Gel Filtration Chromatography of Glutenin in Dissociating Solvents: Effect of Removing Noncovalently Bonded Protein Components on the Viscoelastic Character of Glutenin

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ABSTRACT

Glutenin was chromatographed on Sephadex G-200 using the solvent mixtures of acetic acid, guanidine-HCl, and cetyltrimethyl ammonium bromide; sodium dodecyl sulfate; and phenol, acetic acid, and water in succession. The first two of these three solvents dissociated about 27% protein from glutenin as low molecular weight protein. Removal of this protein by gel filtration gave a residual glutenin (sodium dodecyl sulfate [SDS]-glutenin) from which the third solvent was not able to dissociate any more protein. The residual glutenin could, therefore, be regarded as core glutenin in which all the subunits are probably bound by interpolypeptide disulfide bonds. Viscoelastograph measurements showed that the elastic recovery in residual glutenin (SDS-glutenin) was about half of the starting glutenin (AcOH-glutenin). Thus, the noncovalently bonded polypeptides (low molecular weight protein) increased the elastic character of glutenin. This increase may simply be a result of the increase in the molecular weight of glutenin following accretion of noncovalently bonded polypeptides by residual glutenin (SDS-glutenin).

Glutenin is generally believed to be a mixture of high molecular weight proteins containing subunits bound together by covalent (disulfide) as well as noncovalent (such as hydrogen and hydrophobic) bonds. The idea that glutenin consists of polypeptides joined together by disulfide bonds arose from the work of Pence and Olcott (1952) on the effect of disulfide bond reducing agents on the viscosity of glutenin. Strong support for this view is given by the fact that the molecular weight of glutenin, which is estimated in millions (Jones et al 1961), remains in the millions even in strongly dissociating solvents such as guanidine-HCl and sodium dodecyl sulfate (SDS) (Redman 1973, Huebner and Wall 1980). A model emphasizing the role of interpolypeptide disulfide bonds in glutenin was recently proposed by Graveland et al (1985).

In contrast to the above, Kasarda and co-workers (1976) suggested a model for glutenin having no interpolypeptide disulfide bonds. They proposed that all disulfide bonds in glutenin are of the intrapolypeptide type; the location of these intrapolypeptide bonds is hypothesized to confer glutenin subunit conformations capable of strong noncovalent interactions. Two other models, one proposed by Ewart (1979) and the other by Khan and Bushuk (1979), postulate the presence of both interpolypeptide disulfide bonds and noncovalent bonds between glutenin subunits.

Glutenin is a viscoelastic protein, and although it has been known for a long time that reducing agents bring about a drastic change in its physical character, the effect of removing noncovalent bonded polypeptides on the physical character of glutenin is not known. This paper reports the changes occurring in the physical character of glutenin as measured by visco-elastograph after removal of noncovalently bonded polypeptides. Amino acid compositions and electrophoretic patterns of various isolated fractions are also given.

MATERIALS AND METHODS

Preparation of Glutenin

The strong wheat variety (K65; dough development time 8 min) used in this study was obtained from R. D. Goyal, C. S. Azad University of Agriculture and Technology, Kanpur, India.

One kilogram of wheat was milled in a laboratory Kamas mill (Slagy 200A) to pass through a 52-mesh sieve, and 700 g of flour was obtained. Dough was made from 100 g of this flour. The dough was washed with 0.5*M* NaCl followed by distilled water. The glutenin obtained was cut into pieces and extracted with 1 L of 70% ethanol (three portions) at 4° C by stirring for 12 hr. The residue after final centrifugation was similarly extracted with 1.5 L of 0.1*M* acetic acid. The acid supernatants were combined and lyophilized to give 2.84 g of crude glutenin.

