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The structure and genetic control of a new class of disulphide-linked proteins in wheat endosperm

N. K. Singh and K. W. Shepherd

Department of Agronomy, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond, South Australia 5064, Australia

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Summary. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of unreduced total protein extracts from the endosperm of hexaploid wheat revealed three high molecular weight protein bands (triplet bands) in a zone of heavy background streaking. Electrophoretic examination of 135 hexaploid cultivars showed at least five different patterns of these triplet bands. Nine durum wheat cultivars showed a single band only. Analysis of nullisomic-tetrasomic and ditelocentric lines of 'Chinese Spring' wheat revealed that the slowest moving band (Tri-1) of the triplet was controlled by gene(s) on chromosome arm 1DS and the fastest moving band (Tri-3) by 1AS. The band with intermediate mobility (Tri-2) was found to be a hybrid aggregate of the subunits controlled by 1DS and 1AS. Using a non-reducing/reducing form of 2-dimensional (2-D) electrophoresis, these triplet bands were shown to be heterotetramers of four subunits designated D (M.W. 58,000), δ (22,000), A (52,000) and α (23,000) where Tri-1 = DδDδ, Tri-2 = DδAα and Tri-3 = AαAα. With very low concentrations of 2-mercaptoethanol (ME), the tetramers dissociated into dimeric subunit pairs (Dδ, Aα), the monomers being observed with higher concentrations of ME. The structure of these subunit pairs resembles that of the subunit pairs in the globulin storage proteins of oats and some legumes. The 2-D method employed in this study was useful also for separating low molecular weight (LMW) subunits of glutenin from the monomeric gliadins which have similar electrophoretic mobility in 1-D separation. It was shown that at least four of these LMW glutenin subunits are controlled by genes on 1DS and 1AS and at least one subunit is controlled by gene(s) on 1BS. This electrophoretic separation method has proven useful in understanding the aggregation behaviour of the seed proteins of wheat.

Key words: Wheat – Disulphide-linked proteins – LMW glutenin subunits – Genetic Control – 2-D electrophoresis

1 Introduction

It is widely accepted that aggregated proteins resulting from disulphide linkage between various subunits in the wheat endosperm are important determinants of the bread-making quality of wheat flour (see review by Lásztity 1984). These disulphide-linked proteins, broadly classified as glutenins, are thought to interact with the gliadins to form an elastic but resilient matrix which confers unique visco-elastic properties on dough.

Although several different kinds of forces are involved in the formation of this matrix (Lásztity 1984), it has been demonstrated that disulphide bonds have a major influence on the structure of native glutenin (Pence and Olcott 1952; Nielsen et al. 1962) and on dough properties (Jones et al. 1974; Bloksma 1975). Two major classes of disulphide-linked proteins have been identified in wheat endosperm: firstly, the high molecular weight (HMW) glutenins which are composed of aggregates of HMW and LMW subunits (Bietz and Wall 1973); and secondly, what have been variously referred to as LMW glutenins (Nielsen et al. 1968) or HMW gliadins (Beckwith et al. 1966), which are composed of aggregates of LMW subunits only. Bietz and Wall (1973, 1980) have shown that the LMW subunits present in the HMW glutenins are identical with those present in the LMW glutenins, and these subunits are now referred to as LMW glutenin subunits.

The genes controlling HMW subunits of glutenin are located on the long arms of group 1 chromosomes of wheat (Orth and Bushuk 1974; Bietz et al. 1975; Lawrence and Shepherd 1980, 1981; Payne et al. 1980), and they are closely linked with the centromere (Payne et al. 1982). The LMW glutenin subunits have been more difficult to characterize biochemically and genetically because after reduction they co-migrate with the classical gliadins in 1-D electrophoresis. This problem has been solved, at least partly, by employing 2-D

electrophoretic procedures (Jackson et al. 1983), and it has been shown that genes controlling some of these LMW subunits are closely linked with the *Gli-1* locus on the short arm of group 1 chromosomes (Payne et al. 1984).

Recently, when examining the electrophoretic patterns of unreduced extracts of total endosperm proteins from 'Chinese Spring' wheat by SDS-PAGE, we observed three electrophoretically slow-moving bands in a zone of heavy background streaking. These bands are readily reduced into smaller subunits by trace amounts of ME and, since they are electrophoretically different from the oligomeric bands of HMW glutenin subunits detected by Lawrence and Payne (1983) and the HMW glutenin subunits of 'Chinese Spring', we believe they represent a new class of disulphide-linked proteins in the endosperm of wheat. These three bands, or a similar set of three bands, were found to occur widely among hexaploid wheat cultivars and we have designated them as "triplet" bands until they are more fully characterized.

In this paper we report on the variation, genetic control and subunit composition of these triplet bands. To facilitate the analysis of these triplet proteins we have used a 2-D electrophoretic procedure developed by Wang and Richards (1974), and later used by Sommer and Traut (1975), Hynes and Destree (1977) and Matta et al. (1981), for the analysis of disulphide-linked proteins. This method has also proven to be efficient for separating the subunits of the other disulphide-linked proteins in wheat endosperm, particularly the LMW glutenin subunits, and it has provided a new insight into the aggregation properties of the glutenin subunits of wheat. A brief summary of some of this work was presented at the Second International Workshop on Gluten Proteins held at Wageningen (Singh and Shepherd 1984).

2 Materials and methods

2.1 Wheat stocks analysed

The following ditelocentric (Dt), nullisomic-tetrasomic (TNT) and tetrasomic (T) lines of 'Chinese Spring' wheat (Sears 1954, 1966), kindly donated by Dr. E. R. Sears and now maintained at the Waite Agricultural Research Institute, were used: Dt 1AL, Dt 1BL, Dt 1DL, NT 1A-1B, NT 1A-1D, NT 1B-1A, NT 1B-1D, NT 1D-1A, NT 1D-1B and T 1A. A collection of 135 hexaploid wheat cultivars including representative cultivars from the main wheat growing areas of the world was provided by Dr. A. J. Rathjen of this Institute.

2.2 Extraction of seed proteins

Unreduced proteins were extracted from single kernels of wheat with SDS in a Tris-HCl buffer pH 6.8 as described by Lawrence and Shepherd (1980), except ME was excluded. When reduced proteins were required, 1.5% (v/v) ME was included in the above solvent buffer.

2.3 One-dimensional SDS-PAGE

The discontinuous system of SDS-PAGE was based on the method of Laemmli (1970) as modified by Lawrence and Shepherd (1980), except the gels were more concentrated [10.0% (w/v) acrylamide] and thinner (1.8 mm). The gel slabs were run in pairs (one each side of a perspex stand) at a constant total current output of 80 mA for the first 45 min and then 50 mA until the dye front reached the bottom of the gel.

2.4 Two-dimensional electrophoresis

Proteins were first extracted in the SDS solvent without ME and then separated in SDS polyacrylamide disc gels in glass tubes (120 mm × 5 mm internal diameter). The compositions of the separating (90 mm) and stacking (10 mm) sections of the gel were identical to those of the slab gels. Unless stated otherwise, 50 µl of extract was layered onto the stacking gel and the samples were electrophoresed at 3 mA/tube for 45 min and then 2 mA/tube for 2 h. The gels were then removed from the tubes and incubated at 37 °C for 30 min to 2 h in an equilibration mixture consisting of 1% ME freshly added to a solution of 10.3% (w/v) glycerol, 0.07 M Tris, 2.4% (w/v) SDS made to pH 6.8 with HCl, to achieve protein reduction (c.f. Brown et al. 1979). Control gels were incubated in the same solution but without the addition of ME.

After incubation, the proteins in the disc gels were subjected to electrophoresis in a direction at right angles to that of the first run. The gel rod was loaded horizontally onto the stacking gel of a 3.2 mm thick slab gel. The second dimension of electrophoresis was carried out at a constant current of 75 mA/gel for the first 45 min and at 50 mA for the remainder of the run (about 2 ½ h).

2.5 Staining and destaining

The gels were stained as described by Lawrence and Shepherd (1980), but destaining was carried out in distilled water for 48 h.

2.6 Molecular weight calibration

Apparent molecular weights of wheat proteins were determined from the mobilities of the following proteins used as standards (Pharmacia): phosphorylase b (94,000), bovine albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000).

