

Development of a Panel of Specific Monoclonal Antibodies to High Molecular Weight Glutenin Subunits and Their Application in Genetic Screening

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ABSTRACT

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High molecular weight glutenin subunits (HMW-GS) encoded by different chromosomal loci and alleles (1, 2, 5, 7, 10, and 12) were purified using reversed-phase HPLC from reduced, aqueous propanol extracts of flour from aneuploid or null wheat lines. Unlike previous libraries of monoclonal antibodies developed in our laboratory to SDS-extracted or alkylated HMW-GS, several of the monoclonal antibodies (mAb) developed in this study had a range of specificity patterns for HMW-GS in enzyme-linked immunosorbent assay (ELISA) and on immunoblots. A subset of the mAb bound either x- or y-type HMW-GS but not other gluten proteins, while a few antibodies bound one (mAb 110622, 110421, 140820), or two (mAb 101319, 110804, 140705, 1410460) HMW-GS expressed in each cultivar tested. In most cases, antibodies bound equally to the subunits encoded by different HMW-GS alleles. The more specific antibodies should be useful in research on the quantitative variation of HMW-GS expression and

in studies of the role of particular HMW-GS in dough structure. The mAb 101319, which was prepared to subunit 1, bound to HMW-GS 1Bx subunits in ELISA and on immunoblots. This antibody also provided a higher absorbance value in ELISA with extracts of wheat lines expressing the *Glu-B1e* allele (HMW-GS 20) compared with the *Glu-B1i* allele (HMW-GS 17+18). Another mAb (110622) detected subunit 2 more strongly than subunit 5 in ELISA and produced a higher signal in immunoblots with subunit 2 even though these subunits are >98.7% homologous in amino acid sequence. An ELISA assay using this antibody was optimized for discrimination of wheat lines with the allelic pairs of subunits 1Dx5-1Dy10 from those with 1Dx2-1Dy12, with the former lines providing stronger dough properties and superior breadmaking quality. The performance of this assay was unaffected by other variations at HMW-GS loci and was demonstrated in sets of biotypes, doubled haploid, and cross-bred breeder's lines.

The unique breadmaking quality of wheat is related to the composition and quantity of gluten proteins, in particular the high molecular weight glutenin subunits (HMW-GS). They represent 5–10% of the total seed protein and are encoded by homologous *Glu-1* loci on the long arms of the group 1 chromosomes (1A, 1B, 1D). Based on their electrophoretic mobilities, the HMW-GS are grouped into higher M_r x-type and lower M_r y-type subunits (Shewry et al 1992). In hexaploid wheat, tightly linked pairs of one x-type and one y-type gene are present on each locus of the chromosomes 1A, 1B, and 1D, but because of gene silencing, only three, four, or five subunits are expressed in different cultivars. The composition of HMW-GS is a key property affecting flour processing and product quality, especially the HMW-GS at the *Glu-D1* and *Glu-A1* loci which are particularly associated with the breadmaking qualities (Payne et al 1981, Payne 1987). There is therefore considerable interest in rapid and reliable methods to determine the allelic composition of HMW-GS to assess wheat quality in breeding programs and in industrial processing.

Several methods have been developed to analyze the HMW-GS composition of wheat flour. The HMW-GS are typically separated and analyzed by SDS-PAGE (Wrigley et al 1982, Marchylo et al 1989, Lookhart and Wrigley 1995); capillary electrophoresis has also been used (Werner et al 1994). SDS-PAGE gives reliable results and is the most widely used technique in breeding programs and for varietal identification, but it is time-consuming and relatively low in throughput. Reversed-phase HPLC has also been successfully applied to identification of HMW-GS composition because the subunits differ in surface hydrophobicity (Marchylo et al 1988, Bietz and Huebner 1995). However, even though it can be automated, single samples must be run sequentially (limiting throughput), and the capital cost of the equipment and relatively high per-sample costs limit its wider use in breeding programs. The development of primers for the specific polymerase chain reaction (PCR) amplification of certain HMW-GS has allowed their differentiation on the DNA level. (D'Ovidio and Anderson 1994, Varghese et al 1996, Lafiandra et

al 1997). Although DNA diagnostics is a rapidly evolving field, the methods are not yet robust and high enough in throughput to be the preferred method for allele discrimination in breeding programs.

Immunochemical test systems, such as enzyme-linked immunosorbent assay (ELISA) for measurement of wheat proteins are easy to perform without sophisticated equipment and have the advantage of high sample throughput (Skerritt 1988, Skerritt et al 1994). Due to the high degree of amino acid sequence homologies within and between the families of gluten proteins, it has been difficult to obtain the highly specific antibodies required to differentiate alleles. Howes et al (1998) obtained monoclonal antibodies (mAb) that differentiated the γ -gliadins 42 and 45 that are linked to low molecular weight glutenin subunits (LMW-GS) associated with poor and superior pasta cooking quality, respectively, and later research also isolated mAb that distinguished the two major LMW-GS alleles (Howes et al 1998). The same antigliadin antibody exhibited differences in binding to gliadin extracts of a number of hexaploid wheat cultivars (Dawood et al 1989). We have also developed antibodies that exhibit specificity for gliadins encoded by individual loci (Skerritt et al 1991, Andrews and Skerritt 1996). Antibodies with restricted specificity for small groups of LMW-GS have also been described (Brett et al 1993; Denery-Papini et al 1995; Sissons et al, *in press*).

Because the HMW-GS have been the gluten polypeptides most clearly associated with variation in wheat quality, there have been a number of attempts to develop specific antibody probes for these polypeptides. The original HMW-GS mAb from our laboratory were prepared to alkylated SDS-extracted subunits, and while several antibodies were selective for (total) HMW-GS, they cross-reacted with certain γ - and ω -gliadins and LMW-GS (Skerritt and Underwood 1986). Later, we isolated individual subunits by preparative SDS-PAGE; the antibodies that were prepared using this method were still quite cross-reactive (Skerritt 1991a), although some of them could be used to differentiate durum wheats with differences in HMW-GS composition (Kovacs et al 1992). Intriguingly, certain mAb to isolated rye secalins (Andrews and Skerritt 1996) actually cross-reacted strongly with wheat glutenin subunits and exhibited selectivity for either total HMW-GS (80105; D'Ovidio et al 1997) or x- or y-type HMW-GS (e.g., 80410) and, in one case, one HMW-GS plus a LMW-GS (80603). The x- and y-type subunits exhibit greater sequence differences from one another than individual x- or individual y-type subunits encoded by different loci. It has thus also been possible to isolate rabbit polyclonal antibodies

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Ab with selectivity for either x- or y-type subunits (Curioni et al 1991). Antibodies that selectively bind to *Glu-D1* and *Glu-B1* HMW-GS have been useful for discrimination of wheats differing in dough strength (Skerritt 1991a,b; Andrews et al 1993) but this type of antibody recognizes amino acid repeat regions (Andrews and Skerritt 1994) and thus cannot be subunit-specific.