Gel Filtration on Sephadex G-200

Column chromatography of glutenin was done on Sephadex G-200 (2.1 × 87 cm, volume 300 ml). Eluting solvents were 0.1*M* acetic acid, pH 3.1 (AcOH); 0.1*M* acetic acid, 2.25*M* guanidine-HCl, and 0.01*M* cetyltrimethylammonium bromide, pH 3.2 (AGC); 1% sodium dodecyl sulfate in 0.1*M* acetic acid, pH 3.2 (SDS; Chung and Pomeranz 1979); and phenol-acetic acid and water 1:1:2, pH 1.4 (PAW; Huebner and Rothfus 1971). Solvent AGC was formulated by substituting 2.25*M* guanidine-HCl for 3*M* urea in the solvent mixture acetic acid, urea, and cetyltrimethylammonium bromide of Meredith and Wren (1966). Preliminary runs showed that solvent AGC dissociated more protein from glutenin than acidic guanidine-HCl without cetyltrimethylammonium bromide. Three-milliliter fractions were collected at the rate of 12 ml/hr, and except in PAW chromatography were read at 280 nm. In the case of PAW, chromatography aliquots were subjected to alkaline hydrolysis and the color developed with ninhydrin (Hirs et al 1956). Glutenin (void volume peak) from one chromatography run was prepared for chromatography in the next solvent by dialyzing the pooled fraction against 0.1*M* acetic acid and lyophilization. The lyophilized material was dissolved in the solvent used in the next chromatography and loaded on the column. Glutenin containing SDS was freed from the latter by dialysis against ammonia as described by Huebner and Wall (1980). Glutenin so treated was found to contain less than 2% SDS (Hayashi 1975).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the procedure of Laemmli (1970). The gel slab was 1.5 mm thick and 11 cm long (2 cm stacking gel and 9 cm separating gel). Constant voltage at 130 V was used. The time required was about 4 hr. Staining was done with Coomassie blue as described by Weber and Osborn (1969).

Amino Acid Analyses

For amino acid analyses, the fractions were hydrolyzed according to the procedure of Moore and Stein (1963) and analyzed fluorometrically on a Durrum amino acid analyzer kit MBF using Durrum Fluoropa (*o*-phthaladehyde) as the detecting reagent. Protocol given in the manufacturer's manual for protein

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hydrolysates was followed. The amino acids proline, cystine, and arginine could not be determined. Proline in this system is determined by introducing sodium hypochlorite into the column effluent stream at the time of its emergence. We were unable to get satisfactory results by doing so, and therefore all runs were made without hypochlorite. Cystine could not be determined because of extremely low fluorescent yield and arginine because of the presence of a spurious peak at the same position.

Measurement of Elastic Recovery

Elastic recovery was determined with a visco-elastograph (Visco-Elastographie, Tripette and Renaud, Paris). The machine was set for 200-g load, 20-sec compression time, and 10-sec relaxation time. Glutenin balls were made by wetting weighed amounts of glutenin with water and rolling them between finger tips. After one measurement had been taken, the ball was rounded again and a second reading was taken. No significant difference was observed between the two readings. The following parameters were measured: initial thickness E (mm), thickness under compression e_1 (mm), and thickness after removal of compression e_2 (mm). Elastic recovery was calculated by the equation $ER = 100 \times (e_2 - e_1)/E - e_1$ (Laignelet and Feillet 1979).

RESULTS AND DISCUSSION

Gel Filtration on Sephadex G-200

The successive steps in the chromatography of crude glutenin in solvents AcOH, AGC, SDS, and PAW are shown in Figure 1. Each of the first three solvents gave one high molecular weight (high-MW) and one low molecular weight (low-MW) peak. PAW gave only the high-MW peak. The high-MW peaks are called glutenins, and each is identified by the prefix of the solvent used in its isolations. Thus, the high-MW peak isolated by chromatography in AcOH is called AcOH-glutenin. Similarly, the low-MW peaks are identified as peptides by adding as prefix the solvents used in their isolations. In addition to the above, AcOH-glutenin was also chromatographed directly in SDS, i.e., without the intervening chromatography in AGC. Gel filtration patterns are shown in Figure 2, and the data from them are given in Table I.

