Immunoassay for Wheat Processing Quality: Utilization of a Sandwich Assay Incorporating an Immobilized Single-Chain Fragment

Amanda S. Hill,*[,†,‡] Thomas M. Giersch,[†,‡] Chong S. Loh,[§] and John H. Skerritt[†,‡]
CSIRO Plant Industry and Quality Wheat Cooperative Research Centre Ltd., GPO Box 1600, Canberra, ACT, Australia

A single-chain fragment (scFv) was engineered from a monoclonal antibody to high molecular weight glutenin subunits (HMW-GS), wheat flour polypeptides that play a major role in determining the mixing- and extension strength-related properties of dough and its subsequent baking performance. The scFv was expressed in a thioredoxin mutant *Escherichia coli* strain that allows disulfide bond formation in the cytoplasm and incorporated into a diagnostic test for wheat quality. Although the scFv lacks the more highly conserved antibody constant regions usually involved with immobilization, it was able to be directly immobilized to a polystyrene microwell solid phase without chemical or covalent modification of the protein or solid phase and utilized as a capture antibody in a double-antibody (two-site) immunoassay. In the sandwich assay, increasing HMW-GS concentrations produced increasing assay color, and highly significant correlations were obtained between optical densities obtained in the ELISA using the scFv and the content of large glutenin polymers in flours as well as measures of dough strength as measured by resistance to dough extension in rheological testing. The assay using the scFv was able to be carried out at lower flour sample extract dilutions than that required for a similar assay utilizing a monoclonal capture antibody. This research shows that engineered antibody fragments can be utilized to provide superior assay performance in two-site ELISAs over monoclonal antibodies and is the first application of an engineered antibody to the analysis of food processing quality.

Keywords: *scFv; sandwich ELISA; wheat; dough; quality*

INTRODUCTION

Recombinant antibody technology offers the ability to clone antibody genes to obtain antibody fragments of the desired size, specificity, and sensitivity (Pluckthun, 1991). The smallest antibody fragments that retain affinity for antigen binding, the Fv fragment (variable fragment), are heterodimers of a heavy chain variable domain and a light chain variable domain. Due to the absence of intermolecular disulfide bonds, these fragments easily dissociate (Glockshuber et al., 1992); however, this instability can be removed by producing recombinant Fv fragments that have the heavy and light variable regions linked by a synthetic peptide linker. This recombinant fragment is a single-chain Fv fragment (scFv) and has a molecular weight of 27000 (Bird et al., 1988; Huston et al., 1988; Bird and Walker, 1991; Kitchin et al., 1995).

In the breeding of new wheat varieties, there is a need for selection of new lines for protein quality as distinct merely by protein content. Over the past decade, we have developed monoclonal antibodies (MAb) to wheat gluten proteins for use as diagnostics for screening of large numbers of wheat lines for their suitability for processing into bread and other products (Skerritt, 1991; Andrews et al., 1993) and as research tools for investigating protein structure and function. Enzyme-linked immunosorbent assays (ELISA) using conventional MAbs to flour polypeptides have been used in breeding programs for quality screening. We have previously developed an ELISA for the prediction of dough strength using antibodies to high molecular weight glutenin subunits (HMW-GS) extracted from wheat flour samples. These glutenin subunits are flour polypeptides that are linked by intermolecular disulfide bonds and determine the elasticity of flour doughs. The HMW-GS refer to a group of glutamine- and proline-rich polypeptides of molecular mass 60000−80000, encoded by genes on the long arms of homologous chromosomes 1 and expressed in the endosperm of wheat seed [reviewed in Skerritt (1998)]. They are distinct from the quantitatively major low molecular weight glutenin subunits (LMW-GS), which differ in sequence, size, and location of the encoding genes. The elasticity or "strength" of the doughs can be assessed using specialized rheological testing equipment as the resistance of the dough to extension; differences in dough strength also produce differences in dough development or mixing time. However, wheat breeders require a simple method that offers high-throughput assessment of wheat quality using small samples of grain, wholemeal, or flour, because initial crosses within breeding programs may produce many thousands of progeny, each bearing only small amounts (500 mg−10 g) of grain. As a result, large-scale rheological or other quality testing methods that require many grams of flour and are very labor-intensive cannot be used in early generation quality screening.

The ELISA for dough strength utilizing an MAb reported previously by our laboratory (Skerritt, 1991; Andrews et al., 1993) has several deficiencies that limit its use. Preparation of flour or wholemeal grain samples required pre-extraction to remove interfering proteins in the grain extract, and sample preparation involved

* Author to whom correspondence should be addressed (telephone +61 2 6246 5028; fax +61 2 6246 5351; e-mail A.Hill@pi.csiro.au).
† CSIRO Plant Industry.
‡ Quality Wheat Cooperative Research Centre Ltd.
§ Present address: Therapeutic Goods Administration, GPO Box 100, Woden, ACT, Australia.

10.1021/jf990167f CCC: $18.00
Published on Web 10/01/1999

extensive dilution of grain extracts. Antibody engineering may provide fragments with slightly different specificities and different affinities. In this paper, we describe the development of a recombinant scFv derived from an HMW-GS binding MAb and its incorporation into a two-site (sandwich assay) as the immobilized (capture) reagent. The binding of the recombinant scFv to HMW-GS also correlated significantly with the processing quality of the dough derived from the flour and had the advantage over the MAb assay of requiring a much lower wheat extract dilution, therefore simplifying sample preparation.

