Acid-Polyacrylamide Gel Electrophoresis of Wheat Glutenins: A New Tool for the Separation of High and Low Molecular Weight Subunits[1]

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ABSTRACT

After sequential extraction of gliadins with 50% isopropanol, glutenins were extracted with the same solvent containing dithioerythritol and then alkylated with 4-vinylpyridine. Glutenin subunits were then resolved in a native polyacrylamide gel electrophoresis system buffered by acetic acid (acid-PAGE), which contained 2*M* urea to maintain protein solubility during migration. By two-dimensional electrophoresis (acid-PAGE × sodium dodecyl sulfate [SDS] PAGE), high molecular weight glutenin subunits (HMW-GS) were identified at the top of the acid-PAGE pattern in contrast to low molecular weight glutenin subunits (LMW-GS) that moved further. Twenty cultivars of known HMW-GS compositions in SDS-PAGE were analyzed by acid-PAGE. Most HMW-GS were well resolved; however, bands 2* and 7 tended to comigrate, whereas subunits 7 and 8 presented slight mobility variations in different cultivars. LMW-GS were also accurately resolved using acid-PAGE. In a set of intervarietal substitution lines of the cultivar Courtot, involving five donor cultivars, we identified three LMW-GS patterns controlled by *Glu-3A,* five by *Glu-3B,* and three by *Glu-3D.* Acid-PAGE bands characteristic of each *Glu-3* loci allowed description of allelic variations of the donor cultivars, in contrast to those of the SDS-PAGE patterns, in which subunits controlled by *Glu-B3* and *Glu-D3* loci were frequently superimposed, making determination of the corresponding alleles difficult. The use of acid-PAGE to describe allelic variation among bread wheat cultivars at the three *Glu-3* loci as an alternative to SDS-PAGE was discussed.

Wheat cultivars display considerable differences in breadmaking potential. This is largely due to variation in the composition of endosperm proteins. Pioneering work done by Payne et al (1981) demonstrated a clear relationship between sodium dodecyl sulfate (SDS) sedimentation volume and high molecular weight glutenin subunit (HMW-GS) composition. They identified markers of baking quality that could be used as selection criteria in breeding programs. These results were confirmed and extended to other quality tests using other genotypes in several different countries (Payne et al 1988, Lukow et al 1989, Rogers et al 1989). However, several recent studies (Gazanhes et al 1991, Gupta et al 1991, Khelifi and Branlard 1992) have indicated that a more effective predictive model of dough properties should include the composition of both the low and high molecular weight subunits of glutenin. Moreover, in both Australian and French wheat sets, it was reported that low molecular weight glutenin subunit (LMW-GS) composition correlated even more strongly to maximum dough resistance (Gupta et al 1991) or dough extensibility (Gazanhes et al 1991) than did HMW-GS. Screening for LMW-GS has been restricted because they do not fractionate diversely in SDS-PAGE, and because they have mobilities similar to those of some gliadins. To overcome this problem, and to allow routine analysis of glutenin subunits, several procedures were devised during the last five years. Gupta and Shepherd (1987), Singh and Shepherd (1988), and Gupta and Shepherd (1990) reported a two-step, one-dimensional (1-D) SDS-PAGE procedure that allowed investigation of the relationship between LMW-GS composition and quality. However, it required considerable skill and was time consuming. A rapid, one-step, 1-D SDS-PAGE separation of glutenin subunits after removal of monomeric proteins using Me_2SO and ethanol was also proposed by Gupta and MacRitchie (1991). Based on the sequential extraction method of Marchylo et al (1989), a simplified, 1-D SDS-PAGE was investigated by Singh et al (1991). It was also applied to subunits of wheat and rye by Zhen and Mares (1992). Finally, Branlard et al (1992) proposed a two-step method in which aggregated proteins extracted by 2-chloroethanol were separated from gliadins and albumins at the top of an acid polyacrylamide gel electrophoresis system (acid-PAGE), then reduced with β-mercaptoethanol and subjected to SDS-PAGE.

