Characterization of a Monoclonal Antibody Specific for HMW Subunits of Glutenin and Its Use To Investigate Glutenin Polymers

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A monoclonal antibody, IFRN 1602, has been developed to a synthetic peptide based on the sequence ^{94}GSVTCPQQV^{101} of HMW subunit 1Dx5. The antibody bound strongly to the synthetic peptide based on the cognate sequence of HMW subunit 1Dx2 which contains a serine instead of a cysteine residue. However, it recognized the immunizing peptide by enzyme-linked immunosorbent assay (ELISA) only poorly, probably because the peptide exists as a disulfide-bonded dimer under the assay conditions. From immunoblotting studies against a wide range of wheat varieties, IFRN 1602 was shown to primarily recognize x-type HMW subunits of glutenin encoded on chromosomes 1A and 1D, cross-reacting weakly with the 1A and 1D y-type subunits. It did not bind to any of the 1B-encoded subunits. The Mab also recognized a small number of polypeptides of greater mobility than HMW subunits which were not visible on the stained gels and occurred only in the presence of specific 1A and 1D x-type HMW subunits. Such polypeptides were not present in a preparation of recombinant subunit 2, suggesting that they are modified forms of the subunits which arise in the seed perhaps by processing of the associated subunits. When used to probe partially reduced glutenin, IFRN 1602 bound to 1Dx5−1Dy10 dimers. As the Mab reacted primarily with Cys^{97} of 1Dx5 in a reduced form, these data suggest that this residue is not involved in either intra- or intermolecular disulfide bond in the HMW subunit dimers. Thus, Cys^{97} of 1Dx5 may be present in gluten in a reduced form, involved in intramolecular disulfide bonds, or linking of the HMW subunit dimers into larger polymers.

Keywords: *HMW subunits of glutenin; wheat; monoclonal antibodies; polymers*

INTRODUCTION

The high molecular weight (HMW) subunits of wheat glutenin comprise around 8−10% of the total extractable flour protein (Halford et al., 1992) and represent one of the most intensively studied groups of wheat storage proteins (Shewry et al., 1992). The interest in HMW subunits stems from the correlation of certain subunit alleles with bread-making quality in European wheats (Luckow et al., 1989; Payne et al., 1981, 1987; Moonen et al., 1983; Rogers et al., 1989). The HMW subunits have M_r by SDS-PAGE of about 80 000−146 000, although the analysis of nine isolated genes and mass spectroscopy of seven purified proteins has shown that the true M_r are much lower (~67 000−88 000) (Hickman et al., 1995). Some 30 variants have been identified by SDS-PAGE (Ng and Bushuk, 1989), with three, four, or five individual subunits present in each wheat variety. Six subunit genes, two on each of the long arms of chromosomes 1A, 1B, and 1D, have been identified in wheat. These genes encode two different types of subunit, called x-type or y-type, which differ in their M_r (higher for x-type), number of cysteine residues (four in most x-type subunits compared with six or seven in y-type), and repeat motifs. Not all these genes are expressed, resulting in the variation in HMW subunit number seen in different varieties. Thus, all European cultivars contain 1Dx, 1Dy, and 1Bx subunits, with 1Ax and/or 1By subunits also being present in some cultivars. 1Ay subunits are not usually present, although a subunit of this type has been reported in a Swedish line (Margiotta et al., 1996).

In addition to the variation in subunit number, there is also considerable allelic variation in the electrophoretic mobilities of the HMW subunits, although a relatively small number of such variants are present in commercial wheats. This reflects in part the narrow range of germplasm employed by plant breeders, but may also result from specific allelic forms being strongly associated with good or poor breadmaking performance and therefore being selected for, or against, during the breeding process. The association of HMW subunits and breadmaking quality has enabled Payne and co-workers to assign "quality scores" for different alleles (Payne et al., 1981, 1987), quality being positively associated with specific allelic pairs of subunits encoded by chromosomes 1D and 1B. Thus, the subunit pair called 1Dx5 + 1Dy10 is positively associated with breadmaking quality compared with subunits 1Dx2 + 1Dy12, as are the pairs 1Bx17 + 1By18, 1Bx7 + By8, and 1Bx7 + 1By9, compared with 1Bx6 + 1By8 or 1Bx7 alone. For chromosome 1A the presence of subunits 1Ax1 or 1Ax2*

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are more positively associated with breadmaking quality compared with the null allele. However, because the genes for x-type and y-type subunits are tightly linked, it is not possible to determine quality scores for all the individual subunits present in the allelic pairs.

