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Immunochemical identification of LMW subunits of glutenin associated with bread-making quality of wheat flours

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Abstract. A murine monoclonal antibody (IFRN 0067), one of a library developed against prolamin fractions from *Triticum aestivum*, has been characterised using a combination of immunoassay and immunoblotting techniques. The antibody was specific for two glutenin polypeptides which appeared by 2-dimensional electrophoresis to belong to the B group of LMW subunits. From results of antibody-binding studies with material extracted from genetic stocks, it was deduced that the target polypeptides were encoded on the short arm of chromosome 1D. The antibody was used in an immunoassay of bread wheats with a range of anticipated baking scores and for flours of known baking performance. Significant correlations were found between immunoassay and test-bake results. Indeed, correlation of IFRN 0067 binding with loaf volume was equal or better than that provided by alveograph parameters. The results provide evidence that LMW subunits contribute to the bread-making properties of wheat glutenin, as identified by the use of immunological techniques. The use of particular monoclonal antibodies, such as IFRN 0067, in the further development of simple, rapid diagnostic tests for flour quality predictions is discussed.

Key words: LMW glutenin subunits – Monoclonal antibodies – Baking quality

Introduction

It has long been recognised that of the various components comprising wheat flours, it is the gluten storage proteins that are most important in determining bread-making quality. Gluten proteins have high contents of proline and glutamine, are soluble in aqueous alcohols, and can therefore be considered to be prolamins (Shewry and Tatham 1990). Gluten proteins are traditionally divided into two groups; the monomeric gliadins and the polymeric glutenins. The gliadins are readily soluble in aqueous alcohols and can be classified on the basis of electrophoretic mobility at low pH into α-, β-, γ-, and ω-gliadins. In contrast, many glutenins are only soluble under reducing conditions, and the reduced subunits can be separated by SDS-PAGE into two groups: the high-molecular-weight (HMW; M_rs 95–136,000) and low-molecular-weight (LMW; M_rs 36,000–44,000) subunits.

Much work has been directed towards understanding the contributions that these groups of proteins make to the viscoelastic properties that are so crucial to the technological performance of wheat doughs. Extensive studies have shown that the HMW subunits are closely associated with breadmaking quality (Payne et al. 1981; Payne 1987; Ng and Bushuk 1988; Branlard and Dardevet 1985), but the contributions of other groups of prolamins such as gliadins and LMW glutenins are much less clear (Lagudah et al. 1988; Gupta and Shepherd 1987; Payne et al. 1987). However, a role for LMW subunits in bread-making quality is indicated by correlation studies of allelic variation and dough properties (Gupta et al. 1989; Metakovsky et al. 1990), by their known contribution to gluten viscoelasticity in durum wheats (Pogna et al. 1988; Feillet et al. 1989), and by their presence only in polymers stabilised by inter-chain disulphide bonds.

The LMW subunits make up a large proportion of the total endosperm protein, but are poorly characterised compared with other gluten proteins. They have been divided into three groups, B, C, and D, on the basis of their M_rs and isoelectric points (Jackson et al. 1983). The B subunits comprise the major group and have the most

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basic pIs of all the gluten proteins. Both the D and C subunits possess slightly lower M_rs than the B group, the former having acidic pI values and the latter variable pI values.

Genetic studies have shown that the genes coding for most LMW subunits are present on the short arms of the homoeologous group-1 chromosomes, 1A, 1B, and 1D (Jackson et al. 1983; Payne et al. 1985), although there is evidence that minor C subunits with α-gliadin type N-terminal sequences are encoded by genes on the group-6 chromosomes (Tao and Kasarda 1989). It has also been suggested that some D-type subunits encoded by genes on chromosomes 1B and 1D are associated with glutenins by strong non-covalent forces (not inter-chain disulpide bonds) and are allelic with ω-type gliadins encoded by the same loci in other genotypes (Payne et al. 1988). Relationships between some LMW subunits and ω-gliadins have also been demonstrated using antibody probes specific for the repetitive sequence present in ω-gliadins (Brett et al. 1990). N-terminal sequence analysis of the C-type proteins (Tao and Kasarda 1989) showed that they are related to α-type or γ-type gliadins, while all of the B-type subunits that were not N-terminally blocked had sequences similar to those reported for a mixed LMW subunit fraction (Shewry et al. 1983). Several genes encoding proteins with related but non-identical sequences have also been isolated (Okita et al. 1985; Colot et al. 1989; Cassidy and Dvorak 1991). It appears that both the B and C groups of LMW glutenin subunits are able to participate in inter-chain disulphide bonding, and hence form an integral part of the glutenin polymers.