3 Results

3.1 Detection of triplet bands and their genetic control

The one-dimensional electrophoretic patterns of unreduced and reduced extracts of total endosperm proteins of 'Chinese Spring' wheat are shown in Fig. 1. The triplet bands, with relative staining intensities of approximately 4 : 4 : 1, occur in the cathodal (upper) half of the gel amongst heavy background streaking in the unreduced samples (Fig. 1 a, b). The other faster moving discrete protein bands present are thought to be monomeric gliadins and soluble proteins (albumins and globulins). The triplet bands and the background streaking were absent after reduction with ME (Fig. 1 c), suggesting that disulphide bonding was involved with

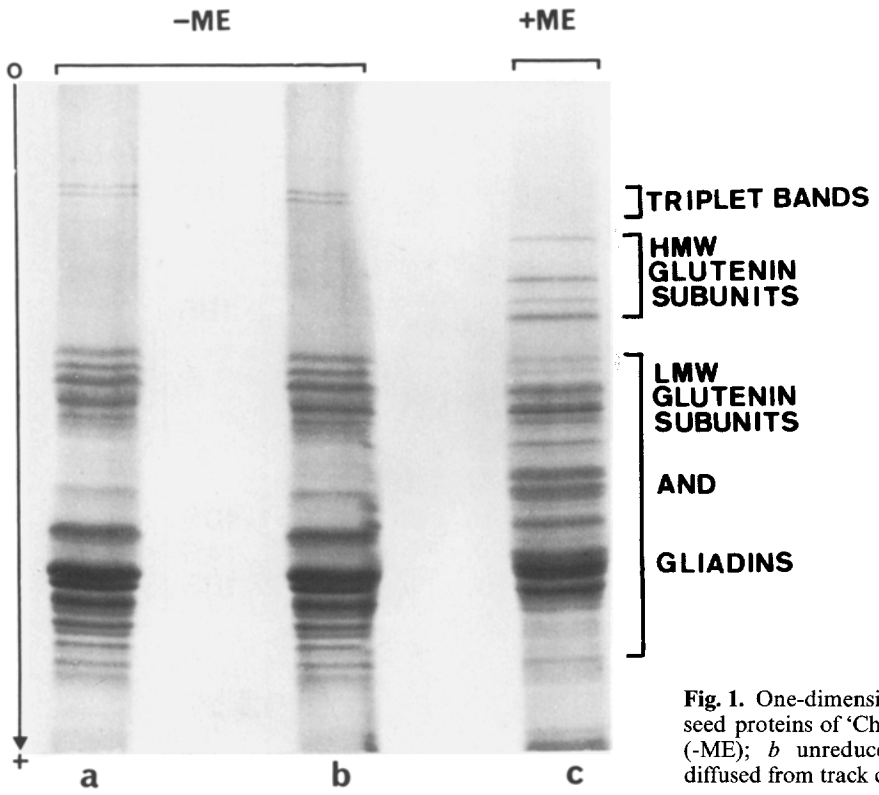


Fig. 1. One-dimensional (1-D) SDS-PAGE patterns of total seed proteins of 'Chinese Spring' euploid. *a* unreduced extract (-ME); *b* unreduced extract showing edge effect of ME diffused from track c; *c* reduced extract (+ME)

both of these features of unreduced extracts. The appearance of many new bands after reduction, including the four HMW glutenin subunits and many less well resolved bands in the LMW region of the gel, was consistent with this conclusion. The triplet bands and the streak are extremely sensitive to reduction by ME, since diffusion of ME from track c has resulted in a pronounced edge effect in track b (Fig. 1).

The chromosomal control of the triplet bands was determined by analysis of 'Chinese Spring' aneuploids. Seeds from stocks lacking chromosome 1A (e.g. NT 1A-1B), or the short arm of 1A (Dt 1AL), possessed only the slowest moving band (Tri-1) of the triplet (Fig. 2 b, c, d), whereas stocks lacking chromosome 1D (e.g. NT 1D-1A), or the short arm of 1D (Dt 1DL), had only the fastest moving band (Tri-3) (Fig. 2 j, k, l). The intermediate band (Tri-2) was absent whenever either chromosome arm 1AS or 1DS was absent. In contrast, the removal of complete chromosome 1B or its short arm did not have any obvious effect on the triplet pattern (Fig. 2 f, g, h). Besides these qualitative changes in pattern, some quantitative changes in band staining intensity were observed when the dosage of chromosomes 1A or 1D in the endosperm was varied. The approximate 4:4:1 ratio of staining intensity of bands Tri-1 : Tri-2 : Tri-3 in euploid 'Chinese Spring' having equal doses of chromosomes 1A and 1D, was changed to 1:2:1 and 2:1:0 (or 16:8:1) ratios when the dosage of these respective chromosomes was changed to 2:1 (NT 1B-1A, Fig. 2 g) and 1:2 (NT 1B-1D, Fig. 2 h), respectively.

These observations indicate that the triplet bands are controlled by genes on the short arms of chromosomes 1A and 1D and that the relative amounts of these proteins are influenced by the dosage of these chromosomes in the endosperm. Furthermore, the observations are consistent with the hypothesis that the Tri-1, Tri-2 and Tri-3 bands are 'dd', 'da' and 'aa' dimers, respectively, produced by random association of 'd' and 'a' monomers controlled by chromosomes 1D and 1A, with the amount of 'd' monomer produced per chromosome being twice that of 'a' monomer. However, later it was shown that this model is an oversimplification since it was found that triplet bands are tetramers rather than dimers (see section *3.5*).

It is known from earlier work using aluminium lactate starch gels at acid pH (Shepherd 1968; Wrigley and Shepherd 1973) that the non-covalently linked prolamins are also controlled by genes on the short arms of chromosomes 1A, 1B and 1D and the patterns obtained with SDS-PAGE are consistent with these findings (see Fig. 2).

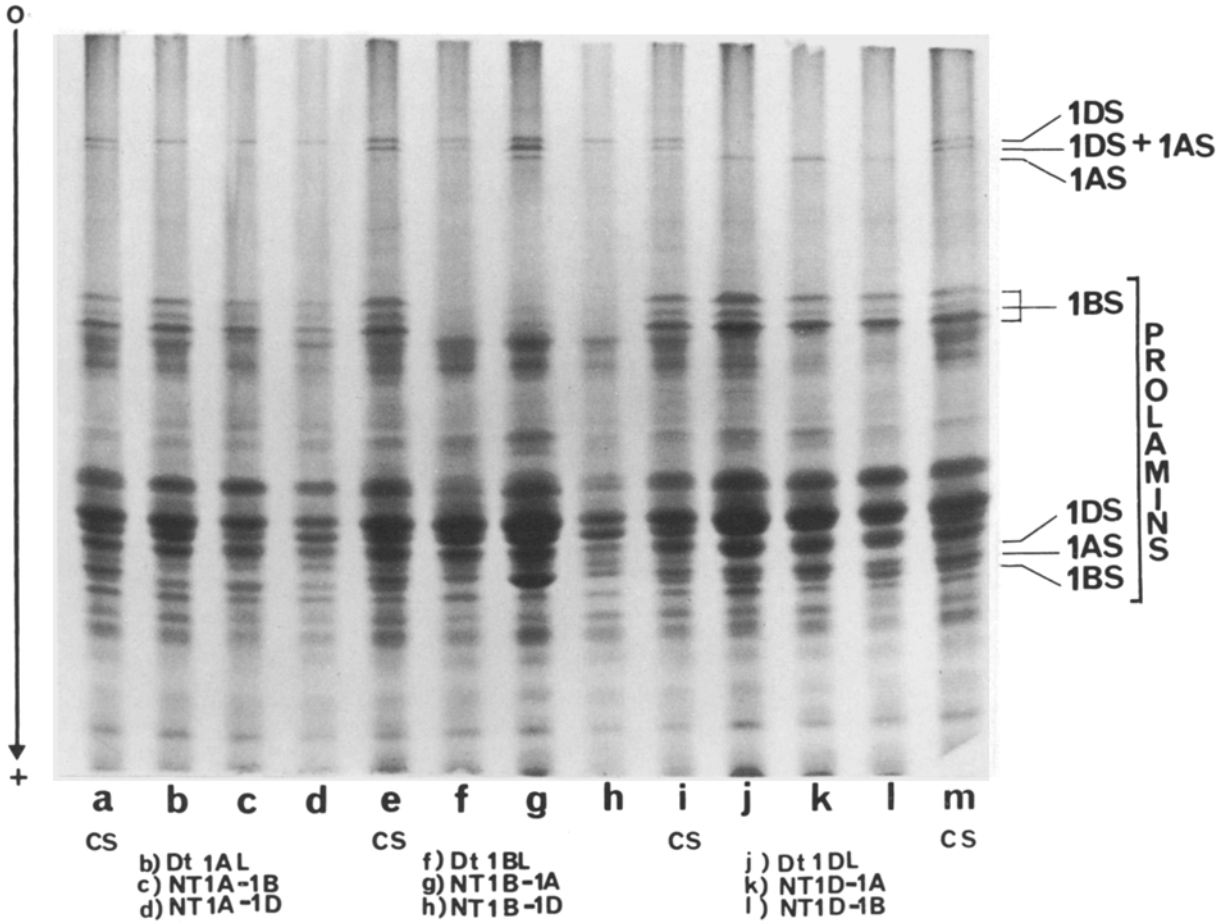


Fig. 2. 1-D SDS-PAGE patterns of unreduced total seed protein extracts of euploid 'Chinese Spring' (CS) and ditelocentric (Dt) and nulli-tetra (NT) stocks

