidence interval for replicate experimental units.

other fractions. Soft wheat gluten together with the starch fraction from a hard wheat gave tablets with intermediate tensile strength. The source of water solubles had little or no influence on tablet tensile strength.

When protein, including the 15-kDa band, was removed from the soft wheat starch fraction by pronase or SDS treatment, resulting tablets had high tensile strength. Therefore, the major factor in wheat hardness was protein associated with the soft wheat starch fraction. Sodium chloride extraction of wheat starch did not change tablet tensile strength. The results supported Greenwell and Schofield's (1986) hypothesis that a 15-kDa protein is responsible for wheat endosperm texture.

Pronase treatment of starch from soft wheat removed essentially all starch granule protein. Because pronase enzymes are too large to enter starch granules, this evidence suggests that no protein is located in the interior of the granule. This conclusion contradicts Schofield and Greenwell's (1987) conclusions that five electrophoretic protein bands come from the interior of the starch granule.

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