

Conformational differences between two wheat (*Triticum aestivum*) 'high-molecular-weight' glutenin subunits are due to a short region containing six amino acid differences

Andrew P. GOLDSBROUGH,*§ Neil J. BULLEID,† Robert B. FREEDMAN† and Richard B. FLAVELL‡

*Institute of Plant Science Research, Cambridge Laboratory, Maris Lane, Trumpington, Cambridge CB2 2JB,

†The Biological Laboratory, The University, Canterbury, Kent CT2 7NJ, and ‡John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

'High-molecular-weight' (HMW, high- M_r) glutenin subunits are protein constituents of wheat (*Triticum aestivum*) seeds and are responsible in part for the viscoelasticity of the dough used to make bread. Two subunits, numbered 10 and 12, are the products of allelic genes. Their amino acid sequences have been derived from the nucleic acid sequences of the respective genes. Subunit 10 has fewer amino acids than subunit 12, but migrates more slowly on SDS/PAGE (polyacrylamide-gel electrophoresis). This anomaly is due to between one and six of the amino acid differences between the subunits, localized towards the C-terminal end of the proteins. This has been established by making chimaeric genes between the genes for subunits 10 and 12, transcribing and translating them *in vitro* and analysing the products by SDS/PAGE. The postulated conformational differences between subunits 10 and 12 are discussed in relation to current hypotheses for the structure of HMW glutenin subunits.

INTRODUCTION

The elastic nature of dough used for breadmaking is largely determined by a group of proteins present in wheat gluten known as the 'high-molecular-weight' (HMW, high- M_r) glutenins. There are two different types of HMW glutenin, termed x and y, encoded by the *Glu-1-1* and *Glu-1-2* loci respectively, present on the long arms of each homoeologous group 1 chromosome [1]. These proteins have a central domain consisting of repeating units, surrounded by non-repetitive N- and C-terminal domains. It has been suggested that the repetitive regions may specify a β -spiral structure in the HMW-glutenin subunits [2] similar to that suggested for elastin [3], and this may contribute to the elasticity of dough.

Several alleles exist at each of the loci, and it has been found that the *Glu-D1-1* and *Glu-D1-2* subunits 2 (x) and 12 (y) are associated with poor breadmaking quality, whereas the allelic subunits 5 (x) and 10 (y) are associated with good breadmaking quality [4,5]. Further evidence has indicated that the y-type allelic subunits numbered 10 and 12 are associated with good and poor quality respectively [6]. Very few amino acid differences exist between these two proteins, as revealed by sequence comparisons. There are just twelve single amino acid substitutions, two hexamer deletions in subunits 10, one 2-mer addition and one 2-mer deletion. All of these differences occur within the repeat domain [7a].

Conformational differences are predicted to exist between the two proteins, as evidenced by the observation that, although subunit 10 is twelve amino acids shorter than subunit 12 [7b], it has lower mobility on SDS/PAGE gels [4] as opposed to the higher mobility which would be predicted on the basis of size differences. It is possible that the same fundamental conformational difference

is responsible both for the observed dough-quality differences and the anomalous electrophoretic behaviour.

In the present paper we describe a series of experiments which identify a region of these proteins containing six single amino acid substitutions as being responsible for the observed SDS/PAGE mobility differences. We also show that these mobility differences are not caused by differences in intramolecular disulphide-bond formation involving cysteine residues, and that, in the absence of secondary structure, these proteins do have SDS/PAGE gel mobilities more in keeping with those expected for their size differences.

The experiments described involved the transcription and translation *in vitro* of genes encoding subunits 10 and 12, of chimaeric genes containing reciprocal exchanges between the two parent genes, and of various truncated versions of these genes.

MATERIALS AND METHODS

The gene for subunit 12 from the wheat (*Triticum aestivum*) variety Chinese Spring was obtained from Dr. R. D. Thompson [8], and the gene for subunit 10 from the variety Hope was isolated and sequenced in this laboratory (A. P. Goldsbrough & R. B. Flavell, unpublished work).