The HMW subunits are components of the glutenin fraction which is responsible for the elastic properties of dough and gluten. Allelic variation in HMW subunit composition is also associated with differences in elasticity, implying that the HMW subunits play a crucial role in the elastic mechanism. However, the molecular basis for this role remains unclear. Biophysical studies have shown that the individual subunits have an extended rodlike shape, resulting from the repetitive domain which comprises some 75−85% of the whole protein and which forms a loose spiral structure (Field et al., 1987; Miles et al., 1991). This structure may contribute to the elastic mechanism but it is probable that interactions (disulfide cross-linking and noncovalent hydrogen bonding) between individual subunits are also involved (Shewry et al., 1992; Belton, 1999). In particular, the ability of the HMW subunits to form disulfide-bonded polymers of M_r several million daltons in size, which also contain other glutenin proteins (Field et al., 1983), may contribute to gluten viscoelasticity. There is evidence that interchain disulfide bonds between HMW subunits are not random (Shewry and Tatham, 1997) and it has been suggested that an extra cysteine residue present in the repetitive domain of 1Dx5 is responsible, in part at least, for the quality associated with subunits 1Dx5 + 1Dy10, when compared with subunits 1Dx2 + 1Dy12. In addition, it is probable that the quantity of HMW subunits present in a variety of wheat is also important in determining gluten viscoelasticity (Halford et al., 1992; Kolster et al., 1993; Sutton, 1991). Such an effect may account for the increased quality associated with the expression of a 1Ax subunit (Halford et al., 1992).

Immunological methods have been increasingly exploited in cereal research (Skerritt and Tatham, 1996) but it has proved difficult to develop antibody preparations specific for particular groups of gluten proteins. This is because of the extensive sequence and structural homologies that exist between gluten proteins (Mills et al, 1995), and while antibodies specific for repeat motifs can be produced, they tend to be broadly reactive with groups of prolamins because of these similarities (Brett et al., 1999). Polyclonal antisera raised to HMW subunits have either been of broad specificity (Festenstein et al., 1985) or required affinity purification to be rendered specific for x-type HMW subunits (Curioni et al., 1991). Synthetic peptides corresponding to the N-terminal sequences and repetitive motifs common to all HMW subunits, together with sequences from the N-terminal domain, have also been used to raise polyclonal antisera with varying degrees of success (Denery-Papini et al., 1996). Monoclonal antibodies have also been produced to gluten proteins (Skerritt, 1987), some of which recognized sequences present in the repetitive domains of HMW subunits (Andrews and Skerritt, 1994). The present report describes the production and characterization of highly specific monoclonal antibodies (Mabs) to a synthetic peptide (^{94}GSVTCPQQV^{101}, based on the sequence of HMW subunit 1Dx5) and their use to probe the accessibility of the thiol group of Cys^{97} of 1Dx5 in wheat glutenin.

MATERIALS AND METHODS

Wheat Varieties, Proteins, and Synthetic Peptides. Varieties of bread wheat (*Triticum aestivum* L.) with different HMW subunit compositions came from in-house collections (IACR-LARS and Advanta Seeds UK) or were supplied by D. Lafiandra (University of Tuscia, Viterbo, Italy). The group 1 nullisomic−tetrasomic lines of wheat cv. Chinese Spring, developed by Sears (1954) were supplied by T. E. Miller (John Innes Centre, Norwich, UK). Total prolamins from wheat were prepared as described by Mills et al. (1990) and purified HMW subunit fractions as described by Hickman et al. (1995). Recombinant subunits were expressed in *E. coli* and purified as described by Shani et al. (1992). Synthetic peptides GSVTC/SPQQV were obtained from Cambridge Research Biochemicals (Northwich, UK). The free thiol groups of synthetic peptides (dissolved in phosphate-buffered saline (PBS; 0.14 M NaCl, 0.0015 M KH_2PO_4, 0.008 M Na_2HPO_4, 0.0027 M KCl)) were determined using dithiobisnitrobenzoic acid, as described by Aitken and Learmout (1996).

Monoclonal Antibody Production. Mice were immunized with the synthetic peptide GSVTCPQQV conjugated to bovine serum albumin (BSA) and monoclonal antibodies (Mabs) produced as described by Mills et al. (1990). Briefly, spleen cells were isolated from immunized mice and fused with the mouse myeloma line X63 Ag 8 653. Antibody-producing cell lines were identified by screening against either GSVTCPQQV conjugated to bovine thyroglobulin (BTG) or a total prolamin preparation from wheat cv. Mercia. All cell culture procedures were performed using Optimem (Gibco, UK Ltd) supplemented with 4% (v/v) foetal calf serum (Advanced Protein Products Ltd, UK) as the culture medium.

