Effect of Disulfide-Bond Cleavage on Wheat Gliadin Fractions Obtained by Gel Filtration[1]

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ABSTRACT

Cleaving the disulfide bonds of the two fractions obtained from classical wheat gliadin by gel-filtration chromatography provides further evidence that structure and properties of these proteins differ. The higher-molecular-weight (MW) fraction, present in small amount in classical gliadin, has properties of a low-MW glutenin and, therefore, is designated as such in this paper. The viscosity of low-MW glutenin decreases drastically upon disulfide cleavage; also its MW drops from about 125,000 to around 37,000. This decline indicates intermolecular disulfide bonds as in glutenin. Also, the starch-gel electrophoretic pattern of reduced and alkylated low-MW glutenin resembles that of glutenin more than it does that of gliadin. The predominant low-MW fraction (purified gliadin) shows no change in viscosity upon disulfide cleavage; however, it does drop appreciably in weight-average MW (27,000 to 22,000), and its molecular units become more asymmetric as indicated by an increase in frictional ratio. Thus the disulfide bonds of purified gliadin are mostly intramolecular and their cleavage allows the molecules to unfold. The starch-gel electrophoretic patterns of reduced and alkylated classical gliadin and of reduced and alkylated purified gliadin are quite similar.

Wheat gluten, the bulk of wheat flour protein, is insoluble in neutral saline but was fractionated by Osborne (1) into classical gliadin, which is soluble in neutral 70% ethanol, and glutenin, which does not dissolve in it. These two classes of proteins have quite different structures, as evidenced by contrasting changes induced in their physical properties during their reaction with such disulfide-cleaving agents as mercaptoethanol.

Glutenin is a series of proteins with molecular weights (MW's) ranging into the millions, and it has a high solution viscosity (2). Disulfide cleavage reduces the MW of glutenin to a fairly homogeneous 20,000 (2) and drastically reduces its solution viscosity (3,4), which indicates that the component polypeptide units of native glutenin are linked by intermolecular disulfide bonds.

In contrast, classical gliadin has a much lower viscosity than glutenin, and gliadin components have fairly low MW's (5,6). From this it was postulated that gliadin is a group of proteins that contain only intramolecular disulfide bonds (4,7). An experimental observation that did not fit this postulate was the small drop in the viscosity of gliadin solutions upon addition of a disulfide-cleaving reagent, such as mercaptoethanol. This small drop in viscosity observed by Pence and Olcott (3) and by Beckwith *et al.* (7), suggested that classical gliadin contained a significant amount of intermolecular disulfide bonds.

Classical gliadin was, therefore, subjected to gel-filtration chromatographic separations to see if it was heterogeneous in MW (8). These separa-

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tions showed that classical gliadin contained a relatively small amount of a high-MW fraction. The moving-boundary electrophoretic pattern of this fraction was quite similar to that of glutenin; however, it moved as a smear in starch-gel electrophoresis, whereas glutenin was immobile. The intrinsic viscosity of this fraction was between that of glutenin and classical gliadin, as was its MW. Also, differences were observed between the amino acid composition of this fraction and that of classical gliadin (8). The above results suggested that the high-MW fraction isolated from classical gliadin by gel filtration chromatography was actually a low-MW glutenin, and it will be referred to as such hereafter.

This paper describes viscosity studies on classical gliadin, low-MW glutenin, and purified gliadin (classical gliadin with low-MW glutenin removed) in the presence of mercaptoethanol, as well as studies of MW before and after disulfide-bond cleavage. These experiments demonstrate that low-MW glutenin contains intermolecular disulfide bonds as does glutenin, whereas most of the disulfide bonds in purified gliadin are intramolecular. Also described are starch-gel electrophoretic investigations on reduced-alkylated glutenin, classical gliadin, purified gliadin, and low-MW glutenin. The electrophoretic studies indicate that peptide units resulting from disulfide cleavage of glutenin and gliadin are different and that the peptide units of the low-MW glutenin fraction are similar to those of glutenin.