3.2 Variation of triplet band patterns in wheat cultivars

In a survey of unreduced protein extracts from seeds of 135 hexaploid wheat cultivars from a world collection, all cultivars except four possessed three electrophoretically slow moving bands in the cathodal portion of the gel. However the fastest moving band (Tri-3) was always faint and difficult to score as in 'Chinese Spring'. The other two bands (Tri-1 and Tri-2) occurred in four distinct patterns referred to as 'broad, slow-moving', 'broad, fast-moving', 'narrow, slow-moving' and 'narrow, fast-moving' (Fig. 3 a, b, c, e, respectively). Four cultivars had a single diffuse band only (Fig. 3 f). The nine durum wheats analysed possessed only one band which had similar mobility to Tri-3 of 'Chinese Spring' (Fig. 3 h). A list of these 144 wheat cultivars and their triplet patterns is available from the authors on request.

3.3 Two-dimensional separation of endosperm proteins

To resolve the structure of the triplet bands it was necessary to identify the subunits released from these bands after reduction and we used a 2-D method of separation, with unreduced proteins in the first dimension and reduced proteins in the second (Wang and Richards 1974), for this purpose. The breakdown products of the triplet bands could be identified by their position vertically below the location of the triplet bands after the first phase of this separation. 'Chinese Spring' T 1A, in which all three triplet bands are clearly visible, was used for this purpose. Unreduced gel rods when run in the second dimension, gave the expected 45° line and the triplet bands were recognizable as dark dots approximately 2 cm from the origin in the streaky region of the gel (Fig. 4 A). The reduced rods gave a very different electrophoretic pattern (Fig. 4 B). The triplet bands separated into four spots, consisting of two slower moving components and two very fast moving components. The dark streak present in the unreduced gels was much less prominent after reduction and at least 20 different subunits (including four HMW glutenin subunits and four subunits of triplet bands) appeared as continuous bands starting from the origin of the rod gel and extending almost to the 45° line. The

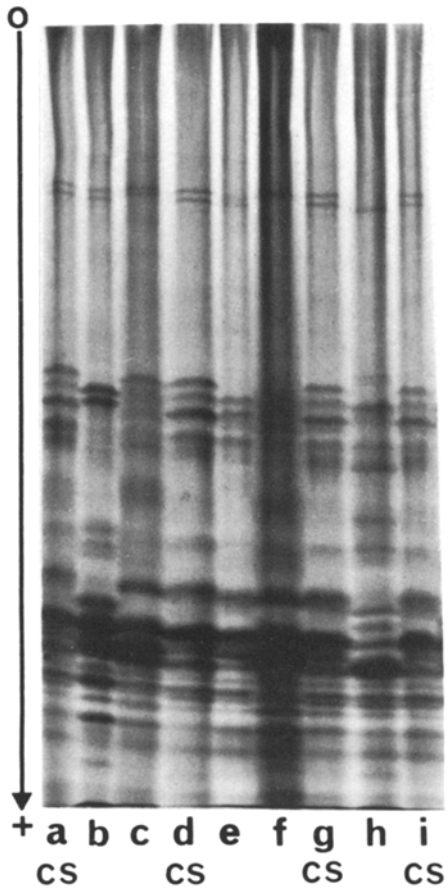


Fig. 3. Variation of triplet band patterns in wheat cultivars. *a, d, g, i* broad, slow-moving ('Chinese Spring'); *b* broad, fast-moving (India-115); *c* narrow, slow-moving ('Hope'); *e* narrow, fast-moving ('Sonalika'); *f* single diffuse band (Tainui); *h* single band ('Durati', a durum wheat)

group of proteins which did not change their mobility upon reduction, remaining as spots on the 45° line, were thought to be monomeric (α, β, γ, ω) gliadins. However, a few of the faster moving gliadin spots (α, β and γ groups) were displaced from the 45° line after reduction, indicating slightly lower mobility in the second dimension; this may be due to the reduction of some intramolecular disulphide bonds in gliadin molecules, resulting in more complete unfolding of the polypeptide chains (c.f. Woychik et al. 1964; Beckwith et al. 1965; Nielsen et al. 1968).

3.4 Genetic control of the triplet and LMW glutenin subunits

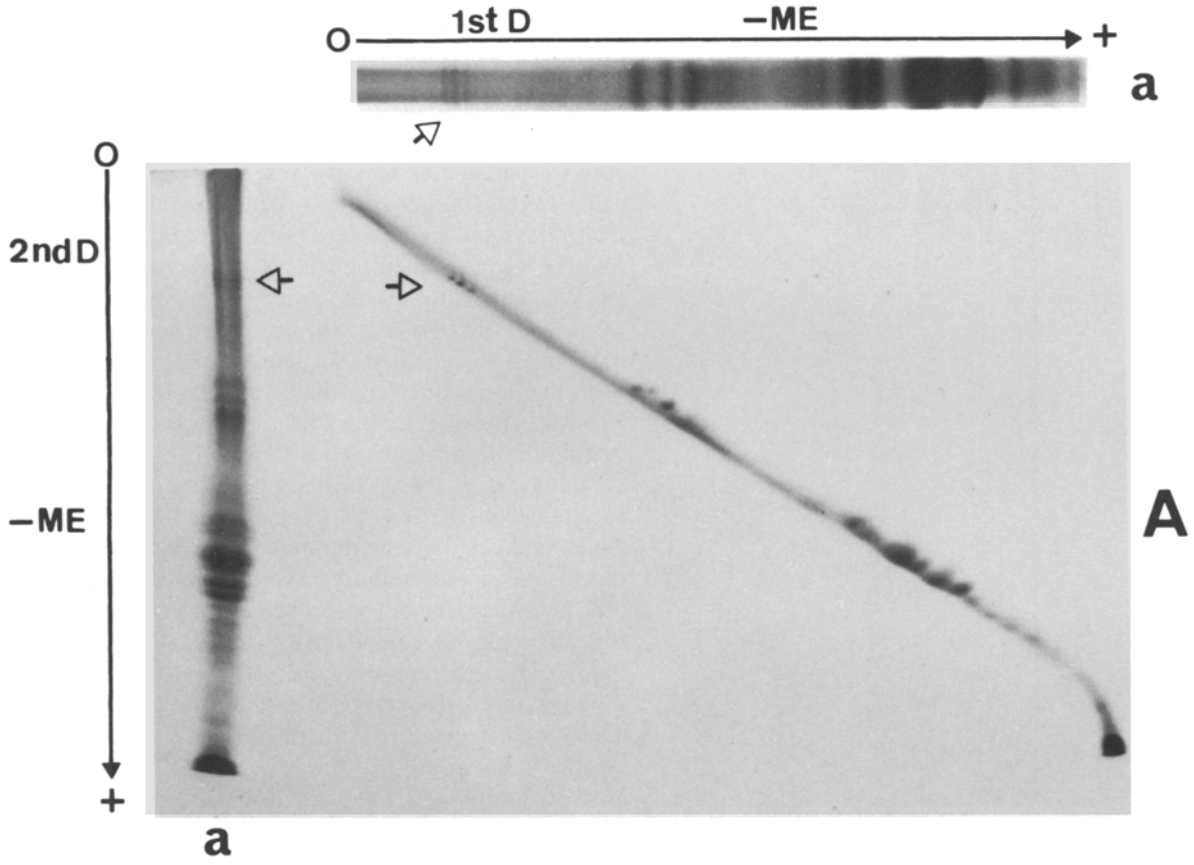
Since the unreduced triplet bands occur in the first 20 mm of the separating gel in the tubes, it was possible to cut out this section from four separate tube gels and after reduction, include all four pieces representing four different samples on a slab gel in the second dimension of electrophoresis. In this way, the subunit composition of the triplet bands present in euploid 'Chinese Spring' could be compared directly with those present in ditelocentric lines 1AL and 1DL (Fig. 5). The slowest moving spot (designated as D) and the fastest moving spot (designated as δ) were absent when chromosome arm 1DS was missing (Fig. 5 c) and therefore these are controlled by gene(s) on chromosome arm 1DS. Similarly, the other two spots (A, α) were absent in the stock lacking chromosome arm 1AS and are controlled by gene(s) on 1AS (Fig. 5 b). Thus, chromosome arms 1DS and 1AS each code for one slow moving and one fast moving subunit of the triplet bands. A lighter 'shadow' spot can be seen below the D spot (Fig. 5 a, b, d) but its significance is not known.

