A Simple Antibody-Based Test for Dough Strength. I. Development of Method and Choice of Antibodies

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ABSTRACT

A monoclonal antibody-based enzyme-immunoassay method was developed to predict dough strength and mixing properties from the analysis of small samples of wheat flour or whole meal. The choice of antibody, grain protein extractant, and extraction technique were important. Solvents such as alkali or acid extracted. Quality-related differences in antibody binding to grain proteins extracted using sodium dodecyl sulfate (SDS) with a reducing agent (dithiothreitol, [DTT]) were noted. Proteins could be extracted by vortex mixing; ultrasonic or high-frequency homogenization, were inferior. Quality-related differences could be measured using grain extracted a single time, although the SDS-DTT extractant provided greater differentiation when a simple preextraction step was performed using SDS. The specificities of the antibody combinations were assessed using gluten protein fractions and genetic wheat stocks, including Chinese Spring aneuploids and near-isogenic wheats that lack certain high and low molecular weight glutenin subunits and gliadins. The best quality differentiation was obtained using antibody combinations that bound to chromosome 1D-encoded high molecular weight glutenin subunits. However, these antibodies bound to glutenin subunits both from varieties having allelic subunits 1Dx5 and 1Dy10 and from varieties having allelic subunits 1Dx2 and 1Dy12, although greater binding to proteins was seen from extracts of wheat flours that yielded stronger doughs.

Variation in the amount and composition of gluten storage proteins is the prime determinant of wheat flour quality (Finney 1943). Early attempts to relate end-use quality to the amounts of various gluten protein families were based on solubility fractionation (Crewe and Bailey 1927, Huebner and Wall 1976). Although initial studies showed that the gliadin-to-glutenin ratio differed little between wheats that varied in quality (Huebner 1970), other studies showed that the amount of least soluble and highest molecular weight fraction, termed "residue protein", correlated positively with dough strength (Pomeranz 1968, Orth and Bushuk 1972, Huebner and Wall 1976). Indeed, the quantity of sodium dodecyl sulfate (SDS)-insoluble protein, termed "gel protein", is measured in the medium-scale SDS sedimentation test for breadmaking quality (Moonen et al 1982). The specific role of the amount of glutenin in determining dough properties has been demonstrated in several flour-reconstitution studies. Mixograph development time (MacRitchie 1987) and dough resistance to extension (Kim et al 1988) are positively correlated with the proportion of glutenin in doughs prepared from reconstituted flours. Other workers, using size-exclusion chromatography (Huebner and Wall 1976, Bottomley et al 1982) found positive correlations between loaf volumes obtained from small sets of wheat flours and the proportion of the highest molecular weight fraction of gluten, found to be mainly high molecular weight glutenin subunits (HMW-GS).

Rapid antibody-based test methods using antibodies specific for quality-related components in flour would provide the potential for developing revolutionary techniques for small-scale testing of wheat quality. Such methods are suited to the simultaneous testing of many dozens or hundreds of small (under 100 mg) flour or whole meal samples, for example in early generation screening for quality in wheat breeding programs. With some modifications, these antibody methods would also have the potential for rapid dough quality prediction of grain when it is received at the bakery, mill, or elevator. After examining several antibody combinations and flour protein extraction techniques, an antibody-based two-site enzyme-immunoassay test was developed to predict dough strength and mixing properties. This article examines the specificity of the test for particular glutenin subunits and its performance with one set of wheats of diverse genotypes. The companion article (Skerritt 1991) discusses the use of the antibody test methods to examine sets of wheats of various genotypes grown under a wide range of environmental conditions and assesses in more detail the relationship between results obtained with the method and various aspects of wheat quality.

MATERIALS AND METHODS

Flour Samples

In initial experiments, white composite flours (65-72% extraction rate) from the wheat varieties Cook (hard, breadmaking type) and Egret (soft, cookie type) at 9 and 13% protein were bulked from individual wheat samples grown at several Australian sites. These samples were used in initial studies to avoid potential environmental biases that could result from using material from a single site only.

Relationships between antibody binding and quality parameters for selected antibody combinations and flour solvent extraction techniques were assessed using a set of flours from 15 wheat cultivars of diverse origin and quality, grown at one site under standard fertilizer conditions (50 kg N/ha) (Horsham, Victoria, Australia) and having moderate flour protein contents (mean, 9.8 ± 0.9%). The detailed quality data for this set of flours are

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published elsewhere (Singh et al 1990).

A number of wheat lines were used to assess the specificities of antibody combinations, including chromosome 1 and 6 nullisomic-tetrasomic lines derived from Chinese Spring (Sears 1954), near-isogenic lines prepared from the variety Olympic but lacking one or more HMW-GS (Lawrence et al 1987), and single and multiple wheat-rye chromosome 1 (short arm) translocation lines, Gabo and/or Chinese Spring mixed backgrounds (Gupta and Shepherd 1987).

Antibodies

Methods used for producing and screening monoclonal antibodies to gluten proteins and some of the specificity properties of individual antibodies are described elsewhere (Skerritt and Underwood 1986, Donovan et al 1989, Skerritt and Robson 1990). Briefly, antibodies 218/17 (IgG) and 237/24 (IgM) bound high and low (B-subclass) molecular weight (LMW) glutenin subunits and at high antibody concentrations, bound to γ- and ω-gliadins on immunoblots. High concentrations of the other three antibodies used (304/13, 405/7, and 412/01, all IgG) bound γ-gliadins and HMW-GS on immunoblots. Antibodies 412/01 and 405/7 also bound LMW-GS (Skerritt and Robson 1990). Using the two-site enzyme-linked immunosorbent assay (ELISA) format, these antibodies were highly glutenin-selective (Table I). For these studies, LMW-GS, HMW-GS, and gliadins were prepared from Timgalen variety flour, as described elsewhere (Skerritt and Robson 1990). The specificities of these antibody combinations (assessed using purified protein fractions) in the two-site assay format is often narrower than that noted on immunoblots after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Hill and Skerritt 1989).