In order to gain new insights into the relationship between flour components and flour quality, we have developed a library of monoclonal antibodies (Mabs) to prolamin fractions from bread wheat (Mills et al. 1990; Brett et al. 1990). One of these antibodies, the characteristics of which are described in the present report, recognises two specific LMW subunits and shows differential binding to flours of different baking qualities.

Materials and methods

Wheat genetic stocks and flour samples

Group-1 nullisomic-tetrasomic lines of wheat cv 'Chinese Spring', developed by Sears (1954), were supplied by T. E. Miller (AFRC Institute of Plant Science Research, Cambridge Laboratory, Norwich, UK). Three lines with double translocations between group-1 chromosomes of wheat and rye (i.e. 1BL-1RS, 1DL-1RS, 1A; 1AL-1RS, 1DL-1RS, 1BL; and 1AL-1RS, 1BL-1RS, 1D) were supplied by K. W. Shepherd (Waite Agricultural Research Institute, Adelaide, Australia). These stocks were produced in a mixed background of cvs 'Chinese Spring' and 'Gabo', with 1BS and 1DS from the former and 1AS from the latter. Pure varieties of bread wheat were provided by J. M. Field (ICI Seeds, Docking, Norfolk, UK) and of durum wheat by J.-C. Autran (INRA, Montpellier, France). The baking quality of the wheat pure varieties was taken as that given by the FMBRA classification (1988). A further 21 flours of unknown variety were obtained from a French working mill. The quality of these flours was determined using the Chopin alveograph and a conventional French-bread test-bake procedure.

Total prolamin extracts of flour

Flours were obtained from genetic stocks by multiplying and milling pure seed. All flours were extracted in ten volumes of 50% (v/v) propan-2-ol, containing 2% (v/v) acetic acid, 2% (v/v) 2-mercaptoethanol by shaking for 2 h at room temperature. After centrifugation at 2,000 rpm for 10 min to remove debris, the supernatant was decanted, dialysed, and freeze-dried. Stock solutions of flour extracts were then prepared at 2 mg/ml in the same solvent mixture used for their extraction.

Monoclonal antibody production and characterisation

The murine Mab IFRN 0067 was 1 of a panel of 28 produced, as described by Brett et al. (1990), using a total glutenin fraction of wheat (cv 'Avalon') both as immunogen and for Mab selection. Enzyme-linked immunosorbent assay (ELISA) procedures were as described previously (Brett et al. 1990) and employed a microtitration plate format onto which purified proteins or flour extracts were coated at a concentration of 1 μg/ml in 0.05 *M* sodium carbonate-bicarbonate buffer. Assays were developed using anti-mouse IgG conjugated with horseradish peroxidase and substrate based on tetramethylbenzidine (Cambridge Veterinary Sciences). Cross-reactivities, expressed as a percentage, were calculated as the ratio of antibody binding observed to a sample protein to the binding to total glutenins (cv 'Avalon'), at a culture supernatant dilution where antibody binding was saturating.

Electrophoretic and immunoblotting procedures

SDS-PAGE was performed essentially as described by Mills et al. (1990). Fractions were extracted sequentially from single seeds of wheat with 70% (v/v) aqueous ethanol followed by the solvent system described by Payne et al. (1980). Two dimensional (2-D) electrophoresis was performed as described by Shewry et al. (1988) using isoelectric focusing (with 6 *M* urea and ampholytes in pH range 3.5–10) as the first dimension followed by SDS-PAGE using a modified Laemmli system with 14% acrylamide and 4 *M* urea as the second dimension (Bunce et al. 1985). Samples for 2-D-PAGE were prepared in 10 m*M* glycine containing 6 *M* urea at a pH adjusted to 8.0 with TRIS.

Immunoblotting was carried out as described previously (Mills et al. 1990) using a transblot apparatus (Biorad). Blots of 2-D gels were double stained for total and immunoreactive proteins using the method of Ono and Tuan (1990). The blotted proteins were initially stained with India ink, which was followed by the detection of immuno-reactive proteins using IFRN 0067.