Enzyme-Linked Immunosorbent Assay (ELISA) Procedures. The direct ELISAs were performed as described by Mills et al. (1990) and the inhibition ELISAs as described by Plumb et al. (1994) using purified HMW subunits 1Bx6 + 1By10, 1Dx5 + 1Dy10 as the solid phase. Briefly polystyrene microtitration plates (Nunc Immunoplate I, Gibco UK Ltd.) were coated using 1μg/mL of protein fraction or peptide-conjugate in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6. ELISA incubation steps were performed using phosphate-buffered saline containing Tween (PBS containing 0.05% (v/v) Tween-20) as diluent and for plate washing. Anti-mouse IgG labeled with horseradish peroxidase (Sigma Chemical Co., UK) diluted 1:2000 (v:v) in PBST was used as the second antibody and substrate was based on 3,3′,5,5′-tetramethylbenzidine (Vetoquinol, Bicester, UK).

Electrophoresis and Immunoblotting Procedures. One-dimensional (1-D) SDS-PAGE was carried out using the Tris-borate buffer system of Koenig et al. (1970) with a 10% acrylamide−0.1% bis separating gel and a 3% acrylamide−0.08% bis stacking gel. Samples were extracted with the aid of sonication into SDS−sample buffer containing 100 mM DTT and heated to 100 °C for 3 min prior to loading. Partially reduced samples for two-dimensional analysis were produced by sonicating crushed grain for 45 min in SDS−sample buffer containing 2 mM DTT and 1 mM EDTA, followed by heating to 100 °C for 30 s. The two-dimensional gel system (partially reduced/fully reduced) used to analyze the preparations was as described by Sing et al. (1984), but using the Tris-borate buffer system described above. Duplicate gels were blotted (see below) or fixed and stained in 0.1% (w/v) Coomassie Brilliant Blue R250 in 20% (v/v) ethanol−8% (w/v) trichloroacetic acid and destained in 8% (w/v) trichloroacetic acid. Prior to blotting gels were soaked for 1 h in transfer buffer comprising 39 mM glycine, 48 mM Tris, 0.038% (w/v) SDS, with or without 20% (v/v) methanol. Transfers were effected in a semidry blotting apparatus (NovaBlot, Pharmacia Biotech, Sweden) at 0.8 mA/cm^2 for 1 h onto nitrocellulose membranes (Sartorius Ltd, Epsom, UK, 0.45 μm pore size) with up to six gels being blotted at one time. Transfer efficiency and selectivity were monitored by staining membranes (Instaview Nitrocellulose, BDH-Merck, Poole, UK) and gels after blotting. Blots were developed as

Table 1. Comparison of Aligned Sequences from the Repetitive Domain of Various HMW Subunits[a]

HMW subunit	sequence
1Dx5	^{90}RYYL **SVTCPQQ** VSYY^{104}
1Ax1	^{46}RYYL **SVTSPQQ** VSYY^{61}
1Ax2*	^{46}RYYL **SVTSPQQ** VSYY^{61}
1Dx2	^{89}RYYL **SVTSPQQ** VSYY^{103}
1Bx7	^{83}RYYP **SVTSSQQ** GSYY^{97}
1Bx17	^{82}RYYP **SVTSSQQ** GSYY^{96}
1By9	^{102}GYYP **SVSSPQQ** GPYY^{116}
1Dy10	^{105}GYYP **GVTSPRQ** GSYY^{119}
1Dy12	^{105}GYYP **SVTSPRQ** GSYY^{119}

[a] Sequence information from Anderson and Greene (1989); Halford et al. (1992); Reddy and Appels (1993); Sugiyama et al. (1985); Thompson et al. (1985).

described by Mills et al. (1990) using alkaline phosphatase labeled anti-mouse IgG and nitroblue tetrazolium-BCIP as the substrate.