DNA manipulations were carried out essentially as described by Maniatis *et al.* [9]. Restriction enzymes phage- T_4 DNA ligase, phage- T_7 RNA polymerase, 7-methylguanosylguanosine (7-mGpppG) were purchased from Boehringer Mannheim. L-[35 S]Methionine and Amplify were purchased from Amersham International. Transcription-translation vectors Bluescribe and Bluescript were from Stratagene (Northumbria

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; HMW glutenin, high- M_r ('high-molecular-weight') glutenin; 7-mGpppG, 7-methylguanosylguanosine.

§ Present address: Plant Breeding International Cambridge, Maris Lane, Trumpington, Cambridge CB2 2LQ, U.K.

Biologicals, Cramlington, Northd., U.K.), and RNA-guard was from Pharmacia.

Dog pancreatic microsomal vesicles were provided by Dr. B. M. Austen [10]. Authentic prolamins were extracted from milled grain of the wheat variety Chinese Spring [11] and ^{14}C -labelled by the reductive methylation of lysine residues [12].

Construction of recombinant plasmids

The entire coding region and approx. 30 bp of upstream flanking sequences and 700 bp of downstream flanking sequences of the gene encoding subunit 12 were subcloned into Bluescribe to give the plasmid pUKC 1000 (Fig. 1a) as described by Bulleid & Freedman [13]. This gene construct has been previously transcribed and translated *in vitro* [13] by using the phage- T_7 promoter. A subunit 10 gene with approx. 70 bp of upstream and 40 bp of downstream flanking sequences was constructed in Bluescript to give the plasmid pWS10 (Fig. 1b), and this too could be transcribed and translated *in vitro*, but with a much lower efficiency than for pUKC 1000 (again using the phage- T_7 promoter present in Bluescript) (results not shown). In order to improve the efficiency of the subunit 10 gene and chimaeric constructs, all constructions were made in a pUKC 1000 derivative so that they all had the same upstream flanking sequences as the subunit 12 gene in pUKC 1000. This derivative included some of the coding sequences of the subunit 12 gene up to an endonuclease-*Bam*HI restriction site 291 bp into the coding sequence (see Fig. 1). However, the amino acid sequence encoded by these 291 bp is identical in both subunit 10 and subunit 12. Each of the constructs also had the same segment to the 3' side of the endonuclease-*Fok*I (F2) site (see Fig. 1), taken from the subunit 10 gene (Fig. 1b), but again the amino acids encoded by this sequence are identical in both subunit 10 and 12.

Owing to a lack of useful unique or rare restriction

sites within the coding region of the glutenin genes, only one reciprocal exchange between the genes encoding subunits 10 and 12 was made in the present work. This involved a 500 bp *Fok*I fragment located towards the 3' end of the coding regions. Although there are two *Fok*I sites in the gene for subunit 10 in pWS 10 (Fig. 1b) and three in the gene for subunit 12 in pUKC 1000 (Fig. 1a), each of these sites within a gene is effectively a single site, because the recognition sequence for *Fok*I is at a distance from the site of restriction. These sites have been designated F1, F2 and F3 in Fig. 1. Six of the amino acid differences between subunits 10 and 12 are encoded within the 500 bp *Fok*I fragment between F1 and F2.

An *Eco*RI-*Sal*I fragment was purified from pUKC 1000 and an *Xba*I-*Asp*718 fragment was purified from pWS 10. These fragments were digested to completion with *Fok*I and the products of digestions mixed together. The mixture of fragments was ligated to Bluescript linearized with *Eco*RI and *Asp*718 and, in a separate reaction, to Bluescript linearized with *Xba*I and *Asp*718. The four possible recombinants were recovered as characterized by their different diagnostic *Pst*I restriction profiles (results not shown). Each recombination has a *Fok*I(F2)-*Asp*718 fragment in common, but, as already mentioned, the amino acids encoded by this DNA are identical in both subunits. Finally, each of the four recombinants was digested with *Bam*HI and *Sal*I, and the resulting fragments were cloned into a pUKC 1000-derived vector linearized with *Bam*HI and *Sal*I. The resulting plasmids, namely pWSH 12, pWSH 12:10, pWSH 10 and pWSH 10:12, are illustrated in Figs. 1(c), 1(d), 1(e) and 1(f) respectively. These four chimaeric genes, when transcribed and translated, give subunit 10, subunit 12 and two chimaeric subunits which have the six amino acid differences encoded by the *Fok*I fragments reciprocally exchanged.