Based on the experience of our laboratory and others, we postulated that there may be a greater chance of obtaining specific mAb if the immunogen subunits were isolated and purified under SDS-free conditions and the cysteine residues were maintained in an unalkylated state. The present report describes the development and characterization of these monoclonal antibodies. Several quite specific mAb were developed, and one was capable of distinguishing subunits 2 and 5, which are considered to have a key role in dough properties.

MATERIALS AND METHODS

Flour Samples

HMW-GS were isolated from wholemeal of four different genetic wheat lines with reduced sets of subunits: Olympic A (only subunit 1) and Olympic D (only subunits 5, 10) (Lawrence et al 1988), as well as nullisomic-tetrasomic lines in Chinese Spring: N1B-T1D (only subunits 2, 12), N1D-T1B (only subunits 7,8). In addition, selected members of a set of biotypes varying at single HMW-GS loci in Australian wheat cultivars were examined (Lawrence et al 1987). To ensure purity of the seed, all of these lines were expanded by single-seed descent in the glasshouse and HMW-GS and LMW-GS subunit compositions checked before preparation of wholemeal. For initial screening of antibodies, we used glutenin subunit preparations from cv. Suneca and Chinese Spring.

Flour from a series of doubled haploid lines derived from a cross between Halberd and Cranbrook, were grown at Roseworthy, South Australia, in 1996, and provided by G. B. Cornish (South Australian Research and Development Institute, Adelaide). These lines were developed under the Australian National Wheat Molecular Marker Program. Finally, we analyzed a set of F8 crossbred lines grown at Narrabri, New South Wales, Australia, in 1996, from a cross between the two Australian Prime Hard (breadmaking quality) wheats, Janz and Hartog. The glutenin composition of the lines is shown in Table I.

Purification of HMW-GS

After three preextractions of 5 g of flour with 45 mL of 50% (v/v) isopropanol by vortex mixing then shaking 30 min at room temperature, the glutenin fraction was extracted using 10 mL of 50% (v/v) isopropanol in 80 mM Tris-HCl, pH 8, containing 1% dithioerythritol (DTE) for 30 min at 65°C. The extracts were concentrated to 2 mL under vacuum. HMW-GS were isolated with RP-HPLC on a Vydac (The Sep/a/rations Group, Hesperia, CA) 218-TP semipreparative reverse phase column (10 × 250 mm). Chromatography conditions were: flow rate 4 mL/min; column temperature 60°C; sample volume 200 µL; detection wavelength 214 nm; mobile phase linear acetonitrile gradient (30–37% acetonitrile-water in 0.1% trifluoroacetic acid [TFA] over 40 min). At the end of the 40-min period, the acetonitrile concentration was increased

to 80% over 2 min, the column was washed for 10 min with 80% acetonitrile then reequilibrated for 20 min with 30% acetonitrile. Peaks corresponding to the different HMW-GS subunits (eluting between 30 and 35% acetonitrile) were collected and protein was freeze-dried.

Production of Monoclonal Antibodies

Groups of 10- to 12-week-old female BALB/c mice were intraperitoneally injected with 50 µg of HMWG subunits in 100 µL of saline emulsified with 100 µL of Freund's complete adjuvant (Sigma, St. Louis, MO). Two injections with Freund's incomplete adjuvant followed at three-week intervals. Serum titers of antibodies to HMW-GS were determined by ELISA one week after the last injection. After a rest period of four to eight weeks, mice with high serum titers received daily booster injections with 400 µg of antigen in 200 µL of saline on the four days before cell fusion (Stähli et al 1980). On the fifth day, half of the spleen cells from each immunized mouse were fused with SP2/0 murine myeloma cell line as described previously (Skerritt et al 1984). After HAT selection (hypoxanthine, aminopterin, thymidine) of hybridoma cells, supernatants from growing cells were tested for glutenin-

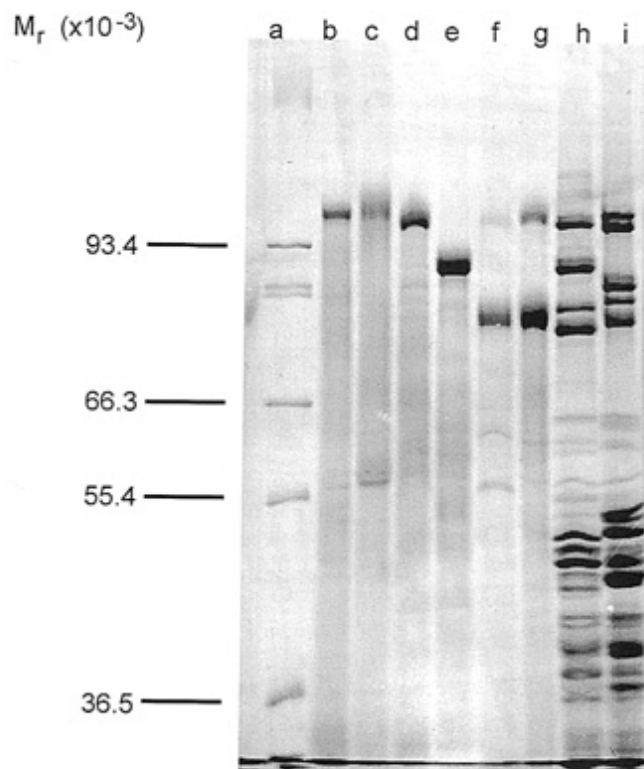


Fig. 1. SDS-PAGE separation of the purified high molecular weight glutenin subunits (HMW-GS) and glutenin extracts from Chinese Spring and Suneca flours. Lanes a–i, from left: molecular weight markers; purified HMW-GS 1; purified HMW-GS 2; purified HMW-GS 5; purified HMW-GS 7; purified HMW-GS 10; purified HMW-GS 12; total glutenin subunits from Chinese Spring flour; total glutenin subunits from Suneca flour.