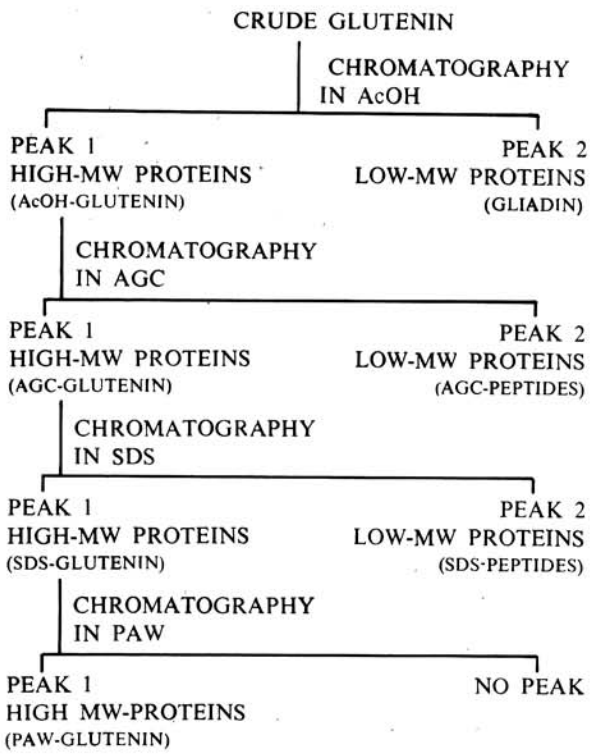

Fig. 1. Scheme for the chromatography of glutenin on Sephadex G-200 using dissociating solvents.

