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# The low-molecular-weight glutenin subunit proteins of primitive wheats. IV. Functional properties of products from individual genes

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**Abstract** Three genes encoding the low-molecular-weight glutenin subunits (LMW-GSs), LMWG-E2 and LMWG-E4, from A-genome diploid wheat species, and LMW-16/10 from a D-genome diploid wheat, were expressed in bacteria. The respective proteins were produced on a relatively large scale and compared with respect to their effects on flour-processing properties such as dough mixing, extensibility and maximum resistance; these are important features in the end-use of wheat for producing food products. The LMWG-E2 and LMWG-E4 proteins caused significant increases in peak resistance and mixing time, compared to the control, when incorporated into dough preparations. The LMWG-16/10 protein was qualitatively less effective in producing these changes. All three proteins also conferred varying degrees of decrease in dough breakdown. LMWG-E2 and LMWG-E4 caused significant increases in dough extensibility, and decreases in maximum resistance, relative to the control. LMW-16/10 did not show a significant effect on extensibility but showed a significant decrease in maximum resistance. The refinement of relating specific features of the structure of the LMW-GS genes to the functional properties of their respective proteins is discussed.

**Key words** Low-weight glutenin subunits · Single proteins · extensibility · Dough properties

## Introduction

The low-molecular-weight glutenin subunits (LMW-GSs) account for 40% of wheat gluten protein content by mass, and these proteins are considered to significantly affect dough-quality characteristics (Gupta and Shepherd 1988; Gupta et al. 1989; Pogna et al. 1990; Gupta et al. 1991; Nieto-Taladriz et al. 1994; Sissons et al. 1997). In particular, LMW-GS fractions have been found to be significantly correlated with dough extensibility (Gupta and Shepherd 1988; Gupta et al. 1989; Metakovsky et al. 1990; Gupta et al. 1991); it has also been shown that these proteins cause increased dough-mixing time (Sissons et al. 1997). Features of these proteins, such as the distribution of the cysteine residues available for intermolecular disulphide bonds (reviewed by Shewry and Tatham 1997), as well as their overall amino-acid compositions involved in non-covalent bonds (Bloksma and Bushuk 1988; Pomeranz 1988), are important in determining the rheological properties of dough. These properties are critical in the food uses of wheat flour.

Using the LMW-GSs produced from a bacterial expression system, 2-g Mixograph and small-scale extensibility tests can be conducted to study the functional behaviour of the LMW-GSs in dough. There have been several studies on the expression of individual wheat storage proteins using various systems; HMW-GSs in *Escherichia coli* (Bartels et al. 1985; Galili 1989), gliadins in the yeast *Saccharomyces cerevisiae* (Neill et al. 1987; Scheets and Hedgcoth 1989; Blechl et al. 1992), LMW-GSs in insect cells using a baculovirus vector (Thompson et al. 1994), and LMW-GSs in *E. coli* (Ciaffi et al. 1998). However, expression of a LMW-GS derived from A- and D-genome diploid wheats has not yet been reported. In the present study three bacterially synthesised LMW-GSs, LMWG-E2, LMWG-E4 (Lee et al. 1998) and LMW-16/10 (Ciaffi et al. 1998), were compared with respect to their effects on flour processing properties such as dough mixing, extensibility and maximum resistance.

Reconstitution studies that combine isolated wheat flour components to form a mixture that behaves as normal flour are a powerful means for determining the

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functionality of certain protein fractions or individual subunit proteins. This approach can define cause-effect relationships between protein structure and final dough properties. The recent development of a small-scale Mixograph has greatly assisted the reconstitutional approach because this apparatus requires only milligrams quantities of purified proteins (Bekes et al. 1994 a). Functional studies of individual glutenin subunits involve the partial reduction of flour proteins and the subsequent incorporation of proteins into the polymeric glutenin proteins of the base flour by re-oxidation. Bekes et al. (1994 a) reported that the reduction re-oxidation procedure is effectively reversible, verifying the reproducibility and reliability of the incorporation experiment. More recently, a micro-extension tester has been developed (Rath et al. 1994) and this can determine other important rheological characteristics of dough, namely extensibility and maximum resistance. The expression of individual LMW-GSs in *E. coli* has been an important development in providing the mg amounts of protein required for small-scale tests to assign functional features to specific proteins.