In general, these procedures allow good resolution of LMW-GS and HMW-GS without significant contamination by gliadins. However, even with high-resolution SDS-PAGE gradient gels, Singh et al (1991) noted that screening for LMW-GS composition and complete description of LMW-GS alleles still proved difficult. The need for a simple method to describe the polymorphism of LMW-GS has led us to develop another electrophoretic system, one that is complementary to SDS-PAGE (Singh et al 1991) or isoelectrofocusing (IEF) (Morel and Autran 1990). In this article, we report a new approach for the assessment of variation in LMW-GS based on the separation of alkylated subunits in an acid-PAGE system.

MATERIALS AND METHODS

Wheat Samples

Intervarietal group I substitution lines of Courtot were produced by M. Bernard, G. Branlard, and M. Rousset in the Station d'Amélioration des Plantes-INRA (Clermont-Ferrand, France), who also provided grains of wheat cultivars from a world collection. Gabo 1B/1R translocations and Gabo 1B/1R, 1D/1R translocations were provided by R. B. Gupta (CSIRO, Australia).

Extraction of Proteins

Glutenin purification and solubilization were achieved according to Singh et al (1991). Before glutenin solubilization, flour (20 mg) was extracted three times with 50% (v/v) propan-2-ol at 60°C for 30 min with agitation every 5 min. Glutenin was then solubilized with 50% (v/v) propan-2-ol, 0.08*M* Tris-HCl (pH 8.5), 20 m*M* dithiothreitol at 60°C for 30 min. The supernatant was diluted with 1 volume of 50% (v/v) propan-2-ol, 0.08*M* Tris-HCl (pH 8.5), 40 m*M* 4-vinylpyridine, and incubated for 3 hr at 60°C. Glutenin was precipitated from 50 μl of this reaction mixture with 200 μl of acetone; the dried pellet was solubilized in 25 μl of 30% glycerol, 6*M* urea, and 25 m*M* acetic acid. Finally, 7-μl samples were loaded into the slots of the acid-PAGE gel.

Acid-PAGE

Composition of gels and electrode buffers were as described by Clements (1988). Polyacrylamide gels (12% acrylamide-0.375%

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bis-acrylamide) contained 2*M* urea, 0.1% ascorbic acid, 0.0014% ferrous sulfate 7 H_2O, and 0.75% glacial acid acetic, pH 3.1. The gels (160 × 180 × 1.5 mm) were cast one day before use and stored at ambient temperature. To cast one gel, 40 ml of the gel solution was deaerated for 5 min under vacuum at ambient temperature, and then 55 μl of 0.6% H_2O_2 (v/v) catalyst was added. Electrophoresis was performed for 3 hr 45 min at 500 volts at 18°C. The gels were stained in 12.5% trichloroacetic acid with 0.14% (w/v) Coomassie Brilliant Blue R250.

1-D SDS-PAGE

The Laemmli buffer system (1970) was used with 10.3% acrylamide gels. Gels (160 × 180 × 0.75 mm) were run at 40 mA per gel for 4 hr 30 min at 18°C. The alkylated glutenin extract (see above) was mixed with an equal volume of a solution containing 2% SDS and 40% glycerol; 20 μl of each sample was loaded. After electrophoresis, the gels were incubated in 15% trichloroacetic acid overnight, rinsed with tap water for 5 min, and then stained as described for acid-PAGE gels.

2-D Electrophoresis (Acid-PAGE × SDS-PAGE)

After the first acid-PAGE migration, the gel was sliced into single tracks (8 × 160 mm) and incubated for 30 min in 62.5 m*M* Tris-HCl buffer (pH 6.8) containing 2% SDS and 40% glycerol. Each track was then loaded onto an SDS-PAGE gel for the second dimension.