MATERIALS AND METHODS

Materials

Classical gliadin is defined as the fraction of wheat gluten soluble in 70% ethanol at neutral pH. Its preparation was described previously (8). The small amount of low-MW glutenin present in classical gliadin was isolated by gel filtration on Sephadex G-100 (8). The portion of gliadin remaining after removal of low-MW glutenin constitutes purified gliadin. Low-MW glutenin and purified gliadin correspond respectively to peaks I and III of Fig. 2 in reference 8.

Classical gliadin, purified gliadin, and low-MW glutenin were reduced and alkylated by the following modification of the procedure of Weil and Seibles (9). A protein sample, 0.1–0.15 g., was dissolved in 4 ml. of 8*M* urea-0.05*M* *tris* buffer, pH 8.0. Mercaptoethanol, 1*M*, was then added and the system was placed under a partial vacuum to reduce oxygen concentration and allowed to react for 4 hr. Acrylonitrile, 2*M*, was then added to alkylate sulfhydryl groups and allowed to react 45 min., after which the sample was dialyzed against 0.03*M* acetic acid overnight and then freeze-dried. Since the reduced and alkylated low-MW glutenin solution was turbid after dialysis against acetic acid, it was decided to reduce and alkylate all the samples again; this time the sulfhydryl reagent was 0.15*M* dithiothreitol (10) and the reaction time, 10 hr.; the other conditions remained the same. After the second treatment, the acetic acid solution of reduced and alkylated low-MW glutenin was clearer. Clarification may have resulted from the cleavage of "resistant" disulfide bonds or from deaggregation.

Aluminum lactate was purchased from K and K Laboratories, Inc.; starch for gel electrophoresis, from Connaught Medical Research Labora-

tories; Sephadex G-100 for gel filtration, from Pharmacia Fine Chemicals, Inc.; bovine plasma albumin from Armour; and ribonuclease from Sigma. Other chemicals were analytical grade from commercial sources.

Viscosity Studies

The solution viscosities of gluten, classical gliadin, purified gliadin, and the low-MW glutenin, as well as the corresponding reduced and alkylated proteins, were measured as a function of time in the presence of the disulfide-cleaving reagent, mercaptoethanol. The initial viscosity of a given protein solution at or near 4% concentration was measured; mercaptoethanol, usually to 0.285*M*, was added; then changes in viscosity were followed for about 250 hr. at 25°C. Control studies in sealed viscometers showed that exposure to air during the measurements had no effect; therefore, most of the experiments were run in open capillary viscometers (Cannon-Manning semimicro size 100) with 0.5-ml. solution samples. Results were expressed as plots of inherent viscosity ($\ln \eta_r/c$) *vs.* a scale linear with respect to the fourth root of time, the zero time point being the viscosity before addition of mercaptoethanol. Inherent viscosity was chosen because it is a close approximation of intrinsic viscosity, and the fourth root of time was used merely to expand the time scale during the first part of an experiment when rapid changes in viscosity are taking place and to compress the time scale when changes are quite slow.

Sedimentation Equilibrium

Measurements were made with a Beckman Model E ultracentrifuge in a solvent of 8*M* urea-0.1*M* formic acid in 30-mm. double-sector cells with 0.4-ml. samples at about 0.5% protein concentration. The rotor temperature was usually controlled at 25°C., and normally runs were at speeds of 12,590 r.p.m. The solvent deaggregated gliadin proteins a little better than the 6*M* guanidine hydrochloride-0.1*M* acetic acid used previously (8), but it is not certain if the proteins are completely deaggregated in a concentrated urea solution.

Weight average MW was derive dfrom plots of $\ln c_r$ *vs.* r^2, as described by Schachman (11); upward curvature, if any, at the bottom of the liquid column was ignored. The initial slope of plots of $(1/r)$ (dc/dr) *vs.* c_r was used to derive z-average MW (11), c_o being adjusted so that the plot would pass through the origin in cases where some protein was thrown from solution during the equilibrium run. Diffusion coefficients were determined by observing the rate at which the system approached equilibrium, according to the procedure of Van Holde as described by Sophianopoulos and co-workers (12).