## Materials and methods

### Proteins used for functional tests

Three bacterially synthesised LMW-GSs, LMWG-E2, LMWG-E4 and LMW-16/10, were used for the functional tests of the proteins. LMWG-E2 and LMWG-E4 were derived from *Triticum boeoticum* (Lee et al. 1998), and LMW-16/10 was derived from *Triticum tauschii* (Ciaffi et al. 1998). A HMW-GS (Bx7) purified from a flour, Galahad-7 (kindly provided by Dr. P. Payne, Cambridge, UK), was used as a positive control.

The method to purify LMW-GS proteins from bacteria expressing them, on a large scale, was modified from Ciaffi et al. (1998). Fresh transformants were inoculated into 5 ml of medium. After overnight incubation this culture was used to inoculate 600 ml of medium in a 2-l flask. Each culture involved the growth of 9 l of media; 600 ml of culture in each of 15 flasks. The cultures were incubated until the cell density reached approximately $1 \times 10^9$ cells/ml ($A_{600} = 1.0$). Protein expression was then induced by adding IPTG to a final concentration of 1.5 mM. The cultures were incubated at 37°C with shaking for 10 h. Expression of the LMW-GSs was checked by analysing the total cell proteins using SDS-PAGE. The cells were harvested at 10 000 rpm at 4°C for 10 min and stored at −20°C.

The cell pellet derived from 1.2 l of culture was re-suspended in 50 ml of water, sonicated for 30 min in the water-bath sonicator and centrifuged at 10 000 rpm for 10 min; the supernatant was discarded and this step repeated. The pellet was then washed several times by repeating cycles of re-suspension of the cells in 300 ml of water and centrifugation until the cell debris and the water-soluble cell proteins were completely removed. The pellet was re-suspended in 50 ml of 55% isopropanol containing 50 mM DTT, and incubated at 65°C for 1 h to extract the LMW-GSs. The sample was centrifuged at 10 000 rpm for 10 min, and the supernatant was then transferred to a new tube and the extraction step was repeated. The supernatant containing the LMW-GSs was assayed by SDS-PAGE to check the purity of the LMW-GSs extracted. The preparation (200 ml) was dialysed in 3.5 l of 0.1 mM acetic acid using dialysis tubing (molecular weight cut-off of 12 000 Da, Sigma) for at least 72 h, changing the dialysing buffer five times. The proteins were freeze dried and stored at −20°C until use.

### Two-gram Mixograph tests

Mixing tests were conducted with a prototype 2 g-Mixograph using a modification of the standard method for 35 g of flour scaled to a 2-g size (Rath et al. 1990). Mixing parameters were determined using a modification of a previously reported computer program (Gras et al. 1990; Gras and Bekes 1996). This modification automatically excised the portions of the recording during which mixing was halted. The parameters determined included the time to peak dough resistance (mixing time, MT), peak dough resistance (PR), and breakdown in resistance (RBD). MT and PR are measures of dough strength while RBD is an inverse measure of dough stabilty.

A reversible reduction/oxidation procedure for incorporating added polypeptides (5 mg of LMW-GSs or a Glu1-Bx7 subunit) into glutenin was employed (Bekes et al. 1994 a). A rye double-translocation-line wheat flour (1RS.1BL, 1RS.1DL) was used as a base flour. This wheat line contained a relatively small number of LMW-GSs, so the addition of the LMW-GSs to be tested in this base flour is expected to show clear effects on the dough properties. Flour was mixed with 1.00 ml of water and 0.10 ml of water containing 50 μg/ml of DTT for 30 s and allowed to react for 4 min. The reduced doughs were then treated with 0.10 ml of oxidant solution containing 200 μg/ml of potassium iodate. Mixing was continued for 30 s and the dough allowed to react for 5 min and then mixed for a further 10 min, as in a conventional Mixograph determination (= 'incorporation'). The same protocol was followed in separate experiments but with 0.10-ml water additions instead of the reducing and oxidising agents (= 'simple addition').