Removal of these chromosome arms was also associated with the loss of some of the LMW subunits which appeared as continuous bands in the second dimension of electrophoretic separation. Chromosome arm 1DS appeared to control two of these bands which, together with the subunits of Tri-1, are labelled 1DS in Fig. 5. When chromosome arm 1AS was absent, there was a marked decrease in the intensity of two other bands which, together with the subunits of Tri-3, are labelled 1AS in Fig. 5. The removal of chromosome arm 1BS, although not affecting the triplet pattern, resulted in the loss of one of the LMW subunits (data not shown). The chromosomal locations of the genes controlling the triplet and the LMW subunits are currently being investigated in linkage mapping experiments (see Singh and Shepherd 1984).

3.5 Subunit composition of triplet bands

From the behaviour of triplet bands displayed in Figs. 1, 2 and 5 it is concluded that Tri-1 is a disulphide-linked aggregate of subunits D and δ controlled by chromosome arm 1DS, Tri-3 is an aggregate of subunits A and α controlled by 1AS, and that Tri-2 is a hybrid aggregate. Furthermore, the structure of the subunit spots observed in the second dimension of separation after reduction of the triplet bands of 'Chinese Spring' T 1A, clearly shows two different subunits coming from each of the Tri-1 and Tri-3 band positions and all four subunits coming from the Tri-2 band position (Fig. 6).

Since Tri-2 is at least a tetramer, Tri-1 and Tri-3 must have a similar composition because of their similar molecular size. Direct evidence for this conclusion came from the detection, in partially reduced gels, of two additional spots with intermediate electrophoretic mobility (Fig. 7 b, c, d). To obtain this gel, unreduced extracts of 'Chinese Spring' T 1A were electrophoresed in 12 rod gels and 1 cm sections were cut from each gel in the region expected to contain the triplet bands.


1st D
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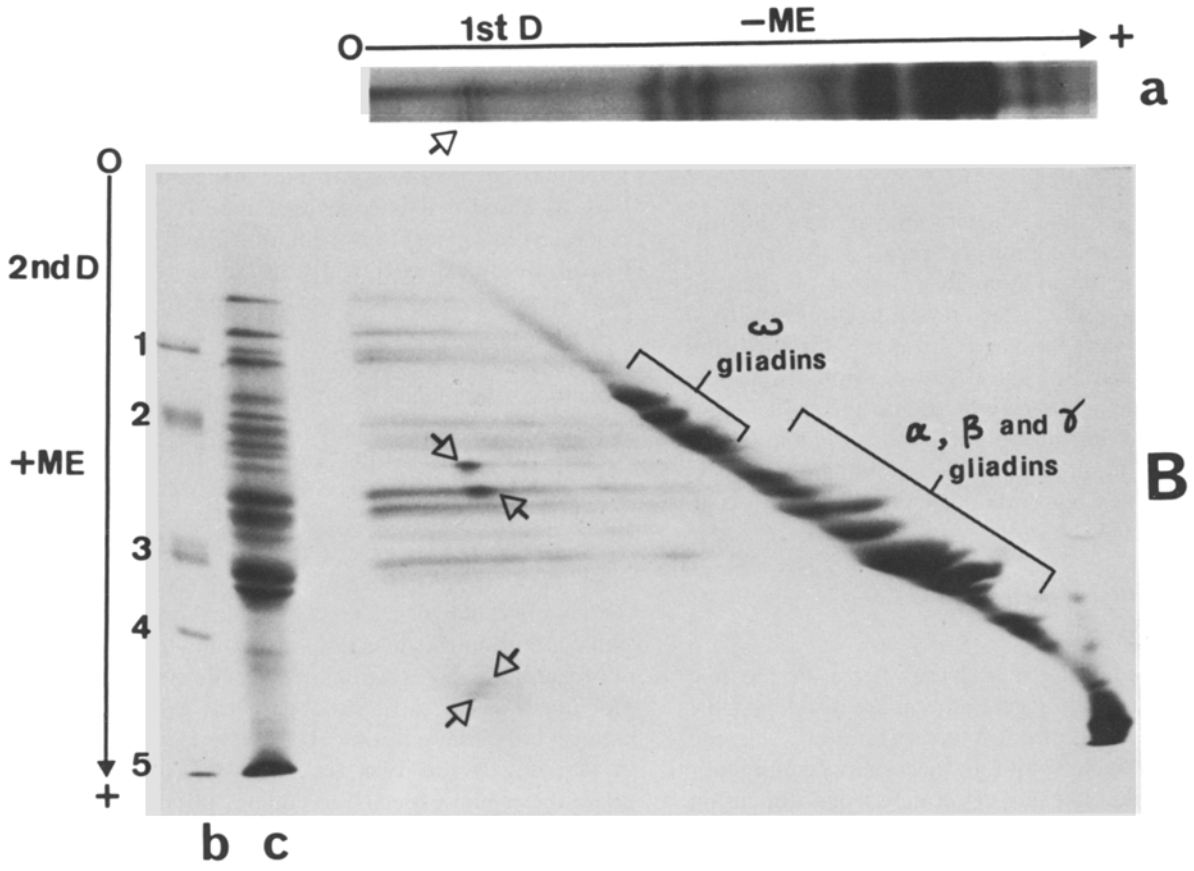
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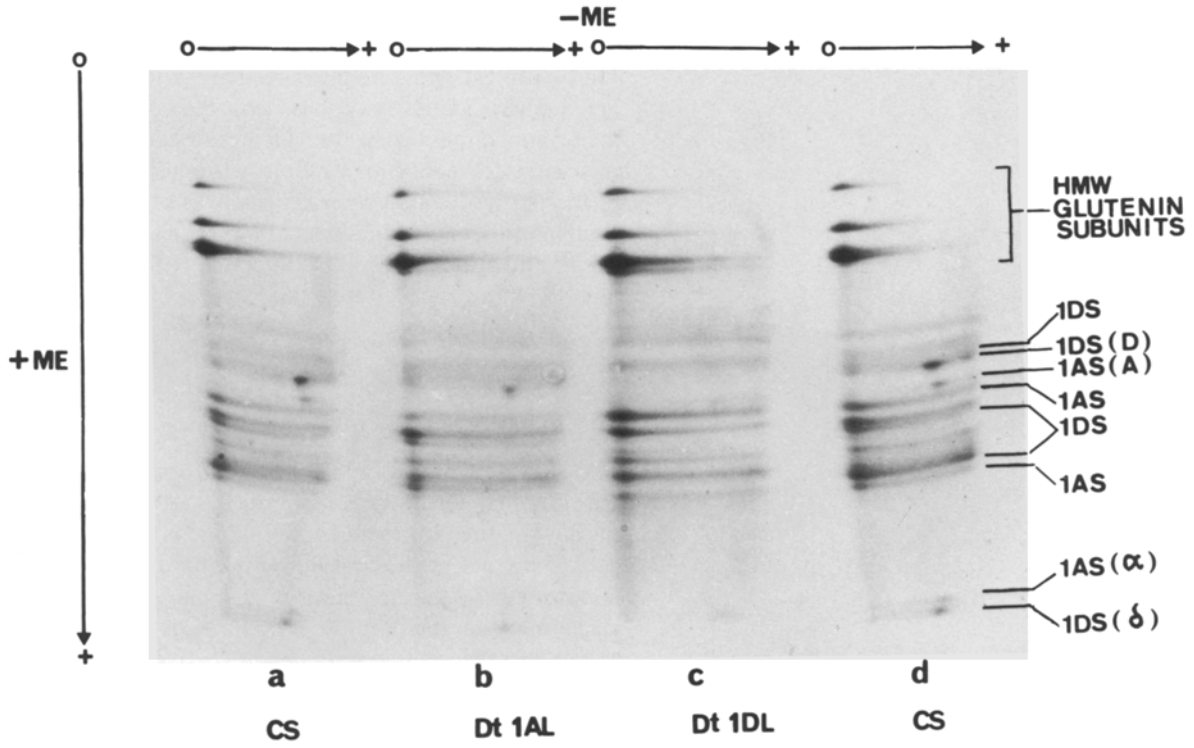