Partial specific volumes were calculated from current amino acid analyses (8,13) by the procedure of Cohn and Edsall as described by McMeekin *et al.* (14) and are: 0.726 for classical gliadin, 0.722 for the low-MW glutenin, and 0.723 for purified gliadin in both intact and reduced-alkylated forms.

Gel Electrophoresis

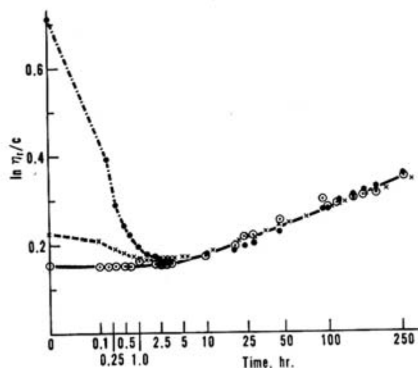
Electrophoretic analysis was carried out in horizontal starch gels in direct contact with the buffer-containing electrode compartment. The buffer was

3*M* urea-0.005*M* aluminum lactate-0.02*M* lactic acid. Details of the procedure were described previously (8).

RESULTS

Viscosity vs. Time Studies

Effects of 0.285*M* mercaptoethanol on the viscosity of low-MW glutenin, purified gliadin, and classical gliadin dissolved in 0.03*M* acetic acid are shown in Fig. 1. Viscosity of low-MW glutenin drops rapidly in a manner similar to that of glutenin (3,4). The best explanation for this rapid drop in viscosity is that low-MW glutenin contains intermolecular disulfide bonds. Large polymer size and high solution viscosity result from intermolecular disulfide bonds, and cleaving them by addition of mercaptoethanol gives much smaller molecular units and low solution viscosity. Purified gliadin shows no drop in viscosity upon addition of the disulfide-cleaving reagent, mercaptoethanol. Such behavior indicates that purified gliadin contains no intermolecular disulfide bonds. The small drop in the viscosity of classical gliadin upon addition of a disulfide-cleaving reagent, observed here and described earlier (3,4), must, therefore, be due to the presence of a high-MW material containing intermolecular disulfide bonds.



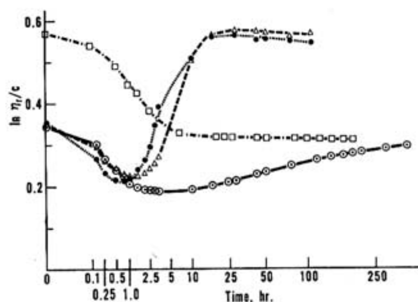


Fig. 1 (left). Inherent viscosity (ln η_r/c) *vs.* time for: low-MW glutenin (● —·—· ●), classical gliadin (x ––– x), and purified gliadin (⊙ ——— ⊙); all dissolved in 0.03*M* acetic acid-0.285*M* mercaptoethanol with mercaptoethanol added at zero time.

Fig. 2 (right). Inherent viscosity (ln η_r/c) *vs.* time for wheat gluten in: 70% ethanol-0.03*M* acetic acid-0.2*M* mercaptoethanol (Δ ––– Δ); 70% ethanol-0.03*M* acetic acid-0.4*M* mercaptoethanol (● ...... ●); 8*M* urea-0.1*M* formic acid 0.4*M* mercaptoethanol (□ —·— □); and 0.03*M* acetic acid-0.2*M* mercaptoethanol (⊙ ——— ⊙). Mercaptoethanol is added at zero time.

