Electrophoretic Study of Some High Molecular Weight Proteins of the Acetic Acid-Insoluble Residue of Wheat Flours[1]

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ABSTRACT

Acetic acid-insoluble (residue) protein fractions of flours of seven wheat varieties were studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretic patterns of unreduced SDS-buffer extracts from flour residue fractions revealed a group of relatively high molecular weight (HMW) proteins having similar electrophoretic characteristics. The HMW proteins of one variety (Neepawa) were characterized further by two-dimensional SDS-PAGE under nonreducing and reducing conditions. The two-dimensional separation showed that the HMW proteins comprised several subunits. Three of these proteins, designated B-2, B-3, and B-4, share some correspondence with the triplet band proteins and appear to be partly composed of four large subunits resolved in pairs with molecular weights of about 56 and 52 kilodaltons.

Considerable evidence exists that residue proteins that remain after extraction of wheat flour with nonreducing solvents play an important role in breadmaking properties. For example, a direct relationship has been observed between the amount of residue protein and functionality, particularly mixing strength of different genotypes (Mecham et al 1962, 1965; Tsen 1967; Orth and Bushuk 1972; Tanaka and Bushuk 1973; Orth and O'Brien 1976). The underlying cause of the observed relationship has been attributed mainly to glutenin that can be released from the residue as polypeptide "subunits" in the presence of reducing agent.

Fewer studies have focused on the composition of the insoluble residue fraction without prior reduction. Singh and Shepherd (1985, 1987), for example, identified a group of relatively high molecular weight (HMW) proteins (150–160 kilodaltons [kDa]) in the unreduced flour and residue fractions. These proteins, originally named triplet band proteins (Singh and Shepherd 1985) and recently renamed triticin (Singh et al 1988), have been impli-

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cated in breadmaking potential (Singh et al 1987).

In this study we attempted to optimize fractionation and electrophoretic conditions for characterization of some of the acetic acid-insoluble (residue) proteins of wheat flour. The paper reports on the results obtained for seven different wheat varieties.

MATERIALS AND METHODS

Wheat varieties used in this study included four hard red spring wheats (Neepawa, Katepwa, Glenlea, Pembina), two soft white spring (Kenya, Fielder), and one soft white winter (Augusta). The grain was milled into straight-grade flour on a Buhler laboratory mill. Flour protein contents (N × 5.7, 14% mb) were as follows: Neepawa, 14.9%; Katepwa, 13.7%; Pembina, 13.4%; Glenlea, 12.9%; Kenya, 12.0%; Fielder, 8.8%; and Augusta, 8.0%.

Extraction and Fractionation of Proteins

Neepawa flour (10 g, 14% mb) was extracted directly with 70% ethanol (40 ml) or with 0.05*M* acetic acid (40 ml) or sequentially with 70% ethanol and 0.05*M* acetic acid (Chen and Bushuk 1970). Additionally, protein fractions were prepared using the modified Osborne procedure of Chen and Bushuk (1970), except that all extractions were done at room temperature.

Acetic acid-insoluble protein fractions of all seven flours were prepared by extracting flour (1 g, 14% mb) with 0.05*M* acetic acid (25 ml) as described by Orth and O'Brien (1976).

Insoluble residues from the above extractions were freeze-dried, weighed, and analyzed for moisture and protein (N × 5. 7). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, flours and residues (on 6-mg protein basis) were extracted with 1 ml of 2% (w/v) SDS buffer solution as described by Ng and Bushuk (1987), except that the 2-mercaptoethanol was excluded. Samples were extracted overnight (16 hr) at room temperature in 15-ml centrifuge tubes, with occasional shaking on a vortex mixer, and subsequently were centrifuged at 20,000 × *g* and 20°C for 20 min. The supernatant was decanted into a microcentrifuge tube, boiled for 3 min, and clarified by centrifuging (10 min, 12,000 × *g*, room temperature). This second clarification step improved the resolution of subsequent electrophoretic analyses. Aliquots were analyzed by SDS-PAGE (see below). When required, mercaptoethanol (50 μl) was added to the extract in the extract in the microcentrifuge tube to obtain reduced proteins for SDS-PAGE.