MATERIALS AND METHODS

Preparation of cDNA and Amplification of Antibody Genes. Total RNA was isolated from hybridoma cells secreting MAb 80105 as follows: 1×10^7 cells were harvested by centrifugation and suspended in 0.5 mL of extraction buffer (3 M guanidine thiocyanate, 0.5% sarkosyl, 0.1 M β-mercaptoethanol, 25 mM sodium citrate, pH 7) using a vortex mixer. The suspension was then added to a tube containing 50 μL of 3 M sodium acetate, pH 5.6, and 0.5 mL of water-saturated acid phenol and vortexed; chloroform was then added and left on ice for 15 min. After centrifugation at 13000*g* for 15 min, the aqueous phase was transferred to a new tube, an equal volume of 2-propanol was added, and the tube was placed at −20 °C for 2 h. After centrifugation at 4000*g* for 10 min, the pellet dissolved in 0.5 mL of 1% sodium dodecyl sulfate (SDS) with occasional vortexing before an equal volume of chloroform was added. After centrifugation at 4000*g* for 15 min, the RNA was precipitated with 2-propanol and salts.

The isolation of m-RNA from total RNA was performed using the Dynabeads Oligo (dT)25 kit (Dynal, Oslo, Norway). Briefly, 75 μg of RNA was heated to 65 °C for 2 min, added to equilibrated Dynabeads, mixed, and hybridized for 5 min. Unbound material was removed by magnetic separation, and the pellet was washed twice with buffer (0.15 M LiCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8). Ten microliters of water was added, and the solution was heated to 65 °C for 2 min before the mRNA was magnetically separated. The mRNA was used as a template for the synthesis of first-strand complementary DNA (cDNA) using deoxynucleotides, random hexadeoxynucleotides as primer, and murine reverse transcriptase (Pharmacia, Uppsala, Sweden). PCR amplification of the variable heavy- and light-chain (VH and VL) genes was performed using Vent polymerase (New England Biolabs, Beverly, MA) and consensus or specific primers, as follows:

- Heavy chain 5′ variable region

Primer 1	SFIHV2	(GC)AGCTTCAGCT(GC)CAGCAG
Primer 2	80105RACE	ACAAGCACAGATCCAGTT

- Heavy chain 3′ variable region

Primer 3	TG(CA)GGAGAC(AG)GTGACCAG
Primer 4	TG(CA)AGAGAC(TG)GTGACCAG
Primer 5	TGAGGAGAC(GA)GTGACTGA
Primer 6	TGCAGAGAC(TG)GTGAGAGT

- Light chain 5′ variable region

Primer 7	GA(CT)ATT(GT)TG(AC)TGAC(AT)CAG

- Light chain 3′ variable region

Primer 8	CCGTTTCAGGTTCAGTTT
Primer 9	CCGTTT(TG)AGGTCCAACTT

The amplified heavy- and light-chain products were purified using a PCR product purification kit (Boehringer-Mannheim, Mannheim, Germany) and assembled into a single gene with a $(Gly_4\ Ser)_3$ linker using the method of splice overlap extension. The fragment was then digested with *Sfi*I, end-filled with T4 DNA polymerase, and digested with *Not*I. This fragment was ligated to a T7 expression vector, pET-32 (Novagen, Madison, WI), previously digested with *Nco*I, end-filled with T4 DNA polymerase, and digested with *Not*I.

Expression, Solubilization, and Refolding of scFv. The recombinant expression vector was transformed using heat shock into *Escherichia coli* strains BL21(DE3) and AD494-(DE3). A single colony was inoculated into LB medium containing 100 μg/mL ampicillin and grown overnight at 37 °C with agitation. The culture was then diluted 1:100 and grown until OD_{600} reached 0.5−0.8. At this point, the culture was induced by the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and incubated at 30 °C for 2 h with agitation. At the end of this period, the induced culture was harvested by centrifugation. The cells were resuspended in 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride (PBS) and sonicated. The resultant lysate was centrifuged, and inclusion bodies were recovered from the pellet. The scFv in the inclusion bodies from 1 L of culture was solubilized with 10 mL of 6 M guanidinium hydrochloride in PBS and loaded onto a 1 mL HiTrap chelating (IMAC) column (Pharmacia). The column-bound denatured scFv was renatured and refolded with a linear gradient of 6 to 1 M urea in PBS containing 20% glycerol. The column was washed further with PBS and the refolded scFv eluted with 200 mM imidazole in PBS.

Immobilization of MAb and scFv in Sandwich ELISA. Refolded and purified scFv from antibody 80105 was dialyzed against 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride (PBS) to remove imidazole. The scFv was diluted to 10 μg/mL in the above buffer, and 100 μL was applied to microwells for ELISA (Nunc Maxisorp, Roskilde, Denmark) for 1 h at room temperature. The original MAb 80105 was immobilized onto microwells at the same concentration. The wells were then washed with 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride and 0.05% Tween 20 (PBS/T), and nonspecific binding was blocked by the addition of 1% bovine serum albumin in PBS. HMW-GS from bread wheat (Suneca cultivar) were dissolved in 50% (v/v) 2-propanol containing 1% dithioerythritol, and concentrations ranging from 0.0025 to 200 ng were added to the wells for 1 h at room temperature. After washing with 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride and 0.05% Tween 20 (PBS/T), peroxidase-labeled MAb to high and low glutenin subunits (from clone 41201; Skerritt, 1991) was added and incubated for 30 min. Following further washes, the chromogen, 2,2′azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide were added and incubated for 15 min at room temperature. After termination of color development with 3% oxalic acid, the absorbance of each microwell was measured at 415 nm.