After the rapid decrease in viscosity, if any, is complete, a slow secondary increase takes place which is virtually identical for classical gliadin, purified gliadin, and low-MW glutenin. This secondary increase in viscosity was observed previously (3,7), and its rate and extent seem to be related to the solvent system. To illustrate this secondary increase, the viscosity of wheat gluten in several solvent systems containing mercaptoethanol is plotted in Fig. 2. Note that there is no rise in viscosity in the solvent system containing 8*M* urea. Thus it appears that the secondary increase in viscosity in

0.03*M* acetic acid and in 0.03*M* acetic acid-70% ethanol is due to aggregation (perhaps via hydrogen bonding) of unfolded peptide chains.

If the small rapid drop in the viscosity of classical gliadin and the large rapid drop in the viscosity of low-MW glutenin upon addition of mercaptoethanol are due to disulfide-bond cleavage, then it would follow that adding mercaptoethanol to reduced-alkylated classical gliadin and reduced-alkylated low-MW glutenin should produce no initial change in viscosity, although there may be a slow secondary change. Similarly, with purified gliadin there should be no initial change in viscosity, but there may be a secondary change. Experiments to check this prediction were run and results are shown in Fig. 3.

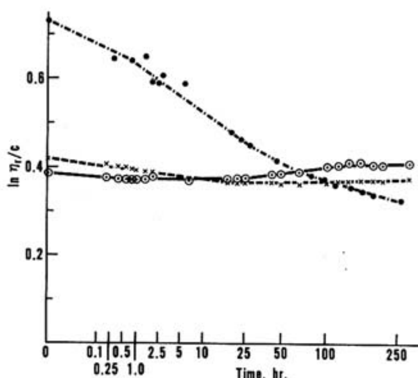


Fig. 3. Inherent viscosity (ln η_r/c) *vs.* time for: reduced-alkylated low-MW glutenin (● —·—· ●), reduced-alkylated classical gliadin (x ---x), and reduced-alkylated purified gliadin (⊙ ——— ⊙), all in 0.03*M* acetic acid-0.285*M* mercaptoethanol with mercaptoethanol added at zero time.

The viscosities of reduced-alkylated classical gliadin and reduced-alkylated purified gliadin show no drop upon addition of mercaptoethanol. The viscosities of these two protein solutions are rather constant for about 300 hr. in the presence of mercaptoethanol, and values are the same as the secondary maximum shown by intact classical gliadin and intact purified gliadin in the presence of mercaptoethanol (Fig. 1.). Since the last step in preparing reduced-alkylated classical gliadin and reduced-alkylated purified gliadin is freeze-drying from dilute acetic acid, it appears that these two proteins are in an aggregated state and remain in the same aggregated state throughout the viscosity *vs.* time experiments.

The initial viscosity of reduced-alkylated low-MW glutenin is rather high. The turbidity of the solution causes the scattered points in the first part of the curve (Fig. 3). After about 10 hr. the solution becomes clear, and viscosity decreases over a period of about 100 hr. to that of reduced-alkylated classical gliadin and reduced alkylated purified gliadin. This slow decrease in viscosity is attributed to low-MW glutenin's going from a highly aggregated state to a less aggregated state. The decrease in viscosity could also be due to cleavage of "resistant" disulfide bonds in low-MW glutenin, but this seems unlikely because the material was twice reduced and alkylated under con-

ditions which should result in complete reaction (see "Materials and Methods").

Sedimentation Equilibrium

The various parameters that can be established from sedimentation equilibrium measurements on low-MW glutenin, classical gliadin, and purified gliadin before and after disulfide-bond cleavage are listed in Table I.

TABLE I
MOLECULAR WEIGHT AND OTHER PROPERTIES OF LOW-MOLECULAR-WEIGHT GLUTENIN, CLASSICAL GLIADIN, AND PURIFIED GLIADIN BEFORE AND AFTER DISULFIDE-BOND CLEAVAGE

	Before Disulfide Cleavage				After Disulfide Cleavage			
Fraction	M_w [a]	s_{20} [b] $\times 10^{13}$	D_{20} [b] $\times 10^7$	f/f_o	M_w	s_{20} $\times 10^{13}$	D_{20} $\times 10^7$	f/f_o
Low-MW glutenin	125,600	2.82	1.96	3.29	36,800	1.12	2.65	3.64
Classical gliadin	42,900	1.93	3.99	2.30	26,200	1.11	3.76	2.88
Purified gliadin	26,900	1.41	4.60	2.33	22,300	0.89	3.51	3.26

[a] Determined in 8*M* urea-0.1*M* formic acid.
[b] Values for s and D are corrected to water at 20°C.

