

# Two-Site Sandwich ELISA for Discriminating Different *Gli-1* (gliadin)/*Glu-3* (LMW-glutenin subunit) Alleles in Hexaploid Wheat

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## ABSTRACT

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A panel of monoclonal antibodies was assessed in a two-site sandwich ELISA format, using both reduced glutenin subunit and gliadin-rich antigen preparations, to develop assays that could potentially discriminate between *Gli-1/Glu-3* allelic variants in hexaploid wheat. Each antibody was assessed as the immobilized and the enzyme-labeled antibody in the sandwich ELISA. A number of antibody combinations were identified which could discriminate different *Gli-1/Glu-3* allelic variants in a population of doubled haploid lines derived from a cross between parents that differed at each of these loci. Certain labeled antibodies consistently detected allelic variation at a particular locus

when used in conjunction with any of several immobilized antibodies. However, the level of discrimination was largely dependent on the choice of immobilized antibody. Two antibody combinations were identified that provided twofold differences in ELISA absorbances in flour extracts from different allelic variants at the *Gli-A1/Glu-A3* and *Gli-B1/Glu-B3* loci. By analyzing the prolamins of the antigen preparations, and the performance of the assays with flour extracts from a set of *Gli-1/Glu-3* biotypes and a range of diverse cultivars, the biochemical basis for the discrimination was determined. The assays may have potential for use in high-throughput screening in wheat breeding programs.

The low molecular weight glutenin subunits (LMW-GS) are encoded on the short arms of group one chromosomes at the *Glu-3* loci. Individual alleles at these loci encode between zero and eight closely related polypeptides (Gupta and Shepherd 1990) and different alleles may encode a subset of polypeptides with the same molecular weight and N-terminal sequence (Gupta and Shepherd 1990; Sissons et al 1999). The *Gli-1* gliadin-encoding loci are tightly linked to the *Glu-3* loci on the short arms of group 1 chromosomes (Singh and Shepherd 1988). Over the last 10–15 years, evidence of the effect of *Gli-1/Glu-3* allelic variation on dough properties in both genetic and correlative studies (Payne et al 1987; Gupta et al 1989, 1991, 1994; Khelifi and Branlard 1992; Gupta and MacRitchie 1994; Morel et al 1994; Nieto-Taladriz and Bouguennec 1994) has identified polypeptides encoded by these loci as being important determinants of grain quality. Highly specific monoclonal antibodies (mAb) may present an alternative to conventional methods for identification of LMW-GS composition. Antibodies have the advantage of being adaptable to high throughput assay formats and require only small quantities of grain for the analysis (Skerritt 1991).

The close genetic linkage and the distinct solubility characteristics of the gliadins present them as an alternative target antigen for assays to discriminate between LMW-GS encoding alleles. A number of antibody-based assays have been described for discriminating between allelic variants at prolamins-encoding loci (Skerritt and Underwood 1986; Dawood et al 1989; Howes et al 1989; Skerritt et al 1991). Glutenin subunit antigens have also been used in antibody assays to discriminate allelic variants. In durum wheat, Kovacs et al (1995) used LMW-GS-specific mAb to sort F<sub>2</sub>-derived F<sub>4</sub> and F<sub>5</sub> families for the allelic variants LMW-GS 2 and LMW-GS 2' associated with good and poor pasta making properties, respectively, while assays that discriminate particular high molecular weight glutenin subunits (HMW-GS) have also been developed (Kovacs et al 1993; Giersch et al 1999). A number of these tests used an indirect immunoassay format that incorporates only one antibody and relies upon recognition of an immobilized target antigen within a diluted

extract of flour or wholemeal, which had earlier been coated onto a microwell. Given the sequence and structural similarity of the LMW prolamins to one another, we wanted to establish whether it was necessary to employ an alternative assay format that made use of the specificity of a pair of mAb and to target different antigen types in assays to discriminate between allele types at the *Gli-1/Glu-3* loci in hexaploid wheat. A panel of mAb, prepared to a range of prolamins fractions, was available in the laboratory. The ability of combinations of these antibodies to discriminate between LMW-GS alleles was assessed in a sandwich ELISA format. Although the gliadins and glutenin subunits are of low aqueous solubility, two-site sandwich assays have been successfully developed for their analysis (Hill and Skerritt 1989; Skerritt et al 1989) and applied in tests for dough strength (Skerritt 1991) and extensibility (Andrews and Skerritt 1996). This assay format involves immobilization of one specific antibody on the surface of a microwell, followed by addition of antigen (diluted extract of flour or wholemeal), then a second specific antibody. Because the assay requires two antibody-antigen interactions, it was postulated that the specificity of the assay and its ability to discriminate different antigens (and thus different LMW-GS alleles) would be enhanced.

## MATERIALS AND METHODS

### Flour Samples

Optimization of sandwich ELISA conditions was performed with glutenin subunit-rich and gliadin-rich preparations extracted from cv. Aroona wholemeal. Analysis of sandwich ELISA performance with *Gli-1/Glu-3* biotypes was performed with antigen from eight Aroona lines and two parental biotypes, Aroona A and Aroona B, differing in HMW-GS composition. These lines were kindly provided by K. W. Shepherd, University of Adelaide, South Australia. Each biotype varied at one locus from the Aroona A parental type (prolamin-encoding allele) designations *Glu-A1a* (1), *Glu-B1c* (7+9), *Glu-D1a* (2+12), *GliA1a/Glu-A3c*, *GliB1b/Glu-B3b*, *GliD1i/Glu-D3c*. The gliadin/LMW-GS-encoding alleles represented in the biotypes are *Gli-A1f/Glu-A3a*, *Gli-A1o/Glu-A3d*, *Gli-A1m/Glu-A3e*, *Gli-B1a/Glu-B3a*, *Gli-B1i/Glu-B3c*, *Gli-B1h/Glu-B3d*, *Gli-B1d/Glu-B3h*, *Gli-D1a/Glu-D3g*. Indirect and sandwich ELISA were performed on extracts of flour from a series of doubled haploid lines derived from a cross between cvs. Cranbrook and Halberd, grown at Roseworthy, South Australia. These lines were provided by G. B. Cornish (South Australian R&D Institute, Adelaide) and were developed under the Australian National Wheat Molecular Marker Program. The glutenin subunit compositions of the parent lines are Cranbrook *Glu-A1b* (2\*), *Glu-B1i* (17+18), *Glu-D1a* (2+12), *Gli-*

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*Alm/Glu-A3e*, *Gli-B1i/Glu-B3c*, *Gli-D1i/Glu-D3c*; Halberd *Glu-A1a (1)*, *Glu-B1e (20)*, *Glu-D1d (5+10)*, *Gli-A1g/Glu-A3b*, *Gli-B1h/Glu-B3d*, *Gli-D1a/Glu-D3a*. Seed from cv. Cadoux (*Glu-3* alleles *bba*), Egret (*ebb*), Janz (*bbb*), Katepwa (*ehc*), Neepawa (*ehc*), RAC 820 (*bhb*), Spear (*chc*), Sunco (*bhb*), Sunstar (*cbh*), Tasman (*bda*), and the CD87 (*bba*) × Hartog (*bhb*) doubled haploid population was provided by P. Brennan, Leslie Research Centre, Toowoomba, QLD, Australia. Seed from cv. Chinese Spring (*aaa*) was provided by R. L. McIntosh, Sydney University Plant Breeding Institute, Cobbitty, NSW, Australia. Seed from cv. Gabo (*bbb*) was provided by G. Lawrence, CSIRO Plant Industry.