Assessment of Wheat Dough Quality Using an Immobilized scFv. The recombinant scFv was first immobilized to the microwell. Refolded and purified MAb 80105 scFv was dialyzed against 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride (PBS) to remove imidazole. The scFv was diluted to 10 μg/mL in the above buffer, and 100 μL was applied to microwells for 1 h at room temperature. The wells were then washed with 50 mM sodium phosphat3, pH 7.2, containing 150 mM sodium chloride and 0.05% (v/v) Tween 20 (PBS/T), and nonspecific binding was blocked by the addition of 1% (w/v) bovine serum albumin in PBS.

Glutenin subunits were extracted from the wheat samples as follows. One milliliter of 0.5% SDS was added to 50 mg of flour and the sample vortexed for 30 s at room temperature. The extract was centrifuged at 14600*g* for 5 min and the pellet resuspended in 2.5% SDS−20 mM dithiothreitol by vortex mixing, mixed overnight, and recentrifuged. The flour extract was initially diluted 1:100 in 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride, 0.05% Tween 20, and

1% bovine serum albumin (BSA-PBS/T), and 3-fold serial dilutions were performed using the same buffer. The diluted extracts were applied to microwells coated with the scFv or MAb and incubated at room temperature for 1 h. Following washes with 50 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride and 0.05 % Tween 20 (PBS/T) to remove nonspecifically bound antigens, peroxidase-labeled MAb (from clone 41201) was added and incubated for 30 min. Following further washes, ABTS and hydrogen peroxide were added and incubated for 15 min at room temperature. After termination of color development with 3% oxalic acid, the absorbance of each microwell was measured at 415 nm.

Analysis of Flour Samples by Dough Testing and Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). Standard small-scale dough tests were used (American Association of Cereal Chemists, 1983), including measurement of the resistance to dough extension (Rmax) and dough mixing time in two types of mixer, including a pin mixer (Mixograph development time, MDT) and a Z-armed mixer (Farinograph development time, FDT). The proportion of large glutenin protein polymers in the flour samples was determined by SE-HPLC, using a Bio-Sep S-4000 column, which, according to the manufacturer's specifications, fractionated globular protein complexes up to a molecular mass of 2000000 (Phenomenex, Torrance, CA). Monomeric flour proteins and small, detergent-soluble glutenin polymers were first removed by 5 min of mixing in 1.5 mL of 1.5% SDS in water per 100 mg of flour, the extract was centrifuged for 5 min at 15600*g*, and then the content of large glutenin polymers in the pellet was suspended by a 30 s probe sonication in 1.5 mL of 1.5% SDS in water per 100 mg of flour before filtration (PVDF Acrodisc filter, Gelman, Ann Arbor, MI) and chromatographic analysis at 30 °C. The mobile phase (0.5 mL/min) consisted of 50% (v/v) acetonitrile (Mallinckrodt, Paris, KY) and 0.1% (v/v) trifluoroacetic acid (Pierce, St. Louis, MO) in water (Batey et al., 1991), and detection was at 214 nm. The content of these large glutenin polymers has been considered to be a major determinant of dough strength properties (Rmax, MDT, FDT).

RESULTS AND DISCUSSION

Cloning of scFv 80105. The mRNA from hybridoma cell line 80105 5F12 (specific for HMW-GS; D'Ovidio et al., 1997) was used as a template for the synthesis of first-strand cDNA using deoxynucleotides, random hexadeoxynucleotides as primer, and murine reverse transcriptase. To allow for variability of framework sequences, degenerate primers homologous for the 5′ and 3′ ends of the antibody variable regions were used for amplification of the variable heavy- and light-chain (V_H and V_L) genes. This approach has been used successfully by other groups (Huse et al., 1989; Clackson et al., 1991; Coloma et al., 1991; Dubel et al., 1994); however, we have found it difficult to amplify the heavy-chain genes from other anti-glutenin MAbs using such "consensus" primers. This is probably due to the limited available sequence data for mouse V_H and V_L regions (Heinrichs et al., 1995). A specific primer for the 5′ end of the variable heavy chain of MAb 80105 was obtained by rapid amplification of cDNA ends (RACE), whereby unknown sequences in the 5′ variable region can be identified using known sequences of the constant region of the heavy chain (Schaefer, 1995). A $(Gly_4\ Ser)_3$ linker was introduced to assemble the heavy- and light-chain genes into a single gene prior to cloning into a T7 expression vector, pET-32. With this vector, protein is expressed as a fusion complex with a 15 amino acid S.tag peptide and is detected using S protein, which interacts with the peptide to form enzymatically active ribonuclease S (Richards and Wyckoff, 1971; Kim and Raines, 1993). Successful cloning of the gene was checked by restriction analysis using *Bgl*II and *Not*I to yield an insert of 750 base pairs when run on a 1% agarose gel.