RESULTS AND DISCUSSION

Mab Characterization. Three Mabs were raised to the synthetic peptide GSVTCPQQV, which corresponds to residues 94−101 of HMW subunit 1Dx5 with an additional C-terminal glycine. This region contains a cysteine residue (Cys^{97}) which is not present in the allelic subunit 1Dx2, which instead contains the sequence SVTSPQQV (Table 1). Two of the Mabs (IFRN 0312,1601) were selected using a GSVTCPQQV-BTG conjugate and one (IFRN1602) using a total prolamin preparation from the breadmaking quality wheat variety Mercia, which possesses the HMW subunits 1Dx6 + 1By8 and 1Dx5 + 1Dy10.

Figure 1A shows the ELISA titration curves obtained with IFRN 1602 against the peptide-BTG conjugate, total prolamin extracts from several bread wheats with different HMW subunit compositions and a HMW subunit preparation from cv. Mercia. Reactivity was greatest with the peptide conjugate and was saturating at dilutions of culture supernatant where binding to the prolamin fractions was only just apparent. Stronger recognition of a synthetic peptide compared with the native protein has been observed previously (Fieser et al., 1987). As might be expected, binding was greater toward the purified HMW subunit fraction than toward the total prolamin extracts. Recognition of the latter also varied, depending on the HMW subunit composition of the wheat variety. Thus, binding to cv. Sicco (HMW subunits 1Ax1, 1By7 + 1By9, 1Dx5 + 1Dy10) was greatest, followed by Moulin (1Bx17 + 1By18, 1Dx2 + 1Dy12) and Broom (1Bx7 + 1By9, 1Dx5 + 1Dy10) which were recognized equally well. The specificity of IFRN 1602 toward the immunizing peptide, GSVTCPQQV, and the corresponding sequence present in subunits 1Dx2 and 1Ax1, GSVTSPQQV, was further defined using an inhibition ELISA (Figure 1B). The Mab preferentially recognized the peptide corresponding to residues 89−103 of 1Dx2 and 46−61 of 1Ax1, which contains the TSP moiety. Thus, 0.16 μg/mL of this peptide, compared with 0.68 μg/mL peptide containing the TCP motif, were required to depress Mab binding by 50%. When the free thiol content of the TCP peptide dissolved in PBS was determined, only 9% of the peptide was present in an unreduced form, the remainder being present in a disulfide-bonded form. The reactivity of the purified iodoacetamide alkylated TCP peptide was around 20% of that observed toward the TSP peptide (data not shown), demonstrating that modification of

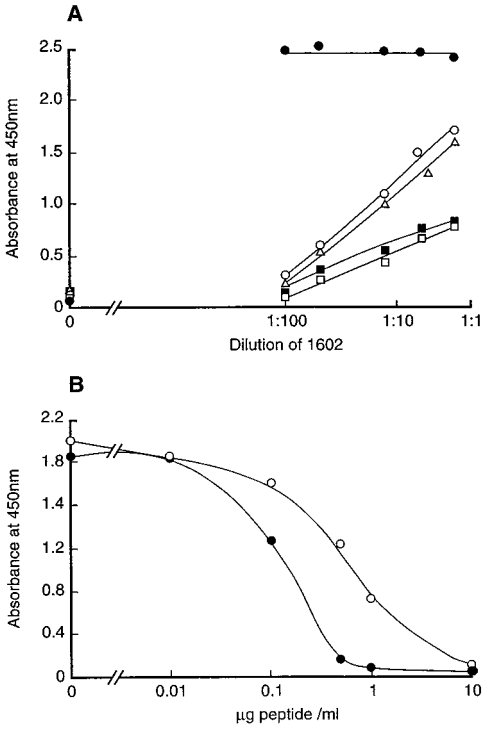

Figure 1. Characterization of Mab IFRN 1602 binding by enzyme-linked immunosorbent assay (ELISA). (A) Titration curves determined by direct ELISA against the peptide conjugate GSVTCPQQV−BTG (-●-); HMW subunit fraction cv. Mercia (HMW subunits 1Bx6 + 1By8, 1Dx5 + 1Dy10) (-○-); total prolamin fractions of wheat cv. Sicco (1A1x,1Bx7 + 1By9, 1Dx5 + 1Dy10) (-△-); cv. Moulin (1Bx17 + 1By18, 1Dx2 + 1Dy12) (-■-); cv. Broom (1Bx7 + 1By9, 1Dx5 + 1Dy10, -□-). (B) Inhibition ELISAs with synthetic peptides corresponding to 1Dx5 residues 94−100 (-○-) and 1Dx2 residues 93−100 (-●-).

the thiol group of the peptide reduced, but did not abolish, Mab binding. It seems likely that the poor recognition of the TCP peptide compared with the TSP peptide is primarily due to the antibody only recognizing the reduced, and not the dimeric, form of the peptide. It was concluded that IFRN 1602 would be expected to cross-react with both 1A and 1D encoded x-type subunits, but would recognize the form of 1Dx5 with a reduced Cys^{97} more strongly than the disulfide bonded form.