### Prolamin Composition in the Doubled Haploid Population

Fifty-five lines from the Cranbrook × Halberd population were selected for screening in the two-site sandwich ELISA. The *Glu-1* and *Gli-1/Glu-3* allele designations for each of the lines were checked by SDS- and acid-PAGE, to confirm the correct allele designations in the lines. For SDS-PAGE separation of glutenin subunits, monomeric components were removed from flour (40 mg) with three preextractions using 1.5 mL of 50% (v/v) aqueous 1-propanol by vortex mixing and then shaking for 30 min at 20°C. Glutenin subunits were extracted from the pellet with 200 µL of 50% (v/v) aqueous 1-propanol, 125 mM Tris-HCl, pH 7.5, containing 1% dithiothreitol (DTT) for 1 hr at 65°C. Polypeptides were alkylated by addition of 4-vinylpyridine to 87 mM final concentration. Extracts were mixed with an equal volume of sample buffer concentrate (250 mM Tris-HCl, pH 7.5, 4% w/v SDS, 20% v/v glycerol) and samples were fractionated on 12% T (total acrylamide plus bisacryl-

amide concentration), 2% C (crosslinked) polyacrylamide gels, run at constant voltage for 1,800 Vhr. Stacking gels were 6% T and 2% C. Gels were stained in colloidal Coomassie G-250 (BioRad, Hercules, CA) in methanol-ammonium sulfate-phosphoric acid (Neuhoff et al 1988). For acid-PAGE separation of gliadins, acrylamide gels (6.3% T, 4.3% C) were pre-electrophoresed in sodium lactate buffer (4.25 mM NaOH buffered with lactic acid to pH 3.1) at 400 V for 2 hr (25–30°C). Proteins were extracted from flour (20 mg) with 1M urea (120 µL). The extract was centrifuged (13,180 × g, 10 min) and aliquots of 3 µL were applied to the wells. Gliadins were separated at 320 V for 5 hr at 25–30°C (Skerritt and Underwood 1986).

### Antigen Extraction and Protein Determination

For glutenin subunit antigen preparations, gliadin and other soluble proteins were removed from flour (20 mg) by three preextractions with 1.5 mL of 50% (v/v) aqueous 1-propanol with vortex mixing. Each extraction took place over 30 min, followed by centrifugation (13,180 × g, 10 min). Glutenin subunits were extracted from the pellet with 50% (v/v) aqueous 1-propanol, 125 mM Tris-HCl, pH 7.5, 100 mM DTT (200 µL, 1 hr, 65°C). The extract was centrifuged and the supernatant collected and used as glutenin subunit antigen. A gliadin-rich fraction was prepared by extracting flour (20 mg) with 1.5 mL of 50% (v/v) aqueous 1-propanol with vortex mixing over 30 min. The total protein concentration of all extracts was determined by Coomassie G-250 dye binding assay (Bradford 1976). To ensure accurate protein determination, samples were assayed in quadruplicate in duplicate experiments each with independent standards (50–500 µg/mL of bovine serum albumin).

TABLE I  
Antibodies Selected for Two-Site Sandwich ELISA with *Gli-1/Glu-3* Biotypes

MAb	Clone	Isotype	Glutenin Subunits <sup>a</sup>			Gliadin
			A	B	C	
Broad LMW-GS selective						
40116	1C9	IgG1	* weak	**	**	broad
40406	1A2	IgG1	–	***	**	β, γ
46489	1A5	IgM	–	broad	–	broad
76133	5D2	IgG1	–	***	***	γ, ω
76136	5A5	IgG1	–	***	**	γ, ω
76336	5B6	IgG1	–	***	***	γ, ω
79115	5C5	IgM	* x-type	**	*	broad
82208	20F6	IgG1	–	***	**	γ, slow ω
B-LMW-GS selective						
21817	7E8	IgG1	*	**	*	γ, 1BS fast ω
42327	5G5	IgG1	–	*	–	α, β
78233	5A10	IgG1	–	**	*	β, γ, fast ω
80112	2E8	IgG1	–	**	–	γ, ω
80217	10A5	IgM	–	**	*	nt <sup>b</sup>
80512	10C2	IgG1	–	**	*	α, β, γ
80818	20E5	IgM	–	**	*	ω
82123	5D5	IgG3	–	**	–	β, γ
92689	5B7	IgM	–	**	–	broad
96355	3G6	IgG1	–	**	*	β, γ, ω
C-LMW-GS selective						
12224	1A9	IgG1	–	*	***	slow ω
40308	3G2	IgG1	*	*	*	β, γ
77811	5B5	IgM	–	*	***	1BS β, γ
Gliadin selective						
12208	4H2	IgG1	–	*	*	ω>γ
22123	8D11	IgG1	–	–	**	α
22205	10B2	IgG1	** weak	*	**	β, γ
23009	1A5	IgG1	*	*	*	6AS α
30413	1B2	IgG1	**	–	–	fast ω
70846	20F6	IgM	–	–	–	α, slow ω
71945	5C6	IgM	–	**	*	α,β,γ, fast ω
73025	10C7	IgM	–	–	–	α, slow ω
75212	5C10	IgM	–	–	–	β
80603 <sup>c</sup>	2B7	IgM	* x-type	*	–	β, 1DS slow ω

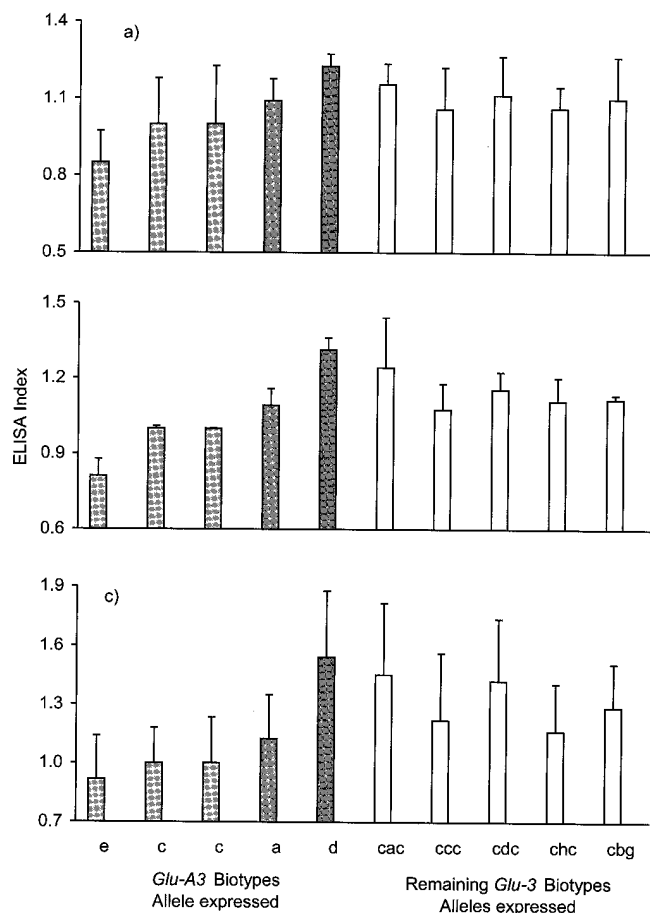
<sup>a</sup> (–) = no reaction, \* = 1–2 bands, \*\* = 3–5 bands, \*\*\* = > 5 bands.

<sup>b</sup> Not tested.

<sup>c</sup> Selective for HMW-GS.

## Two-Site Sandwich ELISA

Antibodies used in preliminary optimization experiments for the two-site sandwich ELISA and the gliadin and glutenin subunit specificities are listed in Table I. Selection of mAb was based on specificity for LMW-GS. Antibodies that cross-reacted strongly with



**Fig. 1.** Recognition of Aroona biotypes varying at the *Glu-A3* locus in two-site sandwich ELISA with mAb combinations a) 82208–22123, b) 82208–82123, and c) 77811–82123. Data are averages of duplicate determinations from three independent experiments ( $\pm$  SD) expressed as a fraction of the OD value obtained with the mean of the Aroona A and B controls. Letters in the legend are *Glu-3* biotypes *Glu-A3e*, *Glu-A3c* (replicate 1), *Glu-A3c* (replicate 2), *Glu-A3a*, *Glu-A3d*, and glutenin allele combinations incorporating *Glu-A3c* (cac, ccc, cdc, chc, cbg).

HMW-GS were avoided, although some HMW-GS reactive antibodies with narrow LMW-GS selectivity were included. Peroxidase labeling of antibodies and the two-site sandwich ELISA format was described earlier by Hill and Skerritt (1989). Assay blanks were determined for both the absence of immobilized antibody and the absence of antigen (diluted flour sample). Preliminary optimization of the sandwich ELISA used antigen extracted from Aroona wholemeal. The mAb were tested in sandwich ELISA as both immobilized “capture” and peroxidase-labeled antibody in a checkerboard format with three antigen concentrations and three labeled antibody dilutions to determine the optimal reaction conditions. Antibody combinations (441) were screened with glutenin subunit antigens and 361 were screened with gliadin-rich antigens. For each mAb combination, antigen concentrations and labeled antibody dilutions at optimal reactivity, defined as 40–70% of maximal color development (i.e., the steep part of the antigen concentration-color response curve) were used for subsequent experiments.