The resultant amino acid sequences for the chimaeric proteins can be deduced from Fig. 2, which gives the sequence of the repeat domains for both subunits 10 and 12 and the common C-terminal domain and shows the positions of reciprocal exchange.

Cell-free transcription

Transcription was carried out essentially as described by Krieg & Melton [14]. Plasmid DNA (2 μg) was linearized with the appropriate restriction enzyme and transcribed in a total volume of 50 μl containing 6 mM-MgCl₂, 2 mM-spermidine, 5 mM-NaCl, 10 mM-dithiothreitol, 0.5 mM-Tris/HCl, pH 7.5, at 37 °C with 100 units of human placental RNAase inhibitor and 30 units of phage- T_7 RNA polymerase. The mixture was incubated at 37 °C for 1 h, then extracted with phenol, followed by two extractions with chloroform/isoamyl alcohol (3-methylbutan-1-ol) (24:1, v/v). The RNA was recovered by adding ammonium acetate, pH 5.0, to 2.5 M and precipitated with 2.5 vol of ethanol at -20 °C for 16 h. The precipitate was recovered by centrifugation and the pellet washed twice with 75% (v/v) ethanol and dried under vacuum. The RNA was resuspended in 20 μl of diethyl pyrocarbonate-treated water.

Cell-free translation

Synthesized RNA was translated in a micrococcal-nuclease-treated rabbit reticulocyte lysate system [15] in the presence of dog pancreatic microsomal membranes as described by Bulleid & Freedman [13].

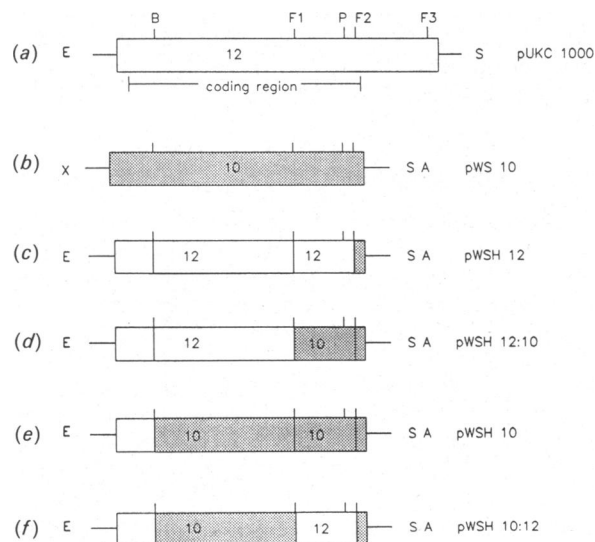


Fig. 1. Subunit 10, 12 and chimaeric-gene constructions

Key to restriction-endonuclease cleavage sites: B, *Bam*HI; E, *Eco*RI; F, *Fok*I; S, *Sal*I; X, *Xba*I; P, *Pvu*II; A, *Asp*718. Further details are given in the text.