RESULTS AND DISCUSSION

Typical results obtained by acid-PAGE fractionation of reduced and alkylated glutenin extracts are presented in Figure 1. Such glutenin extracts yielded well-resolved patterns in a conventional PAGE system in the absence of SDS. To the best of our knowledge, apart from one report of lactate-urea PAGE fractionation of some purified HMW-GS by Shewry et al (1984), this has not been previously reported. The acid-PAGE system is therefore a possible alternative to SDS-PAGE for investigating glutenin subunit composition.

The survey of wheat cultivars revealed extensive variation in both the acid-PAGE (Fig. 1) and the SDS-PAGE patterns (Fig. 2) of slow and fast moving bands. Acid-PAGE patterns of glutenin subunits generally contained more bands than the did the SDS-PAGE patterns, but they were less clearly separated.

Respective Locations of HMW-GS and LMW-GS

On the basis of acid-PAGE, glutenin subunits were assigned to three groups: I, II, and III (Fig. 1). Figure 3 shows the 2-D analyses (acid-PAGE × SDS-PAGE), which were performed to locate the respective positions of LMW-GS and HMW-GS in the acid-PAGE patterns and to assign individual subunits between the two 1-D systems. Most of the protein spots fell on a diagonal line, suggesting that the molecular size parameter could also influence the separation of proteins in the acid-PAGE system. For example, the HMW-GS were the slowest in the two electrophoretic systems. While it is well known from nonequilibrium pH gradient electrophoresis (NEPHGE) × SDS-PAGE analysis that HMW-GS may occur as charge density variants (Shewry et al 1984), these were not detected in the acid-PAGE × SDS-PAGE patterns.

Assignment of HMW-GS Identified in SDS-PAGE × Acid-PAGE Bands

Because the HMW-GS of all the wheat cultivars examined in this study had been previously identified by SDS-PAGE and numbered according to Payne and Lawrence (1983), an initial comparison of the two 1-D systems was possible within the HMW-GS group. For example, the slowest and the fastest bands were assigned to subunits 5 and 10, respectively (Glenlea and Rescue), or to subunits 2 and 12 (Créneau and Chinese Spring). Interestingly, whereas the mobility of subunit 10 is lower than that of subunit 12 in SDS-PAGE without urea (Shewry et al 1992), in the acid-PAGE system, the relative mobilities were in line with the true molecular weights of subunits 10 and 12. Similarly, HMW-GS 1 and 2* (controlled by *Glu-A1* locus) migrated much faster than did subunits 2 and 5 in acid-PAGE × SDS-PAGE. (The corresponding protein spots are clearly above the diagonal line in 2-D patterns). For example, in the patterns of Créneau and Rescler, subunit 1 was between subunits 5 and 7. In the acid-PAGE system, the mobility of proteins reflects both the molecular size and net charge, the latter being determined by the content

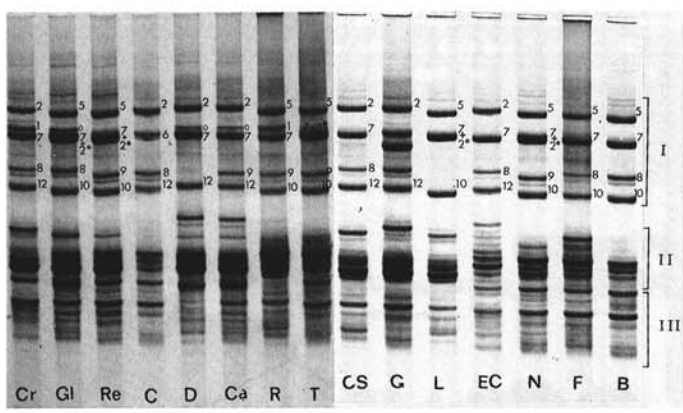

Fig. 1. Acid-polyacrylamide gel electrophoresis patterns of reduced and alkylated glutenin subunits from bread wheat cultivars. Glutenin subunits are assigned to three groups (I, II, and III) and high molecular weight glutenin subunits are labeled according to the nomenclature of Payne and Lawrence (1983). Cultivar abbreviations are: Cr, Créneau; Gl, Glenlea; Re, Rescue; C, Clément; D, Darius; Ca, Capitole; R, Rescler; T, Thatcher; CS, Chinese Spring; G, Gabo; L, Lobo; EC, Etoile de Choisy; N, Neepawa; F, Flambard; B, Bosco.