## RESULTS AND DISCUSSION

Of the 802 mAb combinations tested in the sandwich ELISA, only  $\approx$ 45% functioned (provided OD of  $>0.1$ ) as either immobilized or labeled antibody when screened with antigen extracted from Aroona wholemeal. Lack of reaction may have been due to any of a number of causes such as the alteration or loss of antibody binding epitopes through either denaturation of mAb by passive adsorption to polystyrene solid phase (Butler et al 1992) or following antibody labeling with horseradish peroxidase. Alternatively, combinations may not have functioned because two mAb bound different antigen groups, or because they bound the same or a neighboring epitope causing steric hindrance to binding of the labeled antibody (Skerritt et al 1989). Combinations that functioned well were screened with either gliadin-rich or glutenin subunit preparations extracted from Aroona *Gli-1/Glu-3* biotypes. Of those tested, 26% showed a good degree of differentiation between the lines and were screened with these preparations extracted from flours from the Cranbrook  $\times$  Halberd doubled haploid population.

### Glutenin Subunit Extracts from Biotypes at Individual Loci

The *Gli-1/Glu-3* biotypes each had an individual LMW-GS-encoding allele substituted in an Aroona background (*Glu-A3c*, *Glu-B3b*, *Glu-D3c*). These lines provided a convenient tool for assessing the ability of mAb to detect allelic variation in LMW-GS in a constant genetic background, and allowed analysis of the response of a large number of mAb combinations with a range of allelic variants at a locus. Thirty one LMW-GS-specific antibodies in 162 combinations were tested. The primary criterion for selecting antibody combinations for subsequent experiments with other sets of lines

**TABLE II**  
Differentiation of Alleles at LMW-GS Encoding Loci with Glutenin Subunit Antigen Using Two-Site Sandwich ELISA

Antibodies <sup>a</sup>		Subunit Comparison <sup>b</sup>					
Immobilized	Labeled	<i>Glu-A3b</i>	<i>Glu-A3e</i>	<i>Glu-B3c</i>	<i>Glu-B3d</i>	<i>Glu-D3a</i>	<i>Glu-D3c</i>
12224	22123	0.48 $\pm$ 0.08	0.41 $\pm$ 0.05**	0.43 $\pm$ 0.08	0.45 $\pm$ 0.07	0.45 $\pm$ 0.08	0.43 $\pm$ 0.08
12224	82123	0.43 $\pm$ 0.05	0.32 $\pm$ 0.06**	0.34 $\pm$ 0.08	0.41 $\pm$ 0.07*	0.39 $\pm$ 0.08	0.36 $\pm$ 0.08
22123	82123	0.41 $\pm$ 0.05	0.31 $\pm$ 0.04**	0.34 $\pm$ 0.08	0.37 $\pm$ 0.05	0.36 $\pm$ 0.07	0.36 $\pm$ 0.06
22205	82123	0.33 $\pm$ 0.04	0.24 $\pm$ 0.04**	0.26 $\pm$ 0.06	0.30 $\pm$ 0.05*	0.29 $\pm$ 0.06	0.27 $\pm$ 0.06
40406	22123	0.42 $\pm$ 0.06	0.35 $\pm$ 0.06**	0.36 $\pm$ 0.07	0.40 $\pm$ 0.07	0.39 $\pm$ 0.08	0.37 $\pm$ 0.05
77811	22123	0.50 $\pm$ 0.06	0.39 $\pm$ 0.05**	0.43 $\pm$ 0.08	0.45 $\pm$ 0.08	0.45 $\pm$ 0.07	0.43 $\pm$ 0.08
77811	82123	0.37 $\pm$ 0.06	0.24 $\pm$ 0.05**	0.28 $\pm$ 0.08	0.33 $\pm$ 0.08	0.33 $\pm$ 0.08	0.28 $\pm$ 0.09
80512	22123	0.42 $\pm$ 0.05	0.33 $\pm$ 0.07**	0.36 $\pm$ 0.08	0.39 $\pm$ 0.07	0.37 $\pm$ 0.08	0.38 $\pm$ 0.08
80512	96355	0.77 $\pm$ 0.11	0.78 $\pm$ 0.15	0.73 $\pm$ 0.11	0.83 $\pm$ 0.13*	0.78 $\pm$ 0.14	0.78 $\pm$ 0.12
80603	82123	0.63 $\pm$ 0.10	0.56 $\pm$ 0.10*	0.55 $\pm$ 0.05	0.64 $\pm$ 0.09**	0.59 $\pm$ 0.12	0.60 $\pm$ 0.10
82208	22123	0.53 $\pm$ 0.07	0.40 $\pm$ 0.06**	0.46 $\pm$ 0.10	0.47 $\pm$ 0.09	0.46 $\pm$ 0.09	0.46 $\pm$ 0.10
82208	82123	0.61 $\pm$ 0.07	0.48 $\pm$ 0.08**	0.53 $\pm$ 0.12	0.55 $\pm$ 0.08	0.56 $\pm$ 0.10	0.53 $\pm$ 0.10

<sup>a</sup> Data are averages of duplicate determinations from 30 doubled haploid lines ( $\pm$  SD).

<sup>b</sup> \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , Student's *t*-test. Experiments repeated using an independent set of 30 lines with similar results.

was the degree of discrimination between the various biotypes. One of the most consistent findings was that many antibody combinations had low levels of binding to biotypes expressing the *Glu-A3e* (null) allele. For a number of mAb combinations, the order of increasing ELISA signal corresponded to the number of subunits encoded by each *Glu-A3* allele (Fig. 1), suggesting that the antibodies were detecting subunits expressed by *Glu-A3* allelic variants. However, with the Aroona biotypes, there was no evidence that any of the mAb combinations tested detected variation in the number of subunits expressed by *Glu-B3* alleles ( $c < a < b = d < h$ ) Gupta and Shepherd 1990).

### Glutenin Subunit Extracts from Cranbrook × Halberd Doubled Haploids

Fifty-five lines were selected from the Cranbrook × Halberd doubled haploid population, differing at *Glu-B1*, *-D1*, *-A3*, *-B3* and *-D3* loci. Antibody combinations that exhibited a statistically significant difference ( $P < 0.05$ , Student's *t*-test) in ELISA signal between alleles at any of the glutenin subunit encoding loci with 30 Cranbrook × Halberd lines, were retested with a second set of 30 lines. Five samples were included in both population subsets as they were the only progeny in the population with particular allelic compositions. The results for samples tested twice were averaged for determinations involving 55 lines. Thirty antibody combinations that exhibited discrimination of the Aroona biotypes were applied to the Cranbrook × Halberd population. Twelve combinations (Table II) reproducibly discriminated between alleles at particular loci. In a number of cases, allelic variation at more than one locus had a significant effect on ELISA discrimination. The immunoblot specificities of these mAb are represented in Fig. 2. Some antibodies appeared to detect total LMW-GS, while others detected subgroups of B- and C-groups.

Certain labeled antibodies consistently detected allelic variation at a particular locus when used in conjunction with any of several immobilized antibodies. The mAb 82123 and 22123 were consistently represented as labeled antibody in combinations which discriminated between alleles at LMW-GS-encoding loci (Table II). However, the level of discrimination in these assays was largely dependent on the immobilized antibody. For example, using immobilized 80603-labeled 82123, there was only an 11% difference in ELISA response between *Glu-A3* allelic variants. Using the same labeled antibody with immobilized antibody 82208, there was >20% difference in ELISA response for these alleles. The immobilized antibody in a sandwich ELISA determines the range of LMW-GS available for detection which, in turn, is likely to influence the degree of discrimination between allelic variants. While the immobilized antibody determined the degree of discrimination, the labeled antibody determined the locus at which alleles were discriminated. Immobilized antibody 80512 discriminated between allelic variants with two labeled antibodies, 22123 and 96355. With labeled 22123, the combination discriminated between allelic variants at the *Glu-A3* locus, whereas with labeled 96355, the combination discriminated between allelic variants at the *Glu-B3* locus. Interestingly, mAb 22123 was specific for C-LMW-GS on immunoblots while mAb 96355 recognized mainly B-LMW-GS (Fig. 2).