SDS-PAGE

SDS-PAGE was done on an LKB 2001 unit (Pharmacia LKB) according to a modified Laemmli procedure (Ng and Bushuk 1987). The stacking and separating gel concentrations were 3.03 and 17.3%, respectively. Gel thickness was 1.5 mm. Electrophoresis was carried out for 45 min at 35 mA and for 18 hr at 15 mA ("normal" conditions). To improve resolution of low-mobility proteins in some experiments, electrophoresis was carried out for 45 min at 35 mA, 18 hr at 20 mA, and 1 hr at 30 mA ("extended" conditions).

Two-dimensional SDS-PAGE was performed as described by Singh and Shepherd (1985). For the first dimension, the unreduced protein extract of the acetic acid-insoluble Neepawa flour residue was loaded on a 1.0 mm thick gel and subjected to electrophoresis. The lane containing the resolved proteins was cut out, beginning 0.5 cm from the origin in order to exclude proteins that did not enter the gel. The gel strip was equilibrated in a solution containing 2% (v/v) mercaptoethanol for 1.5 hr at 37°C (Singh and Shepherd 1988) and subsequently was loaded onto a thicker (1.5 mm) gel for electrophoresis in the second dimension. Electrophoresis of the reduced proteins was carried out at 35 mA for 45 min and at 10 mA until the tracking dye migrated to the end of the gel.