TABLE I
Relative Reaction of Different Antibody Combinations with Gluten Protein Fractions[a]

Antibody Combinations	Solvent[b]			
	SDS (2.5%)-DTT (50 mM)		SDS (0.5%)	
	LMW-GS	Gliadin	LMW-GS	Gliadin
C218/17-T412/01	340	410	170	1,200
C237/24-T237/24	5	20	4	3
C243/11-T243/11	43	350	62	160
C304/13-T304/13	6	25	3	3
C405/7-T237/24	330	1,400	150	300
C412/01-T412/01	5	19	3	2

[a] Data are cross-reactions relative to high molecular weight glutenin subunits (= 100) measured using sandwich enzyme-linked immunosorbent assays with the antibody combinations shown.
[b] SDS = sodium dodecyl sulfate, DTT = dithiothreitol, LMW-GS = low molecular weight subunits.

Extraction of Gluten Fractions

In initial experiments, 500 mg of flour was dispersed in 10 ml of solvent by high-frequency homogenization using an Ultraturrax (Janke and Kunkel, Dottingen, Germany) at 15,000 rpm for 1 min and then centrifuged at 2,500 *g* for 10 min. Solvents examined included 1) acids (1 and 10 m*M* HCl), 2) alkalis (10 and 100 m*M* KOH), 3) acidified alcohol (50%, v/v, 1-propanol plus 1%, v/v, of acetic acid), 4) urea (8*M*), 5) SDS (0.5, 1.5, and 2.5%, w/v), and 6) combinations of 1) and 3) or 1) and 4). In some cases these were used in combination with the reducing agent dithiothreitol (DTT) at 10 or 50 m*M*.

In initial studies (Tables II and III) using sequential extractions with differing solutions, the first extraction was performed by vortex mixing for 1 min and then mixing by end-over-end rotation at 45 rpm for 15 min before centrifugation at 50,000 *g* for 30 min. The second extraction was performed by high-frequency homogenization, as described above. In some of these experiments (indicated), a sonifier microtip probe (Branson B-12, Danbury, CT) was used instead at 60% maximal power for 30 sec for the second extraction. Gluten proteins were routinely extracted (Tables III-V) by vortex mixing 50 mg of flour with 1 ml of solvent in a 2-ml microtube (Sarstedt, Germany, Cat. No. 72708). Although standard 1.5-ml microtubes can be used, suspension of flours was faster and more reproducible in the 2-ml tubes, which

TABLE II
Effect of Flour Extractant on Binding of Two Antibody Combinations

Extractants[a]	Antibodies			
	C304/13-T304/13		C412/01-T412/01	
	A 414[b]	Potency[c]	A 414	Potency
A				
Urea (8*M*)	0.196	1	0.164	1
HCl (10 m*M*)	0.355	1.5	0.252	2
Urea (8*M*)-HCl (10 m*M*)	0.407	2	0.300	2.5
Urea (8*M*)-HCl (10 m*M*)-DTT[c] (50 m*M*)	1.071	15	1.866	15
B				
			0.232	1
HCl (10 m*M*)	0.350	1	0.381	2
HCl (10m*M*)-DTT (10 m*M*)	0.631	3	0.485	3
HCl (10m*M*)-DTT (50 m*M*)	0.660	3		
C				
SDS (1%)	0.141	1	0.122	1
SDS (1%)-DTT (10 m*M*)	1.141	24	1.365	16
SDS (1%)-DTT (50 m*M*)	1.077	20	1.520	24
D				
1-Propanol (50%)-acetic acid (1%)	0.253	1	0.167	1
1-Propanol (50%)-acetic acid (1%)-DTT(50 m*M*)	0.672	6	0.667	6
E				
KOH (10 m*M*)	0.999	1	1.335	1
KOH (100 m*M*)	0.786	0.8	1.395	1

[a] DTT = dithiothreitol, SDS = sodium dodecyl sulfate.
[b] Absorbance obtained with a 1:25,000 dilution of flour extract. Similar trends were observed between 1:5,000 and 1:100,000 dilutions.
[c] Reciprocal of the wheat extract producing 50% maximal response in a wheat-extract dilution curve relative to that of the first extractant of each group (A--E).

TABLE III
Effect of Extraction Method on ELISA Absorbances and Discrimination Between Flour Samples[a,b]

Method		Low (9%) Protein Flour		High (13%) Protein Flour	
First Extractant	Second Extractant	Cook A 414[c]	Absorbance Ratio[d]	Cook A 414[c]	Absorbance Ratio[d]
High-frequency homogenization					
1.5% SDS + 50 m*M* DTT	None	0.63	1.24	0.77	1.21
1.5% SDS	1.5% SDS + 50 m*M* DTT	0.45	1.52	0.59	1.40
Vortex mixing					
1.5% SDS + 50 m*M* DTT	None	1.16	1.19	1.34	1.11
1.5% SDS	1.5% SDS + 50 m*M* DTT	0.98	1.23	1.17	1.40
2.5% SDS + 50 m*M* DTT	None	1.15	1.20	1.40	1.20
2.5% SDS	2.5% SDS + 50 m*M* DTT	0.70	1.16	0.95	1.18
0.5% SDS	2.5% SDS + 50 m*M* DTT	1.16	1.29	1.35	1.22

[a] Data are means of two experiments using C412/01 and T412/01; similar results were obtained with C304/13 and T304/13.
[b] ELISA = enzyme-linked immunosorbent assay, SDS = sodium dodecyl sulfate, DTT = dithiothreitol.
[c] ELISA absorbances using flour extracts diluted 1:50,000.
[d] Ratio of ELISA absorbances obtained with Cook to those obtained with Halberd, over the linear part of the concentration-response curve.