Using immobilized antibodies 12224 and 22205, labeled 82123, which was also specific for B-LMW-GS, exhibited a small degree of discrimination between *Glu-B3* allelic variants (Table II). For the same labeled mAb with immobilized mAb 80603 (which also cross-reacted with some B-LMW-GS) (Fig. 2), the discrimination was primarily between alleles at the *Glu-B3* locus. These results suggest that, as labeled antibodies, B-LMW-GS specific antibodies could better discriminate between *Glu-B3* allelic variants and C-LMW-GS specific antibodies could discriminate between *Glu-A3* allelic variants. However, as a labeled antibody, 82123 was most commonly represented in combinations that discriminated between *Glu-A3* allelic variants. This may be related to the difference in low-mobility B-LMW-GS composition associated with allelic variation in *Glu-A3* locus. However, it illustrates that the specificity of labeled antibodies

on immunoblots may not consistently relate to the locus at which alleles were discriminated using a combination of antibodies in a sandwich assay.

### Glutenin Subunit Antigens: Discrimination of Alleles at Particular *Glu-3* Loci

Of the antibody combinations that discriminated between alleles at *Glu-3* loci, the greatest number discriminated between allelic variants at the *Glu-A3* locus. Only one mAb combination discriminated between allelic variants at only the *Glu-B3* locus (80512-96355) (Table II). This combination also discriminated significantly between *Glu-B3* allelic variants in 15 lines from a second doubled haploid population derived from a cross between CD87 and Hartog

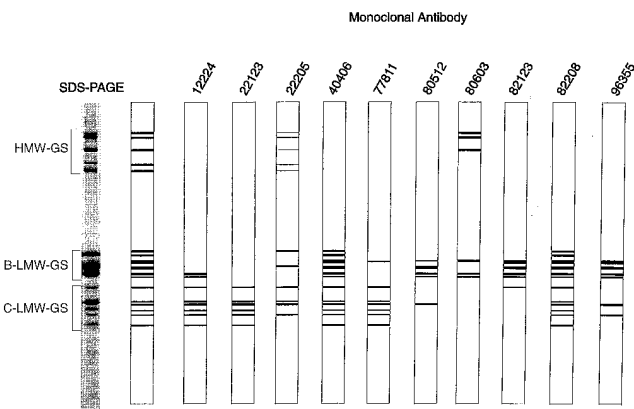


Fig. 2. Antibody recognition on immunoblots of reduced glutenin subunits (cv. Suneca) after SDS-PAGE separation.

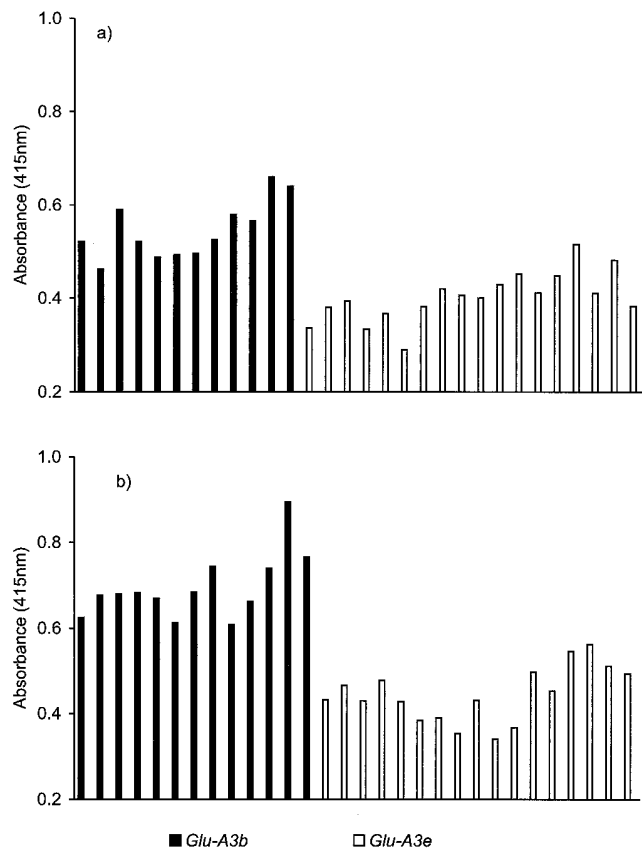
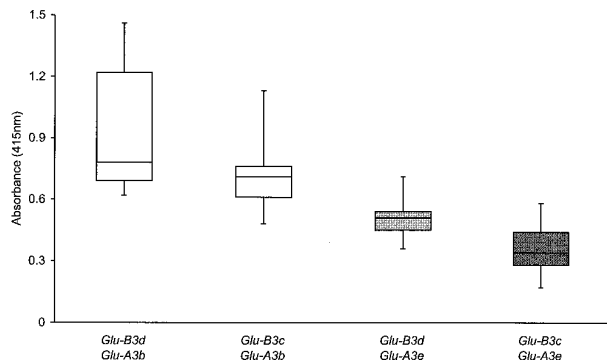
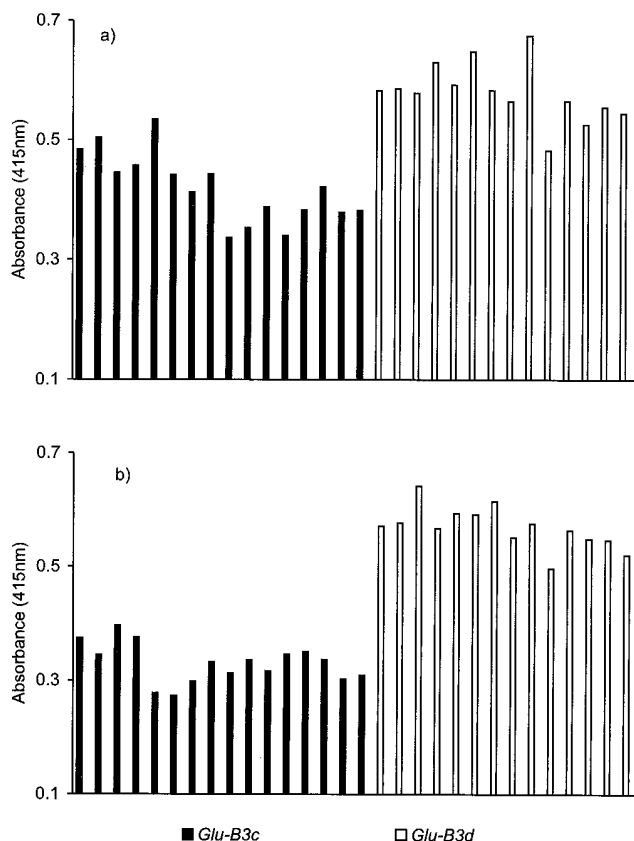


Fig. 3. Two-site sandwich ELISA with immobilized antibody 82208-labeled antibody 22123. Discrimination of *Glu-A3* allelic variants with glutenin subunit antigens extracted a) with and b) without preextraction of monomeric components. Data are averages of duplicate determinations from 30 lines of Cranbrook × Halberd doubled haploid population.

(results not shown). The direction of absorbance change for mAb combinations that significantly discriminated between alleles at either *Glu-A3* or *Glu-B3* loci gave some indication of the biochemical basis for the results. Lower ELISA signal in lines encoding the *Glu-A3e* (null) allele was likely to be due to the greater number of subunits encoded by the *Glu-A3b* allele (Gupta and Shepherd 1990). Fewer polypeptides migrate in the C- compared with B-LMW-GS range (Gupta and Shepherd 1990). Hence, variation in polypeptide number in the C-LMW-GS region may be more easily detected by C-LMW-GS specific mAb. The difference in the number of subunits encoded by *Glu-B3* alleles in the Cranbrook × Halberd population could account for the discrimination observed between alleles at this locus,



**Fig. 4.** Cumulative effect of LMW-GS encoding loci on the two-site sandwich ELISA with immobilized antibody 82208-labeled antibody 22123 using glutenin subunits extracted from 55 lines of Cranbrook × Halberd doubled haploid population. Shaded boxes indicate significant difference from each other ( $P < 0.05$ , Student's  $t$ -test).