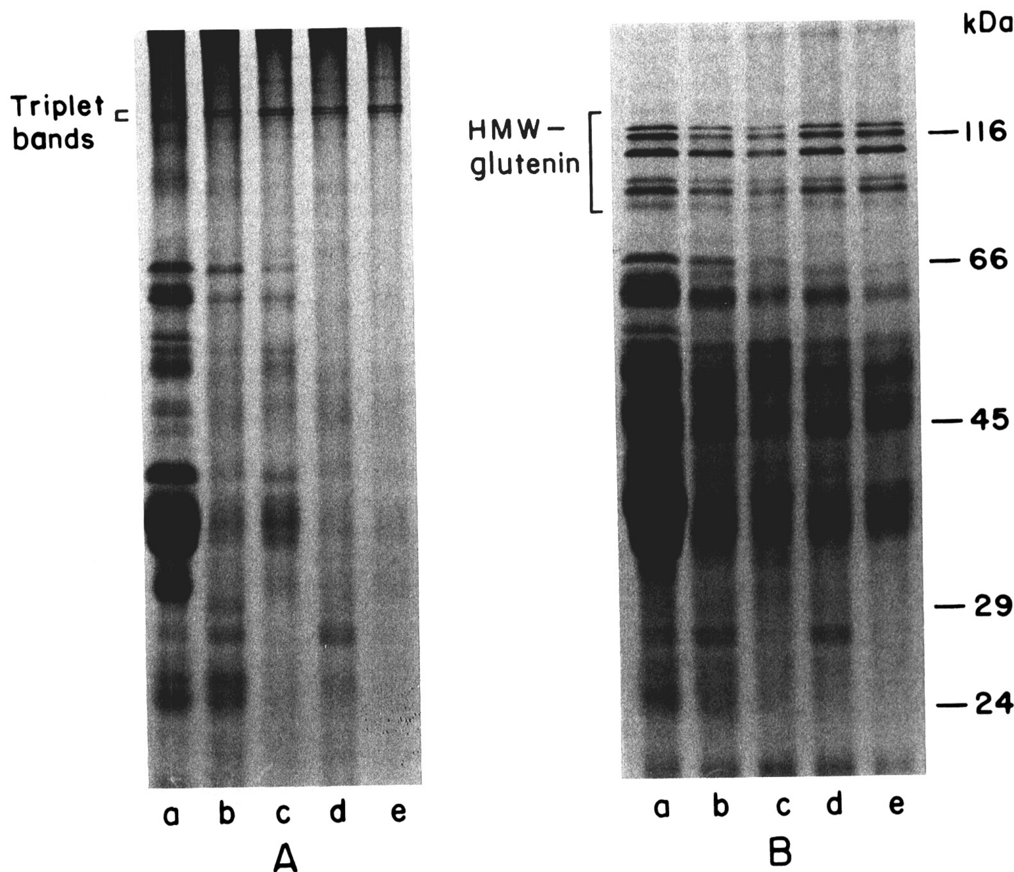

Fig. 1. Electrophoresis of unreduced (**A**) and reduced (**B**) sodium dodecyl sulfate buffer extracts from Neepawa flour (a) and residue fractions obtained after extraction with 70% ethanol (b), 0.05*M* acetic acid (c), sequential extractions with 70% ethanol and 0.05*M* acetic acid (d), and sequential extractions with 0.5*M* NaCl, 70% ethanol and 0.05*M* acetic acid (e).

Apparent molecular weights were determined from SDS-PAGE mobilities using standard reference proteins (Sigma Chemical Co., St. Louis, MO): β-galactosidase (116,000), bovine serum albumin (66,000), egg albumin (45,000), carbonic anhydrase (29,000), trypsinogen (24,000), and lysozyme (14,300). A reduced protein extract of Neepawa flour was used as a secondary molecular weight standard (Ng and Bushuk 1987, 1989).

RESULTS AND DISCUSSION

Protein Extracts of Flour and Residue Fractions

Electrophoretic patterns of SDS-buffer-soluble proteins (without reduction) from Neepawa flour and residue fractions obtained after flour extraction with relatively mild solvents (0.5*M* NaCl, 70% ethanol, and 0.05*M* acetic acid) are shown in Figure 1. As expected, the flour extract (Fig. 1A, lane a) contained a large proportion of endosperm protein resolvable by SDS-PAGE under nonreducing conditions. This group of proteins includes albumins, globulins, and gliadins. Their presence in lesser amounts in the different residue fractions, and virtual absence in the Osborne residue (Fig. 1A, lane e), indicates the degree to which these proteins are extractable by the solvents used. In contrast, the patterns of all the residue fractions clearly showed a tight cluster of bands with low mobility, i.e., relatively high molecular weight proteins. Since these proteins show the characteristic pattern of a distinct doublet preceded closely by a higher mobility band of reduced intensity, they are probably the same as the triplet band proteins described by Singh and Shepherd (1985). These workers also found that addition of mercaptoethanol to the SDS-extracts containing the disulfide-linked triplet band proteins resulted in their disappearance from the gel patterns due to their reduction to lower molecular weight components. This result was confirmed here (compare Fig. 1B and 1A).