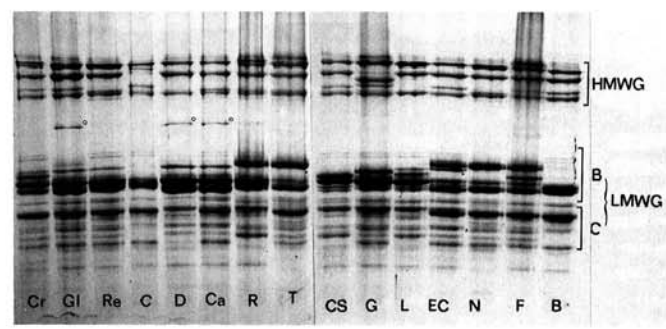

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of reduced and alkylated glutenin subunits from bread wheat cultivars. A possible ω-gliadin component is marked by an open circle. HMWG and LMWG = high and low molecular weight glutenin subunits, respectively. Cultivar abbreviations as in Fig. 1.

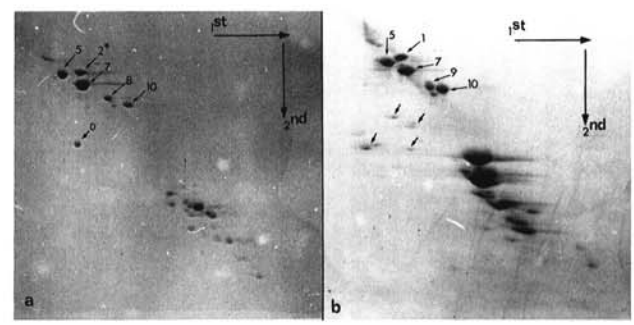

Fig. 3. Two-dimensional (acid-polyacrylamide gel electrophoresis × sodium dodecyl sulfate-polyacrylamide gel electrophoresis) patterns of reduced and alkylated glutenin subunits from bread wheat cultivars Glenlea (**a**) and Rescler (**b**). Numbers on the high molecular weight subunits are the numerical nomenclature of Payne and Lawrence (1983). The contaminating gliadins in **b** are marked by arrows. A possible ω-gliadin component is marked by an open circle.

of basic amino acids and cysteines (converted to a *S*-β-pyridylethyl-cysteine derivative by alkylation). The fact that subunits 1 and 2* contain twice as much arginine as do subunits 2 and 5, which have similar cysteine and lysine contents (Shewry et al 1984), may explain the shift in mobility observed in acid-PAGE.

Subunit 2* comigrated with subunit 7, giving a more intensely stained band in the Glenlea, Rescue, Thatcher, Neepawa, and Lobo cultivars. However, there are some cultivars (e.g., Darius) that lacked subunit 2* but nevertheless still had a strong band 7. The existence of such variation in the intensity of the band of HMW-GS 7 has been already reported by Pogna et al (1989) and Ng et al (1989) as being related to dough strength. Some variations in the migration of particular subunits were also observed. For example, a slight shift in the position of subunit 7 was noted in the patterns of cultivars with allele 7 (Lobo) and with allele 7+8 (Etoile de Choisy). The same phenomenon was observed for subunit 8, depending on whether the allele 6+8 (Clement) or 7+8 (Glenlea) was present. Such variation in the mobility exhibited by subunits 7 and 8, might be relevant to the differences in DNA sequences observed by Anderson and Greene (1989) for subunit 7 and to the qualitative and quantitative differences in subunits 7 and 8 reported by Marchylo et al (1992). In such cases, it seems, therefore, that acid-PAGE has the potential to describe allelic variation of HMW-GS bands as accurately as SDS-PAGE.