**Fig. 5.** Two-site sandwich ELISA with immobilized antibody 77811-labeled antibody 82123. Discrimination of *Glu-B3* allelic variants with gliadin extracted with a) 50% 1-propanol and b) 1M urea. Data are averages of duplicate determinations from 30 lines of Cranbrook × Halberd doubled haploid population.

despite the contrary findings with antigens from the Aroona biotypes. The allele at the *Glu-B3* locus encoding the greater number of subunits, *Glu-B3d* had a higher signal in the sandwich ELISA. There were no mAb combinations that discriminated between alleles at *Glu-D3* locus with glutenin subunit antigen, and the *Glu-D3a* and *Glu-D3c* alleles encoded similar numbers and mobilities of the subunits. It was possible that the presence of different N-terminal sequences in the encoded subunits could also have contributed to the discrimination observed between alleles. However, the fact that N-terminal sequence types are not allele- or even locus-specific (Sissons et al 1999) suggests that this is unlikely.

#### Optimization of Allele Discrimination with Glutenin Subunit Antigens

For routine use in breeding analysis,  $\approx 50\%$  difference in ELISA response between samples is required. Two optimization approaches were tested using antigen from 30 lines from the Cranbrook × Halberd doubled haploid population. For some antibody combinations, there was increased discrimination between samples following addition of extra protein in the sample diluent, which may have reduced nonspecific antigen-solid phase interactions (Giersch et al 1999). With 5% fetal calf serum (CSL, Melbourne, Australia) added to the assay diluent, the immobilized mAb 80512 and labeled mAb 96355 combination exhibited  $\approx 50\%$  difference in ELISA signal ( $OD\ 415\ nm \pm SD$ ) for the two allelic variants ( $Glu-B3c = 0.32 \pm 0.08$ ,  $Glu-B3d = 0.62 \pm 0.17$ ). Omission of the preextraction of monomeric components could potentially simplify sample preparation and in some, such as mAb combination 82208-22123, discrimination was actually improved (Fig. 3). Preextraction of monomeric components selectively solubilizes small glutenin polymers such as those containing HMW-GS 2+12 (Dupuis and Bushuk 1996; Fu and Sapirstein 1996), which may affect the ability of mAb to discriminate between alleles, particularly at *Glu-1* loci.

However, for discriminating between *Glu-1/Glu-3* allelic variants, dividing the extracts into a broad monomeric component and a polymeric component reduced to subunits allowed the antibodies specificity for unreduced gliadins and glutenin subunits to be resolved separately. The total glutenin subunit extract did not partition these protein classes and required the antibodies to discriminate between a group of similar, reduced polypeptides.

#### Assay for *Glu-A3* Alleles Using Immobilized 82208 and Labeled 22123.

In assays with glutenin subunit antigen from 55 lines from the Cranbrook × Halberd population, mAb combination 82208-22123 consistently displayed significant discrimination between allelic variants at the *Glu-A3* locus. Immobilized antibody 82208 and labeled antibody 22123 were represented separately in a number of combinations that discriminated between *Glu-A3* allelic variants. However, there also appeared to be a minor difference in average ELISA signal with 55 lines due to allelic variation at the *Glu-B3* locus. Although the difference just escaped statistical significance ( $P = 0.057$ ) for 55 lines, the effect on ELISA due to variation at the *Glu-B3* locus was significant ( $P < 0.05$ ) for lines expressing *Glu-A3e* allele and may have been additive to the effect observed due to *Glu-A3* allelic variation. The Tukey box plot in Fig. 4 outlines the spread of the data and indicates that all five orientation points for each of the four allele combinations, including the median and the two extreme data points, change in an incremental manner consistent with an additive effect of the two loci on ELISA. Hence, variation at the *Glu-B3* loci may have had some effect on ELISA, although the largest influence with this mAb combination was due to allelic variation at the *Glu-A3* locus. The influence of allelic variation at *Glu-B3* was lessened by using glutenin subunit antigen extracted without preextraction of monomeric components (results not shown).

Seventeen diverse cultivars, many of which were parents of available doubled haploid populations, were analyzed with the mAb 82208-

22123 combination to assess the ability of the sandwich ELISA to detect change in LMW-GS composition in a varied genetic background. The cultivar set was assayed independently four times, each time with fresh reduced glutenin subunit extract obtained after exhaustive preextraction with 50% (v/v) aqueous 1-propanol as antigen. The data was expressed around a mean of 1 as a fraction of the absorbance for the Gabo control (ELISA Index). The cultivars were ranked by *Glu-A3* allele, then *Glu-B3* allele, according to the number of LMW-GS encoded by each as defined by Gupta and Shepherd (1990). The orders are *Glu-A3e* < *c* < *a* < *b* < *d* and *Glu-B3b* < *a* < *c* < *d* < *h*. There was a significant correlation ( $r = 0.70$ ) between ELISA signal and the number of glutenin subunits encoded at the two loci. Furthermore, the ELISA index ( $\pm$  SD) for lines with *Glu-A3* alleles expressing C-LMW-GS (alleles *a*, *b*, and *d* =  $1.18 \pm 0.36$ ) was significantly greater ( $P < 0.05$ ) than for lines with *Glu-A3* alleles not expressing C-LMW-GS (alleles *e* and *c* =  $0.93 \pm 0.35$ ). These data support the assertion that differences in number of glutenin subunits, particularly C-LMW-GS, encoded by each allele was detected by this antibody combination. It is also consistent with *Glu-B3* allelic variation having a minor influence on the discrimination in ELISA.

### Discrimination Between *Gli-1* Allelic Variants Using Gliadin-rich Flour Extracts

Simplicity of sample preparation is important for potential routine use of screening assays in wheat breeding programs. The gliadins can be extracted in a single step using nontoxic solvents such as aqueous alcohol or urea, whereas quantitative extraction of glutenin subunits usually requires the use of reducing agents. Antibody combinations (202) were tested in sandwich ELISA with gliadin extracted from Aroona *Gli-1/Glu-3* biotype wholemeal samples. In general, there was a larger degree of discrimination between the different biotypes with gliadin antigen compared with glutenin subunit antigen. This may be related to differences in the quantity of gliadin expressed or extracted as some solvents may preferentially extract certain gliadin components (Hill and Skerritt 1990; Skerritt and Robson 1990). Although total protein content was similar for extracts from the different biotypes, there may be differences in the concentration of individual gliadin components extracted due to allelic variation at *Gli-1* loci. Antibody combinations that had a large degree of discrimination between various lines, as well as combinations with unusual specificity, were pursued in assays with the Cranbrook  $\times$  Halberd population. Antibodies specific for gliadins encoded by individual genomes (Skerritt et al 1984, 1999; Skerritt and Underwood 1986; Skerritt and Robson 1990) were also included as these mAb may be able to detect allelic variation in polypeptide composition and, hence, discriminate between alleles. Finally, a number of mAb prepared to soluble polymeric glutenin (Partridge et al 1998), which is also present in aqueous alcohol extracts, were tested in the assays as it was thought that the retention of discontinuous epitopes in the polymeric glutenin immunogen may increase the specificity of the derived mAb.

### Discrimination of *Gli-1/Glu-3* Allelic Variants with Two-Site Sandwich ELISA

When gliadin-rich grain extracts were used, eight of 64 antibody combinations tested significantly and reproducibly discriminated between alleles at the *Gli-A1*, *-B1* or *-D1* loci in the Cranbrook  $\times$  Halberd population. However, fewer antibody combinations discriminated between allelic variants at *Gli-1/Glu-3* loci with gliadin than with glutenin subunit antigen. This was unexpected given the large degree of discrimination observed between different Aroona biotypes and the fact that the number of combinations tested with gliadin antigen was twice the number tested with glutenin subunit antigen. This result may indicate that discriminating between *Gli-1/Glu-3* alleles with gliadin antigen is actually more difficult than with glutenin subunit antigen. It may also be partly related to the ability of a considerable number of mAb to discriminate the *Glu-A3e* null allele with glutenin subunit antigen. In acid-PAGE separation of gliadins from the Cranbrook  $\times$  Halberd population (results not shown), there were two major differences in polypeptide composition, among  $\gamma$ - and fast  $\omega$ -gliadins, respectively, marking allelic variation at the *Gli-A1* and *Gli-B1* loci. Both these differences were proposed as potential polymorphisms for discriminating between the alleles at these loci. However, more mAb combinations discriminated between alleles at the *Gli-A1* locus, indicating that this locus may be easier to differentiate in the two-site sandwich ELISA. In contrast, differences in gliadin composition were quantitatively minor for the *Gli-D1* allelic variants, and only one mAb combination discriminated between alleles at this locus.