The average MW of low-MW glutenin drops dramatically upon disulfide-bond cleavage. This decrease is one more aspect in which low-MW glutenin behaves like glutenin (2). Even though the viscosity of purified gliadin does not drop with time upon addition of mercaptoethanol, the MW of purified gliadin does drop slightly upon disulfide cleavage. It may be that gel filtration did not completely remove high-MW contaminants or that subsequent handling resulted in small amounts of disulfide-linked dimers through disulfide interchange. The drop in the average MW of classical gliadin is a compositional mean of the drop in MW's of its components, low-MW glutenin and purified gliadin.

In previous work (8), MW's (M_w) of classical gliadin, purified gliadin, and the low-MW glutenin were determined in 6*M* guanidine hydrochloride-0.1*M* acetic acid with respective values of 46,000, 30,000, and 104,000. The values for the classical gliadin and purified gliadin are a little lower in 8*M* urea-0.1*M* formic acid (Table I) than in 6*M* guanidine hydrochloride-0.1*M* acetic acid, which difference indicates that the strong urea solvent system has a little better deaggregating action than strong guanidine hydrochloride. For low-MW glutenin, the very high end of the MW distribution (approximately 10% of the sample) is thrown out of solution during the sedimentation equilibrium run in both solvents. Less is thrown out when 8*M* urea-0.1*M* formic acid is the solvent; hence, the average MW of low-MW glutenin is higher in 8*M* urea-0.1*M* formic acid than in 6*M* guanidine hydrochloride-0.1*M* acetic acid. The differences in MW's obtained in the two strongly deaggregating solvents show that these proteins have some tendency to aggregate even in a strongly deaggregating solvent system.

Since the solvent systems used in this and a previous paper (8) contained large amounts of urea or guanidine hydrochloride, preferential interaction of the protein with one component of the solvent could cause serious

errors in MW values obtained (15). To test this possibility, sedimentation equilibrium measurements were made on bovine plasma albumin and ribonuclease dissolved in 6*M* guanidine hydrochloride plus 0.1*M* acetic acid. Molecular weights obtained were 66,700 and 14,200 for bovine plasma albumin and ribonuclease, respectively. These results are very close to accepted values for the MW's of these proteins; thus, MW's obtained in these solvent systems are reasonable.

It must be emphasized that the MW values given in Table I are averages and that the proteins range from purified gliadin, which is fairly homogeneous, to low-MW glutenin, which is rather polydisperse. Several tests were made for MW homogeneity. For all the proteins, the plots that yield weight-average MW were straight except for slight deviations at the bottom of the solution columns and weight-average and z-average MW's were quite close together; these are indications of homogeneity. However, some protein was thrown out of solution in every sedimentation equilibrium run except the one on purified gliadin, and the portion of every plot for z-average MW representing the lower portion of the liquid column had a curvature ranging from slight for purified gliadin to rather pronounced for the other samples, which is an indication of polydispersity.

From the polydispersity data and from oher data, it seems reasonable to conclude the following: Low-MW glutenin is a series of molecular species with intermolecular disulfide bonds, but its MW range does not extend as high as that of glutenin. Purified gliadin seems to be a group of proteins with fairly similar MW's. The reduced and alkylated proteins exhibit various degrees of polydispersity, which would seem to be due to their aggregating even in the 8*M* urea-0.1*M* formic acid solvent. The polydispersity of the reduced and alkylated proteins might be due to the disulfide-cleaving procedure's giving rise to a variety of covalently bound species, but this seems unlikely.