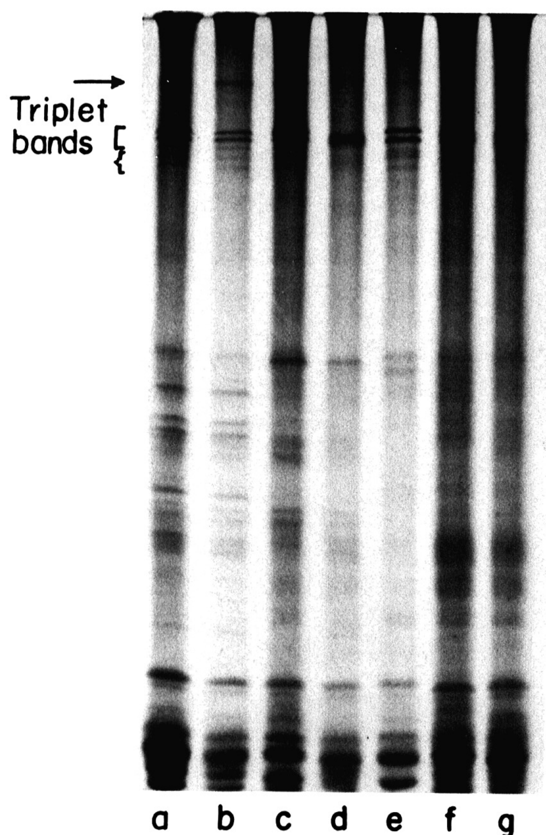

Fig. 2. Electrophoresis of unreduced sodium dodecyl sulfate buffer extracts of 0.05*M* acetic acid-insoluble residues resolved under extended electrophoretic conditions (details in the text). Patterns are for the wheat varieties Neepawa (a), Katepwa (b), Pembina (c), Glenlea (d), Kenya (e), Fielder (f), and Augusta (g).

Under normal electrophoretic conditions the component proteins differed only slightly in mobility (Fig. 1A). To improve their separation, we used extended electrophoresis conditions to examine the unreduced protein extracts of the acetic acid-insoluble residues. This procedure revealed greater heterogeneity in the group of HMW proteins (Fig. 2). In addition to the bands that could represent the triplet band proteins (Fig. 2, bracket), patterns for Neepawa, Katepwa, Pembina, and Kenya clearly showed two additional faint bands with higher mobility (Fig. 2, brace). The appearance of these two bands is likely due to the extended electrophoresis, which would tend to improve the resolution of bands with similar electrophoretic mobility. The extraction procedure might also be a factor, since it included the removal of acetic acid-soluble proteins prior to the SDS-buffer extraction. This procedure would increase the relative concentration of the HMW proteins in the SDS buffer. The pattern for Glenlea (Fig. 2, lane d), showed only a diffuse presence of the higher mobility bands. Interpretation of the patterns of the two soft wheat varieties Fielder and Augusta (Fig. 2, lane f and g) was not possible because of the intense background streaking. Since the extraction with SDS-buffer was based on equal residue protein content, the streaking might be related to the larger amount of residue used to prepare the extracts of these two low-protein wheat varieties.

In addition to the bands described above, patterns of all seven varieties investigated showed the appearance of a neighboring band of lower mobility (Fig. 2, arrow). Although this band was observed in stained gels of the varieties Fielder and Augusta, it is not visible in the photograph due to the heavy background streaking.

Two-Dimensional SDS-PAGE

Two-dimensional electrophoresis was used to separate and identify constituent components of the HMW residue proteins. The pattern of the unreduced SDS-buffer extract of the acetic acid-insoluble residue of Neepawa flour, after electrophoresis in the first dimension, showed the six consecutive bands described above (Fig. 3, lane a). We will refer to them as B-1 to B-6. They included the single band of low mobility (B-1), the three bands (B-2, B-3, B-4) resembling the triplet band proteins of Singh and Sheperd (1985), and the two faint bands with higher mobility (B-5, B-6). After electrophoresis under reducing conditions in the second dimension, these proteins were resolved into several component spots that will be referred to here as subunits (Fig. 3, open and solid single- and double-headed arrows). B-1 separated into at least two subunits (Fig. 3, the spots identified by double-headed arrows) of 56 and 47 kDa. B-2 was resolved into two subunits at 56 kDa, B-3 into four subunits at 56 and 52 kDa, and B-4 into two subunits at 52 kDa. The inset in Figure 3 shows this result more clearly. The poorly resolved spot in the low molecular weight region corresponds to 22 kDa. Because of its faint and diffuse appearance, its correspondence with a specific HMW band could not be determined.