### Optimization of Discrimination of *Gli-1* Alleles Using Urea Grain Extracts

The proportion of glutenin extracted in 1M urea is less than that extracted in 40 or 70% (v/v) aqueous ethanol, or 55% (v/v) aqueous 2-propanol (Hill and Skerritt 1990). Hence, we investigated whether assay discrimination was increased when a more gliadin-specific extractant was used. Seven antibody combinations that exhibited >20% difference in ELISA response in aqueous alcohol extracts of grain encoding different *Gli-1* alleles were tested in two-site sandwich ELISA with 1M urea extracts of 30 lines from the Cranbrook  $\times$  Halberd population (Table III). A significant improvement in discrimination between alleles was observed with immobilized antibody 77811 and labeled antibody 82123, which discriminated between *Gli-B1* variants (Fig. 5), and there was a smaller improvement with immobilized antibody 79115 and labeled antibody 22123, which discriminated between *Gli-A1* variants. Use of 1M urea as a grain extractant primarily affected discrimination between *Gli-B1* allelic variants in the population. Some mAb combinations that discriminated between allelic variants at the *Gli-A1* locus with aqueous alcohol extracts as antigen (e.g., 70846-82123, 71945-82123, 73025-82123, 96355-22123) exhibited a highly significant difference in ELISA signal between *Gli-B1* allelic variants with 1M urea extracts as antigen (Table III). This may be due to the increase in proportion

TABLE III  
Differentiation of Alleles at Gliadin Encoding Loci in Doubled Haploid Lines from Cranbrook  $\times$  Halberd Using Two-Site Sandwich ELISA with Antigen Extracted in 1M Urea<sup>a</sup>

Antibodies		Subunit Comparison <sup>b</sup>					
Immobilized	Labeled	<i>Gli-A1g</i>	<i>Gli-A1m</i>	<i>Gli-B1i</i>	<i>Gli-B1h</i>	<i>Gli-D1a</i>	<i>Gli-D1i</i>
70846	82123	0.29 $\pm$ 0.04	0.27 $\pm$ 0.05	0.25 $\pm$ 0.03	0.31 $\pm$ 0.04**	0.28 $\pm$ 0.06	0.29 $\pm$ 0.04
71945	82123	0.37 $\pm$ 0.06	0.33 $\pm$ 0.05	0.32 $\pm$ 0.04	0.38 $\pm$ 0.06**	0.35 $\pm$ 0.07	0.35 $\pm$ 0.04
73025	82123	0.31 $\pm$ 0.03	0.28 $\pm$ 0.03*	0.28 $\pm$ 0.02	0.31 $\pm$ 0.03**	0.29 $\pm$ 0.04	0.30 $\pm$ 0.03
77811	82123	0.46 $\pm$ 0.13	0.41 $\pm$ 0.12	0.33 $\pm$ 0.04	0.57 $\pm$ 0.04**	0.44 $\pm$ 0.14	0.45 $\pm$ 0.12
79115	22123	0.44 $\pm$ 0.03	0.32 $\pm$ 0.04**	0.35 $\pm$ 0.07	0.39 $\pm$ 0.06	0.37 $\pm$ 0.08	0.38 $\pm$ 0.07
96355	22123	1.33 $\pm$ 0.11	1.07 $\pm$ 0.13**	1.12 $\pm$ 0.16	1.27 $\pm$ 0.16**	1.20 $\pm$ 0.19	1.17 $\pm$ 0.16
96355	82208	0.94 $\pm$ 0.06	0.84 $\pm$ 0.05**	0.87 $\pm$ 0.07	0.91 $\pm$ 0.07	0.89 $\pm$ 0.07	0.89 $\pm$ 0.08

<sup>a</sup> Data are averages of duplicate wells from 30 lines ( $\pm$  SD).

<sup>b</sup> \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , Student's *t*-test. Experiments repeated using an independent set of 30 lines with similar results.

of  $\omega$ -gliadin extracted using 1M urea (Skerritt and Robson 1990) because *Gli-B1* allelic variation accounts for the major difference in fast  $\omega$ -gliadin composition in acid-PAGE separation.

Antibody combinations that discriminated between alleles at the *Gli-B1/Glu-B3* locus were less common than combinations that discriminated between alleles at the *Gli-A1/Glu-A3* locus when using aqueous alcohol extracts as antigen. Using 1M urea extract as antigen with antibody combination 77811-82123, the lowest signal for any line with the *Glu-B3d* allele was higher than the strongest signal for any line with the *Gli-B3c* allele (Fig. 5). The immobilized mAb in this combination, 77811, was initially selected on the basis of narrow specificity for B-genome encoded  $\beta$ - and  $\gamma$ -gliadins with cross-reaction with  $\omega$ -gliadins (Partridge 1999). The discrimination observed for this mAb combination between the *Gli-B1/Glu-B3* allelic variants was consistent with the selectivity of mAb 77811 for B-genome encoded gliadins. However, the Cranbrook *Gli-B1h* and Halberd *Gli-B1i* alleles do not encode  $\beta$ -gliadins and have only minor differences in the mobility of the encoded  $\gamma$ -gliadin (Metakovsky 1991). Hence, it is difficult to fully account for the discrimination between *Gli-B1* encoded alleles.

The major distinction in gliadin composition between the *Gli-B1* allelic variants in acid-PAGE is evident among the fast  $\omega$ -gliadins with the *Gli-B1i* allele encoding a major high-mobility polypeptides missing from the *Gli-B1h* allele. Indeed, the greatest degree of discrimination in ELISA with immobilized mAb 77811 and labeled mAb 82123 was achieved with 1M urea extract, which contains the largest proportion of  $\omega$ -gliadin (Hill and Skerritt 1990). This antibody combination could also discriminate between allelic variants at the *Gli-B1/Glu-B3* locus in 15 lines from a second doubled haploid population derived from a cross between CD87 and Hartog (results not shown). Again, the major distinction in gliadin composition between allelic variants at the *Gli-B1* locus in the CD87  $\times$  Hartog population in acid-PAGE separation was among the high-mobility fast  $\omega$ -gliadins. As with the Cranbrook  $\times$  Halberd population, the fast  $\omega$ -gliadin encoding allele (Hartog *Gli-B1d*) had the lower ELISA signal. At high mAb concentrations, 77811 cross-reacted with  $\omega$ -gliadin on immunoblots. It is possible that, once immobilized on the solid phase, mAb 77811 bound  $\omega$ -gliadins that labeled mAb 82123 did not recognize, resulting in a decreased ELISA signal.

## DISCUSSION

We have identified a number of mAb combinations that could discriminate between allelic variants at the *Glu-A3* and *Glu-B3* loci. One antibody combination (82208-22123) consistently displayed good discrimination between allelic variants at the *Glu-A3* locus in the Cranbrook  $\times$  Halberd population. There was also a positive correlation between the number of subunits encoded at the *Glu-A3* and *Glu-B3* loci and the ELISA signal for 17 cultivars. Use of a total gliadin plus glutenin subunit grain extract as the test sample improved the level of discrimination between *Glu-A3* allelic variants with this mAb combination. It will be important to determine whether this antibody combination can discriminate between *Glu-A3e* null allele and other *Glu-A3* allelic variants in different cultivars. If this can be achieved (analysis of Aroona LMW-GS biotypes suggests this may be possible), then the assay would be a valuable tool for sorting progeny containing the null allele at the *Glu-A3* locus in breeding programs. With the Aroona biotypes, immobilized mAb 82208 and labeled mAb 22123 had a lower response to lines expressing non-C-LMW-GS encoding *Glu-A3* alleles, such as *Glu-A3c*. It may be possible to adapt this assay to discriminate between other *Glu-A3* encoded alleles based on the number of C-LMW-GS encoded.