The extent of changes in the molecular-weight distribution of dough proteins was monitored. Dough pieces after mixing were dropped into liquid Nitrogen and freeze-dried. Total protein extracts were produced by the SDS-sonication method (Singh et al. 1990) and separated using size exclusion (SE)-HPLC (Batey et al. 1991). Four regions of elution profile (P1, P2, P3 and P4) were quantified containing large (Mw > 120 kDa) and small (Mw = 80–120 kDa) polymeric proteins, monomeric gliadins and albumin/globulin, respectively (Batey et al. 1991).

### Small-scale extensibility tests

A micro-extension tester was used to measure the extensibility (EXT) and maximum resistance-rheological parameter, closely related to the baking performance, (Rmax), of the incorporated doughs (Rath et al. 1990, 1994). For the extensibility tests, the dough was mixed until the average peak mixing time, and then weighed and halved. Both pieces of the dough were rolled, placed into individual carrier assemblies, and incubated at 30°C and high humidity for 45 min for stabilisation of the fully developed gluten network. The dough samples were then placed on the carriage of the micro-extension tester, and the carriage pulled the dough samples over the fixed hook. The stretching force of the hook from the transducer and the position of the carriage were recorded electronically 100 times/s. The extensibility (the distance the dough was pulled until rupture) and the maximum resistance of the incorporated doughs were both measured.

### N-terminal amino-acid sequencing

The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane after electrophoretic seperation. The sequencing was performed using an Applied Biosystem amino-acid sequencer, Model 494 Procise, at the John Curtin School for Medical Research, Australian National University. The sequencing data were analysed using Model 16A data-analysis software.

## Results

### Large-scale preparation of LMW-GS proteins

The transformed bacteria, JM109 (DE3), carrying the recombinant plasmids were found to be very unstable, so bacteria had to be freshly transformed each time for protein expression. The amount of protein obtained from a culture inoculated with transformants which had been on a plate for 3 days was less than 15% of the protein amount from the fresh transformants.

The 600-ml cultures used to prepare batches of proteins were collected after 10-h incubation. The results of N-terminal amino-acid sequencing and gel analyses confirmed that there were no modifications; both LMWG-E2 and LMWG-E4 had the N-terminal amino-acid sequence METSCIPGLERPWQEQ. The N-terminal sequence for the LMW-16/10 protein was METRCIPGLERPWQQQ.

### Two-gram Mixograph tests

Characteristic mixing- and extension-curves of control flour without and with incorporated LMW-GSs are shown in Fig. 1, indicating the three mixing parameters (mixing time, peak resistance and resistance at breakdown, see the top panel in Fig. 1) as well as the maximum resistance and extensibility determined from the extension curve (lower panel in Fig. 1). The mean values of three replicate analyses, expressed as the percentage of values for the control experiment, are given in Table 1.

Simple addition of LMW-GS proteins caused a decreased mixing time and a lowered peak resistance (Table 1, $P < 0.05$). As indicated, the differences between the mixing-time values, calculated by using