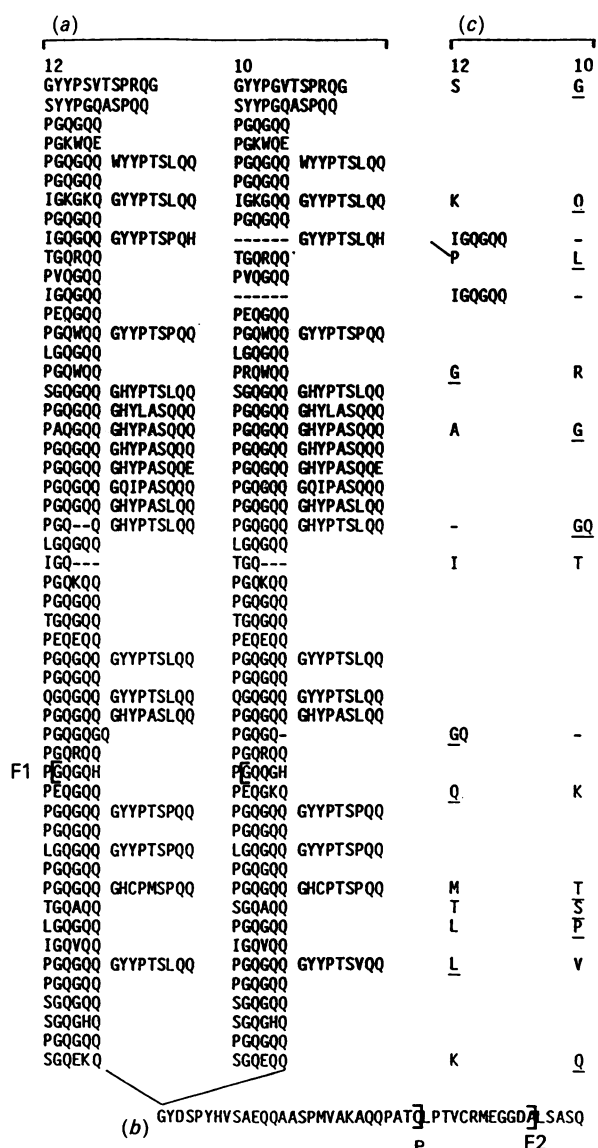


Fig. 2. Amino acid sequences of the repeat and C-terminal domains of subunits 10 and 12

(a) The repetitive regions are written to reveal the repeating subunit structure. (b) The C-terminal domain is common to both subunits. (c) Differences between the two subunits are indicated with consensus residues underlined. Locations of reciprocal exchange and truncations referred to in the text are indicated (F1, F2 and P).

SDS/PAGE

Samples to be separated by conventional SDS/PAGE were mixed with 5 vol of SDS/PAGE sample buffer [20 mM-Tris/HCl (pH 8.0)/20 mM-EDTA/6% (w/v) SDS/20% (w/v) sucrose/0.003% Bromophenol Blue/50 mM-dithiothreitol] and boiled for 4 min. Samples separated by urea SDS/PAGE were prepared as described above, and then urea was added to a final concentration of 4 M.

Electrophoresis was performed through an 11%-polyacrylamide gel (bisacrylamide comprised 1.8% of the total acrylamide) in the presence of SDS as described

by Laemmli [16]. Urea/SDS/PAGE was carried out under the same conditions, except that urea was added to the solutions before polymerization to give a final concentration of 4 M in the gel.

RESULTS

The genes coding for the HMW glutenin subunits 12 and 10 from wheat varieties Chinese Spring and Hope respectively were subcloned into Bluescribe and Bluescript (Figs. 1a and 1b respectively). From these, four hybrid genes were constructed (Figs. 1c-1f). Two of these, pWSH12 and pWSH10, encode subunit 12 and subunit 10 respectively. The other two, pWSH12:10 and pWSH10:12, were constructed by replacing a portion of the subunit 12 gene encoding 171 amino acids from the C-terminal region of the protein with the corresponding portion of the subunit 10 gene and vice versa. Transcription of the cloned genes was brought about by linearizing each plasmid with the appropriate restriction enzyme and transcribing with phage-T₇ polymerase using the phage-T₇ promoter located just upstream of the cloned genes in these vectors.