With glutenin subunit antigen, many mAb combinations primarily detected allelic variation at the *Glu-A3* locus with both the Cranbrook  $\times$  Halberd population and Aroona *Gli-1/Glu-3* biotypes. This was clearly indicated by the large number of mAb combinations that discriminated between alleles at the *Glu-A3* locus compared

with *Glu-B3* and *Glu-D3* loci in the Cranbrook  $\times$  Halberd population and the large number of combinations that had a weak response to antigen from the Aroona biotype expressing the *Glu-A3e* null allele. The biochemical basis for discrimination between LMW-GS-encoding alleles in ELISA may be related to variation in the number of subunits encoded by each allele. However, it was surprising that so few mAb combinations could discriminate between *Glu-B3* allelic variants, given that there was a greater difference in the number of subunits encoded by alleles at that locus when compared with the *Glu-A3* allelic variants.

From immunoblotting evidence, it was difficult to unambiguously establish the specific prolamins components that were responsible for the discrimination observed in ELISA. The mAb selectivity defined in different immunoassay formats may not be directly comparable. For example, SDS is rarely used in ELISA assay diluents, whereas it is routinely used in PAGE separation of glutenin subunits for immunoblotting. Similarly, polypeptides are often alkylated to improve resolution in SDS-PAGE separations and immunoblots, whereas extracts to be used as antigen in ELISA are not. There are a number of reports of such treatments influencing the specificity of prolamins-selective mAb (Skerritt and Robson 1990; Graybosch et al 1993). In sandwich ELISA, the immobilized capture antibody may be partially denatured, and antigen binding sites may be altered that would affect the specificity of the assay (Butler et al 1992). Hence, considerable caution should be exercised when comparing mAb binding with antigen in different assay formats. Discrimination between alleles encoded by loci that are replicated across three genomes (each expressing a complex mixture of polypeptides all closely related in amino acid sequence) is one of the most challenging applications of antibody diagnostics. In the present study, this was achieved for two of the three gliadin/ LMW-GS encoding loci. The specificity provided by a combination of mAb in the sandwich ELISA may be greater than observed in indirect assay with a single mAb. The discrimination observed with the Cranbrook  $\times$  Halberd population tested in sandwich ELISA in this study was greater than that observed in indirect ELISA. The ability to effectively resolve allele-specific reactions from background cross-reactions may be an important benefit of the increased antigen specificity in sandwich immunoassays.

The identification of assays that used different antigen preparations to discriminate between alleles at different loci underscores the value of targeting each antigen type. The ability to alter the proportion of gliadin components in the antigen preparations by changing the solvent may have been a significant factor in the ability to improve the discrimination of *Gli-B1* allelic variants with immobilized antibody 77811 and labeled antibody 82123. Given the complexity of the *Gli-1/Glu-3* loci, both glutenin subunit and gliadin antigen preparations need to be assessed for use in assays to discriminate between allelic variants. Using gliadin-rich antigen preparations, fewer combinations could discriminate between allelic variants at the *Gli-1/Glu-3* loci than with glutenin subunit antigen. This was partly due to the ease with which antibody combinations could differentiate the *Glu-A3e* null allele with glutenin subunit antigen; however, other factors may have influenced this result. The ability of antibodies to discriminate between allelic variants is dependent on the level of polymorphism in the number and mobility of the polypeptides encoded. That there was only one mAb combination that could discriminate (albeit poorly) between allelic variants at the *Gli-D1* locus in the Cranbrook  $\times$  Halberd population. This is an indication of the lack of polymorphism in the polypeptides encoded at that locus. Furthermore, mAb specific for the products of an individual chromosome may not be selective for individual alleles encoded at that locus. For example, mAb 80207 and 80603 were specific for 1DS encoded low-mobility  $\beta$ -gliadins, and  $\beta$ - and 1DS encoded slow  $\omega$ -gliadins respectively (Skerritt et al 1999). However, most of the *Gli-D1* allelic variants have only minor variations in the mobility or quantity of polypeptides encoded (Metakovsky 1991). Hence, only minor changes in

ELISA signal may be evident for the different alleles. This may explain why antibodies that recognized the products of a specific locus were not commonly represented in combinations that could discriminate between *Gli-1* allelic variants. An interesting result was the apparent role of the immobilized and labeled antibodies in the two-site ELISA. With the same labeled antibody, the immobilized antibody, which defines the range of antigens available for detection by the labeled antibody, determined the degree of discrimination. With the same immobilized antibody and, hence, the same range of antigens available for detection, the labeled antibody determined specificity of the assay or the locus at which alleles were discriminated. These results were observed for both antigen types with a number of different antibody combinations and indicate that it is the reaction of both antibodies in combination that determines the specificity for antigen and the degree of discrimination between samples.

The results in this research were based on analysis of biotype and doubled haploid populations, which provided a convenient tool to assess the effect of allelic variation on the ELISA response in a defined and homozygous genetic background. However, the use of doubled haploid populations in cereal research has some inherent disadvantages. First, there are only two allelic variants at each locus, precluding analysis of the effect of other alleles. Second, the parents of the populations are usually selected for diverse characteristics to accentuate the genetic variation. Breeding parents may be more alike in protein composition and, hence, the progeny may be harder to differentiate. The response of these assays to antigen from a number of populations needs to be examined to determine the ability to discriminate between alleles in different segregating populations. It is possible that in some populations, the similarity of polypeptide composition may reduce the degree of discrimination, particularly for combinations detecting variation in the number of subunits expressed by each allele.

## CONCLUSIONS

The assays developed may be able to discriminate between alleles at the *Glu-A3* and *Glu-B3* loci. The ability to differentiate the *Glu-A3e* allele may be particularly important because this allele has been shown in a number of studies (Payne et al 1987; Gupta et al 1991; Khelifi and Branlard 1992; Gupta and MacRitchie 1994), including with the Cranbrook × Halberd population (Cornish et al 1998), to adversely affect the strength and extensibility characteristics of doughs.

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## LITERATURE CITED

Andrews, J. L., Blundell, M. J., and Skerritt, J. H. 1993. A simple antibody-based test for dough strength. III. Further simplification and collaborative evaluation for wheat quality screening. *Cereal Chem.* 70:241-246.

Andrews, J. L., and Skerritt, J. H. 1996. Wheat dough extensibility screening using a two-site enzyme-linked immunosorbent assay (ELISA) with antibodies to low molecular weight glutenin subunits. *Cereal Chem.* 73:650-657.

Andrews, J. L., Blundell, M. J., and Skerritt, J. H. 1996. Identification of wheat-rye translocation lines using antibody probes for *Gli-B1* and *sec-1*. *J. Cereal Sci.* 23:61-72.

Bean, S. R., Lyne, R. K., Tilley, K. A., Chung, O. K., and Lookhart, G. L. 1998. A rapid method for quantitation of insoluble polymeric proteins in flour. *Cereal Chem.* 75:374-379.

Bradford, M. M. 1976. A rapid and sensitive method for the quanti-

fication of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Chem.* 72:248-254.

Butler, J. E., Nessler, R., Joshi, K. S., Suter, M., Rosenberg, B., Chang, J., Brown, W. R., and Cantarero, L. A. 1992. The physical and functional behaviour of capture antibodies adsorbed on polystyrene. *J. Immunol. Methods* 150:77-90.

Cornish, G. B., Bekes, F., Larroque, O., and Appels, R. 1998. The relationship between allelic composition of glutenin subunits and functional properties. Pages 536-539 in: Proc. 48th Australian Cereal Chemistry Conference. L. O'Brien, A. B. Blakeney, A. S. Ross, and C. W. Wrigley, eds. Cereal Chemistry Division, Royal Australian Chemical Institute: Melbourne, Australia.

Dawood, M. R., Howes, N. K., and Bushuk, W. 1989. Preparation of monoclonal antibodies against specific gliadin proteins and preliminary investigation of their ability to discriminate cereal cultivars. *J. Cereal Sci.* 10:105-112.