TABLE I
High and Low Molecular Weight Glutenin Subunits (HMW-GS, LMW-GS) Composition of Wheat Cultivars for Antibody Screening and Parents of Crossbred Lines Used for Assay Development

Wheat	HMW-GS Alleles (subunits)			LMW-GS Alleles		
Suneca	<i>Glu-A1a</i> [1]	<i>Glu-B1i</i> [17+18]	<i>Glu-D1d</i> [5+10]	<i>Glu-A3d</i>	<i>Glu-B3h</i>	<i>Glu-D3e</i>
Chinese Spring	<i>Glu-A1c</i> [null]	<i>Glu-B1b</i> [7+8]	<i>Glu-D1a</i> [2+12]	<i>Glu-A3a</i>	<i>Glu-B3a</i>	<i>Glu-D3a</i>
Halberd	<i>Glu-A1a</i> [1]	<i>Glu-B1e</i> [20]	<i>Glu-D1d</i> [5+10]	<i>Glu-A3e</i>	<i>Glu-B3c</i>	<i>Glu-D3c</i>
Cranbrook	<i>Glu-A1b</i> [2*]	<i>Glu-B1i</i> [17+18]	<i>Glu-D1a</i> [2+12]	<i>Glu-A3b</i>	<i>Glu-B3d</i>	<i>Glu-D3a</i>
Janz	<i>Glu-A1a</i> [1]	<i>Glu-B1b</i> [7+8]	<i>Glu-D1a</i> [2+12]	<i>Glu-A3b</i>	<i>Glu-B3b</i>	<i>Glu-D3b</i>
Hartog	<i>Glu-A1a</i> [1]	<i>Glu-B1i</i> [17+18]	<i>Glu-D1d</i> [5+10]	<i>Glu-A3b</i>	<i>Glu-B3h</i>	<i>Glu-D3e</i>

specific antibodies by ELISA. Hybridomas positive for HMW-GS were expanded and subcloned to ensure uniform cell populations. Isotypes were determined with the mouse-typer subtyping kit (BioRad, Hercules, CA). For production of larger quantities, selected mAb were produced as ascites fluids in BALB/C mice and IgG antibodies purified by Protein G affinity chromatography (Akerstrom et al 1985).

Electrophoresis and Immunoblotting

Freeze-dried glutenin subunits were redissolved in 50% (v/v) isopropanol, 1% (w/v) DTE, and mixed with an equal volume of sample buffer concentrate (250 mM Tris-HCl, pH 7.5, 4% [w/v] SDS, 20% [v/v] glycerol). Samples were fractionated on 1-mm thick 10% T, 2% C polyacrylamide gels at constant voltage for 1,500 Vhr. Gels were stained with colloidal Coomassie Brilliant Blue G-250 (Neuhoff et al 1988). For the preparation of immunoblots from glutenins for antibody screening, whole glutenin subunit extracts from 40 mg of flour of Chinese Spring or Suneca were applied across the gel and fractionated under the same electrophoretic conditions. Glutenin subunits were transferred to nitrocellulose membranes. After electrophoresis, the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.02% [w/v] SDS, 10% methanol) for 20 min. The proteins were transferred to the membrane (Protran 0.2 µm, Schleicher and Schüll, Dassel Germany) in a semidry blotting device at 250 mA using 40 mM 6-aminocaproic acid buffered with Tris to pH 7.5, 20% methanol as cathode buffer. In the two-phase anode buffer system, 25 mM and 300 mM Tris with 20% methanol was used as inner and outer buffer, respectively. After blotting, the proteins were fixed in 1% (w/v) KOH for 5 min and washed twice in phosphate-buffered saline (PBS). The membrane was dried overnight and, after sealing in plastic bags, stored at -20°C until use.

Membranes from glutenin blots were cut into 5-mm strips. Residual binding sites on the membrane were blocked with 3% bovine serum albumin (BSA) (Boehringer Mannheim, Germany) in 50 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS) for 1 hr on a rocker. After washing with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl-0.05% (v/v) Tween 20 (TBST, 4 × 5 min), the membrane strips were incubated with cell culture supernatants for 1.5 hr. After further washing steps (4 × 5 min), alkaline phosphatase-labeled anti-mouse IgG (Promega, Madison, WI) was added at a 1:5000 dilution and the membranes were incubated on a rocker for 1 hr. Further washing was performed (4 × 5 min in TBST) before adding the substrate (0.033% NBT/0.0165% BCIP in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5mM MgCl₂). Specific binding of the mAb was revealed by the appearance of purple-colored bands within 15–20 min of substrate addition.

ELISA Screening for Antibodies to HMW-GS

The extracts of Chinese Spring and Suneca flour were prepared by the same method used for HPLC purification, except that HMW-GS and LMW-GS were then fractionated by acetone precipitation

(Melas et al 1994). ELISA microwell plates (Maxisorp, Nunc, Roskilde, Denmark) were coated for 16 hr at 4°C with 50 µL of HMW-GS or LMW-GS at 10 µg/mL in 50 mM carbonate buffer, pH 9.6. After washing three times with PBST (PBS with 0.05% [v/v] Tween 20), the nonspecific binding sites on the plates were blocked with 100 µL/well 1% BSA in PBS for 1 hr at room temperature. The plates were washed three times in PBS and then 50 µL/well of the cell culture supernatants were incubated for 1 hr at room temperature. After another washing step, 50 µL of HRP-labeled rabbit anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1:2000 in 1% BSA in PBST was incubated in the wells for 1 hr. Microwells were washed three times with PBST and ABTS substrate (2 mM 2,2'-azino-bis-3-ethylbenzthiazoline sulfonic acid [Sigma] in 0.1M sodium citrate buffer, pH 4.5, containing 0.003% H₂O₂) was added. After 30 min, absorbances at 415 nm were measured in a Vmax microplate reader (Molecular Devices). The ELISA to test antibody specificity for individual HMW-GS were performed using the same method, except that the coating was done with HPLC-purified HMW-GS.

Analysis of HMW-GS in Wheat Grain Samples

HMW-GS were either extracted from wholemeal of the HMW-GS biotype samples or flour of the doubled haploid lines. The sample consisted of 400 µL of 50% *n*-propanol added to 20 mg of wholemeal. The sample was mixed by vortexing for 30 sec at room temperature (20–23°C), then the extract was shaken for 5 min and centrifuged at 14,600 × *g* for 5 min. The centrifugation pellet was resuspended in 50% *n*-propanol and 50 mM dithiothreitol by vortex mixing, mixed overnight on a microtube shaker at 37°C, and recentrifuged. Extracts were also prepared without performing the 50% *n*-propanol preextraction step. For ELISA analysis, extracts were diluted in 50 mM carbonate buffer, pH 9.6, and 100 µL of diluted extracts was applied to microwells and incubated 1 hr at room temperature. The optimal dilution of extracts, defined as that producing 40–70% maximal color development (i.e., in the dynamic part of the antigen concentration and color response curve) was determined by analysis of a range of extract dilutions. The wells were then washed three times with PBST and nonspecific binding blocked by the addition of 1% BSA-PBS for 1 hr at room temperature. The mAb tissue culture supernatants were diluted 1:4 or purified antibody diluted to 1 µg/mL with BSA-PBS/T, and 100 µL of either was added to the wells to incubate for 1 hr. The rest of the assay was performed using the methods described above.