The MW of reduced-alkylated low-MW glutenin is much higher than that of reduced-alkylated purified gliadin and is also much higher than that reported for disulfide-cleaved glutenin in a different solvent system (2). The best explanation is that the peptide units of low-MW glutenin form intermolecular aggregates even in 8*M* urea-0.1*M* formic acid, whereas those of purified gliadin do not. Alternatively, the peptide units of low-MW glutenin possibly are larger than those of purified gliadin, or else disulfide cleavage of low-MW glutenin is incomplete. Studies on disulfide-cleaved glutenin in a variety of solvent systems (2,16) demonstrate that the peptide subunits of this protein have a considerable tendency to form a series of intermolecular aggregates, even in systems containing high concentrations of urea and guanidine hydrochloride. These findings with glutenin support the concept that disulfide-cleaved low-MW glutenin also aggregates in urea-containing solvents. Another important point is that the peptide units from disulfide cleavage of low-MW glutenin and from disulfide cleavage of purified gliadin behave differently. This difference in behavior is in agreement with evidence obtained by Beckwith and Wall (17) that the subunits from disulfide cleavage of glutenin are different from those of gliadin similarly cleaved.

The sedimentation coefficients in Table I for the intact proteins are consistent with their MW's. The sedimentation coefficient of each of the proteins drops upon disulfide-bond cleavage; the drop for low-MW glutenin is due to decrease in MW, and the drop for purified gliadin likely is caused by the molecule opening up. The diffusion coefficient of low-MW glutenin increases upon disulfide-bond cleavage due to decrease in MW, whereas that of purified gliadin decreases, probably because the disulfide-cleaved molecules unfold (Table I).

Changes in conformation are more clearly shown by the frictional ratios given in Table I. Disulfide-bond cleavage produces only small changes in the frictional ratio. There is a definite increase in the frictional ratio of purified gliadin and that of classical gliadin upon disulfide cleavage, indicating that disulfide cleavage allows these molecules to open up or become more asymmetric. (It is hardly reasonable to propose that the observed change in frictional ratio for purified gliadin or classical gliadin is due to a change in hydration.) Since all the frictional ratio values given in Table I are quite large, all the proteins are quite asymmetric (18).

Gel Electrophoresis

Starch-gel electrophoretic patterns for several reduced and alkylated proteins are shown in Fig. 4. (Electrophoretic patterns for the same proteins

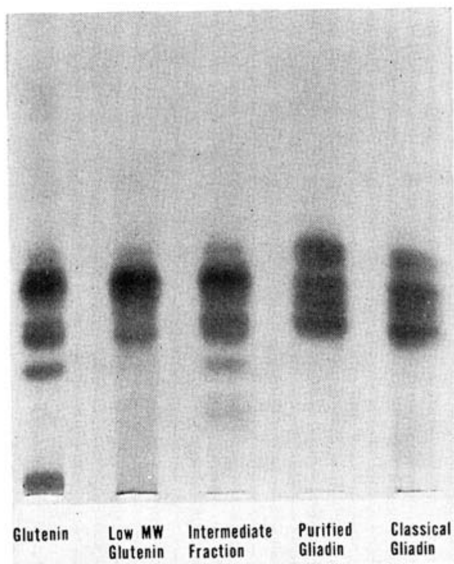


Fig. 4. Starch-gel electrophoretic patterns of reduced-alkylated proteins in a buffer of 0.005*M* aluminum lactate-0.02*M* lactic acid-3*M* urea.

before disulfide cleavage are given by Beckwith *et al.* (8).) The pattern of reduced-alkylated glutenin differs from that of reduced-alkylated classical gliadin. This dissimilarity is another indication that the peptide units obtained by disulfide cleavage of these two proteins are different. The pattern

of reduced-alkylated purified gliadin is almost identical with that of similarly treated classical gliadin, as expected, since purified gliadin comprises the bulk of classical gliadin. There are definite similarities between the patterns of reduced-alkylated low-MW glutenin and reduced-alkylated glutenin. These similarities suggest that low-MW glutenin more closely resembles glutenin than gliadin. The intermediate fraction represents an overlap of the gel filtration peaks representing low-MW glutenin and purified gliadin (8). Thus the gel-electrophoretic pattern of the reduced-alkylated intermediate fraction contains components of reduced-alkylated low-MW glutenin and of reduced-alkylated purified gliadin.