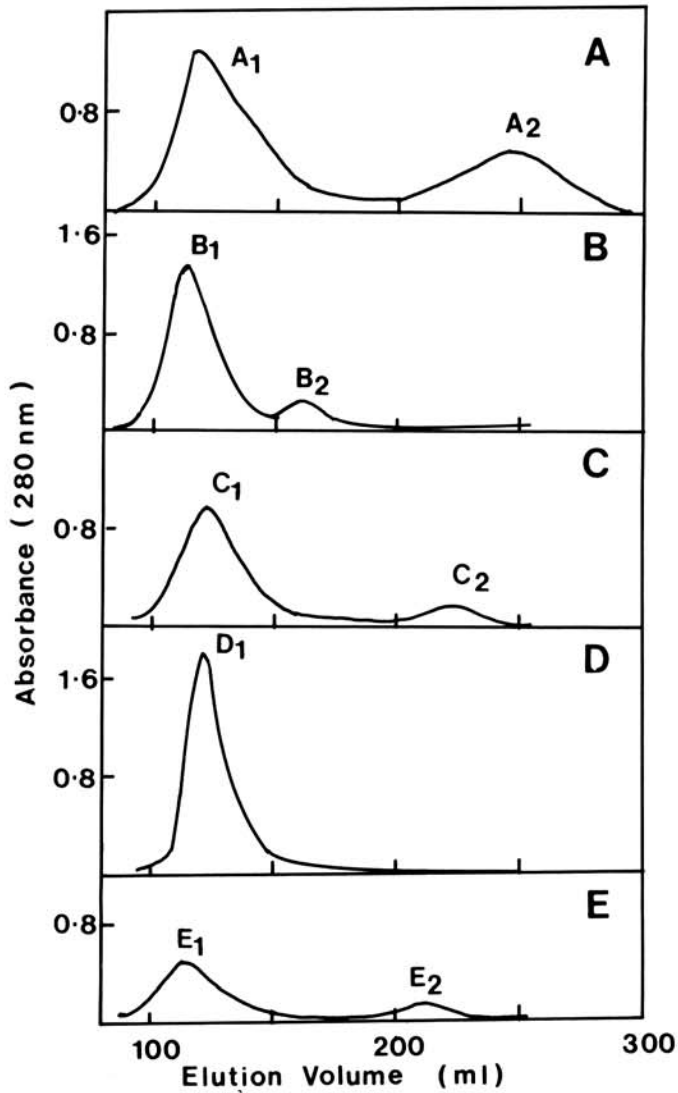

Fig. 2. Gel filtration of glutenin on Sephadex G-200 (2.1 × 87 cm) as outlined in Fig. 1. The chromatograms are as follows: **A,** gel filtration of crude glutenin in solvent acetic acid (AcOH); **B,** gel filtration of the void volume peak A1 in a solvent mixture of acetic acid, guanidine-HCl, and cetyltrimethylammonium bromide; **C,** gel filtration of the void volume peak B1 in solvent sodium dodecyl sulfate; and **D,** gel filtration of the void volume C1 in solvent phenol-acetic acid and water. **E** is the chromatogram of AcOH-glutenin (fraction A1) in SDS.

TABLE I
Chromatography of Glutenin on Sephadex G-200 (Data from Fig. 1)

Peaks of Fig. 1	Solvent[a]	Elution[b] Volume (ml)	Molecular Weight[c]	Yield[d] (%)	Total Recovery (%)
A1	AcOH	116	≥200 K	58	89
A2		248	20 K	31	
B1	AGC	113	≥200 K	80	96
B2		160	51 K	16	
C1	SDS	122	≥200 K	80	91
C2		220	26 K	11	
D1	PAW	122	≥200 K	...	...
D2			...	...	
E1	SDS	114	≥200 K	80	94
E2		210	34 K	14	

[a] AcOH = Acetic acid, AGC = acetic acid, guanidine-HCl, and cetyltrimethylammonium bromide; SDS = sodium dodecyl sulfate; and PAW = phenol-acetic acid and water.
[b] Void volumes determined with blue-dextran were 116 ml in AcOH and 113 ml in AGC and SDS.
[c] Standard graphs in AcOH, AGC, and SDS were made using lysozyme (14.4 K), ovalbumin (45 K), bovine serum albumin (66 K), and bovine serum albumin dimer (132 K).
[d] Percent of loaded optical density recovered in each peak.

In Figure 2A, peak 2 is probably of gliadin (Huebner 1970) and therefore, peak 1 is of gliadin-free whole glutenin (AcOH-glutenin). Rechromatography of peak 1 gave only one peak eluting in the void volume in 93% recovery. Because of this, peak 1 can be considered to be free of any unbound low-MW protein, and the low-MW proteins found in subsequent gel filtration of AcOH-glutenin in dissociating solvents can be regarded as constituents of glutenin bound by noncovalent bonds.

The data in Table I indicate that about 16% protein dissociates from AcOH-glutenin in the presence of AGC, and an additional 11% protein dissociates from the remaining glutenin (AGC-glutenin) in the presence of SDS. Thus, from the start AcOH-glutenin recoverable proteins are AGC dissociable protein (AGC-peptides) 16%, SDS dissociable protein (SDS-peptides) 8.8%, and residual glutenin (SDS-glutenin) 64%. If a correction is made for the experimental loses of 4 and 9% in thcese two gel filtrations, then these values will be 17, 10, and 73%, respectively. The results show that about 27% of AcOH-glutenin is dissociable as low-MW protein.

When SDS-glutenin was chromatographed in PAW, no low-MW polypeptides were found (Fig. 2D). It can, therefore, be assumed that between themselves AGC and SDS are able to dissociate all noncovalently bonded polypeptides from AcOH-glutenin. Also, as up to this point (isolation of SDS-glutenin) no disulfide bond has been broken, all the disulfide bonds that might have been present in AcOH-glutenin must also be present in SDS-glutenin. SDS-glutenin can, therefore, be regarded as core glutenin in which the polypeptides are probably bound together by interpolypeptide disulfide bonds only.