Several faint bands (Fig. 3b, arrows) were also observed in the slow-moving area I of acid-PAGE patterns. In addition, a sharp band that could not be assigned to any previously characterized HMW-GS was found in area I of three cultivars (Glenlea, Capitol, and Darius; open circle in Figs. 2 and 3a). It is possible that these bands correspond to residual ω-gliadin components, because they migrate between HMW-GS and LMW-GS in SDS-PAGE and among HMW-GS in acid-PAGE. Indeed, most ω-gliadin components comigrated with HMW-GS in this acid-PAGE system (results not shown).

Potential of Acid-PAGE for Investigating LMW-GS

As suggested by the 2-D analysis (Fig. 3), bands assigned to groups II and III in Figure 1 were LMW-GS. Spot distribution tended to follow the diagonal line of the gel, suggesting that either the charge density of most glutenin subunits is proportional to molecular size, or that the sieving effect of the gel matrix determines migration velocity. Whatever the case, some spots are seen on the same horizontal or vertical alignments of the 2-D patterns, their superimposition leading to the occurence of apparent single bands in SDS-PAGE or acid-PAGE. Depending on the LMW-GS combination at these bands, the identification of patterns controlled by the three *Glu-3* loci will be more, or less, difficult, depending on electrophoretic system used.

Intervarietal group I substitution lines of Courtot were analyzed in SDS-PAGE and acid-PAGE to determine the bands controlled by the *1A*, *1B*, and *1D* chromosomes. Pattern differences between Courtot and Courtot-Vilmorin 23 or Courtot-Magnif 27 *1A* substitution lines were not very obvious in acid-PAGE (slots 1, 2, and 3, Fig. 4a); SDS-PAGE banding patterns showed a band missing here, but one was present in Courtot (band marked by arrow in slot 1 and star in slots 2 and 3, Fig. 4b). For the Courtot-Prinqual *1A* substitution line, apart from lacking a band present in Courtot (marked by arrow in slot 1 and ☆ in slot 4, Fig. 4a,b), the bands controlled by the chromosome *1A* of Prinqual were more, or less, obvious, depending on the electrophoretic system used. In acid-PAGE, an extra band was present in an area previously blank for Courtot, while in SDS-PAGE, the band most probably corresponding to it was present in a region that already contained several close bands (arrowhead in slot 4, Fig. 4a,b). Substitution of chromosome *1A* of Courtot by those of Magdalena was easily detected in the two systems (band marked by arrowhead in slot 5, Fig. 4a,b). In SDS-PAGE, two extra thin bands among C subunits were also detected. Chromosome *1A* of the Gabo *1B/1R, 1D/1R* translocation line controlled a main band, whose mobility was clearly distinct from the main ones controlled by chromosomes *1A* of Courtot, Magdalena, or Prinqual in both SDS-PAGE or acid-PAGE (bands marked by arrowhead in slots 1, 4, 5, and 6, Fig. 4a,b). In the two electrophoretic systems, the main changes caused by chromosome *1A* substitution usually affected the position of the slowest moving LMW-GS.

Compared to that of Courtot, the acid-PAGE banding pattern of the Courtot-Magdalena *1B* substitution line revealed a small shift (a reproducible occurrence) of one band that seemed to be already present in Courtot (arrow in slots 1 and 2, Fig. 5a). One other band become fainter (star in slot 2, Fig. 5a). In SDS-PAGE, the banding pattern of the Courtot-Magdalena *1B* substitution line lacked at least three bands (arrowhead in slot 1 and star in slot 2, Fig. 5b). One of these bands had previously been shown to be controlled by chromosome *1A* of Courtot (arrow in slot 1, Fig. 4b), and we presumed that two different subunits, controlled by *1A* and *1B* chromosomes, respectively, could have overlapped here. The banding pattern of the Courtot-Prinqual *1B* substitution line was clearly distinct from that of Courtot in acid-PAGE. It showed two additional bands (marked by arrows in slot 3, Fig. 5a). In SDS-PAGE, as for the Courtot-Magdalena *1B* substitution line, the slowest mobility band among the B subunits of Courtot was lacking (star in slot 3, Fig. 5b). Moreover, one extra band was present among the B subunits, while the banding pattern of the C subunits changed with the appearance of a thick band (arrows in slot 3, Fig. 5b). SDS-PAGE patterns of Courtot-Magdalena *1B* and Courtot-Cappelle *1B* substitution lines were similar except for the presence of an extra band among the B subunits that had already been seen in the Courtot-Prinqual *1B* substitution line (band marked by arrowhead in slot 3 and