TABLE IV
Analysis of Antibody Binding to Extracts of Chinese Spring Nullisomic (n) Tetrasomic (t) Lines[a]

Line	Protein (%)	Antibody[b] C237/24 T237/24	C304/13 T304/13	C412/01 T412/01	C218/17 T412/01	C405/7 T237/24
Chinese Spring	14.4	100[a]	100	100	100	100
Chinese Spring, 2x[b]	~(28.8)	265	158	162	NT[c]	NT
n 1A− t 1B	20.9	198	140	160	140	118
n 1A− t 1D	20.8	249	157	211	140	149
n 1B− t 1A	19.2	58	63	51	60	77
n 1B− t 1D	19.1	195	142	135	107	111
n 1D− t 1A	20.5	5	16	16	26	25
n 1D− t 1B	20.9	82	89	92	102	71
n 6A− t 6B	19.8	138	113	117	99	102
n 6B− t 6D	23.8	158	134	133	118	126
n 6D− t 6A	20.9	152	123	129	133	127

[a] Data are enzyme-linked immunosorbent assay absorbances relative to those obtained with Chinese Spring sodium dodecyl sulfate extracts (second extraction) diluted 1:40,000 C and T 237/24, C and T 304/13, or 1:400 C218/17 + T412/01, C405/7 + T237/24; C = solid phase, T = labeled.
[b] Analysis of Chinese Spring extracts at twice the antigen concentration in footnote "a". Thus the apparent protein concentration of the grain would be 28.8%.
[c] Not tested.

TABLE V
Relationships (Correlation Coefficients) Between Antibody Binding and Quality Parameters in 15 Diverse Wheat Cultivars

		Quality Parameter[a]								
		Mixograph	Farinograph			Extensigraph			Loaf Volume	
Antibody[b]	Protein (%)	DT (min)	DT (min)	DB (BU)	WA %	E (cm)	R5 (BU)	Rmax (BU)	(−Bromate) (ml)	(+Bromate) (ml)
C304/13-T304/13										
1-step (SDS-DTT)	0.518*[c]	0.508*	0.542*	−0.440	0.185	0.465	0.399	0.544*	0.584*	0.014
2-step (SDS)	0.230	−0.277	0.214	−0.044	0.297	−0.015	−0.173	−0.178	−0.228	0.268
(SDS-DTT)	0.496	0.739**	0.734**	−0.857***	0.229	0.726**	0.872***	0.854***	0.662**	0.246
C412/01-T412/01										
1-step (SDS-DTT)	0.839***	0.679**	0.856***	−0.770***	0.504*	0.814***	0.605*	0.759***	0.637**	0.280
2-step (SDS)	0.261	−0.219	0.233	−0.112	0.360	−0.014	−0.127	−0.145	−0.189	0.308
(SDS-DTT)	0.657**	0.754***	0.834***	−0.859***	0.414	0.894***	0.825***	0.880***	0.744***	0.395
C218/17-T412/01										
1-step (SDS-DTT)	0.487	0.771***	0.752***	−0.476	0.054	0.748***	0.692**	0.792***	0.581*	0.139
2-step (SDS-DTT)	0.545*	0.525*	0.618*	−0.608*	0.281	0.798***	0.549*	0.679**	0.733**	0.310
C405/7-T2377/24										
1-step (SDS-DTT)	0.540*	0.735**	0.595*	−0.412	0.079	0.708***	0.605*	0.720**	0.586*	0.066
2-step (SDS-DTT)	0.532*	0.758***	0.670**	−0.704**	0.060	0.731**	0.844***	0.865***	0.764***	0.263
Protein										
Total	(1.0)	0.366	0.860***	−0.598*	0.721**	0.667**	0.304	0.460	0.139	0.501*
SDS-extractable, %	−0.646**	−0.518	−0.641**	+0.500	−0.529	−0.758***	−0.469	−0.585*	−0.333	−0.465
SDS-DTT extractable, %	0.368	0.904***	0.639**	−0.630**	−0.032	0.729**	0.857***	0.916***	0.374	−0.023

[a] DT = mixograph development time, DB = dough breakdown, BU = Brabender units, WA = water absorption; E = dough extensibility; R5 = resistance to extension at 5 cm, Rmax = maximal resistance.
[b] SDS = sodium dodecyl sulfate, DTT = dithiothreitol.
[c] * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

have a wider conical base. SDS extracts were prepared by extraction with 0.5% SDS at 20°C by vortex mixing for 30 sec, sitting for 15 min, then mixing for an additional 30 sec before centrifugation in a bench-top microfuge (Beckman, Fullerton, CA) for 15 min at 15,000 *g*. The pellet from centrifugation was re-extracted with 1 ml of 2.5% (w/v) SDS-50 m*M* DTT solution by vortex mixing, as described above. In some cases, the SDS-DTT extracts were instead incubated overnight at 45°C before vortex mixing for an additional 30 sec. In other experiments, the SDS-DTT extraction was performed without initial extraction with SDS alone.

The protein contents of the sequential 0.5% SDS and 2.5% SDS-50 m*M* DTT extracts of selected flours were determined colorimetrically using bicinchoninic acid (BCA, Pierce, Rockford, IL) (Smith et al 1985). Since reducing agents interfere with this assay, DTT was removed by extensive dialysis of the extracts against 0.05% SDS-0.1% NaN_3. The protein contents of each fraction were expressed relative to the total (Kjeldahl) protein in the flour.

SDS-PAGE

SDS-PAGE analysis was performed on selected extracts using either 10% polyacrylamide gels run for 1,000 Vhr or 15% polyacrylamide gels run for 1,400 Vhr; other details are described elsewhere (Skerritt and Underwood 1986). All samples were reduced with a final concentration of 5% 2-mercaptoethanol, SDS was added to a final concentration of 2.5% and glycerol to 20%, and extracts were boiled for 5 min before analysis. Acidic and alkaline samples were neutralized first.