RESULTS AND DISCUSSION

Purification of HMW-GS

The HMW-GS from various genetic wheat lines were purified by preparative RP-HPLC to obtain pure preparations for immunization of mice and for the mAb screening. The use of genetic lines with a limited set of HMW-GS simplified the task of fractionation

TABLE II
High Molecular Weight Glutenin Subunits (HMW-GS) Selective Monoclonal Antibodies

Immunogen	Cell Line ^a	Specific for Subunits	Isotype
HMW-GS 1	101319 B3#	<i>Glu 1Ax1, Glu-1Bx7, Glu-1Bx17</i>	IgM, kappa
HMW-GS 2	110208 H9#	<i>Glu-1Ax, Glu-1Bx, Glu-1Dx</i>	IgG1, kappa
	110421 B3#	<i>Glu-1Ax,</i>	IgM, kappa
	110804	<i>Glu-1Ax, Glu-1Bx17, Glu-1</i>	IgM, kappa
	110510 D4#	<i>Glu-1Ax, Glu1Bx7, Glu1Dx</i>	IgG1, kappa
	110524	<i>Glu-1Ax, Glu1Bx17, Glu 1By18, Glu1Dx</i>	IgG1, kappa
	110622 G7#	<i>Glu-1Dx</i>	IgG1, kappa
	110924 E3	<i>Glu-1Ax, Glu-1Bx, Glu-1Dx5</i>	IgG1, kappa
HMW-GS 10	130612 B1#	<i>Glu-1Bx, Glu-1Dx2</i>	IgG1, kappa
HMW-GS 12	140820	<i>Glu-1Bx</i>	IgM, kappa
	141046 D10#	<i>Glu-1By</i>	IgG1, kappa
	140705 C3#	<i>Glu-1By</i>	IgG1, kappa

^a # = Subclones from selected cell lines of the corresponding number (Fig. 2).

of the subunits, providing a higher yield of the single subunit protein amount (total glutenin subunit loadings on the HPLC column could be higher) compared to lines with a more complex mixture of HMW-GS. After preliminary experiments to optimize the HPLC parameters, the profiles generally showed clearly distinguishable peaks of the particular subunits that could be collected separately. However, even when collecting the middle of the peaks, some overlapping of protein from the peak bases could not be excluded. The analysis of the different fractions by SDS-PAGE (Fig. 1) showed generally high purity of the different subunits. The purified individual subunits exhibited a slightly different mobility when compared to those in the total glutenin extracts. This is possibly due to slight acidic deamidation of the purified subunits that were dried from solutions containing TFA (Lookhart 1987, Shih 1990). Resulting differences in the binding of SDS used in electrophoresis could then be responsible for the differences in mobility between purified HMW-GS and glutenins from total extracts. Subunit 12 showed some contamination with subunit 2. HPLC profiles of the extract from Chinese Spring N1D-T1B (which has only HMW-GS 7 and 8) showed a peak with a shoulder which was collected together. The peak analyzed by SDS-PAGE contained only subunit 7. Extracts from 10 g of flour yielded 5–10 mg of the different purified subunits after freeze-drying. These amounts were sufficient for the immunization of mice and for specificity determinations by ELISA.

mAb Production and Specificities for Individual HMW-GS

After immunizing mice with the different subunits, the spleen cells from the immunized mice were isolated and fused with myeloma cells. The supernatants from growing cells (200 wells/fusion averaged) were tested for reaction with HMW-GS from Chinese Spring and Suneca and tested for reaction with LMW-GS from Suneca. The test for LMW-GS was included to select mAb which react only with HMW-GS. Immunoblots of glutenin subunits from two cultivars were examined because they had different HMW-GS allelic compositions. In the screening, ~40% of the cell supernatants contained antibodies that bound glutenin subunits. Cell lines binding HMW-GS were propagated for further characterization and subcloning. The specificities of the monoclonal antibodies reacting with HMW-GS were

examined in separate experiments on immunoblots after SDS-PAGE separation of total glutenin from both Chinese Spring and Suneca flour. As expected from the degree of sequence homology between the different glutenin subunits (Shewry et al 1992), many mAb detected the full range of HMW-GS as well as several LMW-GS. A limited number of antibodies showed selectivity for HMW-GS or a subset of HMW-GS.

The main specificities and subtypes of lines selected from the different fusions are summarized in Table II. Most of the cell lines secreting antibody with greater specificities were derived from immunization with subunit 2. The fusions from immunizations with subunits 5 and 7 resulted in cell lines producing mAb of broad reactivity with all HMW-GS, thus examples of antibodies from these fusions are not listed in Table II. The mAb 140820 was not further investigated due to its poor secretion level. Cell lines with specificities for only a limited number of HMW-GS were selected, and the specificity further examined on blots of SDS-PAGE separated glutenins and by ELISA with the purified subunits. Figure 2 shows the results from Chinese Spring and Suneca glutenin subunit blots of 10 mAb. Several of these mAb detect only one or two of the subunits present on these blots. The ELISA using the purified HMW-GS available from the initial immunogen isolation showed similar specificity patterns (Fig. 3). The cross-reaction of the mAb 110622 and mAb 110208 in ELISA with subunit 12 was not noted on immunoblots. It is likely to be a reaction with contaminating subunit 2 that was present in this preparation. There were some discrepancies between both test systems. For instance, the lack of binding of subunits 2 and 7 in blots with mAb 110421 could have been caused by protein modification during preparation (SDS treatment of blotted glutenins) and different presentation of epitopes on nitrocellulose membranes compared with polystyrene surfaces. These influences have been observed before (Skerritt and Martinuzzi 1986) and therefore have to be considered in the final characterization and selection of a test format.