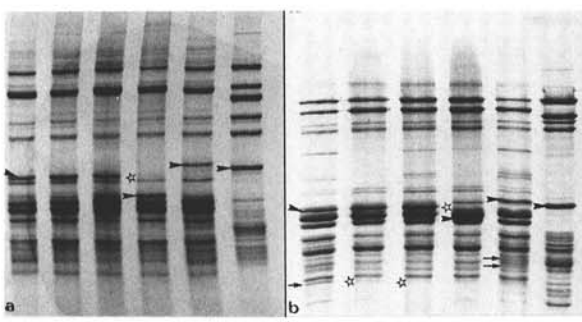

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**a**) and acid-polyacrylamide gel electrophoresis (**b**) of reduced and alkylated glutenin subunits from some intervarietal *1A* chromosome substitution lines of Courtot and from Gabo *1B/1R, 1D/1R* translocation line. Cultivars for lanes from left to right are: Courtot, Courtot-Vilmorin 23 *1A*, Courtot-Magnif 27 *1A*, Courtot-Prinqual *1A*, Courtot-Magdalena *1A*, Gabo *1B/1R 1D/1R*. Bands marked by arrows and stars are explained in text.

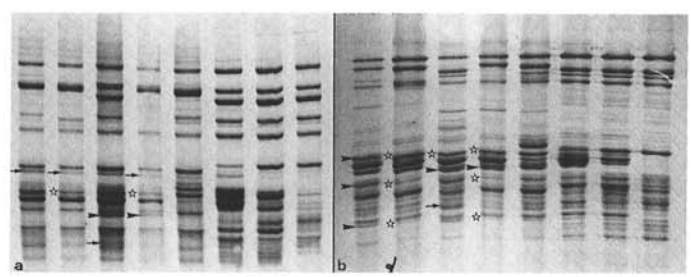

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**a**) and acid-polyacrylamide gel electrophoresis (**b**) of reduced and alkylated glutenin subunits from some intervarietal *1B* chromosome substitution lines of Courtot and from Gabo and some Gabo translocation lines. Cultivars for lanes from left to the right are: Courtot, Courtot-Magdalena *1B*, Courtot-Prinqual *1B*, Courtot-Cappelle *1B*, Courtot-Magnif 27 *1B*, Gabo, Gabo *1B/1R*, Gabo *1B/1R, 1D/1R*. Bands marked by arrows and stars are explained in text.

4, Fig. 5b). This band is likely to be the one that is common in the Courtot-Cappelle *1B* and Courtot-Prinqual *1B* substitution lines in acid-PAGE (arrowhead in slots 3 and 4, Fig. 5a). In SDS-PAGE, differences between the Courtot-Cappelle *1*B and Courtot-Prinqual *1B* substitution lines arose from the banding patterns of the C subunits. In acid-PAGE, the two patterns were distinguished by the mobility shift of one band (arrow in slot 4, Fig. 5a). The banding pattern of the Courtot-Magnif 27 *1B* substitution line was obviously different from that of Courtot or other *1B* substitution lines previously examined using the two electrophoretic systems (slot 5, Fig. 5a,b). From these results, it appeared that either SDS-PAGE or acid-PAGE allowed the different chromosome *1B* controlled patterns examined here to be identified.