There is a possibility that the multiple bands in the starch-gel electrophoretic patterns are due to one or a few proteins interacting with components of the aluminum lactate-lactic acid buffer system to form multiple charged species (19). This is not the case. Moving-boundary electrophoretic patterns of gluten proteins in aluminum lactate-lactic acid buffers are symmetrical with respect to ascending and descending limbs (8,20), which precludes species formed via interactions with buffer components. Furthermore, Huebner and Wall (21) have chromatographically isolated gliadin components which migrate as single gel-electrophoretic bands.

DISCUSSION

Since the work of Osborne (1), wheat flour proteins have been classified into fractions obtained by differences in solubility in various solutions. Although recent techniques have shown these fractions to be heterogeneous, they have also shown that they do represent fairly distinct groups of proteins (4). Despite large differences between glutenin and classical gliadin in composition and properties, some investigators have suggested that wheat gluten is a continuum of proteins (22) and that the glutenin-gliadin separation is arbitrary. Recently, gel filtration chromatography (8,23,24) demonstrated that there are two distinct molecular-size distributions in gluten. These two size distributions do not completely correspond to the glutenin and gliadin of Osborne. The protein soluble in 70% ethanol was mostly in the lower molecular-size distribution but was contaminated with some higher-MW component (8).

The higher-MW fraction of gliadin has properties of a low-MW glutenin and was so designated in this paper. Since disulfide cleavage causes the MW and viscosity of low-MW glutenin to decrease drastically, the disulfides are primarily intermolecular as in glutenin. In addition, its moving-boundary electrophoretic pattern and amino acid composition were previously shown to resemble those of glutenin more closely than those of gliadin (8). The component polypeptide chains of the reduced-alkylated low-MW glutenin have mobilities in starch-gel electrophoresis like that of similarly treated glutenin, which indicates further that the polypeptide chains may be the same as those found in glutenin. Also, both reduced-alkylated low-MW glutenin and reduced-alkylated glutenin exhibit tendencies to aggregate in solution, even in high concentrations of urea. The average MW of this low-MW glutenin is only about 125,000, compared to 300,000 to 2,000,000 reported for ordinary glutenin (2,5). The lower MW of this glutenin contaminant in clas-

sical gliadin preparations probably accounts for its solubility in 70% neutral ethanol in contrast to the insolubility of ordinary glutenin in that solvent. Also, the small molecular size of low-MW glutenin permitted it to migrate into starch gel during electrophoresis, although it streaked rather than giving discrete bands as does purified gliadin (8). This streaking and other properties indicate that it consists of a spectrum of components differing in MW and in combination of disulfide-linked polypeptides.

The isolation of purified gliadin and determination of its composition and physical characteristics have substantiated the concept that the properties of purified gliadin arise because its disulfide bonds are primarily intramolecular, whereas the contrasting properties of glutenin results come from its disulfide bonds' being primarily intermolecular. Little change in the MW or viscosity of the purified gliadin occurred after cleavage of its disulfide bonds. Previous observations that significant changes in these properties in the classical gliadin followed splitting of the disulfide bonds are now definitely attributable to the presence of low-MW glutenin contaminants. With availability of purified gliadin, it was possible to observe those physical changes due solely to the unfolding of gliadin polypeptide chains upon disulfide cleavage. Changes in sedimentation and diffusion coefficients confirmed that the disulfides maintained the protein in a folded conformation. The contrasting properties of the low-MW glutenin and the purified gliadin demonstrate that they are distinctly different proteins, although both are extracted with 70% ethanol from wheat gluten.

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