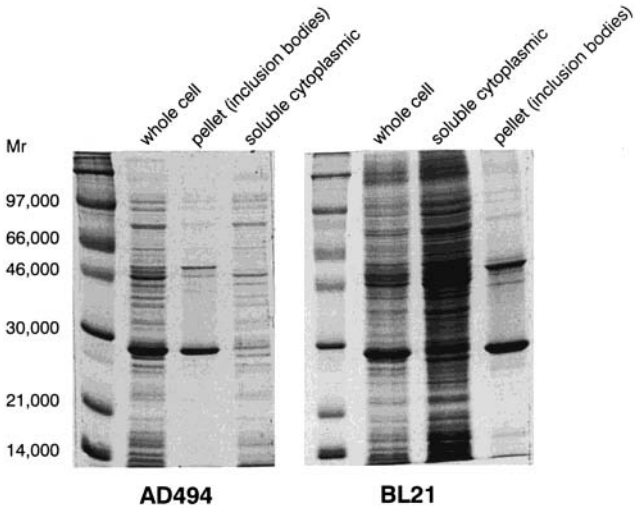

Figure 1. Presence and cellular location of expressed scFv in *E. coli* strains AD494 and BL21.

Expression, Purification, and Refolding of scFv. For expression of the scFv fragment, the recombinant expression vector was transformed into *E. coli* strains BL21 (DE3) and AD494 (DE3) for protein expression. The BL21 strain was used to determine if protein degradation was occurring during the induction process as this strain lacks both the lon protease and the ompT outer membrane protease, which are associated with protein degradation. To determine the cellular location of the expressed protein, cultures of *E. coli* AD494 and BL21 were induced and both whole cell and (after sonication) cellular soluble and insoluble fractions were analyzed by SDS−PAGE. The majority of the M_r = 28000 scFv was associated as insoluble inclusion bodies with little cytoplasmically soluble protein expressed (Figure 1). The pET32 vector does not contain a prokaryotic leader sequence for transport of expressed protein to the periplasm, where disulfide bond formation occurs and protein folding mechanisms can be subsequently activated to form soluble and functional protein. As a result, the protein was extracted from the insoluble pellet with guanidine hydrochloride and immobilized onto a nickel-charged chelating column for refolding into active scFv. The activities of soluble and solubilized and renatured scFv expressed in these bacterial strains were tested by probing immunoblots containing transferred glutenin subunits and by indirect ELISA (data not shown). Although the yields of expressed cytoplasmically soluble antibody in both strains were similar, the expressed cytoplasmically soluble scFv in BL21(DE3) showed considerably less antigen binding activity than the scFv expressed in AD494 (DE3), which has a mutation that allows disulfide bond formation in the cytoplasm (Derman et al., 1993). Because there are only intramolecular disulfide bonds present in a scFv, it appears these bonds are critical for correct folding of the protein and therefore antigen binding activity.

Immobilization and Activity of scFv in Sandwich ELISA. Refolded and purified scFv from MAb 80105 and original MAb was immobilized onto 96-well plates (Nunc Maxisorp) at 1 μg/well. An anti-influenza-neuraminidase recombinant scFv (Malby et al., 1993) was also immobilized at the same concentration to determine nonspecific binding effects of the recombinant single-chain preparation. The limit of detection (0.1 OD

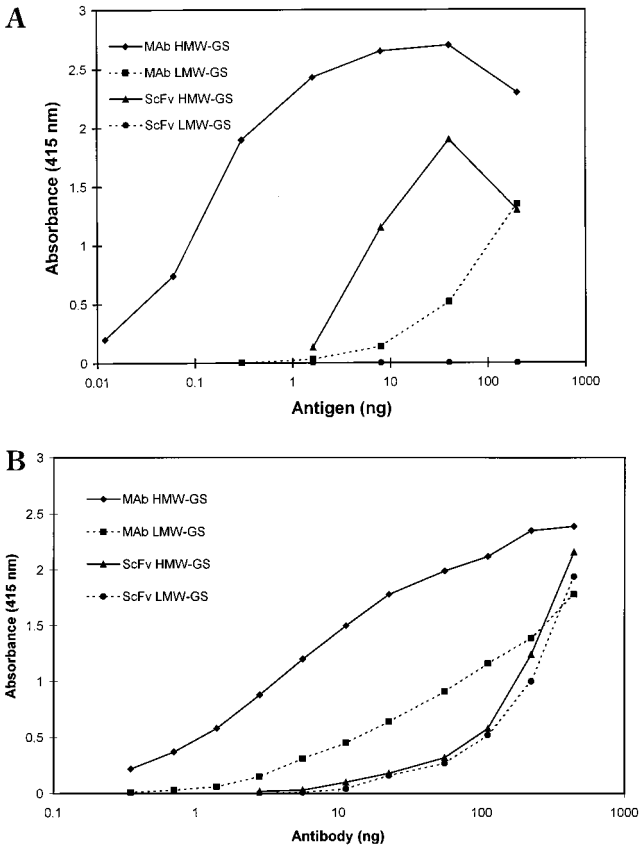

Figure 2. Relative specificity and sensitivity of MAb and scFv in different assay formats: (A) specificity of capture MAb and scFv for HMW- and LMW-GS; (B) titration of MAb 80105 and scFv against glutenin subunits (indirect ELISA).