ELISA

Microwell ELISA plates (Immulon B, Dynatech, Chantilly, VA) were coated with 1 μg of solid phase-bound "capture" (C) monoclonal antibody (per 100-μl microwell) diluted in 50 m*M* sodium carbonate buffer, pH 9.6, for 60 min at 20°C. After three washes with 50 m*M* sodium phosphate—154 m*M* NaCl, pH 7.2 (PBS) containing 0.05% (v/v) Tween 20—microwells were postcoated with 1% (w/v) bovine serum albumin (BSA, Boehringher-Mannheim, Germany) in PBS for 60 min at 20°C. These steps can be performed either immediately before the ELISA, or batches of plates may be prepared for up to one year before use. Either gluten protein fractions (Skerritt and Robson 1990) at fourfold dilutions starting from 50 μg/ml or clarified extracts of flour samples were diluted serially in 1% BSA-PBS 0.05% Tween, and 100-μl aliquots were added to replicate microwells. After 30 min of incubation at 20°C, microwells were washed four times with PBS-0.05% Tween. Then 100 μl of horseradish peroxidase-labeled "tag" (T) monoclonal antibody per well, diluted in 1% BSA-PBS-0.05% Tween, was incubated for 30 min at 20°C. In certain cases, the same antibody could be used as C and T, without and with peroxidase label, respectively. In other cases, different monoclonal antibodies were used. The wells were washed five more times with PBS-0.05% Tween, then 150 μl of substrate (3-azino bisthiazolin-ethylsulfonic acid-0.003% H_2O_2) per well was added. After 20 min of incubation at 20°C, further color development was stopped by adding 50 μl of 6% oxalic acid per well.

RESULTS AND DISCUSSION

Specificities of Antibody Combinations

The specificities of several antibody combinations, which from preliminary experiments had been demonstrated to function in the two-site assay format with gluten proteins (Hill and Skerritt 1989), were investigated using purified gliadin, LMW-GS and HMW-GS (Table I). To analyze the cross-reaction of different antibody combinations with the three protein fractions, the displacements of the linear sections of the (logarithm) antigen dilution-absorbance curves were calculated for gliadin and LMW-GS relative to HMW-GS. To ascertain whether the solvent affected the specificity of the assay, fractions were dissolved (with sonication) both in 2.5% SDS-50 m*M* DTT and in 0.5% SDS. Effects on specificity were minor, although many antibodies showed a trend toward greater relative binding to HMW-GS compared with the other fractions in SDS. Because dough strength has been related to the proportion and composition of glutenin subunits (Payne et al 1987, Gupta et al 1989), especially the HMW-GS, antibody combinations binding strongly selectively to these subunits (e.g., C237/24-T237/24, C304/13-T304/13, and C412/01-T412/01) were selected for further study. Certain combinations (C218/17-T412/01 and C405/7-T237/24) bound relatively well to LMW-GS and were included for comparison. Other antibody combinations (e.g., 222/5-221/23) (Hill and Skerritt 1989), bound specifically to gliadin or bound each gluten protein fraction roughly equally (e.g., 243/11-243/11) (Table I) and therefore were not examined further in the current study.

Effects of Different Extractants and Extraction Methods

Initially, a number of different solvents known to extract gliadins and/or glutenins were examined. A relatively vigorous extraction technique (1-min treatment with a high-frequency homogenization probe) was chosen; flour from Suneca, a strong-gluten variety, was used. Full wheat extract dilution-response curves (Table II) were prepared for each extractant, using two antibody combinations (C304/13-T304/13 and C412/01-T412/01), shown in Table I to bind selectively to HMW-GS. Adding the reducing agent (DTT) to each extractant produced higher absorbance values, which suggested greater extraction of glutenin antigens. This was confirmed by electrophoretic analysis of the extracts (not shown). Extractants producing the greatest absorbance values in the ELISA were SDS-DTT, HCl-DTT, urea-HCl-DTT, and KOH (Table II). Because the presence of urea caused marked swelling of the flour (probably due to gelatinization of starch granules), which made collection of the centrifugation supernatants difficult, it was not studied further.

The three remaining extractant combinations were studied using samples of medium strength flour (Halberd) and a higher strength flour (Cook) blended to yield samples of approximately 9 and 13% protein for each variety. For both farinograph development time and loaf volume, the flours were ranked in decreasing order: Cook 13%, Halberd 13%, Cook 9%, and Halberd 9%. Flours that differed only moderately in strength were chosen to ensure that the discriminatory power of the test under development would be sufficient for practical use. SDS-DTT was the only extractant that discriminated the flours in the correct order, with the strongest Cook sample yielding the highest ELISA absorbances (Table III).

Table III shows a comparison of the results obtained with high-frequency homogenization of 0.5 g of sample with 10 ml of extractant and the results obtained with the small-scale method (vortex mixing of 0.05 g of sample and 1 ml of extractant) using various SDS concentrations and combinations with 50 m*M* DTT. In each case, the Cook sample gave higher absorbances in the ELISA than did Halberd, as indicated by the absorbance ratios greater than 1.0. Discrimination between the two varieties was slightly greater when preextraction with 1.5% SDS was used for both methods of extraction. This may be due to removal of the gliadin fraction (present in greater amounts than HMW-GS), with which the antibody combinations cross-react weakly (Table I). The higher protein samples of each variety gave higher absorbance values for each treatment. Vortex mixing, although less rigorous, was more efficient than homogenization, as indicated by the higher absorbance values obtained with each Cook sample. Vortex mixing is faster, and because it is suitable for use with small samples, it is a more appropriate method than high-frequency homogenization for wheat breeding quality assessment. Therefore, vortex mixing was studied further using differing SDS concentrations. With 1.5% SDS-50 m*M* DTT and 2.5% SDS-50 m*M* DTT, a preextraction step using the same concentration of SDS (i.e., 1.5 and 2.5%, respectively) reduced the absorbance values obtained with the second extraction, indicating that glutenin antigen was being removed by the initial treatment, which was undesirable. This was confirmed by analysis of these SDS extracts by SDS-PAGE under reducing conditions (not shown). Pretreatment with 0.5% (w/v) SDS did not result in the loss of significant amounts of glutenin, whereas it enhanced discrimination between the Cook

and Halberd samples. Therefore, these conditions (vortex mixer used for preextraction with 0.5% SDS followed by extraction with 2.5% SDS-50 m*M* DTT) were chosen for further work with each antibody combination (Skerritt 1991).