Fig. 5. Modified 2-D SDS-PAGE patterns of euploid 'Chinese Spring' (CS) and its ditelocentrics (Dt). The four rod pieces loaded onto the slab gel were reduced with ME before running in the second dimension. The chromosome arms controlling the LMW subunits of glutenin and triplet band subunits (D, A, α, δ) are indicated

These gel sections were reduced for two hours in separate equilibration tubes containing ME concentrations ranging from 0–2% (see legend Fig. 7, for details), before loading them onto a slab gel for electrophoresis in the second dimension.

Although four (c, i, k, l) of the gel sections were loaded with reverse polarity and one section (e) did not contain the triplet bands, Fig. 7 clearly shows that in the presence of trace amounts of ME the triplet bands are reduced to two intermediate-sized products, and in two of these cases (Fig. 7 c, d) the four monomeric subunits appear as well. At higher concentrations of ME, these intermediate products disappear and only the four subunits are present. These intermediate spots are interpreted to be dimeric products Dδ and Aα, coming from the partial reduction of tetrameric triplet bands DδDδ (Tri-1), DδAα (Tri-2) and AαAα (Tri-3).

There are two other features of interest in Fig. 7. First, the LMW glutenin subunits tend to show decreased mobility with increasing concentrations of ME up to a threshold of 0.1% (Fig. 7 i), and it is thought that this might be due to the disruption of intramolecular disulphide bonds with high concentrations of ME resulting in greater unfolding of these subunits, just as was found with some of the classical gliadins (Fig. 4). Second, the shadow bands of the D subunits described earlier (Fig. 5) are more pronounced in this gel and there appear to be shadow bands under the A subunits as well.

A model of the structure of the triplet bands based on the above observations is given in Fig. 8. The molecular weights of subunits D, δ, A and α are estimates

Fig. 4 A, B. 2-dimensional (2-D) SDS-PAGE patterns of total seed proteins of 'Chinese Spring' tetrasomic 1A. **A** Non-reducing conditions in both dimensions (−ME × −ME). Only 25 µl of extract was loaded for better resolution of triplet dots. **B** Non-reducing conditions in the first dimension and reducing conditions in the second dimension (−ME × +ME). *a, b, c* reference samples: *a* 1-D separations of unreduced extracts; *b* molecular weight standards (Pharmacia); *1* phosphorylase b (94,000), *2* bovine albumin (67,000), *3* ovalbumin (43,000), *4* carbonic anhydrase (30,000), *5* trypsin inhibitor (21,000); *c* 1-D separation of reduced extracts. Arrows indicate the positions of bands and spots related to triplet proteins

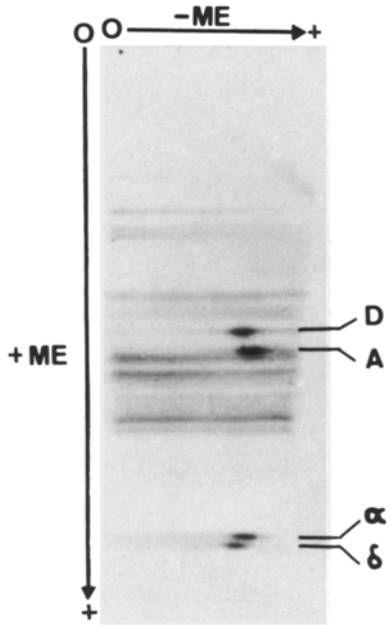

Fig. 6. 2-D SDS-PAGE pattern of 'Chinese Spring' tetrasomic 1A. Note the overlap of four subunit spots (D, A, α and δ) in central position of the bands and overlap of only two subunits on *left* (D, δ) and *right* (A, α) corresponding to breakdown of Tri-2, Tri-1 and Tri-3, respectively

based on comparison with the mobilities of standard protein markers (Fig. 4B, b). However, the values given for the triplet bands and the partial breakdown products are deduced values only, based on the assumed tetrameric and dimeric structure of these bands. However, in several gels (not shown) the electrophoretic mobility of the partial breakdown products, was observed to be similar to that of ω-gliadins (MW = 65,000–80,000: see Booth and Ewart 1969; Bietz and Wall 1972; Charbonnier 1974 and Fig. 4B, b of this paper), indicating that their actual molecular weights were consistent with the deduced values.

3.6 Origin of the streak and parallel lines

Two striking features of this 2-D electrophoretic procedure applied to total protein extracts of wheat endosperm are the pronounced streaking in the cathodal half of the gel in the first dimension and, after reduction with ME, the appearance of many parallel lines extending from the origin to a point at, or near, the 45° line in the second dimension (Fig. 4).

The streaking in the first dimension is not due to continuous and prolonged entry of one or a few proteins into the gel, because it is maintained as a well

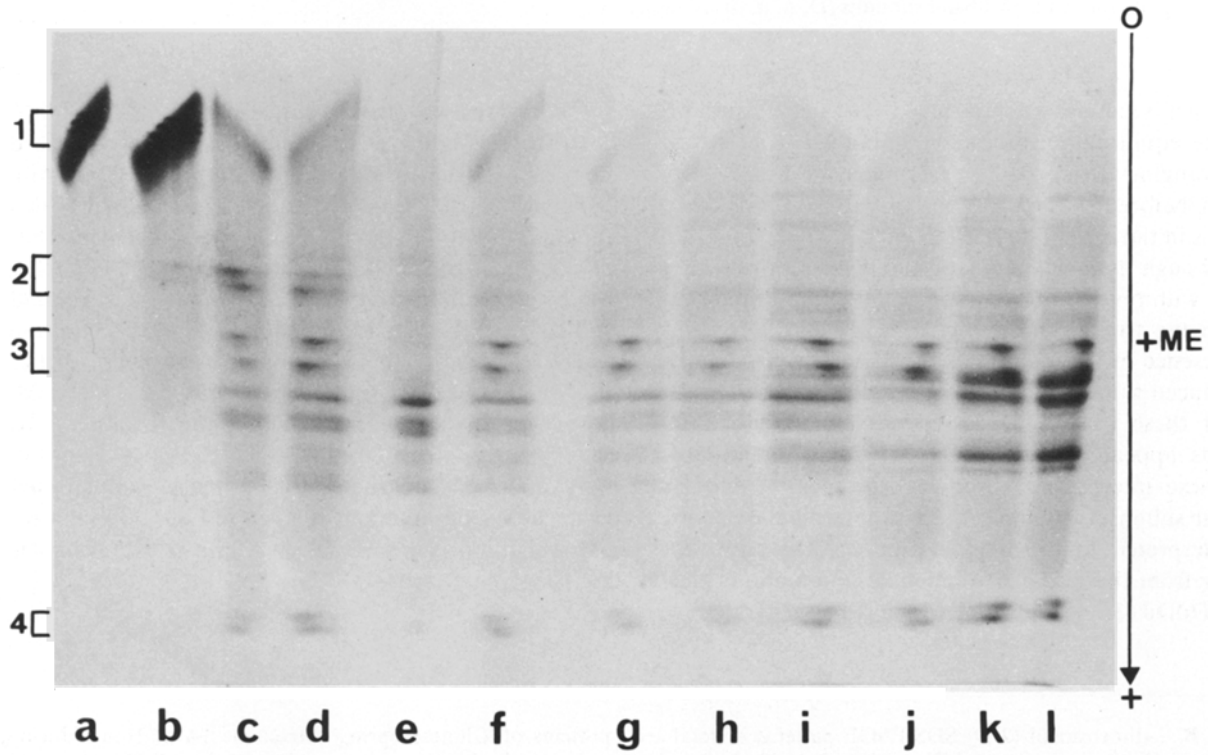

Fig. 7. Modified 2-D SDS-PAGE patterns of 'Chinese Spring' tetrasomic 1A. The 12 rod pieces (*a–l*) loaded on to the slab gel were equilibrated with a range of ME concentrations [% (v/v)] as follows: *a* 0; *b* 0.001; *c* 0.01; *d* 0.0125; *e* 0.015; *f* 0.0175; *g* 0.02; *h* 0.05; *i* 0.1; *j* 0.5; *k* 1.0; *l* 2.0. *1* triplet bands (tetramers); *2* intermediate breakdown products (dimers); *3* large subunits – D, A (monomers); *4* small subunits-α, δ (monomers)