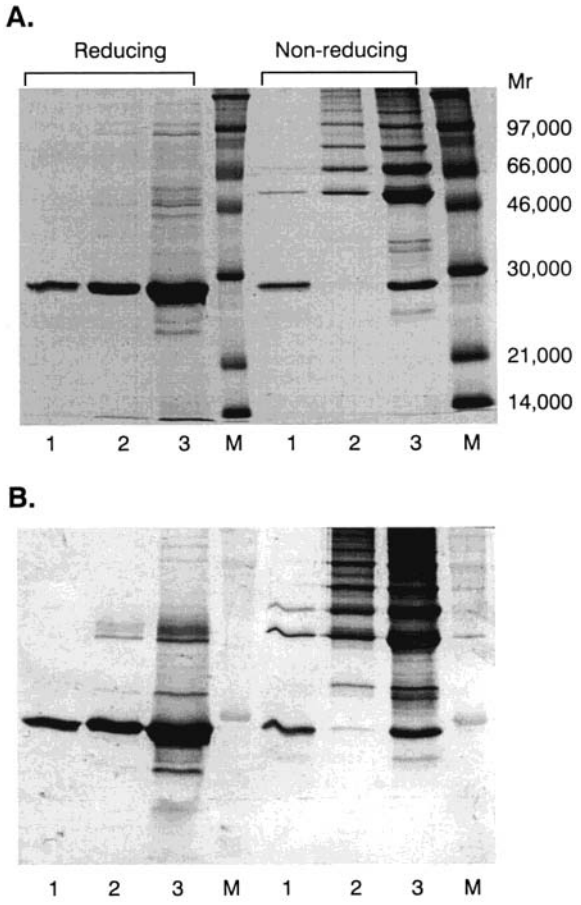

Figure 3. SDS−PAGE and immunoblot of monomeric and oligomeric 80105 scFv under reducing and nonreducing conditions: (A) SDS−PAGE; (B) immunoblot detection using alkaline phosphatase-labeled S protein; monomeric scFv (lanes 1), oligomeric scFv (lanes 2), column load (lanes 3), and marker (lanes M).

unit above background) for binding of recombinant scFv to HMW-GS was 1.6 ng compared with >0.12 ng for binding of the MAb (Figure 2A). This result is not unexpected as the lower affinity of recombinant antibody fragments compared to the parent MAb has been previously reported (Kramer and Hock, 1996). The immobilized scFv did not show nonspecific binding to an unrelated protein (bovine serum albumin), nor did it show binding to HMW-GS when peroxidase-labeled antibody to wheat α-amylase was used as the detecting reagent. The apparent affinity of the immobilized recombinant scFv and parent MAb for LMW-GS was low, enabling this assay format to be used to quantify HMW-GS in sample extracts that also contain LMW-GS. When these antigens were immobilized onto microwells and the activity of the fragment assessed using an indirect ELISA, the scFv showed similar cross-reactions with both HMW-GS and LMW-GS (detection limits of 20 ng), whereas the MAb showed greater sensitivity for HMW-GS than LMW-GS (detection limits of >0.4 and 3 ng, respectively; Figure 2B).

On nonreducing SDS−PAGE, the expressed scFv fractionated as a series of oligomers of a subunit of $M_r \sim 28000$, with dimeric, trimeric, and monomeric species predominating (Figure 3A); the presence of the expressed scFv in the monomer and each of the oligomeric species was confirmed using immunoblotting and detection using alkaline-phosphatase labeled S-protein (Figure 3B). After reduction with 100 mM dithiothreitol, the oligomers were fully converted to monomers. Other workers have also observed the aggregation of bacterially expressed scFv to oligomeric species (Griffiths et al., 1993; Fipula et al., 1995) but have suggested that the association is noncovalent, because the oligomers converted to monomers in the presence of SDS. Our results may be suggestive of an intermolecular disulfide bond between scFv or, alternatively, reduction of the intramolecular disulfide bond between cysteine residues in the N-terminal conserved region, and the third complementarity determining region (CDR-3) of the heavy-chain sequence may permit SDS to more fully denature the structure of individual scFv, leading to dissociation of monomers.

The effect of scFv oligomerization on immobilization (and antigen capture) efficacy was determined by size-based separation of refolded scFv using Sephacryl S100. Over a range of coating concentrations (0.2−1 μg/ well), separate scFv preparations containing either predominantly monomer or predominantly oligomer exhibited similar detection sensitivities, with 50% maximal binding between 9 and 12 ng of HMW-GS per well. However, the monomer preparation provided 20−50% higher absorbances in the assay at each HMW-GS concentration. The abilities of different coating concentrations of the MAb and of the scFv preparation to capture antigen were compared using a range of HMW-GS antigen concentrations (Figure 4). Increasing the coating amount of scFv from 0.1 to 10 μg increased the assay absorbance at each HMW-GS antigen concentration (Figure 4), whereas a similar increase in the amount of MAb coated had relatively little effect. With the scFv, the limit of detection ($A_{415nm} > 0.1$) was lowered at the higher

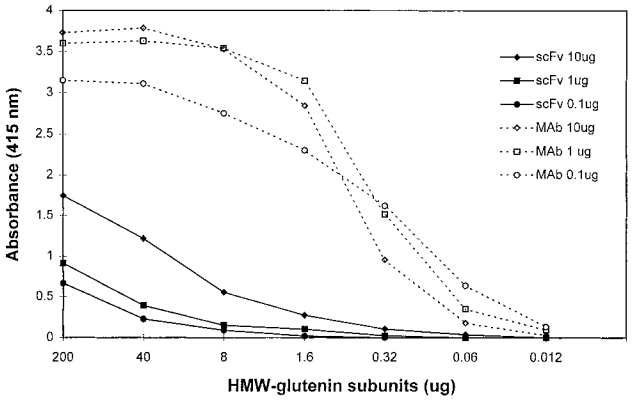

Figure 4. Effect of MAb and scFv coating concentration in sandwich ELISA.