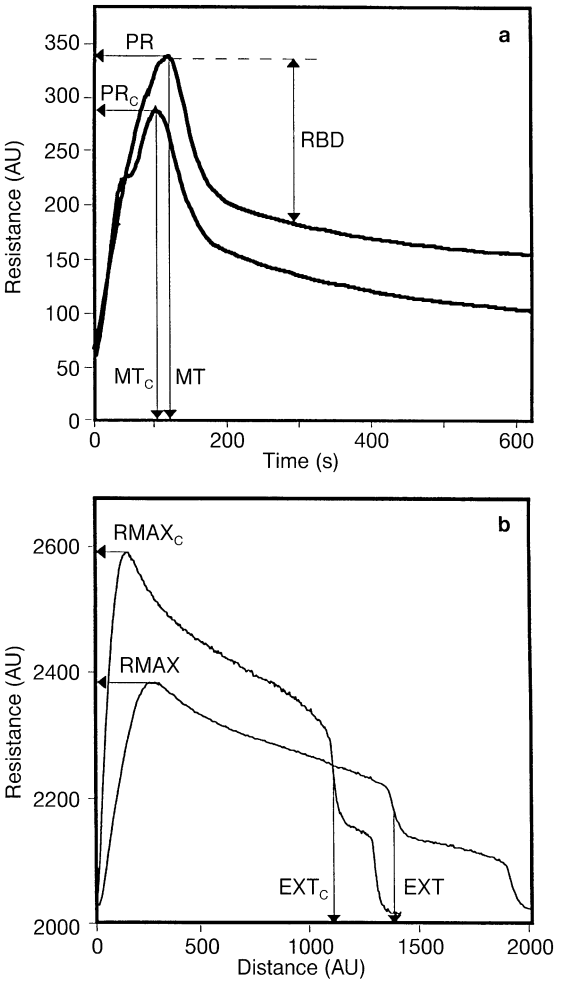


**Fig. 1** Mixing (**a**) and extension (**b**) curves of base flour without (control) and with 10 mg of LMWG-E2 in an incorporation experiment using the 2-gram Mixograph and the micro-extension tester. $MT$ = mixing time, $PR$ = peak resistance, $RBD$ = breakdown in resistance, $RMAX$ = maximium resistance and $EXT$ = extensibility. The superscript C indicates the values for the control

**Table 1** Mixing time (MT), peak resistance (PR), breakdown for resistance (RBD), extensibility (EXT) and maximum resistance (RMAX) values of doughs mixed with LMWG-E2, LMWG-E4, LMWG-D (= LMW-16/10) and HMW-GS (Bx7) in simple addition and incorporation experiments. Mean values of the parameters, determined from three replicate mixing and extension experiments, are shown as a percentage of the control flour. Statistically significant differences between the mean values were determined using a Student's $t$-test ($P < 0.05$); the coefficients of variance (CV) are shown for each parameter measured

| Item | Control | LMWG-E2 | LMWG-E4 | LMWG-D | HMW-GS(Bx7) | CV |
|---|---|---|---|---|---|---|
| Simple addition experiment | | | | | | |
| MT | 100 | 83 | 87 | | | 4 |
| PR | 100 | 98 | 92 | | | 7 |
| RBD | 100 | 96 | 99 | | | 15 |
| Incorporation experiment | | | | | | |
| MT | 100 | 109 | 131 | 129 | 212 | 16 |
| PR | 100 | 121 | 117 | 112 | 119 | 13 |
| RBD | 100 | 91 | 93 | 93 | 87 | 4 |
| EXT | 100 | 146 | 132 | 101 | 80 | 18 |
| RMAX | 100 | 42 | 48 | 56 | 71 | 13 |

Student's *t*-test, were statistically significant. Addition of LMWG-E4 caused a significant decrease in peak resistance compared to the control, but the effect of LMWG-E2 on this parameter was marginal (significant at $P<0.1$ but not at $P<0.05$). However, the trend of the LMWG-E2 values is consistent with a simple addition of the proteins causing decreased peak resistance values. No statistically significant differences in resistance at breakdown were observed in the addition experiments.

When the LMW-GS proteins were incorporated into the base flour, both of them caused an increased mixing time and peak resistance (Table 1, $P < 0.05$). LMWG-E2 was less effective in increasing mixing time and peak resistance than LMWG-E4; while LMWG-E2 did not give a statistically significant increase in peak resistance, the trend was consistent with the incorporation of LMW-GSs giving increased values for this parameter. The HMW-GS (Bx7) was included as a positive control, and showed the highest mixing time, as expected. LMWG-E4 caused a similar level of increased mixing time and, based on the mean values of the Mixograph tests, LMWG-E4 and Bx7 were assigned to the same class. LMWG-E4 and the HMW-GS (Bx7) gave significant increases (in the order of 20%) in the peak resistance of the doughs. There were no statistically significant changes observed in resistance breakdown in these incorporation experiments.

The incorporation of LMW-GSs into the polymeric glutenin proteins of the base flour were confirmed by a size-exclusion (SE) HPLC analysis of the total proteins of the incorporated doughs. The SE-HPLC profile of the incorporated dough with LMWG-E2 was compared with those of the incorporated dough with the HMW-GS (Bx7) and the dough with LMWG-E2 simply added. The P1 and P2 regions in the SE-HPLC profile are considered to contain polymeric protein fractions, while the P3 and P4 regions are monomeric protein fractions. Results of the quantitative comparison of the P1–P4 regions are summarised in Table 2. The first column for each fraction represents the relative amount of the fraction as a percentage of the total protein. The second column shows the amount of the fraction calculated from these percentages and the amount of protein present (protein content of the base flour plus added individual polypeptides), while the third column indicates the distribution of the added polypeptides. The analysis of the distribution of the LMW-GS proteins within the SE-HPLC profile confirmed their incorporation into the polymeric fractions of the dough (P1 and P2). The HMW-GS (Bx7) protein was entirely accounted for within the largest polymer fraction (P1), while the LMW-GS proteins were distributed between the P1 and P2 region indicating that a certain amount of the incorporated polypeptides were part of the smaller glutenin polymers.