Studies Using Genetically Modified Stocks

The specificities of five antibody combinations for different gliadins and glutenin subunits were analyzed using a number of wheats that lacked certain groups of storage proteins. Earlier studies had indicated that specificity in the two-site ELISA assay for individual glutenin subunits could not be predicted from immunoblotting results (Hill and Skerritt 1989). Because purification of individual glutenin subunits requires manipulations that may include acidic extraction, chromatographic separation, and freeze-drying, and these treatments may modify protein structure (e.g., by deamidation), it was decided that analysis of extracts of genetically modified stocks would provide more reliable results.

The first set of stocks under analysis lacked HMW-GS from one or more genomes and were in a near-isogenic background from the variety Olympic (Lawrence et al 1987). Analysis of extracts of these stocks by two-step SDS-PAGE (Skerritt and Robson 1990) indicated that the LMW-GS composition was not affected by the presence or absence of particular HMW-GS in these flours. Extracts produced by (A) 0.5% SDS extraction, (B) single 2.5% SDS-DTT (50 m*M*) extraction, and (C) after SDS-DTT extraction of the residue from (A) were analyzed by SDS-PAGE (Fig. 1) and in the immunoassays (Fig. 2). While each of the major polypeptide species present in the grain was extracted by the single 2.5% SDS-50 m*M* DTT treatment (Fig. 1B), clear fractionation of the endosperm proteins was seen with SDS treatment followed by SDS-DTT extraction of the residue (Fig. 1C). With all HMW-GS present in the flour, only a minor proportion of these and other glutenin subunits is extracted by 0.5% SDS solution (Fig. 1A). Most of the subunits (A- or HMW-GS) together with B- and C- LMW-GS and certain disulfide-bonded albumins and globulins (Gupta and Shepherd 1987) were extracted by the second SDS-DTT treatment (Fig. 1C). Results were similar with flours having subunits encoded by any two of the three genomes, although for the wheat lacking chromosome 1D-encoded subunits, more HMW-GS were extracted by 0.5% SDS. In the three wheats with subunits from a single genome only, contrasting results were seen. Only small amounts of 1D-encoded subunits but moderate amounts of 1B HMW-GS, and virtually all of the 1A HMW-GS (Fig. 1A) were extracted by 0.5% SDS. The proposed role of HMW-GS in forming the "backbone" of the glutenin polymer (Graveland et al 1985) is reinforced by the absence of not only the HMW-GS but also many of the LMW-GS in the second SDS-DTT extract (after SDS preextraction) of the lines that lacked all HMW-GS or had only the 1A subunit (Fig. 1C). Similarly, Marchylo et al (1989) found that the extractabilities of 1A- and 1B-encoded HMW-GS in 50% propanol (without reducing agent) increased in the absence of 1D-encoded subunits.

ELISA analysis of extracts (Fig. 2A) produced by a single SDS-DTT extraction (Fig. 1B) revealed that combinations using the same C and T antibodies (237/24, 304/13, and 412/01) bound two to three times better to extracts of wheats having D-genome-encoded HMW-GS than to the A-genome- or B-genome-encoded HMW-GS. Deletion of the A-genome HMW-GS did not significantly reduce antibody binding, whereas deletion of either the B- or D-genome subunits caused a 12–29% decrease. The functional specificity of these three antibody combinations for D-genome HMW-GS was increased by initial SDS extraction followed by SDS-DTT extraction (Fig. 2C). Binding to HMW-GS 1 (A-genome-encoded) was minimal, as seen from the analyses of lines having only this subunit and from comparing the results obtained with lines having either A- and B- or A- and D-HMW-GS with results obtained with lines having only the B- or D-genome HMW-GS. Binding to D-genome HMW-GS was two to five times that of the B-genome glutenin subunits (Fig. 2C). Antibody combination 237/24 was the most specific and 304/13 was the least specific. Although the combinations C218/17-T412/01 and C405/7-T237/24 bound very little to SDS-extracted proteins (not shown), differences in the specificity patterns were noted after preextraction with SDS (Fig. 2C). With flour samples extracted directly with SDS-DTT, binding to the different subunits was closer to equal (Fig. 2A). Apart from grain lacking all of the HMW-GS, only the material with HMW-GS 1 had decreased binding, compared with the euploid Olympic wheat. After SDS preextraction, selective (and equal) binding to B- and D-HMW-GS was noted, and only (10%) binding to A-genome-encoded subunits occurred.

The initial SDS extracts were also examined (Fig. 2B) with antibodies C+T 237/24, C+T 304/13, and C+T 412/01. A fivefold higher antigen concentration was analyzed for the SDS extracts than for the SDS-DTT extracts because of the lower binding of these antibodies to the SDS-extracted proteins. Antibodies were only weakly selective for wheats containing D-genome-encoded HMW-GS. In all cases, far less binding occurred when each HMW-GS was absent. For antibody combinations based on C+T 237/24 and C+T 412/01, binding was 2–9% of Olympic

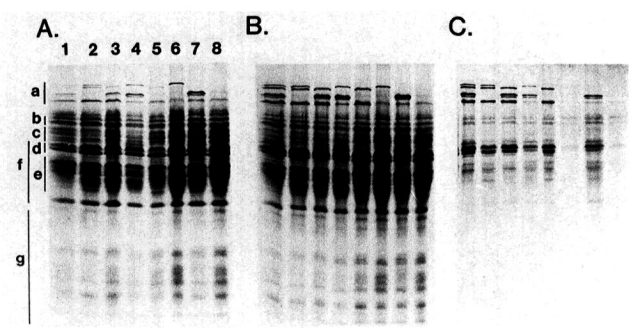

Fig. 1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic analysis of extracts of near-isogenic Olympic wheats lacking various HMW-GS. **A,** SDS (0.5%) extracts; **B,** SDS (2.5%)-dithiothreitol (DTT) (50 m*M*) extracts; **C,** SDS-DTT extraction of the residue from **A.** Polypeptide groups are as follows: a, high molecular weight glutenin subunits (HMW-GS); b, disulfide-bonded albumins; c, ω-gliadins; d, B-LMW-GS; e, C-LMW-GS; f, α-, β-, and γ-gliadins; g, LMW albumins and globulins. Lanes are as follows: 1, HMW-GS encoded by 1A, 1B, 1D present; 2, 1A and 1D present; 3, 1B, and 1D present; 4, 1A and 1B present; 5, 1D present; 6, 1A present; 7, 1B present; 8, no HMW-GS present.