Table 1. Relationships between Antibody Binding, Glutenin Content Analyses, and Quality Parameters in 14 Diverse Wheat Cultivars

antibody	extract dilution	large glutenin polymers	dough quality parameter Rmax	MDT	FDT
MAb	72000	0.534*	0.822***	0.759***	0.870***
scFv	300	0.680**	0.722**	0.708**	0.568*
scFv	1000	0.690**	0.843***	0.686**	0.749**

[a] Data shown are linear correlation coefficients from a single experiment; similar values were obtained in two repeat experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

coating concentrations; it was not possible to determine whether the antigen concentration required for maximal binding was altered at low scFv coating because of the aqueous insolubility of HMW-GS above 2 μg/mL.

ELISA for Dough Strength. The relationship between binding of the scFv or of the parent MAb to HMW-GS-containing extracts of flour samples and Rmax, MDT, and FDT was established for a set of flours from 15 wheat cultivars grown at one site (Horsham, Victoria, Australia) and having moderate flour protein content (mean = 9.8 ± 0.9%). Detailed dough quality data for these flours have been published elsewhere (Singh et al., 1990). Significant correlations were noted between both antibody binding and the content of large glutenin polymers (measured by SE-HPLC) (Gupta et al., 1995) and each of these dough strength parameters (Table 1); however, higher correlations were observed between the ELISA data for the scFv and Rmax and MDT. The relationship between antibody binding and the content of large glutenin polymers confirms that the antibodies are detecting HMW-GS, because the content of these subunits directly determines the content of the large polymers (Gupta et al., 1995). The correlation between scFv binding and FDT improved when the sample extract dilution was increased from 1/300 to 1/1000, because the latter dilution was near the center of the dilution−absorbance curve rather than at 65−75% maximal absorbance, and in further studies this dilution resulted in consistently higher correlation values for these parameters. The correlation between scFv binding and Rmax, which is a major predictor of dough strength, improved from 0.722 to 0.843. The lower affinity but similar specificity of the immobilized recombinant antibody compared with the MAb enabled flour sample preparation to be simplified by using much lower dilutions of the flour extract (Figure 5A,B). The relative slopes of the plots of the relationships between ELISA absorbance and quality parameters were similar for both scFv and MAb. For example, a 100 BU difference in Extensograph Rmax gave a 13−16% difference in color development for both the scFv and MAb-based ELISAs.

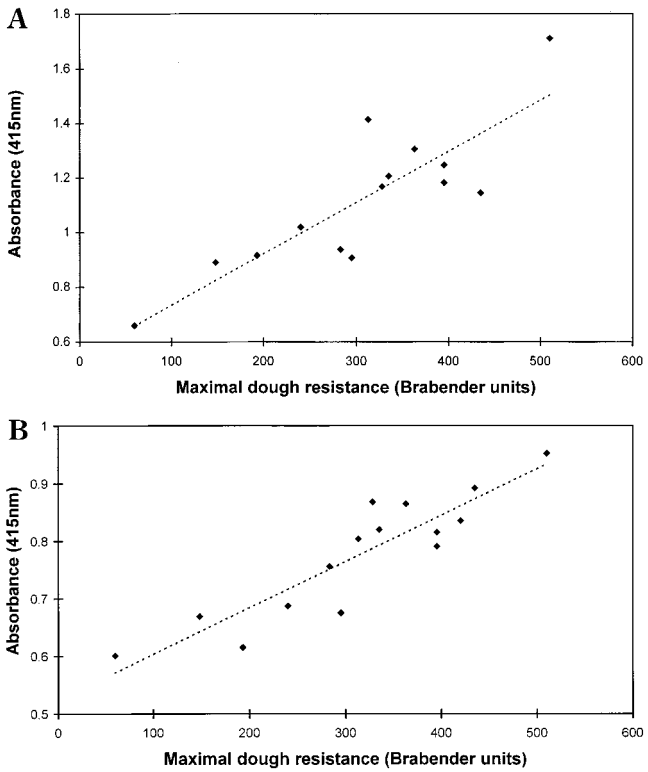

Figure 5. Relationship between MAb and scFv binding and Extensograph maximal dough resistance: (A) flours diluted 1/72000 ($r = 0.825$); (B) flours diluted 1/1000 ($r = 0.902$).

Pre-extraction of the flour samples with 0.5% SDS to remove the monomeric gluten protein (gliadin) fraction from flour samples gave improved discrimination between strong and weak flours with Rmax correlation values increasing from 0.649 to 0.843. The effects of pre-extraction were similar for Rmax and FDT, but correlation coefficients for MDT were not affected. This effect has been shown earlier in a detailed study using a number of MAbs (Skerritt, 1991).

GENERAL DISCUSSION

The use of an antibody in a sandwich ELISA requires its immobilization to a solid support; however, scFv are lacking the more highly conserved constant regions usually involved with immobilization. Generally, to enable immobilization to a solid phase, a recombinant scFv has been fused with the more highly conserved regions of the antibody constant region (Morrison and Coloma, 1995) fused with a human kappa light-chain constant domain (Graham et al., 1995) or modified by the addition of sugar residues to the recombinant fragment, and then the complex is immobilized through this moiety (Hansen et al., 1995). Recombinant antibody fragments have also been used as tracers to detect the presence of a particular antigen in ELISA and protein blots (Kuroki et al., 1995) or as conjugates produced as fusions with *E. coli* alkaline phosphatase or staphylococcal protein A (Gandecha et al., 1994).