Results

Characterisation of an antibody specific for a LMW subunit

IFRN 0067, a Mab of the IgG_1 isotype, was generated using a total glutenin fraction from the good bread-making quality cv 'Avalon' as immunogen and for selection. Initially specificity was determined by ELISA using a

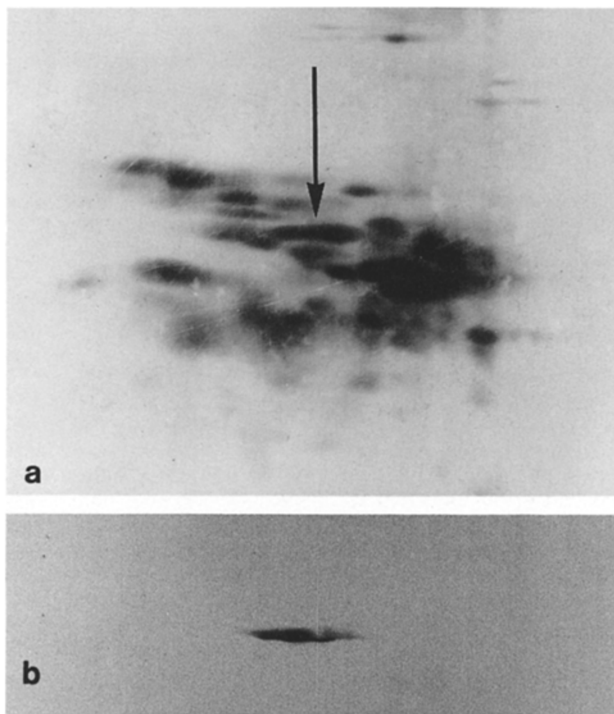

Fig. 1 A, B. Two-dimensional electrophoresis (IEF, pH range 3.5–10 followed by SDS-PAGE) of total gliadins from wheat cv 'Chinese Spring' extracted with 50% (v/v) propan-1-ol, 2% (v/v) 2-mercaptoethanol, 2% (v/v) acetic acid **A** India ink-stained blot, visualising transferred proteins; **B** immunoblot developed with IFRN 0067. Initially, proteins transferred to a nitrocellulose membrane were stained with India ink, before visualising those proteins recognised by IFRN 0067 using an alkaline-phosphatase nitro blue-tetrazolium-based immunodetection system. The two immunoreactive proteins are *arrowed*

range of purified protein fractions and individual proteins, and the Mab was found to be specific for the glutenin fraction of wheat.

To confirm the identity of the polypeptide(s) recognised by IFRN 0067, an immunoblot was performed using a 2-D separation of total prolamins from cv 'Chinese Spring', the filter being double stained to visualise total proteins and immunoreactive proteins (Fig. 1). This showed reactions with two polypeptides with similar M_rs but different pIs. These appear to correspond to the B-type LMW subunits described by Jackson et al. (1983).

In order to determine the genetic control of the protein(s) recognised by IFRN 0067, a range of genetic stocks was analysed by immunoblotting. Two types of stock lines were used: the group 1-compensating nulli-tetrasomics of Sears (1954), and three lines with double translocations between the group-1 chromosomes of wheat and rye (Gupta and Shepherd 1988). Initially, single seeds of these stocks were extracted with 70% (v/v) aqueous ethanol separated by SDS-PAGE under non-reducing conditions, blotted, and reacted with IFRN 0067.