Figure 6a,b shows SDS-PAGE and acid-PAGE banding patterns of the donor cultivars from which the Courtot *1A* or *1B* chromosome substitution lines were developed. Some main subunits controlled by chromosome *1D* were easily identified in acid-PAGE or SDS-PAGE (bands marked by arrowheads in Fig. 6a,b). In acid-PAGE, three distinct patterns controlled by chromosome *1D* could be identified, one for Courtot and Cappelle, another one for Magdalena and Prinqual, and a third one for Magnif 27. Vilmorin 23 might have the Courtot pattern for LMW-GS controlled by *Glu-D3* locus and an original pattern for subunits controlled by *Glu-B3* locus. The comparison of Gabo *1B/1R* and Gabo *1B/1R, 1D/1R* translocation line patterns led us to conclude that subunits controlled by chromosome *1D* occurred among the fast-moving B subunits and among the C subunits in SDS-PAGE (slots 7 and 8, Fig. 5b). This situation complicates the interpretation of SDS-PAGE patterns of unknown cultivars. The accurate determination of *1B*-controlled banding patterns could be very difficult depending on the presence of overlapping LMW-GS bands from chromosome *1D* or even from chromosome *1A,* among the fast moving B subunits. Thus, in SDS-PAGE, differences between Magdalena and Cappelle banding patterns controlled by *Glu-B3* locus are more difficult to detect than those between Courtot-Magdalena and Courtot-Cappelle *1B*-substitution lines patterns, which have the same Courtot background pattern. This problem was not encountered with the acid-PAGE method, because identification of allelic variations of *Glu-B3* locus was not obscured by either of the banding patterns controlled by *Glu-A3* and *Glu-D3* loci.

CONCLUSION

The resolution provided by the two electrophoretic systems, SDS-PAGE and acid-PAGE, allows the identification of each allelic pattern of LMW-GS controlled by the group I chromosomes. Nevertheless, allele identification might remain difficult in SDS-PAGE because of the complexity of the banding pattern among the fast-moving B subunits. A careful analysis will be necessary to segregate the LMW-GS bands controlled by chromosomes *1B* and *1D* among these subunits to achieve allele identification. On other hand, simpler acid-PAGE patterns allow rapid identification of LMW-GS patterns controlled by chromosomes *1A* and *1D,* as well as those controlled by chromosome *1B,* even when the patterns are less sharp than those obtained from SDS-PAGE. Because of the complexity of LMW-GS fraction and the occurrence of overlapped bands, it will most probably be necessary to use 2-D electrophoresis to identify and then determine the genetic control of all individual polypeptides. For that purpose, acid-PAGE, which is a more familiar and more commonly used method than either isoelectric focusing (IEF) or NEPHGE, can be combined with SDS-PAGE. We expect that the 2-D patterns will be readily understandable, as we know the chromosomal control of the LMW-GS bands fractionated in the two systems taken separately, whereas little knowledge regarding the chromosomal control of LMW-GS separated by IEF or NEPHGE is available.

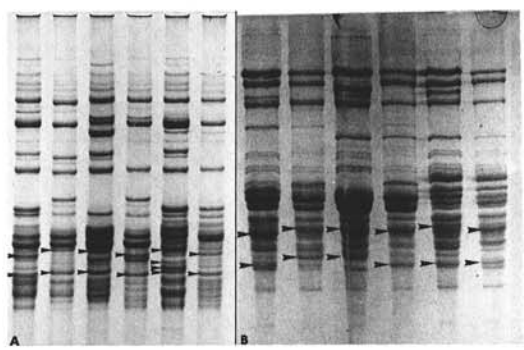

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**a**) and acid-polyacrylamide gel electrophoresis (**b**) of reduced and alkylated glutenin subunits from the previous donor cultivars. Cultivars in lanes from left to right: Courtot, Magdalena, Prinqual, Cappelle, Magnif 27, Vilmorin 23. Bands marked by arrows are explained in text.

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