As presented in Table I, the elution volumes of AcOH-glutenin, AGC-glutenin, and SDS-glutenin are 116, 113, and 122 ml, respectively. The void volume of the column is 113 ml. At 122 ml, the elution volume of SDS-glutenin, although still within the range of void volume, is more than the elution volumes of AcOH- and AGC-glutenins. This may be indicative of a significant decline in the molecular weight of AcOH-glutenin by successive gel filtration in AGC and SDS. It is speculative, but is nevertheless possible, that the molecular weight of SDS-glutenin is of the order of few hundred thousands rather than a few millions.

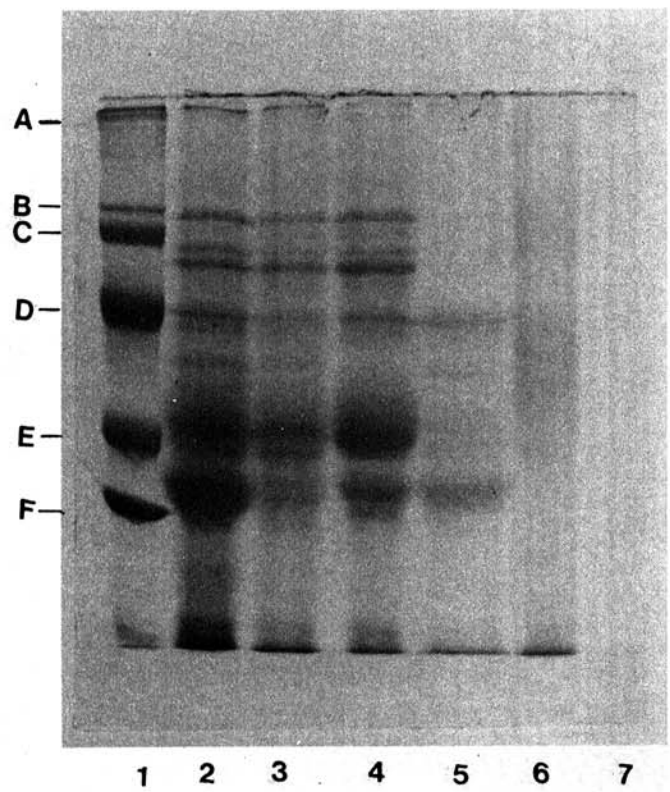

Fig. 3. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis patterns of standards (**1**); crude glutenin (**2**); acetic acid (AcOH)-glutenin (**3**); acetic acid, guanidine-HCl, and cetyltrimethylammonium bromide (AGC)-glutenin (**4**); AGC-peptides (**5**); SDS-glutenin (**6**); and SDS-peptides (**7**). Standards used were myosin, 205 K (**A**); β-galactosidase, 116 K (**B**); phosphorylase-B, 97.4 K (**C**); bovine serum albumin, 66 K (**D**); ovalbumin, 45 K (E); and carbonic anhydrase, 29 K (**F**).

For two reasons, the greater elution volume of SDS-glutenin is not likely to result from the effect of SDS on the elution behavior of glutenin independent of its effect on glutenin's molecular weight. First, the elution volume of SDS-glutenin remains the same 122 ml in PAW (Table I). Secondly, when AcOH-glutenin is chromatographed directly in SDS, the glutenin peak elutes at 114 ml (Table I, E1). Elution of a major proportion of glutenin in the void volume when chromatographed separately in AGC and SDS (Table I) is in agreement with reports in the literature. Glutenin is known to give a void volume peak in individual dissociating solvents not only on Sephadex gels, but also on agarose gels, which have much higher exclusion limits (Meredith and Wren 1966; Huebner and Rothfus 1971; Redman 1973; Huebner and Wall 1976, 1980; Hamauzu et al 1979; Bottomley et al 1982; Graveland et al 1985).