The RNA transcripts obtained were translated individually in a rabbit reticulocyte lysate system in the presence of dog pancreatic microsomal vesicles. The translation of individual storage proteins and the demonstration of signal-peptide cleavage and translocation to the microsomal-vesicle interior has been described elsewhere [13]. The translocation products obtained were separated by SDS/PAGE (Fig. 3). The upper series of bands are the products of the wheat gene. The lower bands in this Figure and Figs. 4-6 are proteins endogenously labelled in the reticulocyte system. The subunit 12 prepared *in vitro* migrated similarly to subunit 12 isolated from wheat seeds (Fig. 3, lanes 1 and 2). Translation product subunit 10 migrated more slowly

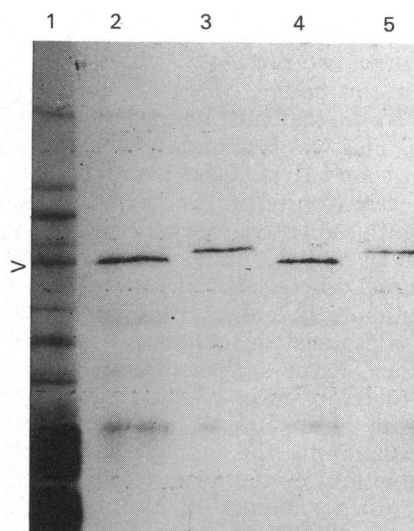


Fig. 3. Cell-free translation products of mRNA derived from the hybrid genes, separated by SDS/PAGE

Lane 1, authentic ¹⁴C-radiolabelled prolamines of the wheat variety Chinese Spring. Subunit 12 is indicated by a chevron (>); lane 2, pWSH 12; lane 3, pWSH 12:10; lane 4, pWSH 10:12; lane 5, pWSH 10.

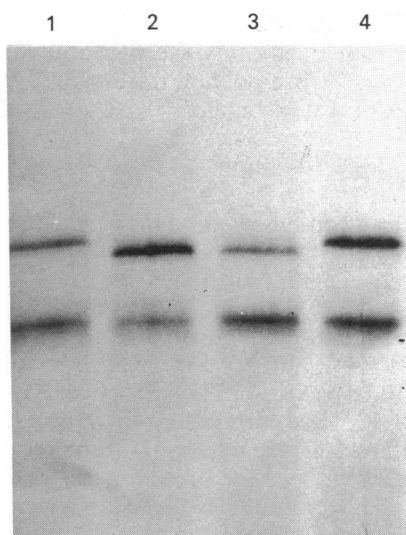


Fig. 4. Cell-free translation products of mRNA derived from *FokI*-truncated hybrid genes, separated by SDS/PAGE

Lane 1, pWSH 12; lane 2, pWSH 10; lane 3, pWSH 10:12; lane 4, pWSH 12:10.

than subunit 12, even though subunit 12 has a higher molecular mass than subunit 10. This result is consistent with earlier results comparing the mobilities of the proteins extracted from seeds [4]. Interestingly, the hybrid proteins showed relative mobilities which are consistent with their molecular masses calculated from the deduced amino acid sequences. Thus the hybrid 12:10 protein, which contains most of subunit 12 with 171 amino acids from subunit 10, migrated more slowly than subunit 12. The hybrid protein 10:12, which contains most of subunit 10 with 171 amino acids from subunit 12, migrated faster than subunit 10. Thus this C-terminal region containing these 171 amino acids appears to determine the anomalous relative electrophoretic mobility of these two proteins.

To confirm this finding, the genes encoding subunits 10 and 12 were truncated to remove the C-terminal 176 amino acids. This was brought about by linearizing the plasmids with *FokI* (F1) (Figs. 1 and 2). The resulting truncations were transcribed as described above. When the translation products were separated by SDS/PAGE, the polypeptides migrated in the order predicted by their molecular-mass differences (Fig. 4). Thus the *FokI*-truncated subunits 12 and 12:10 migrated more slowly than the *FokI*-truncated subunits 10 and 10:12. The removal of the C-terminal 176 amino acids from subunits 10 and 12 therefore reversed their relative mobilities, confirming the observations that the C-terminal 176 amino acids determine the anomalous mobility.