The specificities of some of the mAb reflect the higher sequence homology within x- or y-type subunits rather than between these groups (Smith et al 1994). For example, mAb 110208, 110421, 110510, 110622 and 110924 detect only subunits of the x-type in blots, whereas mAb 140705 and 141046 detect only representatives

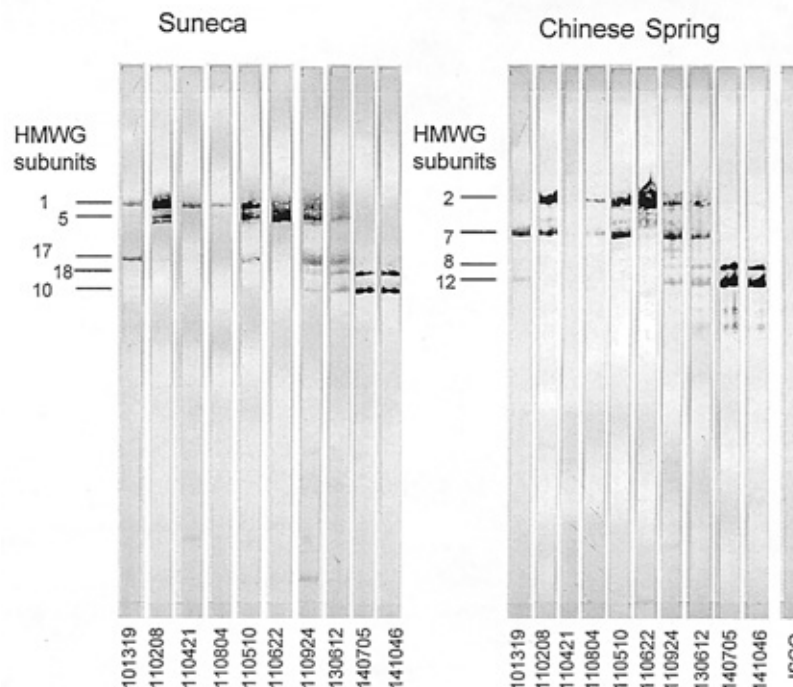


Fig. 2. Analysis of the specificities of selected monoclonal antibodies (mAb) on immunoblotting of total glutenin subunit extracts of Suneca and Chinese Spring. Mobilities of the major high molecular weight glutenin subunits (HMW-GS) are shown.

of the y-type. Interestingly, there was not a general link between the particular subunits used for immunization and the specificity of the resulting mAb. This reflects the broad specificity of the immune response. Some of the more common epitopes could be more immunogenic and the rare subunit-specific antibody producing cells could be missed during cell fusion and screening. The band intensities on blots and the ELISA results (Figs. 2 and 3) indicate that antibodies detecting several subunits vary in their apparent affinity for the subunits. Due to these differences in apparent affinity, it was considered likely that some antibodies are able to discriminate between wheat samples of differing HMW-GS compositions.

Differentiation of Wheat Flours

The full panel of antibodies was assessed with a set of 32 biotype flours from the Halberd × Cranbrook doubled haploid set. Because it comprised four samples of each allelic combination of HMW-GS, pooling the results from groups of 16 flours with a similar allele at a given locus, the ability of the antibodies to distinguish the products of different HMW-GS alleles could be established. Similar results were seen with extracts of flours prepared in 50% isopropanol and 50 mM dithiothreitol (DTT) with or without gliadin preextraction in 50% isopropanol. For brevity, only results obtained without preextraction are shown (Table III). The mAb 110622 and 101319 significantly discriminated individual glutenin subunits encoded by different alleles, and in one case (110804), discrimination of marginal statistical significance was observed. The mAb 101319 raised to HMW-GS 1 displayed some interesting properties, it recognized some 1Bx subunits and subunit 1Ax on immunoblots. In the ELISA with flour samples, it bound the products of the *Glu-B1e* locus better than the products of the *Glu-B1i* locus (Table III). Several alleles of *Glu-B1* locus are known, expressing a number of HMW-GS patterns. The ability of this antibody to discriminate products of other alleles requires further investigation. The mAb 110622 prepared to HMW-GS 2 always showed a stronger signal with bands of subunit 2 than with subunit 5 on blots. In ELISA, this antibody reacted more strongly with purified subunit 2 than with subunit 5, and in wheat flour samples, the discrimination between subunits 2+12 versus 5+10 was even stronger and statistically significant (Table III).

A final type of specificity pattern was noted for mAb 110208 and 110510. These antibodies did not show selectivity for a single subunit but did discriminate groups of wheats with particular combinations of two alleles. When the data was grouped on the basis of variation at single loci (Table III), this type of specificity pattern manifests as high standard deviations in ELISA absorbance readings, but the variation in binding to extracts of different flours was not random (Table IV). For example, mAb 110208 bound less strongly to glutenin extracts of lines that contained both the *Glu-A1a* and *Glu-B1i* loci than to those with other *Glu-A1* and *Glu-B1* combinations (Table IV), while 110510 showed lower binding two of the four combinations (*Glu-A1a/B1i* and *Glu-A1b/B1e*).

The mAb 110421 which appeared to show selectivity on Suneca immunoblots for subunit 1 and mAb 110924 which detected subunits 1 and 5 and to a lower extent 17 on Suneca and subunits 2 and 7 on Chinese Spring blots were tested with flour extracts from biotype lines for specificity for *Glu-A1* products because biotypes were available in three cultivars that express none of the *Glu-A1* HMW-GS. However, these antibodies did not differentiate wheat lines expressing subunits 1 or 2* from the null biotypes (which did not express of *Glu-A1* products). The lack of discrimination at this locus is probably due to the detection of *Glu-B1* locus products by both antibodies (110421 and 110924).

Optimization of the *Glu-D1* Allele Assay

These results indicate that of the antibodies tested, only mAb 110622 was able to discriminate wheats differing in allelic composition at *Glu-D1*, namely the *Glu-D1d* allele (HMW-GS 5+10) from wheats expressing the *Glu-D1a* allele (HMW-GS 2+12). Because these alleles are the most clearly associated with dough properties (Payne et al 1987), the method for their discrimination using ELISA with mAb 110622 was further optimized. Initial investigations were intended to establish whether gliadin preextraction was required for optimal discrimination of samples. Analysis of six pairs of *Glu-D1a/d* biotypes (Fig. 4) suggested that preextraction was not only unnecessary, but discrimination was actually slightly improved by omitting this step. This result probably arises from selective, partial solubilization of the smaller glutenin poly-