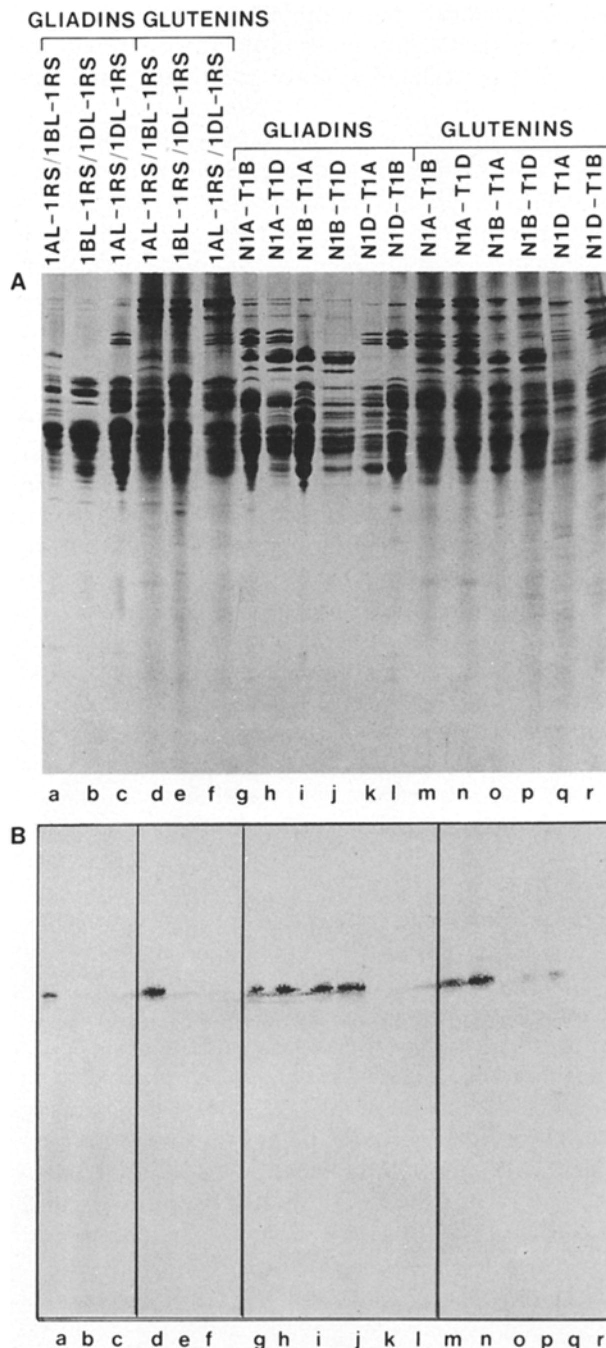

Fig. 2 A, B. SDS-PAGE (**A**) and immunoblot using IFRN 0067 (**B**) of extracts from single seeds of the double-translocation lines of Gupta and Shepherd (*tracks a–f*) and the compensating nulli-tetrasomics of Sears (1954). Proteins were extracted sequentially with 70% (v/v) aqueous ethanol (*tracks labelled gliadins*) followed by the solvent of Payne et al. (1980) (*tracks labelled glutenins*)

The only reactions were with poorly resolved bands in the high M_r region (data not shown). However, when the same fractions were separated under reducing conditions, a single immunoreactive band of M_r about 45,000 was observed (Fig. 2, fractions labelled gliadins). A band

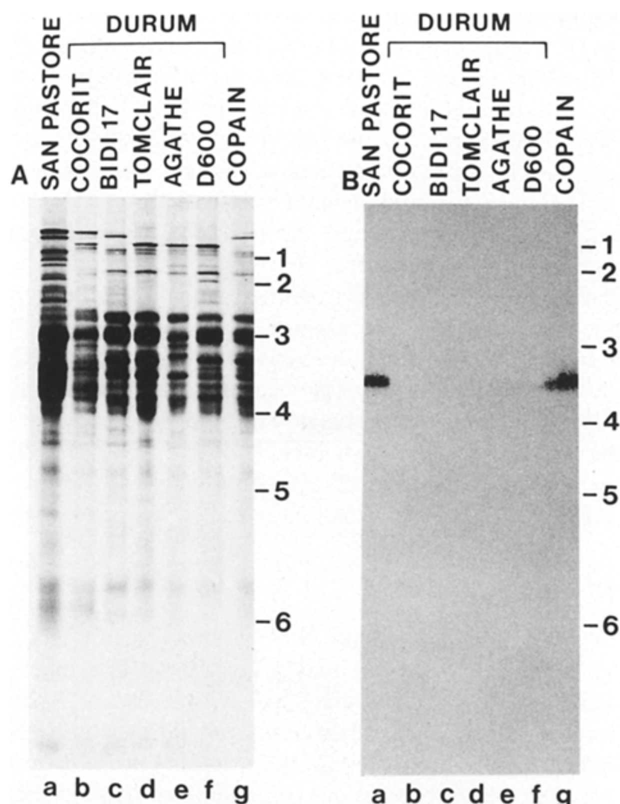

Fig. 3 A, B. SDS-PAGE (**A**) and immunoblot with IFRN 0067 (**B**) of total protein fractions extracted from seeds of bread (*tracks a, g*) and pasta wheat (*tracks b–f*) using the solvent systems of Payne et al. (1980). *Numerals 1–6* indicate the positions of the molecular weight markers (see legend to Fig. 4)