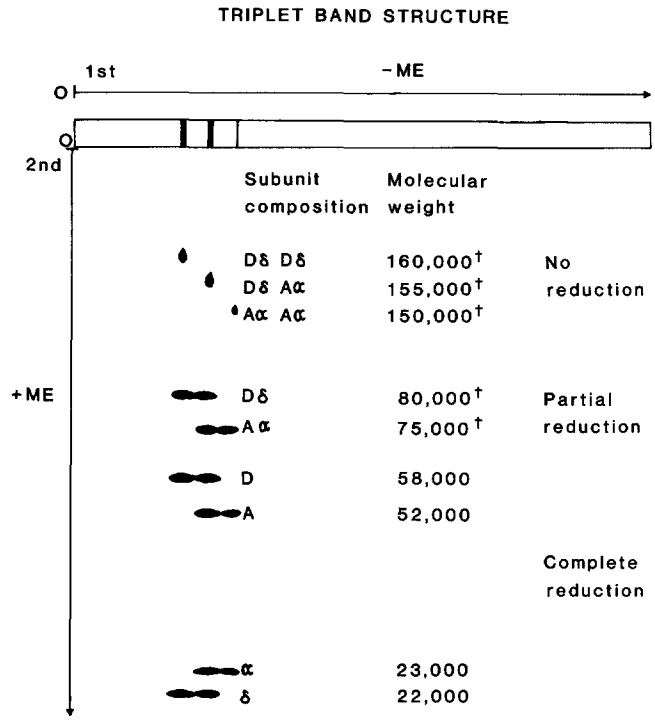

Fig. 8. Model for the structure of triplet bands. †Deduced values based on the assumed dimeric and tetrameric structure of intermediate breakdown products and triplet proteins, respectively

defined narrow diagonal line in the second dimension of separation of unreduced extracts. Thus the position of each component of the streak in the first dimension reflects a particular electrophoretic mobility which is maintained in the second dimension. The streak is thought to represent the electrophoretic separation of a complex mixture of wheat proteins having a continuous array of sizes, which is consistent with the conclusions drawn earlier from gel filtration studies (Crow and Rothfus 1968; Danno et al. 1974; Huebner and Wall 1976; Payne and Corfield 1979; Bottomley et al. 1982).

In seeking an explanation for the parallel lines, it was necessary first to eliminate the possibility that protein subunits had diffused along the rod during the equilibration procedure or the second dimension of electrophoresis. This was considered unlikely since the lines were restricted to the origin side of the gel and did not extend beyond the 45° line. However, proof that diffusion was not involved was obtained by cutting the rod gel into seven ordered pieces after the first run and equilibrating each piece separately. When the reduced pieces were loaded onto the slab gel end-to-end so as to reconstitute the original rod and electrophoresed, it was observed that the adjacent pieces had the same bands appearing as parallel lines, but the bands did not extend beyond the cut ends of the gel (Fig. 9).

Thus it is concluded that the bands occurring as parallel lines represent the array of subunits which make up the different-sized protein aggregates forming the cathodal streak in unreduced rod gels. The four slowest moving of these bands correspond in size to the HMW glutenin subunits of 'Chinese Spring' (Bietz and Wall 1972). The other bands, because of their size and control by genes on the short arm of group 1 chromosomes, are thought to be the LMW glutenin subunits (c.f. Jackson et al. 1983) and the four triplet subunits. The lines corresponding to the A and D triplet subunits can be seen in Figs. 4 and 9 but the lines corresponding to the α and δ subunits, although clearly present in the original slab gels, are too faint to show up in the photographs. It is of interest that the α and δ lines do not extend completely to the 45° line on the slab gel but terminate as well defined spots in the positions expected for reduced $\alpha\alpha$ and $\delta\delta$ dimers, respectively.

The continuous nature of the bands forming the parallel lines is thought to result from two main sources. The main contribution to continuity is expected to come from the formation in the endosperm of protein aggregates of different sizes due to disulphide bonding between and within the many different LMW and HMW subunits of glutenin and the four triplet band subunits. In this model a given glutenin subunit may be present in a wide array of different-sized aggregates ranging from very large multimers to a small dimer involving just this subunit and the smallest interacting subunit, possibly the α and δ subunits. Another factor contributing to the continuity of these lines could be the occurrence of conformational isomers among the protein aggregates, due to differences in the number and sites of disulphide linkage between any two interacting subunits, or to differences in degree of unfolding of a given dimer or multimer in the presence of SDS.

As shown earlier, some proteins remain as spots on the diagonal line, and these are thought to be the classical α, β, γ and ω gliadins. Thus this simple 2-D procedure separates wheat endosperm proteins into those that occur as projections below the diagonal line and those which remain as spots on this line, corresponding with protein subunits forming disulphide-linked aggregates and those which remain as monomers, respectively.

3.7 Analysis of 'pure' gliadin and glutenin

We decided to apply this new method for distinguishing between monomeric proteins and disulphide-linked protein aggregates to determine the composition of gliadin and glutenin fractions prepared by the classical method of differential solubility (Osborne 1907). Mr. J.

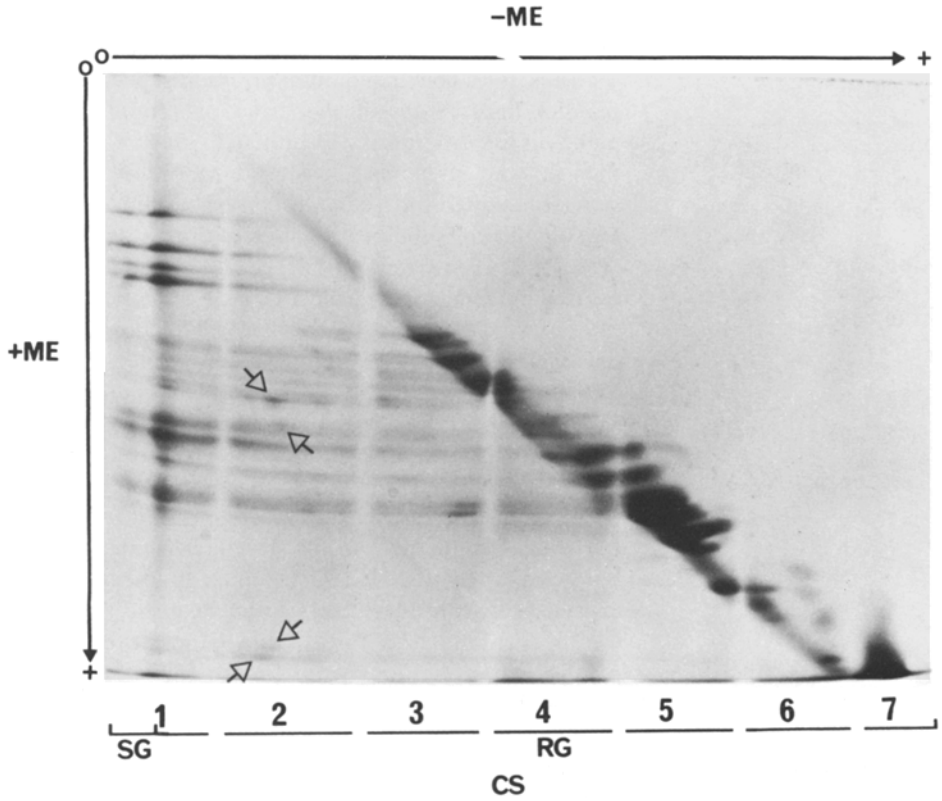

Fig. 9. 2-D SDS-PAGE patterns of 'Chinese Spring' (CS) – a disc gel, after electrophoresing unreduced proteins in it, was cut into seven pieces and each was equilibrated separately with ME before running in the second dimension. *1–7* refer to the sequential pieces of the cut disc gel. SG = stacking gel portion of the disc gel; RG = running gel portion. The four *arrows*, ordered from top to bottom, indicate the positions of the D, A, α and δ subunits of triplet bands, respectively

A. Bietz, U.S.D.A., Peoria, Illinois, kindly donated samples of gliadin and glutenin which had been prepared by sequential extraction of flour with 0.04 M NaCl, 70% ethanol, and 0.7% acetic acid. The glutenins solubilized in acetic acid were finally precipitated by adding ethanol to 70% concentration and adjusting the pH to 6.6–8.0 in order to remove traces of gliadins not removed by the earlier treatments (see Bietz et al. 1975).