**Table 2** Distribution of added/incorporated polypeptides in the SE-HPLC regions of total-protein extracts isolated from doughs after 10 min mixing

| Sample | Treatment | P1 | | | P2 | | | P3 | | | P4 | | | Total protein present mg[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | %[a] | mg[b] | mg[c] | %[a] | mg[b] | mg[c] | %[a] | mg[b] | mg[c] | %[a] | mg[b] | mg[c] | |
| Control | | 23.00 | 65.3 | | 13.30 | 37.9 | | 47.94 | 136.1 | – | 15.76 | 44.7 | – | 284 |
| + 10 mg LMWG-E2 | Addition | 22.00 | 64.7 | – | 12.75 | 37.2 | – | 50.15 | 147.4 | **10** | 15.20 | 44.7 | – | 294 |
| + 10 mg LMWG-E2 | Incorporation | 23.97 | 70.5 | **5** | 14.61 | 43.3 | **5** | 15.05 | 136.2 | – | 15.05 | 44.7 | – | 294 |
| + 10 mg LMWG-E4 | Incorporation | 24.62 | 72.4 | **7** | 13.82 | 40.7 | **3** | 46.36 | 136.2 | – | 15.07 | 44.7 | – | 294 |
| + 10 mg LMWG-D | Incorporation | 24.65 | 72.5 | **7** | 13.82 | 40.6 | **3** | 46.36 | 136.2 | – | 15.07 | 44.7 | – | 294 |
| + 10 mg HMW-GS | Incorporation | 26.26 | 75.2 | **10** | 13.09 | 38.0 | – | 45.76 | 136.1 | – | 15.07 | 44.7 | – | 294 |

[a] Relative amount of fraction as a percentage of the total protein content of the sample
[b] Amount of fraction in dough mixed from 2 g of flour
[c] Amount of added/incorporated polypeptide found in the fraction
[d] Total amount of protein in dough mixed from 2 g of flour

Small-scale extensibility tests

Doughs with LMWG-E2, LMWG-E4, LMW-16/10 and HMW-GS (Bx7) incorporated were tested using a micro-extension tester in order to examine the effects of each subunit on dough extensibility and maximum resistance. The mixing time to make doughs for the extensibility tests was determined from the average values of the peak mixing time measured in the Mixograph. Extensibility curves and values of the extensibility and maximum resistance are shown in Fig. 1 (lower panel) and Table 1, respectively. The mean values of the incorporated doughs with respect to extensibility and maximum resistance were compared statistically in order to investigate significant changes in these two parameters. LMWG-E2 and LMWG-E4 caused significant increases in dough extensibility relative to the control. LMW-16/10 did not show a significant effect on extensibility. The HMW-GS (Bx7) resulted in significantly decreased extensibility values.

Maximum resistance ($R_{max}$) values of the incorporated doughs were significantly decreased compared to those of the control doughs, which had been reduced and re-oxidised (Table 1). LMWG-E2 caused the greatest decrease in $R_{max}$, followed by LMWG-E4, which showed a marginal difference to LMWG-E2. LMW-16/10 resulted in significant decreases in $R_{max}$ values relative to the control, but with less decreases than LMWG-E2 and LMWG-E4. In these experiments HMW-GS (Bx7) also caused a significant (and unexpected) decrease in $R_{max}$ but much less so than the LMW-GSs; the reason for the drop in $R_{max}$ after the addition of Bx7 to this in vitro system is currently under investigation.