SDS-PAGE of Glutenins and Low-MW Proteins

Figure 3 gives the SDS-PAGE patterns of crude glutenin, AcOH-glutenin, AGC-glutenin, AGC-peptides, SDS-glutenin, and SDS-peptides. The patterns are well defined for crude glutenin, AcOH-glutenin, AGC-glutenin, and AGC-peptides but not for SDS-glutenin and SDS-peptides. SDS-glutenin gave a very broad diffuse stain, whereas SDS-peptides gave a faint (not visible in this picture) band around 14,000. The SDS-PAGE patterns for crude-glutenin, AcOH-glutenin, and AGC-glutenin are very similar; each has 16–18 bands. The pattern for AGC-peptides is different from that of AGC-glutenin in not having the high-MW (above 66,000) bands. It has nearly all the bands corresponding to medium and low-MW polypeptide bands of AGC-glutenins. However, from this it does not follow that the medium- and low-MW bands of AGC-glutenin and AGC-peptides are of the same peptides. As the nature of bonding of AGC-peptides in glutenin is different, the electrophoretically similar bands of AGC-peptides and AGC-glutenin may also be of different polypeptides. Assuming this, the total number of polypeptides in AcOH-glutenin comes to 24, 16 for AGC-glutenin and eight for AGC-peptides. It may be noted that molecular weights of the polypeptides found in AGC-peptides range from 66,000 to 29,000. This range is the same as for the polypeptides of unreduced glutenin that enter the gel on SDS-PAGE (Khan and Bushuk 1979).

The above total of 24 polypeptides would have been even more had SDS-glutenin and SDS-peptides given good electrophoregrams. Separation of noncovalently bonded polypeptides from glutenin before reduction offers another approach for studying glutenin subunit composition. In earlier studies, although the presence of noncovalently bonded protein in glutenin was noted, its separation was not attempted (Khan and Bushuk 1979, Huebner and Wall 1980). Other approaches reported are fractionation of reduced and alkylated glutenin by gel filtration (Huebner and Wall 1974) and solubility fractionation (Bietz and Wall 1973) prior to electrophoresis. The latter technique according to Bietz (1979), indicates the presence of 40–45 unique subunits of glutenin.

Amino Acid Analysis of Glutenin and Low-MW Proteins

Table II gives the amino acid compositions of all the protein fractions isolated (Fig. 1). For comparison, the amino acid composition of crude glutenin reported by Orth and Bushuk (1973) is also given. The amino acid compositions of the final three fractions, i.e., AGC-peptides, SDS-glutenin, and SDS-peptides, although typical of glutenin, are different enough to suggest unique polypeptide make-up of these fractions. The amino acid compositions of AGC- and SDS-peptides show that their components are indeed glutenin constituents and not some nongluten impurities. It may be noted that SDS-peptides are relatively low (31%) in glutamic acid compared to AGC-peptides (43%).

TABLE II
Amino Acid Compositions of Various Glutenin Fractions (from Table I) in Mole Percentages of Recovered Amino Acids (averages of two analyses)

Amino Acid	Crude Glutenin	AcOH-Glutenin[a]	AGC-Glutenin[a]	AGC-Peptides[a]	SDS-Glutenin[a]	SDS-Peptides[a]	Osborne Glutenin[b]
Alanine	3.9	4.0	3.7	4.6	4.0	5.9	4.3
Aspartic acid	3.5	3.1	2.5	4.4	2.4	4.7	3.4
Glutamic acid	43.0	42.0	43.0	43.4	45.9	30.9	41.7
Glycine	6.4	7.9	8.9	6.7	8.9	10.5	9.1
Histidine	2.3	2.2	2.3	2.2	2.4	2.9	2.0
Isoleucine	4.7	4.2	4.2	4.1	3.7	5.0	3.9
Leucine	8.4	7.9	8.4	7.1	7.6	8.8	8.2
Lysine	1.6	1.6	0.9	1.4	1.0	3.6	1.7
Methionine	2.5	2.5	2.4	2.1	1.7	2.4	1.5
Phenylalanine	5.2	4.7	4.3	5.4	3.7	4.6	4.7
Serine	6.3	6.4	7.2	7.0	7.0	7.2	7.5
Threonine	3.4	3.7	3.1	3.5	3.4	3.8	3.5
Tyrosine	2.8	2.9	3.1	2.5	2.9	3.0	3.4
Valine	6.2	6.8	6.2	5.7	5.6	7.5	5.1