Dupius, B., and Bushuk, W. 1996. Variation in high molecular weight glutenin subunit composition in various solubility fractions of flours of diverse dough strength. Pages 262-266 in: Gluten '96. Proc. 6th International Gluten Workshop. C. W. Wrigley, ed. Cereal Chemistry Division, Royal Australian Chemical Institute: Melbourne, Australia.

Fu, B. X., and Sapirstein, H. D. 1996. Procedure for isolating monomeric proteins and polymeric glutenin of wheat flour. *J. Cereal Sci.* 24:241-246.

Giersch, T. M., Hill, A. H., and Skerritt, J. H. 1999. Development of a panel of specific monoclonal antibodies to high molecular weight glutenin subunits and their application in genetic screening. *Cereal Chem.* 76:380-388.

Graybosch, R., Seo, Y. W., and Peterson, C. J. 1993. Detection of wheat-rye chromosomal translocations using an antiscalin monoclonal antibody. *Cereal Chem.* 70:458-463.

Gupta, R. B., Singh, N. K., and Shepherd, K. W. 1989. The cumulative effect of allelic variation in LMW and HMW glutenin subunits on physical dough properties in progeny of two bread wheats. *Theor. Appl. Genet.* 77:57-64.

Gupta, R. B., and Shepherd, K. W. 1990. Two-step one-dimensional SDS-PAGE analysis of low molecular weight subunits of glutenin. I. Variation and genetic control of the subunits in hexaploid wheats. *Theor. Appl. Genet.* 80:65-74.

Gupta, R. B., Bekes, F., and Wrigley, C. W. 1991. Prediction of physical dough properties from glutenin subunit composition in bread wheats: Correlation studies. *Cereal Chem.* 64:328-333.

Gupta, R. B., Paul, J. G., Bekes, F., Cornish, G., and Rathjen, A. J. 1994. Allelic variation in glutenin subunit and gliadins loci, *Glu-1*, *Glu-3* and *Gi-1* of common wheats. I. Its additive and interaction effects on dough properties. *J. Cereal Sci.* 19:9-17.

Gupta, R. B., and MacRitchie, F. 1994. Allelic variation at glutenin subunit and gliadin loci, *Glu-1*, *Glu-3* and *Gli-1* of common wheats. II. Biochemical basis of effects on dough properties. *J. Cereal Sci.* 19:19-29.

Hill, A. S., and Skerritt, J. H. 1989. Monoclonal antibody based two-site enzyme immunoassays for wheat gluten proteins. I. Kinetic characteristics and comparison with other ELISA formats. *Food Agric. Immunol.* 1:147-160.

Hill, A. S., and Skerritt, J. H. 1990. Determination of gluten in foods using a monoclonal antibody-based competition enzyme immunoassay. *Food Agric. Immunol.* 2:21-35.

Howes, N. K., Kovacs, M. I., Leslie, D., Dawood, M. R., and Bushuk, W. 1989. Screening of durum wheats for pasta making quality with monoclonal antibodies for gliadin 45. *Genome* 32:1096-1099.

Khelifi, D., and Branlard, G. 1992. The effects of HMW and LMW subunits of glutenin and of gliadins on the technological quality of progeny from four crosses between poor breadmaking quality and strong wheat cultivars. *J. Cereal Sci.* 16:195-209.

Kovacs, M. I. P., Howes, N. K., Leisle, D., and Skerritt, J. H. 1993. The effect of high-molecular weight glutenin subunit composition on tests used to predict durum wheat quality. *J. Cereal Sci.* 18:43-51.

Kovacs, M. I. P., Howes, N. K., Leisle, D., and Sawistowski, J. 1995. Effect of two different low molecular weight glutenin subunits on durum wheat pasta quality parameters. *Cereal Chem.* 72:85-87.

Metakovsky, E. V. 1991. Gliadin allele identification in common wheat II. Catalogue of gliadin alleles in common wheat. *J. Genet. Breed.* 45:325-344.

Morel, M. H., Bonicel, J., Melas, V., and Autran, J. C. 1994. Multiple approach (IEF, SDS-PAGE, and A-PAGE) of the composition of glutenin and its effect on dough properties. Pages 244-254 in: Gluten Proteins 1993. Proc. 5th Int. Gluten Workshop. Association of Cereal Research:

- Detmold, Germany.
- Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9:255-262.
- Nieto-Taladriz, M. T., and Bouguennec, A. 1994. The effect of the allelic variation at the 1B and 1D encoded proteins on bread wheat quality. Pages 262-269 in: *Gluten Proteins 1993. Proc. 5th Int. Gluten Workshop. Association of Cereal Research: Detmold, Germany.*
- Partridge, M. A. K. 1999. Immunoassays for discriminating between Gli-1/Glu-3 alleles in hexaploid wheat. PhD thesis. University of Sydney: Sydney, Australia.
- Partridge, M. A. K., Blundell, M. J., and Skerritt, J. H. 1998. Antibody probes for unreduced glutenins. Pages 35-40 in: *Proc. 48th Cereal Chemistry Conference. Cereal Chemistry Division, Royal Australian Chemical Institute: Cairns, QLD, Australia.*
- Payne, P. I., Seekings, J. A., Worland, A. J., Jarvis, M. G., and Holt, L. M. 1987. Allelic variation of glutenin subunits and gliadins and its effects on breadmaking quality in wheat: Analysis of F5 progeny from "Chinese Spring" x "Chinese Spring (Hope 1A)". *J. Cereal Sci.* 6:103-118.
- Singh, N. K., and Shepherd, K. W. 1988. Linkage mapping of the genes controlling endosperm proteins in wheat. I. Genes on the short arms of group 1 chromosomes. *Theor. Appl. Genet.* 75:628-641.
- Sissons, M. J., Hac, L., and Skerritt, J. H. 1999. Antibodies to N-terminal peptides of low  $M_r$  subunits of wheat glutenin. 2. Detection of subunits encoded by different loci. *J. Cereal Sci.* 30:267-281.
- Skerritt, J. H., Smith, R. A., Wrigley, C. W., and Underwood, P. A. 1984. Monoclonal antibodies to gliadin proteins used to examine cereal grain protein homologies. *J. Cereal Sci.* 2:215-224.
- Skerritt, J. H., and Underwood, P. A. 1986. Specificity characteristics of monoclonal antibodies to wheat storage proteins. *Biochim. Biophys. Acta* 874:245-254.
- Skerritt, J. H., Martinuzzi, O., and Wrigley, C. W. 1987. Monoclonal antibodies in agricultural testing: quantification of specific wheat gliadins affected by sulfur deficiency. *Can. J. Plant Sci.* 67:121-129.
- Skerritt, J. H., Jenkins, K. L., and Hill, A. S. 1989. Monoclonal antibody based two-site enzyme immunoassays for wheat gluten proteins. 2. Specificity analysis. *Food Agric. Immunol.* 1:161-171.
- Skerritt, J. H., and Robson, L. G. 1990. Wheat low molecular weight glutenin subunits—Structural relationship to other gluten proteins analysed using specific antibodies. *Cereal Chem.* 67:250-257.
- Skerritt, J. H. 1991. A simple antibody-based test for dough strength. I. Development of method and choice of antibodies. *Cereal Chem.* 68:467-474.
- Skerritt, J. H., Martinuzzi, O., and Metakovsky, E. V. 1991. Chromosomal control of wheat gliadin epitopes: analysis with specific monoclonal antibodies. *Theor. Appl. Genet.* 82:44-53.
- Skerritt, J. H., Andrews, J. L., Blundell, M., Beasley, H. L., and Bekes, F. 1994. Applications and limitations of immunochemical analysis of biopolymer quality in cereals. *Food Agric. Immunol.* 6:173-184.
- Skerritt, J. H., Andrews, J. L., Stoddard, F. L., Gupta, R. B., and Howes, N. K. 1996. A rapid antibody-based test for *Sec-2*, a marker for the short arm of chromosome 2 of rye (2RS). *Genome* 39:1006-1012.
- Skerritt, J. H., Hill, A. S., and Andrews, J. L. 2000. Antigenicity of wheat prolamins: Detailed epitope analysis using a panel of monoclonal antibodies. *J. Cereal Sci.* 32:259-279.

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