## Discussion

The main objective of this work was to compare the functional properties of the products from three LMW-GS genes giving rise to proteins with known primary structure, and to account for some of the observed functional differences of these proteins; the functional properties of the proteins are summarised in Fig. 2. The technique for examining the functional properties of the glutenin subunit proteins utilised, firstly, a bacterial expression system to obtain sufficient quantities of the proteins and, secondly, a reduction/oxidation protocol for incorporating the proteins into flour in a reconstitution-type experiment. This approach has been extensively discussed (Bekes et al. 1994 a, b, c, 1995, 1996; Anderson et al. 1996; Sissons et al. 1997). The most reliable evidence for the applicability of such reconstitution-type experiments to evaluate the functional properties of the polypeptides in wheat dough is that the properties of the Glu1-Ax1 and Glu-Dx5 subunit proteins in reconstitution-type experiments correspond

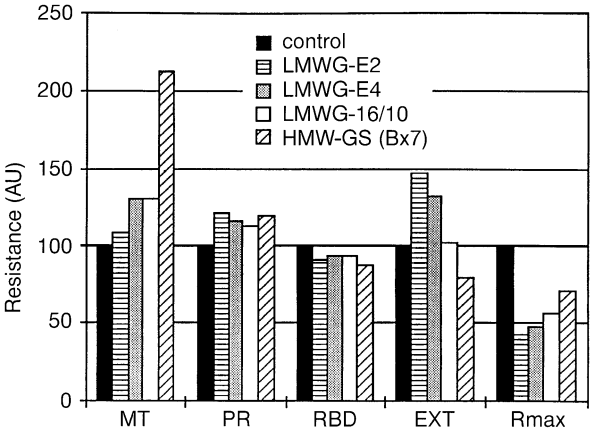


**Fig. 2** A summary of the functional properties of the glutenin subunit proteins studied in this paper

exactly to their properties in vivo in transgenic wheats (Barro et al. 1997).

The structural differences between the LMW-GS gene products studied in the present paper are summarised in Fig. 3. The individual LMW-GSs produced consistent trends in modifying the behaviour of flour-mixing in the 2-g Mixograph. The results obtained using 10 mg of the proteins showed excellent reproducibility and significant effects on the dough properties, suggesting that 10 mg is a sufficient amount of protein for the 2-g Mixograph studies. The incorporation of the proteins into the polymeric glutenin proteins of the doughs was successful as judged from the SE-HPLC analysis of the total proteins of the doughs incorporated with the LMW-GSs and HMW-GS (Bx7). These results confirmed that reduction and re-oxidation treatments rearranged the gluten network to allow incorporation of the additional subunits into the polymeric glutenin proteins of the doughs.

When the LMW-GSs were simply added to the base flour the doughs showed decreased mixing time and peak resistance. This has been interpreted to be similar to the addition of monomeric proteins which also cause decreases in dough-mixing parameter values; Comparable results have been obtained for the addition of unincorporated gliadins (Gras and Bekes 1996) and HMW-GSs (Bekes et al. 1994 b). Therefore, in these experiments, the LMW-GSs added to the base flour simply act as monomeric proteins, although it is possible that a small percentage (less than 10%) of the proteins may link to other adjacent proteins by spontaneous re-oxidation during dough mixing.

Incorporation, using a reduction/oxidation cycle as discussed above, of both the three different LMW-GSs and the HMW-GS (Bx7) resulted in statistically significant increases in mixing time, which were consistent in all experiments. The LMWG-E2-encoded protein caused a statistically significant increase in mixing time when 10 mg of the protein was used, but with 5 mg the

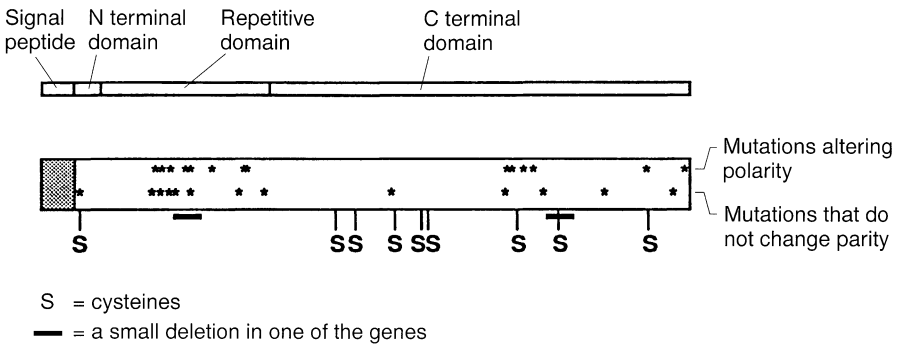


**Fig. 3** A summary of the differences between the amino-acid sequences of LMW-E2, LMWG-E4 and LMWG-16/10; the detailed differences that may be related to functional differences between the proteins are discussed in the text