The gliadin and glutenin fractions were dissolved separately in 4% SDS at pH 6.8 and subjected to 2-D electrophoresis using both unreduced (Fig. 10 b, f) and reduced (Fig. 10 d, h) proteins in the second dimension of electrophoresis. The rods containing unreduced gliadins (Fig. 10 b) and unreduced glutenin (Fig. 10 f), both gave pronounced streaking along the 45° line in the second dimension of separation, but whereas there were many darkly stained protein spots on the gliadin diagonal line there were only a few lightly stained spots on the glutenin diagonal. After reduction, the streaky portion of the diagonal line almost disappeared in both samples. The reduced gliadin sample (Fig. 10 d) contained a trace of HMW components, a few LMW subunits appearing as parallel lines, and many dense spots on the diagonal, which had mobilities identical to the α, β, γ and ω-gliadins (compare Fig. 10 c and d). In contrast, the glutenin sample contained the full spectrum of HMW and LMW subunits (Fig. 10 h). The LMW subunits of glutenin had similar electrophoretic mobilities to those from the gliadin sample, except one of the prominent LMW subunits in the glutenin appeared to be absent from the reduced gliadin sample. Three prominent spots in the region of ω-gliadins remained on the diagonal line of the reduced glutenin sample, and these showed no indication of being involved in aggregation (Fig. 10 h). Finally, it should be noted that there was no indication of triplet bands in either of these gliadin and glutenin samples prepared by sequential extraction of flour with NaCl, ethanol and acetic acid.

These results indicate that the fractions obtained by differential solubility are not pure, since there is some overlap of components in each fraction as observed in gel filtration studies (Payne and Corfield 1979; Bietz

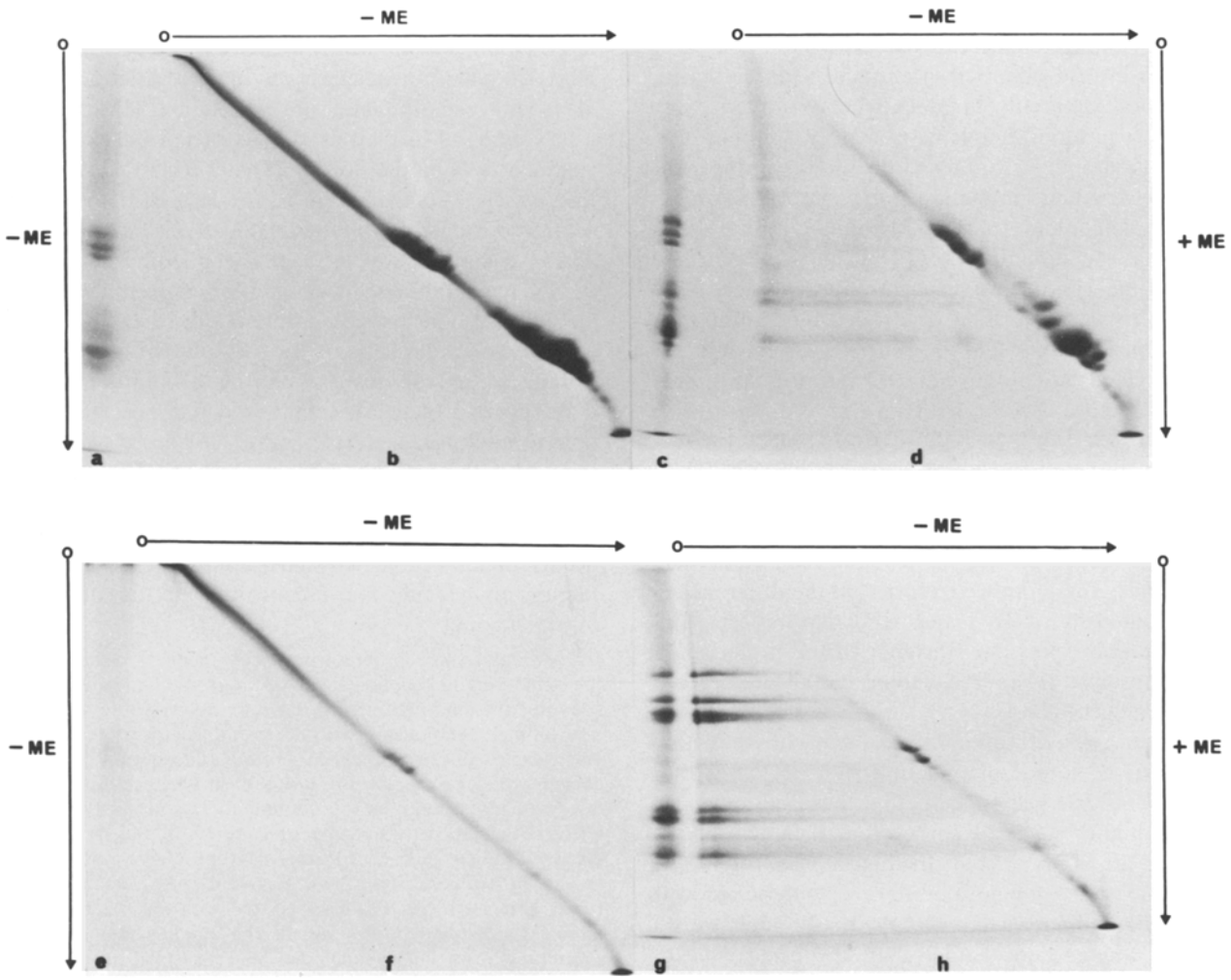

Fig. 10. SDS-PAGE patterns of gliadin and glutenin samples. *a–d* gliadin: *a* 1-D separation of unreduced proteins (–ME) included for reference; *b* 2-D separation (–ME × –ME); *c* 1-D separation of reduced proteins (+ME) for reference; *d* 2-D separation (–ME × +ME); *e–h* glutenin: *e* 1-D separation of unreduced proteins (–ME) for reference; *f* 2-D separation (–ME × –ME); *g* 1-D separation of reduced proteins (+ME) for reference; *h* 2-D separation (–ME × +ME)

and Wall 1980). However, the present approach is much simpler to apply and moreover the overlap of components can be visualized in the gels.

4 Discussion

The electrophoretic mobility of the triplet bands is different from that of the other known wheat endosperm proteins and therefore we consider them to be a new class of endosperm proteins. In this study we have attempted to characterize these bands further by determining their structure and genetic control and their relationship to the other wheat endosperm proteins.