of the same size was also present in the fraction extracted sequentially from the same seeds using the reducing solvent system of Payne et al. (1980) (Fig. 2, tracks labelled glutenins). These results demonstrated that the antibody reacted with a LMW subunit(s) present in the polymers, some of which were soluble in 70% (v/v) aqueous ethanol. This was confirmed by immunoblotting against purified gliadin and glutenin fractions. The former had been fractionated to remove alcohol-soluble glutenin polymers, which were then bulked with the glutenins for immunisation. The antibody reacted with only a single band in the glutenins. Comparison of the fractions from the different stocks demonstrated that the immunoreactive protein was only present in the lines in which the short arm of chromosome 1D was present and was increased in the nulli-tetrasomic lines with four copies of this chromosome. There was also some variation in the proportions of immunoreactive protein present in alcohol-soluble and alcohol-insoluble polymers, but this may have been due to experimental variation in extraction yield. The presence of the controlling genes on chromosome 1D was confirmed by the absence of immunoreactivity as determined by ELISA (results not shown) and

Table 1. Cross-reaction of IFRN 0067 against wheat prolamins from a selection of wheat varieties

Variety	Bread-making score	Cross-reaction (%)	IBL/IRS
Timmo	A	34	No
Avalon	B	64	No
Brimstone	B	55	No
Broom	B	31	No
Mercia	B	88	No
Mission	B	56	No
Moulin	B	53	No
Rendezvouz	B	89	No
Sicco	B	0	No
Fortress	C	46	Yes
Norman	C	35	No
Slejpner	C	60	Yes
Aquila	D	34	No
Galahad	D	0	No
Hustler	D	0	No
Longbow	D	80	No
Rapier	D	0	No
Stetson	D	0	Yes

immunoblotting analyses of total protein extracts from several cultivars of pasta wheat (*T. durum*, genome constitution AABB) lines (Fig. 3).

Correlation of the LMW subunit recognised by IFRN 0067 with bread-making qualitiy

Table 1 gives a summary of the ELISA reactions observed for IFRN 0067 with flour extracts of 18 cultivars of *Triticum aestivum* varying in bread-making quality from very good (A) to poor (D). In general, the binding was higher for bread-wheat cultivars classified as good as quality (A and B) than for those classified as poor (C and D). Some exceptions were apparent – most notably 'Longbow', which has poor quality but gave high binding, and 'Sicco', for which the converse was observed. In the case of 'Slejpner' and 'Fortress', poor bread-making quality is probably related to the presence of the 1BL/1RS translocation. Immunoblotting against total grain extracts essentially confirmed the ELISA results (Fig. 4). No reaction with 'Stetson', 'Rapier', Galahad', 'Sicco', and 'Rektor' was observed, and in some varieties (e.g. 'Mission', 'Moulin', and 'Avalon') the antibody reacted with one or two minor bands of M_r about 30,000 as well as the major band (M_r about 45,000).

These results indicated a possible correlation with bread-making quality, but the material used had been grown under glasshouse conditions and had not been directly tested for bread-making quality. We therefore analysed 21 routine mill intake flour samples of unknown varietal composition, which had been tested for bread-making performance using the Chopin alveo-