increase was not statistically significant (data not shown). These results are in agreement with the data of MacRitchie and Gupta (1993) who suggested that an increased HMW-GS to LMW-GS ratio provides positive effects on dough mixing time and maximum resistance. Experiments of this type, using other flour proteins such as gliadins, show no significant effects (Fido et al. 1997).

The HMW-GS (Bx7) incorporation caused longer mixing times, as expected, because there are several differences between the LMW-GSs and HMW-GSs. The first is the molecular-weight difference where the HMW-GSs show double the molecular weight of the LMW-GSs. In the case of the gluten network, larger glutenin proteins may affect more strongly the rheology of the gluten network (Bekes and Gras 1993). The relative molecular-size distribution of the polymeric proteins is shifted by the presence of larger-sized monomeric units in the polymer when the HMW-GSs are incorporated as compared to LMW-GSs. The secondary structures of the proteins are also likely to be important, as discussed below.

There was marked variation in the effect on mixing time among the LMW-GSs. LMWG-E4 contributed most notably to an increased mixing time, followed by LMW-16/10 and LMWG-E2. The sizes of these proteins are very similar and it has been concluded that the secondary structure of the LMW-GSs (Lee et al. 1998) is the key factor responsible for the variation in their mixing-time, as these proteins contain amino-acid substitutions that affect polarity, charge and side-chain structures (see also Shewry and Tatham 1990; Shewry et al. 1992, 1995). The cysteine-residue positions, for example, in the LMW-GSs studied in this paper are highly conserved. However, substitutions of the neighbouring amino-acid residues of the cysteines may affect the availability of the cysteine residues for intermolecular linking. Some non-covalent bonds in these LMW-GSs may also assist in creating a favourable arrangement of the proteins which affects the mixing time. LMWG-E2 and LMWG-E4 showed very high overall homology in their amino-acid sequences, whilst LMW-16/10 showed less similarity to each of these two proteins. The differences between LMWG-E2 and LMWG-E4 in their mixing time may be explained by the fact that the two proteins contain several amino-acid substitutions and two glutamine-residue deletions in LMWG-E4. More particularly, an amino-acid substitution near the last cysteine residue (threonine/methionine) in the C-terminal domain may have a significant impact on disulphide bond formation as this substitution changes the polarity of the residue. This may result in altering the secondary structure of the proteins. The substitution of a histidine residue by an arginine residue, near the third cysteine residue in the C-terminal domain, may also be as important as the side chains of these residues because arginine has a butyl-ammonium side chain while histidine carries a ring-structured imidazolium moiety. These factors may affect protein interaction in the dough system and in gluten network formation, and thereby change the physical properties of the doughs. Evidence for the importance of minor structural changes in determining the secondary structure and reactivity of cysteine residues in glutenin analog polypeptides have been shown recently in functional studies where the products of genetically modified C-hordein and Glu1-Dx5 genes with cysteine residues in an altered position have been compared (Buonocore et al. 1998; Tamas et al. 1997 a, b).

According to the results from the dough-extensibility tests using the four subunits, LMWG-E2 and LMWG-E4 contributed positively to dough extensibility, LMWG-16/10 did not contribute to increased dough extensibility, while the HMW-GS (Bx7) had a negative impact on this dough property. This implies that particular structural features of LMWG-E2 and LMWG-E4 allow these proteins to promote dough extensibility, whereas, surprisingly, the incorporation of LMWG-16/10 did not measurably affect dough extensibility. Since it contains two partial-repeat unit additions and a large deletion shifting the position of a cysteine residue in the C-terminal domain. Alterations of this type may have an affect on the structure of the proteins although these structural characteristics did not affect dough mixing time in the same manner among the three LMW-GSs.

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