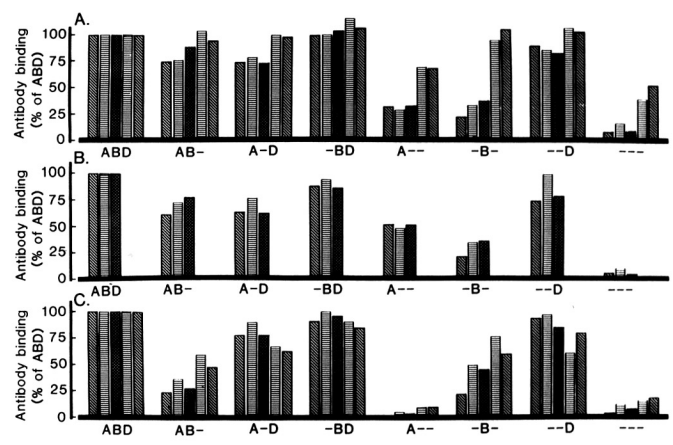

Fig. 2. Enzyme-linked immunosorbent assay (ELISA) analysis of extracts of near-isogenic Olympic wheats lacking various high molecular weight glutenin subunits (HMW-GS). The data shown indicate antibody reaction relative to the mean of three euploid Olympic lines grown at the same site (Lawrence et al 1987). **A,** sodium dodecyl sulfate (SDS) (2.5%)-dithiothreitol (DTT) (50 m*M*) extracts; **B,** SDS (0.5%) extracts; **C,** SDS-DTT extraction of the residue from **B.** Each bar indicates a different antibody combination. From left: C237/24-T 237/24, C304/13-T 304/13, C412/01-T 412/01, C218/17-T 412/01, and C405/7-T 237/24. Letters below each histogram indicate that HMW-GS are present from the genomes indicated.

control, whereas for C+T 304/13, binding of up to 15% was seen. For the combinations C218/17-T412/01 and C405/7-T237/24, quite high binding to SDS-DTT extracts of wheats lacking HMW-GS was seen; this was reduced by preextraction with SDS.

These results, taken together with the results of analysis of SDS extracts of nullisomic-tetrasomic lines of Chinese Spring, would suggest that most binding in SDS extracts was to the traces of HMW-GS (and perhaps LMW-GS) that were extracted in the absence of a reducing agent. These subunits could be seen on high-sensitivity staining of SDS-PAGE gels (not shown). Binding of the antibody combinations to extracts of the chromosome 1 aneuploids of Chinese Spring also indicated that chromosome 1D had a major effect and 1B had a minor effect on antibody binding to SDS-DTT extracts. The protein contents of the aneuploid grains were about 50% (on average) higher than those of euploid Chinese Spring. Analysis of the effect of protein content on the binding of these antibodies was performed by examining the absorbance values produced by an extract of Chinese Spring that had been diluted only half as much as the control sample (i.e., the values for Chinese Spring 2x in Table IV). The major effect of chromosome 1D-encoded HMW-GS and a secondary effect of chromosome 1B-encoded HMW-GS on antibody binding to SDS-DTT extracts were confirmed on analysis of these Chinese Spring aneuploids. Binding was decreased on removal of chromosome 1D and was increased in tetrasomic 1D lines. These effects were reduced when chromosome 1B dosage compensation was present, for example, in nulli 1D-tetra 1B (Table IV). Modifying the dosage of homeologous chromosomes 6 did not alter antibody binding to SDS-DTT extracts.

Attempts were made to investigate the role of specific LMW-GS by analyzing chromosome 1 short-arm wheat-rye translocations. However, interpreting these results was complicated by 1) replacement of the wheat chromosome arms with rye arms, since some antibody combinations bind rye prolamins, and 2) in some cases, use of material with a mixed background of Chinese Spring (low dough strength and thus low antibody binding) and Gabo (high strength and thus high antibody binding). Binding to rye proteins extracted by SDS-DTT was depressed to 50–72% of Chinese Spring wheat proteins, for each of the three combinations having the same C and T antibody. Some of the translocation lines (most notably 1AL/1RS-1BL/1RS) showed decreased binding, which could indicate that some antibody binding to 1AS and 1BS LMW-GS occurred.

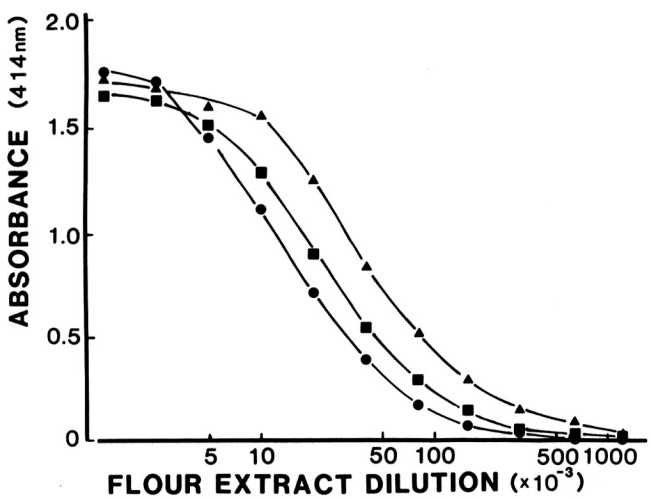

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) absorbances at differing dilutions of a strong wheat flour extract (Gabo, ▲), a medium strength wheat flour (Halberd, ■), and a weak-doughed wheat flour (Chile, ●), using antibody combination C401/21-T401/21 and sodium dodecyl sulfate dithiothreitol (SDS-DTT) extracts made after a prior SDS extraction.