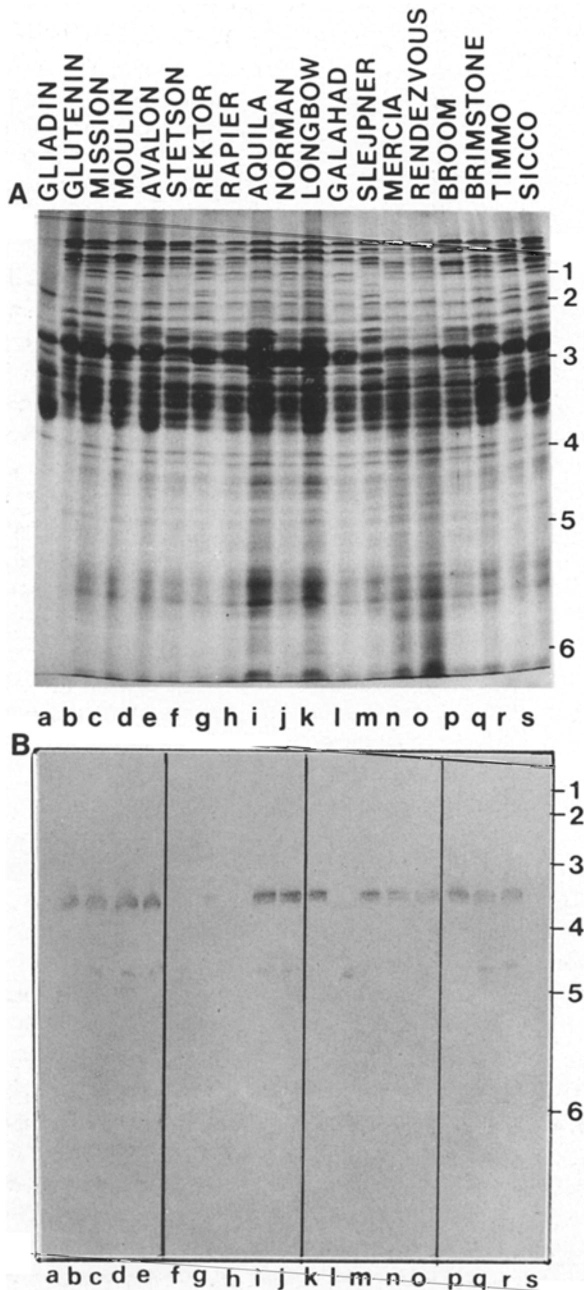

Fig. 4 A, B. SDS-PAGE (**A**) and immunoblot using IFRN 0067 (**B**) of total protein fractions extracted from cultivars of wheat using the solvent system of Payne et al. (1980). *Numerals 1–6* indicate the positions of the molecular weight markers as follows: *1* β-galactosidase (M_r 115,000), *2* phosphorylase b (M_r 97,000), *3* bovine serum albumin (M_r 66,000), *4* egg albumin (M_r 45,000), *5* carbonic anhydrase (M_r 29,000), *6* myoglobin (M_r 17,000)

graph, by test-baking, and for which a number of other measurements had been made (Table 2). The calculation of correlation coefficients (Table 3) showed that alveograph parameter W showed no significant correlation with loaf volume, whilst parameters G and PL showed significant positive and negative correlations, respectively ($P < 0.05$). The magnitude of the correlation coefficients for G and PL were the same as the significant positive correlation observed between Mab IFRN 0067 binding with loaf volume (also $P < 0.05$; Table 3). Samples 20 and 21 presented bread-making problems because of sticky doughs, and the data were therefore recalculated without these samples. As a result the correlation between loaf volume and alveograph parameter P became significantly correlated, the correlation with parameters G and PL was abolished, whilst parameter W remained uncorrelated. Notably, the binding of IFRN 0067 remained significantly – and equally – correlated ($P < 0.05$) with loaf volume. The samples had a fairly narrow range of protein content (11.5–12.55%), and this parameter was not correlated with quality with or without the inclusion of samples 20 and 21 in the calculations.

Discussion

Considerable efforts have been made to identify those gluten components that play a key role in determining baking quality. One of the goals of this effort is to gain an understanding of the molecular interactions involved in bread-making. However, the complexity of the materials and of their interactions has hampered these investigations. The results described in this paper show that using an immunological approach has allowed gluten polypeptides to be identified that were not previously known to be associated with bread-making quality. An additional benefit of the use of such immunological techniques is the consequent availability of monoclonal antibody probes that can be used in robust diagnostic tests. It is tempting to speculate that as the molecular interactions of gluten proteins are further studied and the multifactorial elements of bread-making potential identified, then panels of antibody probes will become available covering such factors. Application of these will allow plant breeders and millers to predict bread-making potential with greater assurance than currently possible. The fact that antibodies can be used in rapid, simple, and comparatively cheap tests is an added attraction.