[a] Prefixes are solvents used in chromatography: AcOH = acetic acid, AGC = acetic acid, guanidine-HCl, and cetyltrimethylammonium bromide; and SDS = sodium dodecyl sulfate.
[b] From Orth and Bushuk (1973), who reported the values of Arg and Pro in addition to the amino acids listed in this table. To make their values comparable to ours we recalculated their percentages after deleting Arg and Pro.

TABLE III
Visco-Elastograph Data for K65 Wheat Flour Dough, Gluten, Glutenin, and Glutenin Fractions

Sample	Weight[a] (mg)	Elastic Recovery[b]
Dough	100	5.35 ± 0.04
Dough	50	4.91 ± 0.01
Gluten	50	26.3 ± 0.05
Crude glutenin	50	13.25 ± 0.21
AcOH-glutenin[c]	50	23.19 ± 1.93
AGC-glutenin[c]	50	18.99 ± 0.51
SDS-glutenin[c]	24	13.33 ± 0.07

[a] Milligrams of dry weights used for making balls.
[b] Equation for calculating elastic recovery is given in the experimental section.
[c] AcOH = Acetic acid, AGC = acetic acid, guanidine-HCl, and cetyltrimethylammonium bromide; and SDS = sodium dodecyl sulfate.

Elastic Recovery of Glutenin Fractions

The visco-elastograph data are given in Table III. Beginning with AcOH-glutenin, there is a decline in elastic recovery from 23.2 to 19 for AGC-glutenin and to 13.3 for SDS-glutenin. Thus, at each step, removal of noncovalently bonded polypeptides results in decreased elastic recovery; however, SDS-glutenin still has about the same value as crude glutenin (13.25).

Elastic recovery values for dough, gluten, and crude glutenin are also given in Table III. The values fluctuate widely between dough, gluten, crude-glutenin, and AcOH-glutenin. These fluctuations are consistent with the changes in the composition of these fractions during their isolation. At 26.3 gluten has the highest value. The low value for dough (4.9) is caused by the dilution of gluten by other dough constituents. In gluten, glutenin is the elastic and gliadin the viscous component (Huebner and Wall 1974). Thus, glutenin will increase elastic recovery and gliadin will decrease it. In going from gluten to crude-glutenin, all the insoluble glutenin (42% in this variety, Ram and Nigam 1979) and most of gliadin (32% in this variety) are removed, leaving behind crude glutenin. Thus, there is a much greater decrease in the quantity of insoluble glutenin than of gliadin. This is reflected in decrease in elastic recovery value from 26.3 for gluten to 13.3 in crude glutenin (Table III).

Similarly, in going from crude glutenin to AcOH-glutenin the contaminating low-MW protein (gliadin) is removed (Fig. 2A). This results in the increase of elastic recovery value from 13.3 for crude glutenin to 23.2 for AcOH-glutenin (Table III). It may be noted that the elastic recovery value for AcOH-glutenin (23.2) is not very different from the elastic recovery value for gluten (26.3). This is suggestive of opposite functional roles for gliadins and insoluble glutenin in gluten.

CONCLUSIONS

The results suggest that noncovalently bonded polypeptides increase the elastic character of covalently bonded portion of glutenin, which by itself is also elastic. The increase may simply result from the increase in the molecular weight of covalently bonded polypeptides. The covalently bonded portion of glutenin can be regarded as core glutenin.

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