Quality-Antibody Binding Correlations in a Set of Genetically Unrelated Wheats

Several antibody combinations were examined for correlations between antibody binding and each of several quality parameters. Linear regression of ELISA absorbance results obtained with a well-characterized set of 15 wheat varieties that varied markedly in dough properties was performed. Significant correlations between several strength-related quality parameters of dough and antibody binding to SDS-DTT extracted proteins were seen for each antibody combination studied. Over a range of dilutions of the wheat extracts, strong-doughed wheats provided greater ELISA absorbance values than did weak-doughed wheats (Fig. 3); a 1:50,000 dilution was chosen for further analyses (Table V). Correlations were not seen for the SDS extracts using antibody combinations C304/13-T304/13 and C412/01-T412/01. Preliminary experiments showed that very little binding of antibody combinations C218/17-T412/01 and C405/7-T237/24 occurred with SDS extracts, thus these data are not shown.

In general, SDS-DTT extracts made without prior extraction with 0.5% SDS (Table V) did not yield as good correlations between binding of the antibodies and dough strength parameters as did the two-step assay (using SDS preextraction) performed with the same antibodies. In the latter case, positive correlations were found with mixograph mixing and farinograph development times, and negative correlations were found with farinograph dough breakdown values. These were indicative of greater dough stability observed in flours with more glutenin and/or more of the particular HMW-GS leading to the formation of a stronger gluten network. Correlations with farinograph water absorption were typically not observed, as this parameter relates to milling starch damage, which itself is related to grain hardness rather than to gluten protein composition. For most antibody combi-

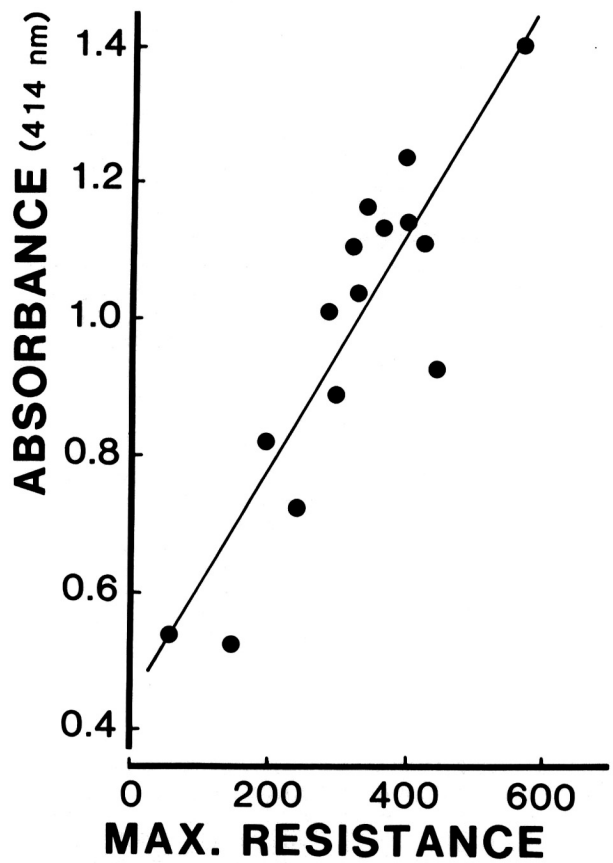

Fig. 4. Relationship between extensigraph maximal resistance and enzyme-linked immunosorbent assay (ELISA) absorbance for 15 wheat varieties using antibody C412/01-T412/01. Data shown are for 2.5% sodium dodecyl sulfate (SDS)-50 m*M*-dithiothreitol (DTT) extracts prepared after preextraction with 0.5% SDS.

nations, very high correlation coefficients were found between antibody binding and extensigraph dough resistance (Fig. 4); loaf volumes (from loaves baked with and without bromate) and extensibility were also correlated. The binding of antibody combinations C304/13-T304/13, C218/17-T412/01, and C405/7-T237/24 were only weakly correlated with flour protein content; binding of C412/01-T412/01 was more closely correlated with protein, especially for the one-step assay. In addition to the selectivity of this antibody combination for HMW-GS (Table I), the closer correlation with protein content may be partially responsible for the superior correlations between antibody binding and quality parameters that were seen for C412/01-T412/01 using the one-step extraction method. As protein content is itself positively correlated with each of the quality parameters, its effect is hard to dissociate from that of quality-related differences in antibody binding. However, the following points should be noted:

1. Quite large alterations in protein content in samples of each of two varieties (Cook and Halberd) caused only minor increases in antibody binding.

2. The protein contents in the series of 15 wheats showed only moderate variation (8.1–11.7%).

3. In general, the correlations between the various quality parameters and antibody binding were higher than they were with protein content (Table V).

Protein contents of the sequential 0.5% SDS and 2.5% SDS-50 m*M* DTT extracts from the set of 15 flours were also determined. The relationship between these amounts and the various quality parameters are shown in Table V. Significant positive correlations between the proportion of protein that is SDS-DTT extractable (comprising both HMW-GS and LMW-GS) and several of the strength parameters and dough extensibility were seen. However, the farinograph development time and breakdown values were better correlated with antibody binding (e.g., C+T 304/13, C+T 412/01), and, unlike antibody binding, correlations between loaf volume and SDS-DTT-extracted protein were not seen. Variation in strength was associated with greater differences in antibody binding than in the amount of SDS-DTT-extracted glutenin subunits. For example, a doubling in maximal resistance was associated with a 48% increase in C+T 412/01 antibody binding; that is, a 75% difference in the amount of HMW-GS reaction (Fig. 3), but only a 35% increase in SDS-DTT-extracted glutenin subunits. Some statistically significant negative correlations were observed between certain strength and extensibility parameters and the proportion of SDS-extracted protein. However, the magnitudes of the differences between the fractions were small; a doubling in maximal resistance was associated with only a 9% decrease in SDS-extracted protein.