One explanation for the relative anomalous mobility observed between subunits 10 and 12 could be that conformational differences result from intramolecular disulphide-bond formation, which is resistant to reduction, within the C-terminal 176 amino acids. In both proteins there are two cysteine residues within this region (see Figs. 1 and 2), and both proteins have been shown to form intramolecular disulphide bonds using this cell-free system (results not shown). To test this potential source of the anomalous mobilities we prepared truncated

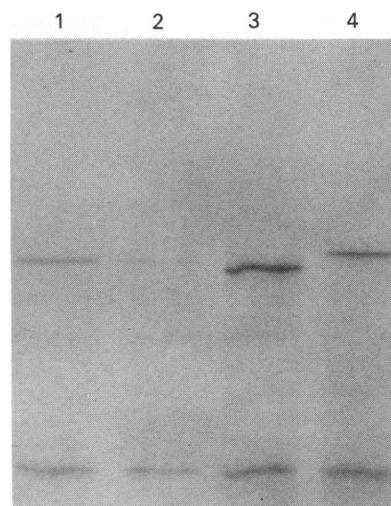


Fig. 5. Cell-free translation products of mRNA derived from *PvuII*-truncated hybrid genes, separated by SDS/PAGE

Lane 1, pWSH 10; lane 2, pWSH 12; lane 3, pWSH 10:12; lane 4, pWSH 12:10.

genes in which the C-terminal 17 amino acids, including one of the cysteine residues, were removed. This was brought about by linearizing the plasmids with *PvuII* (Figs. 1 and 2). The resulting truncations were transcribed and translated. When the products of translation were separated by SDS/PAGE, the anomalous relative mobility was again seen for the hybrid subunits 10:12 and 12:10 relative to subunits 10 and 12 (Fig. 5). Thus the *PvuII*-truncated 10:12 protein migrated faster than subunit 10, and the *PvuII*-truncated 12:10 protein migrated more slowly than subunit 12. This shows that, although there was no possibility of an intramolecular disulphide bond forming in this region, anomalous relative mobilities of these proteins were still apparent.

When the products of translation of the intact genes were separated by SDS/PAGE in the presence of urea, their relative mobilities were in the same order as their molecular masses (Fig. 6). Thus subunit 10 migrated faster than subunit 12, 12:10 migrated with a similar

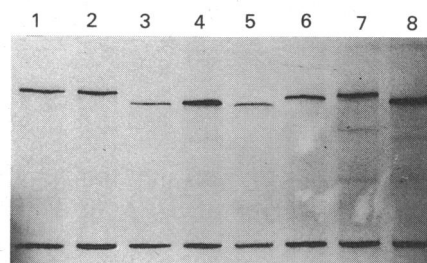


Fig. 6. Cell-free translation products of mRNA derived from the cloned genes separated by SDS/PAGE in the presence of 4 M-urea

Lane 1, pUKC 1000; lane 2, pWSH 12; lane 3, pWSH 10; lane 4, pWSH 10:12; lane 5, pWSH 10; lane 6, pWSH 12:10; lane 7, pWSH 12; lane 8, pWSH 10:12.

mobility to subunit 12, and 10:12 migrated with a similar mobility to subunit 10. This suggests that some secondary structure remains within the protein in the presence of SDS which affects its electrophoretic mobility, by altering either the shape of the molecule or the binding of SDS. In the presence of urea this secondary structure is broken down and the proteins are separated according to their relative molecular masses.

DISCUSSION

The lower mobility of subunit 10 relative to subunit 12 in SDS/PAGE after extraction from wheat seeds can be seen in the paper by Payne *et al.* [4], but not until the genes for subunits 10 and 12 were sequenced and it was discovered that subunit 10 was twelve amino acids shorter than subunit 12 did the relative mobility of subunits 10 and 12 appear anomalous. In the present paper we have confirmed, by transcription and translation of the isolated genes *in vitro*, the observation that subunit 10 has a lower mobility in SDS-containing gels than subunit 12. These results indicate that the products of translation *in vitro* have the same physical properties, in terms of electrophoretic mobility, as the authentic proteins (see Fig. 3).