Mab specificity was further investigated by immunoblotting analysis of various wheat varieties with different HMW subunit compositions (Figure 2). Figure 2A shows a 1D SDS-PAGE separation of a total protein extract of a wheat cultivar with HMW subunits 1,5+10, 14+15 (track 1), together with the corresponding probed blot from a duplicate gel (track 2). These results demonstrate that the Mab only recognized polypeptides migrating in the HMW glutenin subunit region. Figure 2B−E show the upper portion of gels (B,D) and the corresponding probed blots (C, E) of 31 lines and varieties of wheat containing a range of HMW subunits, including some types that only occur rarely. The individual subunits are labeled using the nomenclatures of Payne and co-workers (Payne and Lawrence 1983; Payne et al., 1980, 1981) and Lafiandra et al. (1994) which are based on the relative electrophoretic mobilities of HMW subunits by SDS PAGE. These results show that the Mab has greatest affinity for 1Ax subunits (i.e., 1 and 2*) and 1Dx subunits (i.e., 2, 2.2, 2.2*, 2**, 3, 4, 4_1, 5, 5*, and 5**). A much lower level of binding was observed to 1Dy (10, 10*, 12, 12*, and 12_1) and 1By

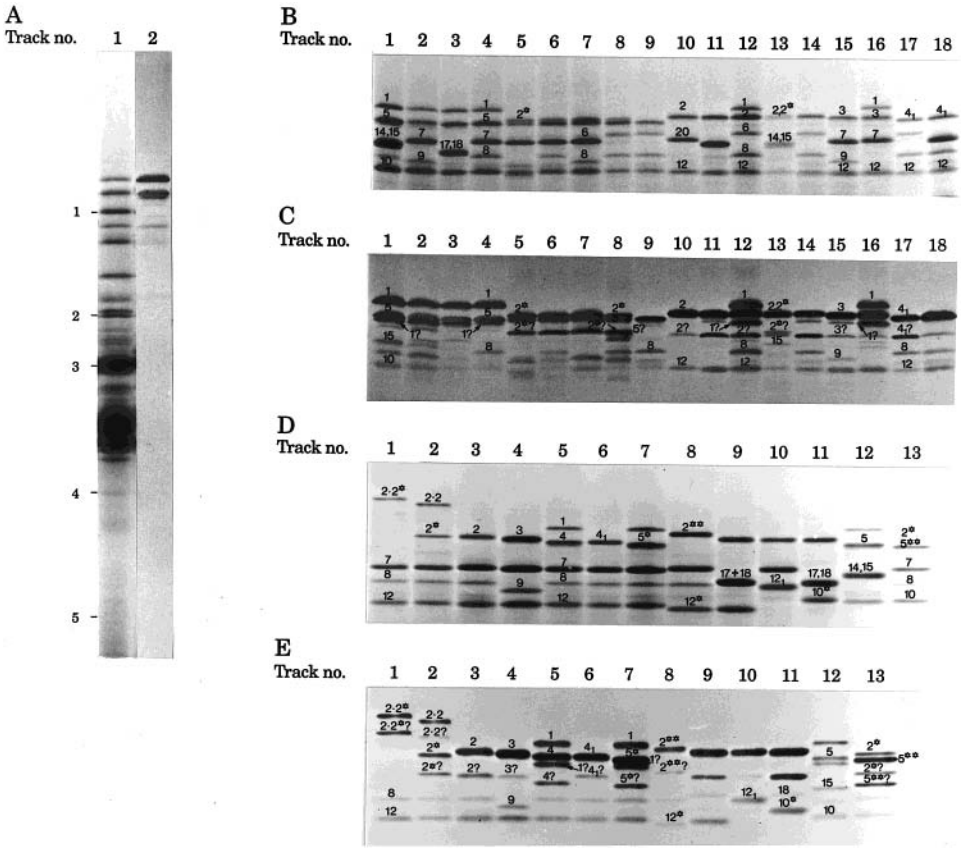

Figure 2. (A−E) SDS-PAGE and immunoblot analysis of 31 wheat lines and varieties containing a range of HMW subunits. The immunoblots were developed using Mab IFRN 1602. (A) Shows entire track of an SDS-PAGE gel (track 1) and corresponding immunoblot (track 2) of a total protein extract of a single wheat seed. (B−E) Shows only the upper portions of SDS-PAGE gels (B, D) and corresponding immunoblots (D, E) of total protein extracts of a collection of wheat varieties with different HMW subunit compositions. The HMW subunits on the gels and corresponding bands on immunoblots have been marked using the nomenclature of Payne and Lawrence (1983), Payne et al. (1980, 1981), and Lafiandra et al. (1994).