These results share some correspondence with the 2-D electrophoretic characterization of Chinese Spring triplet band proteins by Singh and Shepherd (1985). They showed that triplet band proteins, designated Tri-1, Tri-2, and Tri-3 in order of increasing mobility, have a tetrameric structure, which upon complete reduction comprises two large (58 and 52 kDa) and two small (23 and 22 kDa) subunits. Tri-2 was reported to be a hybrid containing subunits of Tri-1 and Tri-3. In our study, B-3 appeared to be a hybrid containing subunits of B-2 and B-4. Accordingly, the B-2, B-3, and B-4 bands observed here seem to correspond to the Tri-1, Tri-2 and Tri-3 bands respectively. If true, our results suggest that the triplet band proteins of the variety Neepawa contain four large subunits resolved in two pairs with molecular weights of approximately 56 and 52 kDa, respectively. However, our observations are based on a sample of one cultivar, and more work is required to investigate the heterogeneity in subunit composition of these HMW proteins in different varieties.

Subunits from the faint pair of bands B-5 and B-6 were not observed after reduction. This might be due to their original low concentration in the unreduced gel strip (see Fig. 3, lane a) and/

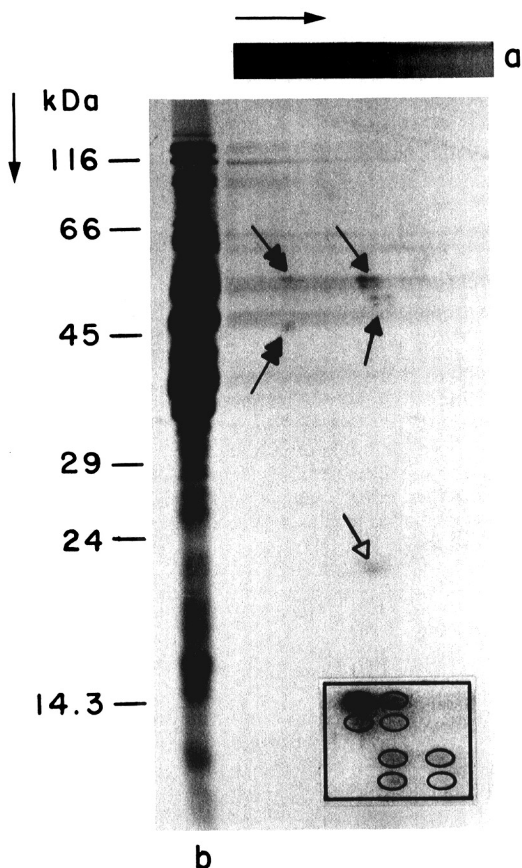

Fig. 3. Two-dimensional electrophoresis of sodium dodecyl sulfate buffer extract of acetic acid-insoluble residue from Neepawa flour: **A,** first-dimension separation of proteins resolved under nonreducing conditions, and **B,** reduced protein extract from Neepawa flour used as the secondary standard. The inset is a photographic enlargement of the protein spot cluster in the 52–56 kDa region (flanked by solid single-headed arrows).

or to the horizontal streaking that appeared under reducing conditions, which could mask the subunits of the B-5 and B-6 proteins (Fig. 3). The 2-D separation (Fig. 3) showed that the horizontal streaking is probably caused by other subunits of disulfide-linked proteins of the flour (e.g., high and low molecular weight glutenin subunits). Disulfide-linked proteins were shown to be involved in formation of the dark background streaking that appears in electrophoregrams under nonreducing conditions (Singh and Shepherd 1985, Payne et al 1986).

CONCLUSION

SDS-PAGE analysis of unreduced SDS-extracts from acetic acid-insoluble residues of seven wheat varieties revealed the presence of relatively HMW proteins. Some of these proteins appear to correspond to the triplet band proteins reported by Singh and Shepherd (1985). Further work however, is needed to establish the identity of the HMW proteins and the triplet band proteins and to investigate the subunit composition of the HMW proteins in different wheat varieties. Singh and Sheperd (1985) suggested that triplet band proteins might be involved in formation of larger protein aggregates. Hence, the appearance of more than three bands in the HMW region under nonreducing conditions might be related to the association of triplet band proteins with other proteins in the residue fraction.

ACKNOWLEDGMENTS

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