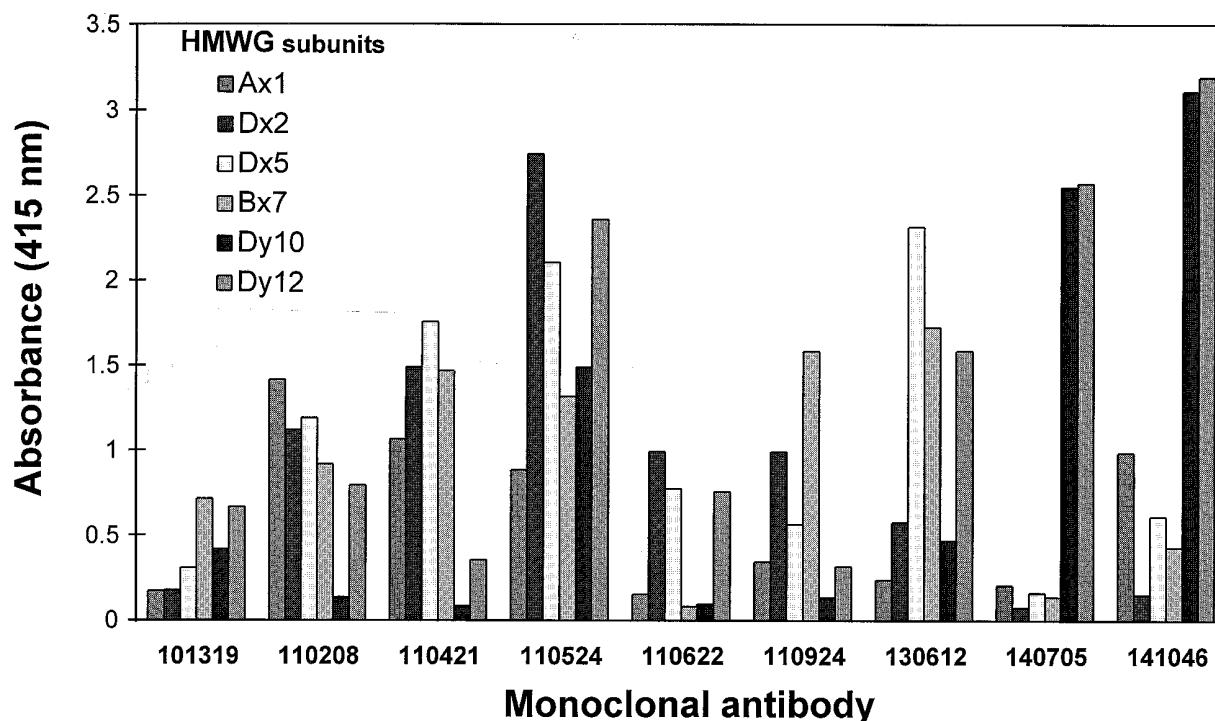


Fig. 3. Reaction of selected monoclonal antibodies (mAb) in enzyme-linked immunosorbent assay (ELISA) using purified high molecular weight glutenin subunits (HMW-GS) as coating antigen.

mers containing HMW-GS 2+12 (Bangur et al 1997) by the aqueous propanol preextraction step and their resultant removal from the sample. The influence of gliadins on the results can be excluded because the mAb exhibited no cross-reactivity with purified gliadins from either Suneca and Chinese Spring wheats. Because the antibody discriminated the HMW-GS alleles through detection of HMW-GS 2, the selective depletion of this subunit reduces discrimination of samples. A similar result was obtained in two experiments using extracts of 16 Halberd × Cranbrook doubled haploid lines: optical density for preextracted 2+12 lines = 1.01 ± 0.16, and for 5+10 lines = 0.70 ± 0.11; without preextraction 2+12 lines = 1.49 ± 0.12, 5+10 lines = 0.88 ± 0.12 (means ± standard deviation of eight lines per set). Subsequent analyses of flour samples were performed without an aqueous propanol preextraction step. Dilution of sample extracts and antibody concentration was varied. Discrimination of 2+12 and 5+10 lines, as determined by the ratio of assay color for the 2+12 lines to that produced by the 5+10 lines, was constant within the range 1:20 to 1:250 sample extract dilution. At dilutions <1:10 discrimination was poor, while at >1:250 the color development in the assay was insufficient. Variation of antibody concentration also had little effect, although discrimination appeared optimal at 1µg/mL.

Because it appeared that sample extraction conditions could affect the discrimination of samples, other extractants were assessed. Flour samples were extracted using either 50% *n*-propanol, 70% ethanol, and 50 mM acetic acid; centrifuged; and the extracted protein in the supernatant was reduced by addition of DTT to 50 mM. However, although these solvents have reportedly provided some selectivity in their extraction of glutenin subunits (Dupuis and Bushuk 1996), none of these treatments provided superior discrimination over that obtained using direct extraction with aqueous propanol and DTT. Next, variation in the concentration of DTT was examined because stepwise reduction studies have shown that subunits encoded by *Glu-D1* are extensively involved in oligomer formation (Lawrence and Payne 1983) and the differences in the reductive solubilization of different HMW-GS exist between flours (Kim and Bushuk 1995),

especially at >1 mM DTT (Lindsay and Skerritt 1998). Flours from a set of eight doubled haploid lines were extracted using 1, 5, and 50 mM DTT (Fig. 5). Decreasing the reductant concentration did not alter the response obtained with the HMW-GS 2+12 lines but caused stepwise decreases in the response of the HMW-GS 5+10 lines, thus leading to an increase in the ratio of absorbances obtained and an improvement in the discrimination of the samples. The lower color obtained with the HMW-GS 5+10 lines at low DTT concentrations could either arise from a decrease in the extraction of HMW-GS or, more likely, incomplete reduction of these subunits (and shielding of the antibody binding sites) at 1 mM DTT. We have found that unreduced glutenin is poorly recognized by mAb 110622.

The final optimization experiments examined the role of the nature of the protein in the sample diluent, with 1% BSA being compared with 1% BSA plus 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) with a set of 16 flour samples from the doubled haploid population (Fig. 6). The higher protein diluent slightly (but consistently) increased the differentiation of the flours with different *Glu-D1* alleles, again decreasing the average color produced by the HMW-GS 5+10 samples but not the HMW-GS 2+12 samples. Increasing the concentration of BSA or using high concentrations of other proteins such as gelatin had similar effects (data not shown). Added proteins often reduce interference of unspecific binding of antibodies to the polystyrene surface and thus enhance discrimination efficiency of ELISA tests.

If the assay using mAb 110622 is to successfully discriminate lines with different *Glu-D1* alleles, it is important that variation in other *Glu-1* alleles does not affect this discrimination. This was examined by analysis of flours from 32 lines of the Halberd × Cranbrook doubled haploid population, which provided all eight possible allelic combinations at each of the three *Glu-1* loci (Fig. 7). It was evident that only variation at *Glu-D1* was detectable, and that variation in the other alleles did not affect the result. The assay also discriminated lines with 5+10 from 2+12 in a population of F8 lines from a cross between parents of similar dough strength (Janz and Hartog) but with HMW-GS 2+12 and 5+10, respectively (Fig. 8).