We have demonstrated that a recombinant scFv can be immobilized to a solid phase without chemical or covalent attachment chemistry and used for detection and quantification of antigen in a two-site or double-

antibody sandwich ELISA. The advantage of noncovalent immobilization is that it provides a means of immobilizing the scFv without the use of chemical reagents that modify the amino acid sequence and structure of the scFv with corresponding potential for a loss of binding or an alteration in its binding properties. The mechanism for immobilization is not fully understood as yet. We have investigated whether the formation of multimeric scFv was required for immobilization; however, predominantly monomeric scFv was successfully immobilized. Due to the potential increase in valency of scFv dimers compared to monomers, aggregation should play a role in the immobilization as a multimeric scFv would have more available binding sites for antigen interaction that are not involved in immobilization of scFv to the solid phase. An earlier paper described the immobilization of an scFv via a hydrophobic linking group (Davis et al., 1990). We are currently assessing the role of hydrophobic and hydrophilic sequences expressed as fusion proteins with the scFv in immobilization of these fragments and whether scFv are immobilized when there is no peptide tag present. The reduced binding of the scFv compared with that of the MAb could be the result of orientation of the adsorbed protein or a loss of antigen capture efficiency of the immobilized scFv. For the wheat industry, sandwich assays utilizing an immobilized recombinant single-chain antibody of appropriate specificity and affinity can be incorporated into test methods for the analysis of grain or cereal products to predict particular aspects of wheat end-use processing quality, yield, or disease resistance. The improved ELISA for predicting dough strength using the immobilized scFv would enable early generation quality screening to reject lines of unacceptable quality earlier in the breeding process, which would therefore speed and simplify the development of new wheat varieties.

ABBREVIATIONS USED

HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; scFv, single-chain variable fragment; MAb, monoclonal antibody; SDS, sodium dodecyl sulfate; MDT, Mixograph development time; FDT, Farinograph development time; Rmax, maximal dough resistance to extension; PCR, Polymerase Chain Reaction; IPTG, isopropylthio-β-D-galactoside; PBS, phosphate-buffered saline; SE-HPLC, size exclusion high-performance liquid chromatography.

ACKNOWLEDGMENT

The anti-neuraminidase scFv was kindly supplied by Prof. Peter Hudson and Dr. Robert Irving, CSIRO Molecular Science, Parkville, VIC, Australia.

LITERATURE CITED

American Association of Cereal Chemists. *Approved Methods*, 8th ed.; St. Paul, MN, 1983.

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

Batey, I. L.; Gupta, R. B.; MacRitchie, F. Use of size-exclusion high performance liquid chromatography in the study of wheat flour proteins: An improved chromatographic procedure. *Cereal Chem.* **1991**, *68* (8), 207−209.

Bird, R. E.; Walker, B. W. Single chain antibody variable regions. *Trends Biotechnol.* **1991**, *9*, 132−137.

Bird, R. E.; Hardman, K. D.; Jacobson, J. W.; Johnson, S.; Kaufman, B. M.; Lee, S.-M.; Lee, T.; Pope, S. H.; Rior, G. S.; Whitlow, M. Single chain antigen-binding proteins. *Science* **1988**, *242*, 423−426.

Clackson, T.; Hoogenboom, H. R.; Griffiths, A. D.; Winter, G. Making antibody fragments using phage display libraries. *Nature* **1991**, *3352*, 624−628.

Coloma, M. J.; Larrick, J. W.; Ayala, M.; Gavilondo-Cowley, J. V. Primer design for the cloning of immunoglobulin heavy-chain leader-variable regions from mouse hybridoma cells using the PCR. *Biotechniques* **1991**, *11*, 152−156.

Davis, P. J.; Verhoeyen, M. E.; De Winter, R. F. J. Antibody variable domain conjugates. World Patent Appl. WO 91/08483, 1991.

Derman, A. I.; Prinz, W. A.; Belin, D.; Beckwith, D. Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science* **1993**, *262*, 1744−1747.

D'Ovidio, R.; Anderson, O. D.; Masci, S.; Skerritt, J. H.; Porceddu, E. Construction of novel wheat high-molecular weight glutenin gene variability: modification of the repetitive domain and expression in bacteria. *J. Cereal Sci.* **1997**, *25*, 1−8.

Dubel, S.; Breitling, F.; Fuchs, P.; Zewe, M.; Gotter, S.; Welschof, M.; Moldenhauer, G.; Little, M. Isolation of IgG antibody Fv-DNA from various mouse and rat hybridoma cell lines using the polymerase chain reaction with a simple set of primers. *J. Immunol. Methods* **1994**, *175*, 89−95.

Fipula, D.; Rollence, M.; Essig, N.; Nagle, J.; Achari, A.; Lee, T. In *Antibody Expression and Engineering*; Wang, H. Y., Imanaka, T., Eds.; ACS Symposium Series 604; American Chemical Society: Washington, DC, 1995; pp 70−85.

Gandecha, A.; Owen, M.; Cockburn, W.; Whitelam, G. Antigen detection using recombinant, bifunctional single chain Fv fusion proteins synthesized in *Escherichia coli*. *Protein Express. Purif.* **1994**, *5*, 385−390.