Thus it is quite unlikely that differences in the amount of total protein or in the amount of protein extracted by the (second) SDS-DTT extraction were the main origin of quality-antibody binding correlations. This aspect is further examined in another study (Skerritt 1991), first by statistical separation of protein content and antibody binding contributions to quality variation using multiple linear regression analysis, and then by analysis of a number of sets of wheat samples that did not vary significantly in protein content.

GENERAL DISCUSSION

An ELISA assay format and several antibody combinations were identified that have the potential to discriminate wheat flour samples differing in dough properties. The most useful antibodies bound selectively to the particular HMW-GS thought to exert a major influence on dough strength. However, since other gluten proteins (e.g., LMW-GS) can influence dough strength (Gupta and Shepherd 1988), the extent of the correlations between antibody binding and strength may vary in different sets of wheats (Skerritt 1990). The solvent used for flour extraction also had a major effect on the performance of the assay. Although acids, alkalis, and urea solutions were efficient extractants of HMW-GS, especially in the presence of a reducing agent, they did not discriminate sufficiently between samples in the ELISA despite findings by different groups that the proportions of acetic acid-insoluble (Mecham et al 1962, Orth and Bushuk 1972), dilute HCl-insoluble (MacRitchie 1987), or SDS-insoluble protein fractions (Orsi and Pallagi 1989) correlated positively with dough strength. SDS, the detergent that is widely used in wheat protein research for gluten fractionation and dissolution, was also the most useful solvent in these studies. Measurement of the amount of protein aggregates formed in the presence of SDS is the basis of the small-scale sedimentation volume test for predicting dough quality (Axford et al 1978). SDS solutions have been reported to solubilize a large proportion of glutenin in the absence of a reducing agent (Danno 1981, Bottomley et al 1982), especially after high-frequency sonication of extracts (Singh et al 1990). Although the proportion of glutenin polymers in such extracts has been demonstrated to be higher in flours with strong glutens (Singh et al 1990), quality discrimination was not observed with the current ELISA test unless a reducing agent was used. The ability of reducing treatments to enhance antibody binding to gluten proteins may arise from exposure of additional antibody-binding sites on individual HMW-GS, unavailable to the antibody on the unreduced disulfide-bonded glutenin complex. It is relevant that many of the subunit-specific regions of amino acid sequence on HMW-GS are located near cysteine residues (Anderson and Greene 1989), which would be exposed only after reduction. Another possibility is that the most suitable antibody combinations bind to repeat motifs within the HMW-GS sequences. This is supported by the observation that the antibody combinations providing the greatest HMW-GS specificity (Table I) and dough strength correlations were those based on pairs of identical monoclonal antibodies. For such a monoclonal antibody pair to be able to bind an individual polypeptide, it follows that a repeating or multiple peptide sequence is recognized. It is likely that HMW-subunit-related differences in dough strength arise from differences in the number and location of repeat motifs within HMW-GS (Flavell et al 1989).

Extraction of antigen by vortex mixing was found to be as efficient as high-frequency probe homogenization and more efficient in terms of exposure of antibody-binding sites (when a reducing agent is present) than ultrasonic probe treatment. It is also more suited to handling large numbers of small flour samples, since all the extraction manipulations can be performed using microcentrifuge tubes. Sample suspension using a vortex-type mixer is also fast and can be done in a sealed tube. The results indicate that the extractant as well as the antibody combination chosen had major effects on ELISA specificity. For example, results from analysis of extracts of Olympic-derived near-isogenic wheats lacking particular HMW-GS indicated that binding of the three antibody combinations based on 237/24, 304/13, and 412/01 was roughly equal to A-, B-, and D-genome-encoded subunits present in SDS extracts, especially if the relative amounts of these subunits (Lawrence et al 1987) were taken into account. However, selective binding to B- and D-HMW-GS was found in SDS-DTT extracts, and especially pronounced binding to D-HMW-GS after SDS preextraction was performed.

These results, obtained with the modified Olympic lines, were confirmed after the analysis of chromosome 1 aneuploids of Chinese Spring. Analysis of these lines, using the SDS-sedimentation test (Rogers et al 1988), also showed that the line with highest antibody binding, nulli 1A-tetra 1D, had potentially the best quality, and lines with depressed antibody binding (lacking 1D) also had decreased SDS-sedimentation volumes compared with euploid Chinese Spring. These results, while also indicating that chromosome 1D had the greatest influence on quality, must be interpreted with some caution, since Chinese Spring is null for the Glu-A1 allele. Analysis of these lines also showed that chromosome 6-encoded gliadins did not affect antibody binding to specific antigens in these SDS-DTT extracts. It was harder to ascertain whether antibody binding to LMW-GS played a significant role. With the antibody combinations 237/24-237/24, 304/13-304/13, and 412/01-412/01, some binding to purified LMW-GS was seen. However, effects of rye proteins complicated

the interpretation of the results with extracts of genetically modified stocks, such as the double and triple chromosome 1 (short arm) translocation lines. The decreases in binding observed with the 1AS and 1BS translocation may be due to a less extensive glutenin polymer being formed in the absence of certain LMW-GS (Graveland et al 1985). Indeed, others have demonstrated significant effects of LMW-GS on physical dough properties (Gupta and Shepherd 1988, Gupta et al 1989). The relationship between HMW-GS and dough strength is well documented in earlier genetic studies (Payne et al 1979, 1984, 1987; Burnouf and Bouriquet 1980; Branlard and Dardevet 1985), and the specificities of and results obtained with the antibody combinations used in this study correlate with these genetic findings.

In conclusion, a simple antibody-based test was developed for predicting strength-related properties of wheat dough. Several antibody combinations were assessed, using a set of 15 wheat varieties grown at a single site. In general, a two-step extraction method and use of the same antibody on the solid phase and in solution gave the best results. The companion article (Skerritt 1991) discusses in greater detail the effects of environment, fertilizer treatment, genotype, and glutenin subunit composition on the performance of the test with the different antibody combinations.

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