subunits (8, 15, 18, and 19), while the 1Bx subunits (6, 7, 14, 17, and 20) were not recognized at all. However, other polypeptides, migrating in the same region as the 1By-type subunits, were also recognized. Furthermore, the Mab also bound to a small number of polypeptides which were not visible on the stained gels. Although the origin and nature of these polypeptides are not known, examination of Figure 2 shows that they only occur in the presence of particular HMW subunits. Thus, 1Ax subunits (1 and 2*) and the 1Dx subunits (2, 2.2, 2.2*, 2**, 3, 4, 4_1, 5 (in one instance only, see Figure 2C track 8), 5* and 5**) are each accompanied on the probed blots by an additional band of greater mobility. Because of this clear association with previously identified HMW subunits, the additional bands are labeled 1?, 2*? etc. Furthermore, immunoblot analysis of a series of aneuploid lines of cv. Chinese Spring (Figure 3) showed that one of these bands (2?) was encoded on the same chromosome as the associated HMW subunit (1Dx2). It also appears that the additional bands are specific to proteins synthesized in the wheat grain, as immunoblot analysis of recombinant subunit 2 expressed in *E. coli* (Figure 3D track 2) gave only a single immunoreactive polypeptide which corresponded to the full length subunit. It is possible, therefore, that the ? bands present in endosperm protein preparations may be minor processing products of the associated subunits.

Epitope Accessibility in Glutenin Polymers. It has been suggested that the good breadmaking quality associated with HMW subunit 1Dx5 results from an extra cysteine residue (Cys^{97}) present at the beginning of the repetitive domain, which is not present in the allelic "poor quality" subunit 1Dx2 (Anderson and Greene, 1989). Therefore, IFRN 1602 was used to probe partially reduced glutenin by immunoblotting (Figure

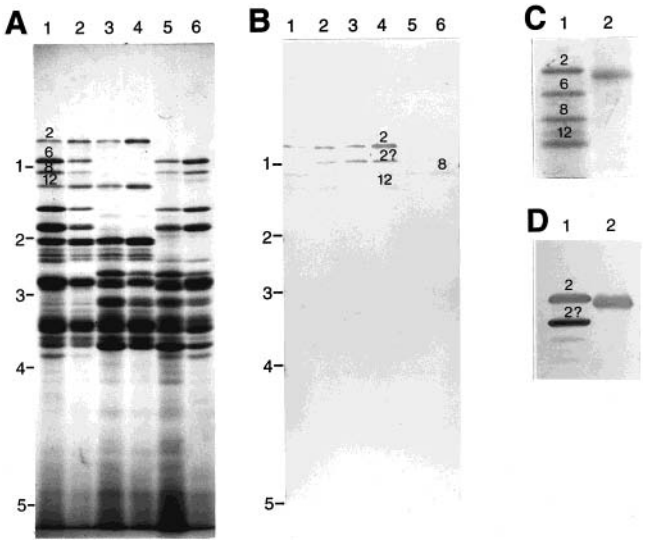

Figure 3. SDS-PAGE (A, C) and immunoblot (B, D) analysis of total protein extracts of single seeds of the compensating nulli-tetrasomics of Sears (1954) developed in cv. Chinese Spring and recombinant HMW subunit 1Dx2. The immunoblot was developed with IFRN 1602. Tracks were as follows for A, B: 1, N1A−T1D; 2, N1B−T1A; 3, N1B−T1D; 4, N1D−T1A; 5, N1D−T1B. C,D: 1, wheat cv. Riband; 2, recombinant HMW 1Dx 2.