Glockshuber, R.; Schmidt, T.; Plückthun, A. The disulfide bonds in antibody variable domains: effects on stability, folding in vitro, and functional expression in *Escherichia coli*. *Biochemistry* **1992**, *31*, 1270−1279.

Graham, B. M.; Porter, J. R.; Harris, W. J. Cloning, expression and characterization of a single chain antibody fragment to the herbicide paraquat. *J. Chem. Technol. Biotechnol.* **1995**, *63*, 270−289.

Griffiths, A. D.; Malmqvist, M.; Marks, J. D.; Bye, J. M.; Embleton, M. J.; McCafferty, J.; Baier, M.; Holliger, K. P.; Gorick, B. D.; Hughes-Jones, N. C.; Hoogenbloom, H. R.; Winter, G. Human anti-self-antibodies with high specificity from phage display libraries. *EMBO J.* **1993**, *12*, 725−734.

Gupta, R. B.; Popineau, Y.; Lefebvre, J.; Cornec, M.; Lawrence, G. J.; MacRitchie, F. Biochemical basis of flour properties in bread wheats. II. Changes in polymeric protein formation and dough/gluten properties associated with the loss of low Mr or high Mr glutenin subunits. *J. Cereal Sci.* **1995**, *21*, 103−116.

Hansen, H. J.; Leung, S.; Shevitz, J.; Griffiths, G. L.; Govindan, S. V. Preparation and use of immunoconjugates. World Patent Appl. WO 95/15769, 1995.

Heinrichs, A.; Milstein, C.; Gherardi, E. Universal cloning and direct sequencing of rearranged antibody V genes using C region primers, biotin captured cDNA and one-side PCR. *J. Immunol. Methods* **1995**, *178*, 241−251.

Huse, W. D.; Sastry, L.; Iverson, S. A.; Kang, A. S.; Alting-Mees, M.; Burton, D. R.; Benkovic, S. J.; Lerner, R. A. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **1989**, *246*, 1275−1280.

Huston, J. S.; Levinson, D.; Mudgett-Hunter, M.; Tai, M. S.; Novotny, J.; Margolies, M. N.; Ridge, R. J.; Bruccoleri, R.; Haber, E.; Crea, R.; Opperman, H. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5879−5883.

Kim, J.-S.; Raines, R. T. Ribonuclease S-peptide as a carrier in fusion proteins. *Protein Sci.* **1993**, *2*, 348−356.

Kitchin, K.; Lin, G.; Shelver, W.; Murtaugh, M.; Pentel, P.; Pond, S. M.; Oberst, J. C.; Humphrey, J. E.; Smith, J. M.; Flickinger, M. C. Cloning, expression and purification of an anti-desipramine single chain antibody in NS/0 myeloma cells. *J. Pharm. Sci.* **1995**, *84*, 1184−1189.

Kramer, K.; Hock, B. Recombinant single chain antibodies against s-triazines. *Food Agric. Immunol.* **1996**, *8*, 97−109.

Kuroki, M.; Matsumoto, Y.; Arakawa, F.; Haruno, M.; Murakami, M.; Kuwahara, M.; Ozaki, H.; Senba, T.; Matsuoka, Y. Reducing interference from heterophilic antibodies in a two-site immunoassay for carcinoenbryonic antigen (CEA) by using a human/mouse chimeric antibody to CEA as the tracer. *J. Immunol. Methods* **1995**, *180*, 81−91.

Malby, R.; Caldwell, J.; Gruen, L.; Harley, N.; Ivancic, N.; Kortt, A. A.; Lilley, G. G.; Power, B. E.; Webster, R. G.; Colman, P. M.; Hudson, P. J. Recombinant anti-neuraminidase single chain antibody: expression, characterisation and crystallisation in complex with antigen. *Proteins: Struct., Funct. Genet.* **1993**, *16*, 57−63.

Morrison, S.; Coloma, M. J. Production of bispecific tetravalent antibody by recombinant expression of DNA encoding variable heavy and light immunoglobulin chains. World Patent Appl. WO 95/09917, 1995.

Plückthun, A. Antibody engineering: Advances from the use of *Escherichia coli* expression systems. *Bio/Technology* **1991**, *9*, 545−551.

Richards, F. M.; Wyckoff, H. W. In *The Enzymes*; Boyer, P. D. Ed.; Academic Press: New York, 1971; Vol. IV, pp 647−806.

Schaefer, B. C. Revolutions in rapid amlplification of cDNA ends: new strategies for polymerase chain reaction cloning of full-length cDNA ends. *Anal. Biochem.* **1995**, *227*, 255−273.

Singh, N. K.; Donovan, G. R.; MacRitchie, F. Use of sonication and size exclusion HPLC in the study of wheat proteins. II. Relative quantity of glutenin as a measure of bread-making quality. *Cereal Chem.* **1990**, *67* (7), 161−170.

Skerritt, J. H. A simple antibody-based test for dough strength. 1. Method development and choice of antibodies. *Cereal Chem.* **1991**, *68* (8), 467−474.

Skerritt, J. H. Gluten proteins: Genetics, structure and dough quality—a review. *AgBiotech News Inf.* **1998**, *10*, 247N−270N.

Received for review February 17, 1999. Revised manuscript received July 15, 1999. Accepted July 19, 1999.

JF990167F