Some of the factors known to influence the bread-making quality of flours relate to protein composition, such as the amount of gluten, the amount and composition of the HMW glutenin subunits (Payne 1987), and the proportion of the high M_r glutenin polymers (Field et al. 1983). The detrimental effects of the 1BL/1RS chromosome translocation may also be due, at least in part, to the substitution of the secalins encoded by 1RS for the γ-gliadins, ω-gliadins, and LMW subunits encoded by 1BS. In addition other grain components, most notably carbohyrates and lipids, also influence bread-making quality, possibly by interacting with gluten (Chung 1985; Lásztity et al. 1987). The results of the present work

Table 2. Analysis of 21 flour samples for baking quality

Sample number	% Moisture	% Protein	Alveograph parameters[a]				Test bake loaf (volume cm^3)	IFRN 0067 cross-reaction %
			P	G	W	PL		
1	12.1	12.55	61	18.3	151	0.89	2030	47
2	12.1	12.24	64	18.3	162	0.95	2025	59
3	12.9	11.05	109	16.0	207	2.10	1982	100
4	12.6	11.75	102	17.3	226	1.68	1950	60
5	13.3	12.05	87	17.8	105	1.36	1907	84
6	13.0	11.25	88	16.8	185	1.54	1863	66
7	12.4	11.30	68	18.4	167	0.99	1863	124
8	13.5	11.10	71	19.0	173	0.98	1875	60
9	12.5	11.85	72	19.3	177	0.96	1807	67
10	12.3	12.80	101	18.8	255	1.40	1719	100
11	12.5	10.55	89	16.3	170	1.65	1725	52
12	12.7	11.75	103	18.3	237	1.52	1719	66
13	11.3	11.05	107	15.0	183	2.32	1557	87
14	13.0	11.25	110	15.2	176	2.34	1544	34
15	11.5	12.35	82	18.3	199	1.20	1563	62
16	11.6	12.25	95	17.8	205	1.48	1582	23
17	12.2	12.30	94	17.9	206	1.44	1550	22
18	11.6	12.00	95	17.2	194	1.58	1500	26
19	12.3	12.00	96	18.2	215	1.43	1468	23
20	12.4	11.60	90	16.3	164	1.67	1375	39
21	12.3	11.10	81	14.4	126	1.93	1275	65

[a] P, Over pressure; G, square root of the volume of air used to blow gluten bubble (cm^3); W, mechanical work in blowing bubble (10^3 ergs/g dough); PL, measure of dough extensibility

Table 3. Correlation coefficients between loaf volume and other baking quality parameters for 21 flour samples

Baking quality parameters	Samples 1–21	Samples 1–19
Alveograph[a]		
P	− 0.352	− 0.497*
G	0.451*	0.223
W	− 0.026	− 0.352
PL	− 0.451*	0.391
% Protein	0.070	0.075
% Cross-reaction IFRN 0067	0.445*	0.497*

* $P < 0.05$ [correlation coefficients > 0.433 (samples 1–21) or 0.456 (samples 1–19)]
[a] See Table 2 for definitions

indicate that the protein(s) recognised by the Mab IFRN 0067 also make a substantial contribution to quality in many currently grown European cultivars and that from their characterisation described here are probably B-type LMW subunits of glutenin.

The LMW subunits account for about 40% of the total gluten proteins, although they have not been studied in detail due to their complexity and the difficulty of isolating single components. In addition, they are inadequately resolved from other gluten proteins by one-dimensional electrophoresis, making it difficult to carry out correlative studies of the type pioneered by Payne and co-workers for the HMW subunits (Payne et al. 1981; Payne 1987). Nevertheless, Gupta et al. (1989) recently carried out detailed analyses of allelic variation using a specially developed two-stage electrophoretic procedure and special genetic stocks and suggested that allelic variation in the LMW subunits encoded by chromosome 1A was related to extensibility. Similarly, Metakovsky et al. (1990) reported a correlation between LMW subunit composition and extensibility in 28 Australian wheats. Our results show that individual subunits within the mixture encoded by a single chromosome are correlated with bread-making quality (IFRN 0067 did not react with other LMW subunits encoded by chromosome 1D of 'Chinese Spring') and also provide a tool to screen for these proteins in breeding programmes and at mill intake.

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