The anomalous relative mobility of these proteins has been shown to be due to conformational differences between the proteins, because the anomalous behaviour is destroyed by the addition of the strong denaturant urea to the SDS-containing gels. In the presence of urea and SDS, the proteins display relative electrophoretic mobilities more in agreement with that expected from their size differences (Fig. 6). Moreover, through the construction of chimaeric genes, it has been possible to pinpoint a 171-amino-acid region containing just six amino acid substitutional differences between the two subunits as being responsible for the anomalous relative mobilities in the absence of urea. The region contains two cysteine residues which may form intramolecular disulphide bonds. Three of the six amino acid differences occur in the vicinity of one of these cysteine residues and might theoretically alter its availability for disulphide-bond formation. However, transcription and translation of truncated genes which are lacking one of these cysteine residues indicates that different structures arising from intramolecular disulphide-bond formation do not occur because the anomalous mobilities are still evident for the truncated proteins (Fig. 5). The cause of the mobility differences must therefore be the amino acid differences themselves.

The amino acid differences between subunits 10 and 12 are confined to the central domain which consists of repeating units. This domain is composed of two different repeating motifs: one a hexamer of consensus sequence P/SGQGQQ and the other a nonamer of consensus sequence GYYPTSLQQ. The repeating unit structure and the amino acid composition of the units are likely to produce an unusual protein conformation. Tatham *et al.* [2] and Field *et al.* [17] have proposed that the repeating structure imposes regular β -turns on the amino acid chain and the central part of the molecule therefore adopts a β -spiral structure similar to that of elastin. This sort of structure (or others based on a regular repeating structure) is likely to produce an anomalous electrophoretic mobility in SDS/PAGE relative to globular proteins. The molecular masses of subunits 10

and 12 calculated from their amino acid residues are 69600 and 70839 Da respectively. However, the molecular masses estimated relative to standard markers in SDS/PAGE are about 90000 Da [18]. This anomalously lower mobility of the subunits relative to globular proteins supports the hypothesis that the central domain of repeating units imposes an unusual structure.

The 171 amino acids in the C-terminal segment of subunit 10, which are responsible for its additionally reduced electrophoretic mobility relative to subunit 12, contain six amino acid differences compared with subunit 12 (Fig. 2). Of these, two are amino acid changes away from the consensus of all the repeat units, whereas the remaining four are changes to this consensus. Thus the slower migration of subunit 10 relative to subunit 12 may be because it has longer regions adopting a structure imposed by the consensus repeat units which in general are likely to be the reason for the slower migrations of glutenin subunits in SDS/PAGE. In predictions of protein structure devised by Chou & Fasman, described by Goldsbrough *et al.* [7a] and Flavell *et al.* [7b], the two amino acid substitutions away from the consensus sequence are likely to have no effect on β -turn structure, whereas the amino acid changes to the consensus sequence are predicted to increase the regularity of β -turns. Thus, if glutenins adopt β -spirals, subunit 10 may have longer stretches of amino acids in a continuous β -spiral conformation.

Single amino acid changes can affect the mobility of proteins in SDS/PAGE [19], but we suggest that the individual differences between subunits 10 and 12 are unlikely to have a major effect separate from their contribution to the dominating effect of the repeat unit structure.

Subunit 10 is believed to confer better breadmaking quality (viscoelasticity) on dough than subunit 12 [6]. The relationship of protein structure to viscoelasticity is not yet understood. However, the studies in the present paper, which have shown, for the first time, conformational differences, at least in SDS/PAGE, between two closely related subunits, raise the possibility that the observed conformational differences are the basis of the viscoelasticity differences in dough. Such a hypothesis would be based upon the premise that viscoelasticity of the gluten protein aggregate is dependent, in part, upon the lengths of the HMW glutenin proteins that adopt a regular repeating structure.

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