Their behaviour when reduced with ME indicates that they are composed of subunits held together by intermolecular disulphide bonds just as proposed for the glutenin protein complex (see review by Lasztity 1984). However, in contrast to the models showing more or less random associations of many different subunits to form native glutenin, the triplet bands have been shown to have a tetrameric structure, composed of two large (A = 52,000 MW, D = 58,000) and two small (α = 23,000, δ = 22,000) subunits. The simplest model consistent with the available evidence depicts the large and small subunits associating by disulphide linkage into Aα and Dδ subunit pairs and these combining in all possible pairwise combinations, and again by disulphide linkage, to give tetramers DδDδ, DδAα, and AαAα which correspond to the triplet bands Tri-1, Tri-2 and Tri-3, respectively. Since there is no evidence of α subunits in Tri-1 nor δ subunits in Tri-3 (see Fig. 6), it is concluded that subunit pairs Aδ and Dα

are not involved in the triplet formation. The much stronger staining intensity of the Tri-1 band compared to the Tri-3 band observed in euploid 'Chinese Spring' also occurs commonly in other wheat cultivars, even when the equivalent bands have slightly different electrophoretic mobilities to those in 'Chinese Spring'. The observed variations in staining intensity of the triplet bands in aneuploid stocks of 'Chinese Spring', suggested that the subunits (D, δ) controlled by genes on chromosome 1D are produced in approximately twice the quantity of those (A, α) controlled by chromosome 1A. One possibility is that the genes for triplet subunits are duplicated on chromosome 1D but present in only one dose on chromosome 1A. However, if duplicate loci are present on 1D, then following mutation we would expect to find more complex patterns than just three bands, where two different alleles are present at the duplicate loci. In the cultivar survey allelic variants of Tri-1 were observed but no cultivar had more than three bands. Therefore we believe that the difference in staining intensity of Tri-1 and Tri-3 may reflect a difference in the expression efficiency of the triplet genes on chromosomes 1D and 1A, rather than the occurrence of duplicate genes on 1D.

The presence of continuous bands with the same mobility as all four triplet subunit spots in the second dimension of 2-D gels suggests that these subunits also occur in higher states of aggregation than tetrameric. However, no bands or spots having the electrophoretic mobility expected for hexameric or octomeric associations of the subunits were observed. Although we do not have any evidence other than the occurrence of these continuous bands, it is suspected that the subunits of the triplet band interact with the subunits of glutenin to give a large number of higher order protein aggregates covering a wide range of sizes. If this is correct, the triplet proteins might also influence the functional properties of dough.

The genes controlling the large and small subunits of the triplet bands have been located on chromosome arms 1AS (A and α) and 1DS (D and δ), each of which also carries a tightly linked complex of genes controlling gliadins and LMW glutenin subunits (Payne et al. 1984; Singh and Shepherd 1984). Recently we have used wheats showing allelic variation for triplet bands and ω-gliadins as parents, to map the genes controlling the large subunits of the triplet bands. These genes showed $40.1 \pm 2.9\%$ and $36.5 \pm 3.6\%$ recombination with the ω-gliadin genes present on chromosome arms 1AS and 1DS, respectively, and were closely linked ($11.0 \pm 1.8\%$ and $10.1 \pm 2.2\%$) with their respective centromeres (Singh and Shepherd 1984). However, except for their chromosome arm location, we do not have any precise information on the location of the genes controlling the small subunits of the triplet bands.

Although the genes on 1AS and 1DS controlling the large subunits of the triplet bands are clearly homoeoallelic, we have not detected any homoeoallele on 1BS. However, recently Galili and Feldman (1984) detected a protein band in total protein extracts from the endosperm of a 'Chinese Spring' (Thatcher 1B) substitution line, which is controlled by a gene located between the centromere and the ω-gliadin locus on 1BS, showing 25.5% recombination with the ω-gliadin genes and 23.5% recombination with genes controlling HMW glutenin subunits on 1BL. Although this location is not directly comparable with the position of genes controlling triplet subunits on 1AS and 1DS, its intermediate position suggested that it could possibly be a homoeoallele of the triplet genes. We have examined the 2-D pattern of this 'Chinese Spring' (Thatcher) 1B substitution line and while it is clear that the protein band detected by Galili and Feldman (1984) normally occurs in an aggregated state until reduced by ME, there is no evidence that it contributes to the formation of triplet bands.

Although some progress has been made in determining the structure and genetic control of the triplet bands and their subunits, the data presented so far provide very little information on their relationship with other endosperm proteins and, particularly, their significance in the endosperm. Thus, in current work, we are seeking to establish whether they are in fact storage proteins and to define their solubility characteristics. Very recently we separated protein bodies by sucrose density gradient ultracentrifugation from 21-day old endosperms of 'Chinese Spring' wheat and it was found that the triplet bands are specifically associated with the fraction containing the protein bodies, as are the gliadins and glutenin subunits. Thus we have concluded that the triplet proteins are also part of the storage protein complex of wheat (Singh and Shepherd, unpublished).

In solubility tests it was found that the triplet proteins are not present in distilled water, 0.04 M salt solution, 70% aqueous ethanol or 0.1 M acetic acid extracts from wheat endosperm at room temperature. These proteins were recovered only in the presence of strong dissociating agents such as SDS and to a much lesser extent 6M urea (Singh and Shepherd, unpublished). Although these proteins could be extracted from washed gluten balls, they were not present in either the glutenin or the gliadin fractions prepared by the differential solubility and precipitation methods (see section *3.7*). To account for the difficulties in extracting triplet proteins it is postulated that they occur in the wheat endosperm as high molecular weight aggregates in the glutenin complex, held together by hydrophobic or hydrogen bonds, so that they are not extracted in the normal Osborne (1907) fractions. Strong dissociating agents such as SDS are thought to disrupt this hydrophobic or hydrogen bonding and thus release the triplet proteins for extraction. The solubility of the triplet proteins when present in free state, is likely to be quite different from that of the postulated aggregates, but we do not have any information on this.

Thus on solubility criteria the triplet proteins do not correspond to either glutelins or prolamins. Instead, because of a striking common feature in the subunit structure of the triplet proteins and that of the subunit pairs making up the globulin storage proteins of several plant species, we need to consider

whether the triplet proteins could be globulin storage proteins. It has been shown that the subunit pairs of the 11S globulins of *Glycine max* (Moreira et al. 1979), *Pisum sativum* (Gatehouse et al. 1980), *Vicia faba* (Matta et al. 1981) and *Avena sativa* (Peterson 1978) each consist of a large and a small subunit joined by disulphide linkage. The size of these subunits is variable but of the same order as the large and small subunits of the triplet bands. It has been shown that there is much amino acid sequence homology between the small subunits present in such diverse plant species as legumes (Casey et al. 1981) and oats (Walburg and Larkins 1983), and the latter authors suggest that the genes controlling the small subunits of oat and legume globulins may have been derived from the same ancestral gene. Clearly additional work needs to be done to characterize more fully the triplet proteins of wheat. Determination of their amino acid content, and particularly their amino acid sequence, would provide the most definitive evidence for deciding whether they also might be globulin storage proteins.

Our 2-D method has demonstrated that the streak observed when total protein extracts from wheat endosperm are separated by SDS-PAGE, is due to the presence of a complex mixture of disulphide-linked aggregates of glutenin and triplet protein subunits. Further, the method has provided evidence, in the form of well defined continuous bands when reduced samples are separated in the second dimension, that a given glutenin or triplet subunit is present in a wide range of different-sized aggregates. Our results support the earlier conclusions of Payne and Corfield (1979) and Bietz and Wall (1980) that the HMW and LMW subunits of glutenin combine at random to give the continuous array of different-sized aggregates which make up glutenin. As an extension of this model, it is postulated that LMW glutenin molecules result when, by chance, only LMW subunits are included in an aggregate, whereas HMW glutenins include both types of subunits.

In general, our results parallel those obtained with combined gel filtration and electrophoresis of subfractions (Crow and Rothfus 1968; Danno et al. 1974; Huebner and Wall 1976; Payne and Corfield 1979; Bottomley et al. 1982). However, the 2-D approach has the clear advantage that it can be applied simply and quickly to protein extracts from single kernels. The disadvantage is that this analysis is restricted to only those aggregates, up to a molecular weight of approximately one million, which will enter a 10% acrylamide gel, and since glutenin is thought to contain aggregates with a molecular weight of several millions, a large proportion will be excluded from the 2-D separation. However, this is not a problem if these higher molecular weight aggregates of glutenin are composed of the same subunits as those which enter the 10% acrylamide gels, as seems likely from the results of gel filtration studies.

This 2-D method has proven particularly useful for separating the LMW subunits of glutenin from classical gliadins, which have similar electrophoretic mobilities in the gels. Recently we have developed a two-step 1-D procedure for separating LMW glutenin subunits by first separating unreduced protein extracts by normal 1-D SDS-PAGE in slab gels, and then cutting a 1 cm strip from the cathodal end of the separating gel. After reduction of the proteins, this strip is then loaded onto another slab gel and reelectrophoresed. This procedure allows 20 samples to be analysed per gel and it has greatly facilitated the analysis of large numbers of progeny for mapping the genes controlling LMW glutenin subunits (Singh and Shepherd 1984).

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