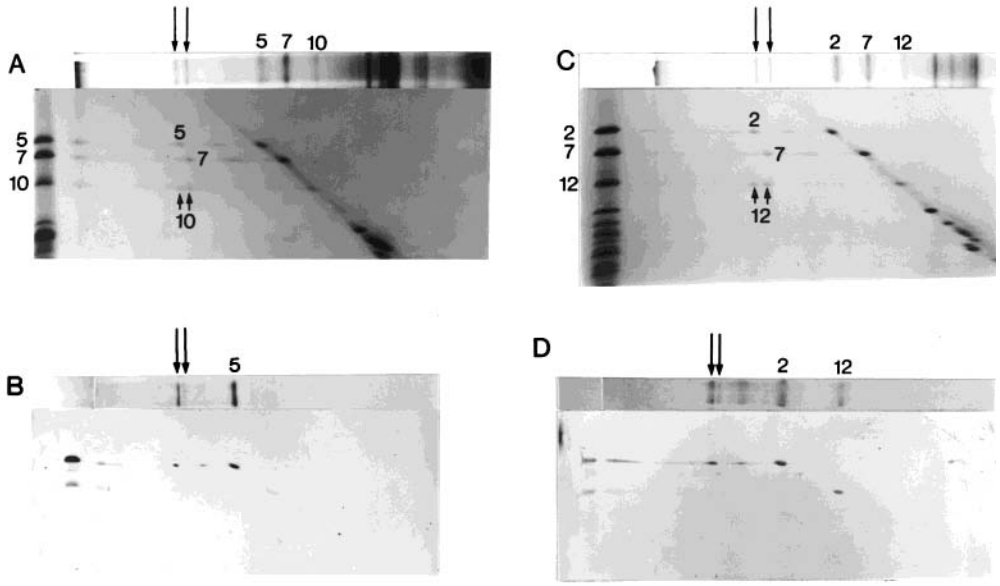

Figure 4. (A, B) Two-dimensional (nonreduced/reduced) SDS-PAGE analysis (A, C) and immunoblots (B, D) of partially reduced extracts of single seeds of wheat cv Bouquet (A, B) and Galahad (C, D). The 1-D SDS-PAGE patterns under nonreducing conditions are shown along the top, and those under reducing conditions are shown to the left of each 2-D separation. Arrows indicate the position of HMW subunit dimers in the 1D separations performed under nonreducing conditions. HMW subunit monomers are numbered.

4). Sample extraction conditions were chosen such that the solubilized material contained a significant proportion of HMW subunit dimers, as indicated on the 1-D gel strips in Figure 4, A and C. Complete reduction and subsequent SDS-PAGE of this material revealed that the dimers comprised HMW subunits 1Bx7 + 1By10 and 1Dx5 + 1Dy10 in cv. Bouquet (Figure 4A) and 1Bx7 + 1By12 and 1Dx2 + 1Dy12 in cv. Galahad (Figure 4C). When the corresponding blots (Figure 4, B and D) were probed with IFRN 1602 it could be seen that antibody bound to the 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12 dimers but not, detectably, with the 1Bx7 + 1By10 and 1Bx7 + 1By12 dimers. This implies that binding was restricted to subunits 1Dx5 and 1Dx2, indicating that Cys^{97} of subunit 5 was accessible to the antibody and therefore not involved in dimer formation. An additional dimer band, presumably corresponding to 2?-1Dy12, and the 2? monomer are also apparent on the Galahad immunoblot (Figure 4D). Streaks were also evident on the immunoblots, particularly for Galahad (Figure 4D), running between the HMW subunit dimers and monomers. These may represent HMW-LMW subunit dimers.

GENERAL DISCUSSION

The strong binding to the 1A and 1D x-type HMW subunits displayed by the Mab IFRN 1602 indicates that it recognized sequences present in these subunits which correspond to the synthetic peptide used as the immunogen (namely residues 94−101 of subunit 1Dx5). Table 1 shows the homologous sequences for those subunits which have been sequenced to date. From these data it can be seen that Cys^{97} of 1Dx5 has been substituted by a serine in subunits 1Dx2, 1Ax1, and 1Ax2*. Cysteine and serine have R-groups which are very similar in size and electronic configuration, which may account for the observed cross-reactivity of the Mabs with these subunits. The results also indicate that the Mabs recognize 1Dx5 with a reduced Cys^{97}much more strongly than the disulfide bonded form. The recognition of the other 1A and 1D x-type subunits for which sequence information is not yet available (i.e., 2.2, 2.2*, 2.2**, 3, 4, 4_1, 5*, and 5**) suggests that these subunits also contain epitopes consisting of the sequences SVTCPQQ or SVTSPQQ. The only sequences of 1B x-type HMW subunits described to date are for subunits 7 and 17. These have additional substitutions compared with 1Dx5, with Pro^{98} being replaced by serine. By virtue of being an imino acid, proline introduces kinks in the polypeptide backbone and the loss of such a residue would introduce considerable change to the architecture of the epitope. Such an alteration could account for the lack of recognition of these subunits by IFRN 1602. The lack of Mab reaction toward the other 1B encoded x-type subunits suggests they may also contain major sequence changes compared to 1Dx5.