TABLE III
Differentiation of Doubled Haploid Lines from Halberd × Cranbrook Using Monoclonal Antibodies

Immunogen Antibody	Sample Extract Dilution	Subunit Comparison ^a					
		<i>Glu-A1a</i> 1	<i>Glu-A1b</i> 2*	<i>Glu-B1e</i> 20	<i>Glu-B1i</i> 17+18	<i>Glu-D1a</i> 2+12	<i>Glu-D1d</i> 5+10
HMW-GS 1							
101319	1:20	0.61 ± 0.20	0.59 ± .18	0.78 ± 0.06	0.43 ± 0.04**	0.58 ± 0.16	0.64 ± 0.20
HMW-GS 2							
110421	1:20	0.37 ± 0.06	0.34 ± 0.04	0.39 ± 0.04	0.32 ± 0.02	0.34 ± 0.04	0.37 ± 0.05
110804	1:20	0.22 ± 0.03	0.19 ± 0.03	0.23 ± 0.03	0.18 ± 0.02*	0.20 ± 0.03	0.22 ± 0.04
110924	1:500	1.49 ± 0.07	1.52 ± 0.09	1.48 ± 0.07	1.53 ± 0.09	1.48 ± 0.11	1.52 ± 0.06
110208	1:1000	1.82 ± 0.40	2.06 ± 0.24	2.16 ± 0.20	1.73 ± 0.34	1.90 ± 0.33	1.98 ± 0.36
110622	1:200	1.09 ± 0.28	1.14 ± 0.32	1.13 ± 0.25	1.10 ± 0.33	1.36 ± 0.09	0.81 ± 0.11**
110510	1:200	1.54 ± 0.30	1.67 ± 0.46	1.54 ± 0.32	1.66 ± 0.44	1.58 ± 0.39	1.66 ± 0.39
HMW-GS 10							
130612	1:5000	0.81 ± 0.12	0.87 ± 0.09	0.78 ± 0.09	0.90 ± 0.08	0.84 ± 0.10	0.84 ± 0.11
HMW-GS 12							
140705	1:1000	2.40 ± 0.26	2.35 ± 0.21	2.43 ± 0.25	2.33 ± 0.21	2.28 ± 0.22	2.50 ± 0.19
141046	1:200	2.24 ± 0.28	2.43 ± 0.26	2.44 ± 0.25	2.20 ± 0.30	2.20 ± 0.28	2.47 ± 0.26

^a * = *P* < 0.05, ** = *P* < 0.01, Student's *t*-test. Data for samples extracted directly in 50% isopropanol and 50 mM dithiothreitol (DTT).

TABLE IV
Differentiation of High Molecular Weight Glutenin Subunits (HMW-GS) Expressed by Combinations of *Glu-A1* and *Glu-B1* Alleles

Antibody	Sample Extract Dilution	Subunit Combinations ^a			
		<i>Glu-A1a</i> and <i>Glu-B1e</i> 1 and 20	<i>Glu-A1a</i> and <i>Glu-B1i</i> 1 and 17+18	<i>Glu-A1b</i> and <i>Glu-B1e</i> 2* and 20	<i>Glu-A1b</i> and <i>Glu-B1i</i> 2* and 17+18
110208	1:1000	2.21 ± 0.15a	1.48 ± 0.14b	2.10 ± 0.24a	2.02 ± 0.25a
110510	1:200	1.80 ± 0.19a	1.31 ± 0.15b	1.28 ± 0.18b	2.06 ± 0.29a

^a Means denoted by different letters (for a particular antibody) are significantly different from one another (*P* < 0.05) by the Student's *t*-test. Samples are doubled haploid lines from Halberd × Cranbrook using monoclonal antibodies. Data for samples extracted directly in 50% isopropanol and 50 mM dithiothreitol (DTT).

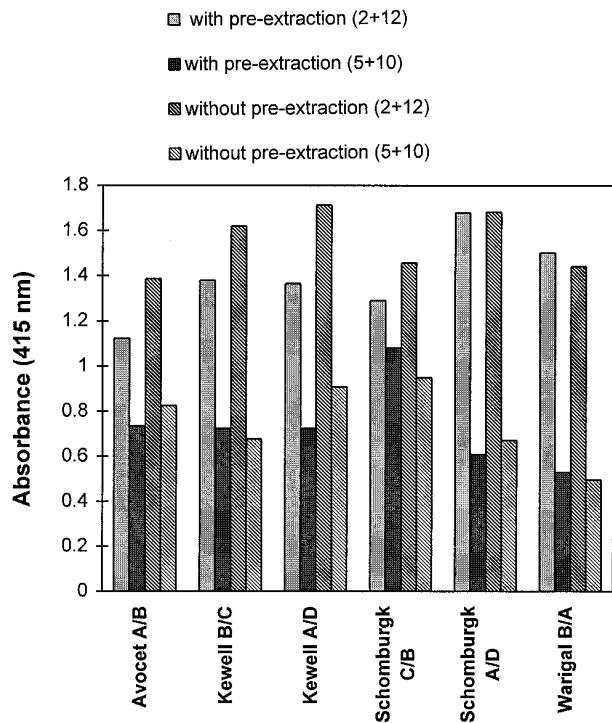


Fig. 4. Effects of aqueous propanol preextraction of flours on discrimination of biotypes varying in *Glu-D1* high molecular weight glutenin subunits (HMW-GS) composition. Data are from triplicate determinations, experiment was repeated with similar results. HMW-GS composition of the biotypes: Avocet A (7+8, 2+12), Avocet B (7+8, 2+12), Kewell B (1, 20, 2+12), Kewell C (1, 20, 5+10), Kewell A (2*, 20, 2+12), Kewell D (2*, 20, 5+10), Schomburgk C (1, 7+9, 2+12), Schomburgk B (1, 7+9, 5+10), Schomburgk A (1, 7+8, 2+12), Schomburgk D (1, 7+8, 5+10), Warigal B (7+8, 2+12), Warigal A (7+8, 5+10).