The weak binding observed with the y-type subunits probably results from the larger number of substitutions present in the epitopes of these proteins. Thus, Thr^{96} is replaced by a serine in subunit 9, and Gln^{96} by an arginine in subunits 10 and 12, with subunit 12 also containing a glycine instead of Ser^{94} at the N-terminal end of the epitope. These alterations, unlike the loss of Pro^{98} in subunit 1Bx7, are not sufficient to abolish Mab binding but nevertheless greatly reduce the affinity of the antibody for the y-type HMW subunits. Similar types of alteration in the epitope must also be present in the other 1D and 1B y-type subunits, for which no sequence data are available, but which are recognized only weakly by the antibody.

The observation that the Mab recognized additional polypeptides of greater mobility which are associated with the 1A and 1D x-type HMW subunits was unexpected. They were not an artifact of the electrophoretic separation as recombinant 1Dx2 expressed in *E. coli* did not show an associated band after immunoblotting. Furthermore, these additional polypeptides did not result from the partial reduction of the original subunit as they were present when dithiothreitol concentrations, ranging from 1 to 100 mM, were employed for sample extraction (results not shown). They may be modified

forms of the HMW subunits, which resemble the peptide immunogen more closely in terms of secondary structure than the native HMW. Since the additional bands were not observed in the recombinant subunit 2 preparation, these modified forms of the subunits clearly arise in the seed. Evidence of truncated versions of HMW subunits being present in the seed has been found from sequencing studies (Lew et al., 1992). These workers found a polypeptide with a N-terminal sequence which corresponds to the N-terminal region of HMW subunit 1Dx5 minus the first 60 residues. Such a fragment would still contain the epitope region recognized by IFRN 1602 and is likely to correspond to the additional low-mobility polypeptide observed on immunoblots of seed extracts.

The formation of glutenin polymers, linked by intermolecular disulfide bonds, is thought to be important in determining the viscoelastic properties of flour doughs (Ewart, 1968, 1990; Gupta et al., 1992). Thus, the role of HMW subunits in determining glutenin polymer formation and properties may account for their effects on breadmaking quality. Subunit 1Dx5 is positively associated with quality and is the only HMW subunit characterized to date which has a cysteine residue (Cys^{97}) at the N-terminal end of the repetitive domain. It has been suggested that this residue may play a role in giving rise to more highly cross-linked, and hence viscoelastic, gluten (Shewry et al., 1992). Secondary structure prediction suggests that Cys^{97} is located in a β-turn and is solvent exposed (Greene and Anderson, 1991). It might therefore be able to participate in either intra- or intermolecular disulfide bond formation. Tao et al. (1992) and Werner et al. (1992) have indicated that subunit 1Dx5 may form at least one intramolecular disulfide bond but that it is the cysteines in the C-terminal domain which are involved in forming disulfide bonds with the N-terminal region of the y-type subunits. Furthermore, Cys^{97} of 1Dx5 has not yet been identified as a component of disulfide-linked peptides isolated from gluten digests, although other HMW subunit cysteine residues have (Shewry and Tatham, 1997). These findings are consistent with the observation made in this study that Cys^{97} was accessible to antibody binding and hence not involved in the formation of dimers in partially reduced glutenin.

The Mab described here reacted primarily with Cys^{97} in the reduced form and also bound to 1Dx5−1Dy10 dimers. Therefore, this residue is not involved in either intra-or intermolecular disulfide bond formation in these dimers. However, it is possible that Cys^{97} is either involved in interchain disulfide bonds which are cleaved by the partial reduction procedures used to release the dimers from gluten, and/or be present in gluten in a reduced form. Such free sulfhydryl groups are highly reactive and have been shown to participate in disulfide interchange reactions (Freedman, 1979) which are thought to occur during dough mixing and proving. Thus, Cys^{97} of HMW subunit 1Dx5 could be involved in a process whereby disulfide bonds in polymers are rearranged during processing. Confirmation will depend on the isolation of peptides from glutenin which contain Cys^{97} in either a disulfide bonded and/or reduced form.

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