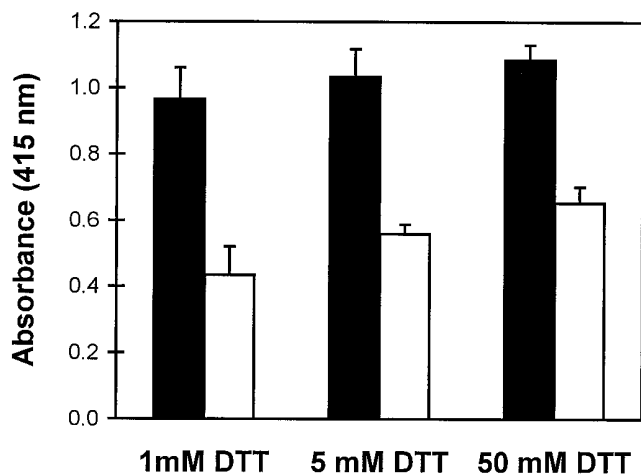


Fig. 5. Effects of variation in dithiothreitol (DTT) concentration on discrimination of eight Halberd × Cranbrook flours with different *Glu-D1* allelic compositions. Data are means ± standard deviation from five flours with high molecular weight glutenin subunits (HMW-GS) 2+12 (light gray) and three flours with HMW-GS 5+10 (dark gray). Experiment was repeated with similar results.

CONCLUSION

The use of purified HMWG subunits (which were unalkylated and had not been exposed to SDS detergent) for immunization resulted in several mAb being isolated that were quite selective for particular glutenin subunits. These should be useful in quantitative analysis of the expression of different subunits and in

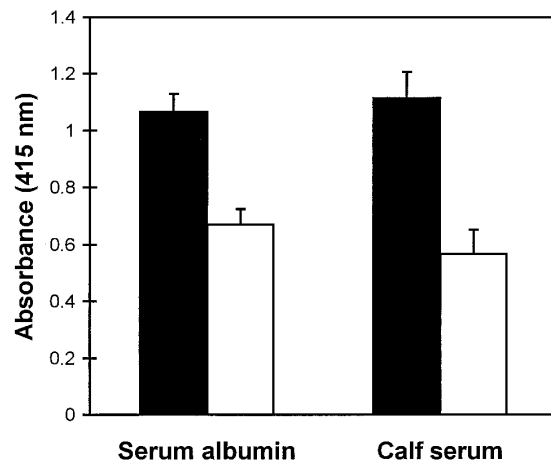


Fig. 6. Comparison of 1% bovine serum albumin (BSA) with 1% BSA plus 5% fetal calf serum for discrimination of 16 Halberd × Cranbrook flours with different *Glu-D1* allelic compositions. Data are means ± standard deviation from eight flours with high molecular weight glutenin subunits (HMW-GS) 2+12 (light gray) and eight flours with HMW-GS 5+10 (dark gray). Experiment was repeated twice with similar results.

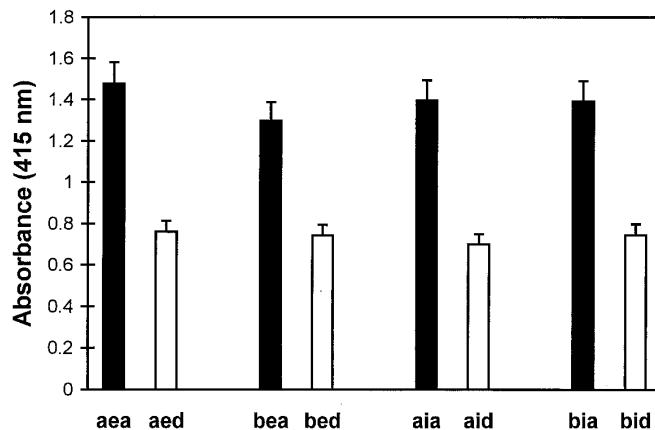


Fig. 7. Variation in the composition of other high molecular weight glutenin subunits (HMW-GS) alleles do not affect discrimination of *Glu-D1* alleles for 16 Halberd × Cranbrook flours with different *Glu-D1* allelic compositions. Data are means ± standard deviation from 32 flours, four of each possible HMW-GS composition. Experiment was repeated with similar results.

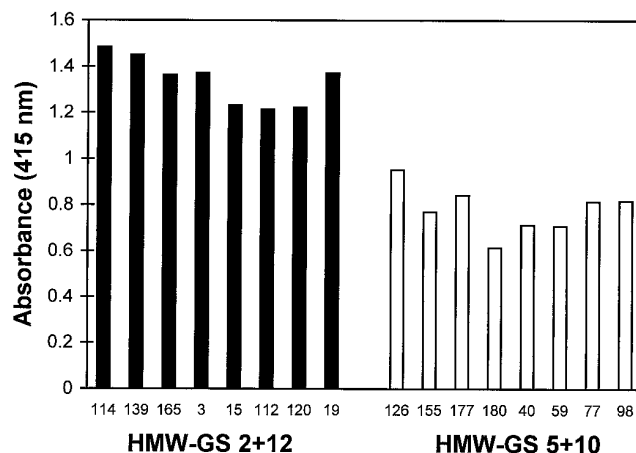


Fig. 8. Application of the *Glu-D1* assay to cross-bred lines from a Janz (high molecular weight glutenin subunits [HMW-GS] 2+12) × Hartog (HMW-GS 5+10) cross. Experiment was repeated with similar results.

studies of the structure of the glutenin polymer. However, because many of the antibodies bound similarly to HMW-GS expressed by different alleles at the locus, these mAb are not useful in diagnostics for those alleles.

Given the >98.7% sequence homology between subunits 2 and 5 (Shewry et al 1992), we were fortunate to isolate one mAb (110622) that could discriminate wheat cultivars with the *Glu-D1* allelic pairs of subunits 5+10 and 2+12. This distinction is of special interest because allelic variation at *Glu-D1* has the most marked effect on dough properties. Another mAb (101319) also discriminated wheats differing in *Glu-B1* alleles 17+18 and 20, and the ability of this antibody to discriminate other *Glu-B1* alleles requires further study.

We have adapted mAb 110622 into a simple ELISA for analysis of flour or wholemeal and demonstrated its ability to discriminate wheats in three sets of samples. In its optimized form, the assay provides ≈2:1 discrimination between lines with 5+10 and 2+12, although this can be poorer in sets of wheats with widely varying protein concentration or if the extraction of HMW-GS varies (A. S. Hill, D. Iglesias, and J. H. Skerritt, unpublished data). While further optimization of sample preparation for the assay will be undertaken, we are attempting to manipulate the antibody itself through isolation of the genes encoding the variable domains of the antibody (Hill et al 1996) and mutagenesis of the antigen-binding (complementarity-determining) regions. This approach could potentially be used to modify or improve the specificities of other selective, but not specific, antibodies to develop a new generation of diagnostic tests for the wheat industry.

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