Selective Reduction of Interpolypeptide and Intrapolypeptide Disulfide Bonds of Wheat Glutenin from Defatted Flour

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ABSTRACT

Selective cleavage of interpolypeptide (intersubunit) SS bonds of wheat glutenin was accomplished by treatment with 32 m*M* 2-mercaptoethanol (2-ME) at pH 4.0 and 20° C for 4 hr. The subunits liberated by this partial reduction had uncleaved intrapolypeptide SS bonds, according to the following criteria: 1) Relative mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis of the subunits with low molecular weight from partial reduced glutenin was greater than that from the completely reduced glutenin, showing the compact conformation of subunits by uncleaved intrapolypeptide SS bonds. 2) With further reduction by 2-ME in the presence of 8*M* urea, these bands decreased their mobility and shifted to the position of the completely reduced glutenin subunits, indicating complete unfolding and expansion of the subunit polypeptides that may cause the decrease of electrophoretic mobility. The amount of cysteine residue involved in interpolypeptide and intrapolypeptide SS bonds was determined by selective modification with iodoacetamide and vinylpyridine respectively. All subunits contained 2 moles of interpolypeptide SS bonds per mole of subunit, whereas the number of intrapolypeptide SS bonds was 2–3 moles per mole of subunit. Wheat glutenin may be a linear polymer protein consisting of several subunits that link together through SS bonds.

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Wheat glutenin is thought to be a polymeric protein that consists of several kinds of subunit polypeptides linking together through SS bonds (Nielsen et al 1962, Woychik et al 1964). Such a structure of glutenin molecules is considered to be a main cause for the unique viscoelastic property of wheat flour dough (Pence and Olcott 1952, Jones et al 1974). How many SS bonds are present in each glutenin subunit and how each subunit links to adjacent subunits through SS bonds has not been elucidated clearly. Two structural models of glutenin molecules are illustrated in Figure 1. If each subunit has two interpolypeptide SS bonds, glutenin may have a linear structure (Ewart 1977); if each subunit has more than three interpolypeptide SS bonds, glutenin may have a three-dimensional network structure through SS bonds (Bietz 1979, Wall 1979).

To determine the number of interpolypeptide and intrapolypeptide SS bonds of each subunit, we first isolated main glutenin subunits and determined cysteine content for each (Matsumura et al 1984a,b). We tried to find the condition of selective cleavage of only interpolypeptide SS bonds by reducing glutenin with various concentrations of 2-mercaptoethanol (2-ME) at pH 6.0 (Matsumura et al 1984b), but we could not detect sulfhydryl (SH) groups that seemed to be involved in interpolypeptide SS bonds. However, SH groups liberated in protein seem to reform SS bonds easily because glutenin aggregates rapidly at pH 6.0; also, the concentration of 2-ME probably was fairly low in the precipitate of glutenin, and alkylation of SH groups might have been incomplete owing to the precipitate, resulting in low value of SH content.

In the present study, to exclude the effect of precipitation, we accomplished reduction of glutenin and alkylation of SH groups liberated in solution at pH 4.0. This enabled us to cleave and modify interpolypeptide and intrapolypeptide SS bonds of each subunit selectively. Then the number of interpolypeptide and intrapolypeptide SS bonds of each subunit was determined.

MATERIALS AND METHODS

Wheat flour (60% extract of no. 1 Canadian Western wheat, not a single variety, milled by a test mill) was provided by Nisshin Flour Milling Co. Ltd., Kobe. Iodoacetamide (IAM) was purchased from the Protein Research Foundation. Other reagents were obtained from Wako Pure Chemical Ind. Ltd. or Nakarai Chemicals Ltd.

Preparation of Glutenin

Wheat flour was defatted by 10 volumes of *n*-butanol at room temperature for 12 hr and was air dried. The defatted flour was made into dough and kneaded by hand thoroughly in a 1% NaCl solution at 0° C to remove starch and water-soluble proteins. The

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wet gluten thus obtained was dispersed in 0.01 *M* acetic acid and centrifuged at 20,000 × *g* for 30 min. The supernatant was heated in boiling water for 15 min to inactivate proteases (Olcott et al 1943). Crude glutenin was separated from gluten by the alcohol fractionation method described by Woychik et al (1964). Glutenin was further purified with CM-Sephadex as described by Mita and Yonezawa (1971). The glutenin thus obtained was lyophilized and stored for use.

Reduction and Alkylation

Partial reduction of SS bonds of glutenin and carboxymethylation of SH groups was performed as follows. Glutenin was dissolved completely in 0.01 *M* acetic acid in a protein concentration of 0.04% at pH 4.0. The glutenin solution was freed of oxygen by nitrogen (N_2), and 2-ME was added to the solution at various concentrations. After reduction at 20° C for 4 hr, a twice molar excess of IAM over SH groups originating from glutenin and 2-ME was added, and alkylation (carboxymethylation) was accomplished at 20° C for 16 hr at pH 4.0.

During the carboxymethylation, iodide was liberated and the pH of the solution fell gradually, particularly in the solution containing high concentrations of 2-ME and IAM. Hence, pH was sometimes adjusted to 4.0 with 0.1 *N* NaOH. In spite of carboxymethylation for 16 hr, some of the SH groups of glutenin and of 2-ME may have remained unmodified, so denaturant was added to the solution and the pH was raised to modify the SH groups in glutenin completely. However, because addition of denaturant and raising of pH may cause cleavage of SS bonds of glutenin owing to unmodified 2-ME, the reaction mixture was dialyzed against 0.1 *M* acetic acid under N_2 bubbling, to remove unmodified 2-ME, and then dialyzed against 0.2 *M* Tris HCl buffer, pH 8.0, containing 8 *M* urea, 1 m*M* ethylenediaminetetraacetic acid (EDTA), and 10 m*M* IAM for 16 hr, to modify the unreacted SH groups in protein completely. Then the reaction mixture was dialyzed against 0.1 *M* acetic acid to remove the reagents and lyophilized. Partially reduced and carboxymethylated glutenin was thus obtained.

Complete reduction and pyridylethylation of SH groups was accomplished as follows: Glutenin was dissolved in 0.3 *M* Tris-HCl buffer (pH 8.0), containing 8 *M* urea and 1 m*M* EDTA in a protein concentration of 1%. The glutenin solution was deaired by N_2 gas, and 5 mM of 2-ME was added. After reduction at 20° C for 4 hr, 10 mM of vinylpyridine was added. Pyridylethylation of SH groups was accomplished at 20° C for 16 hr. The reaction mixture was dialyzed against 0.1 *M* acetic acid and lyophilized.

SH Determination by Amino Acid Analysis

S-Alkylated glutenin and glutenin subunits were hydrolyzed with 6 *N* HCl in a sealed and evacuated tube at 110° C for 22 hr. Cysteine residues in the hydrolysate were determined as S-carboxymethyl cysteine (CM-Cys) and S-pyridylethyl cysteine (PE-Cys) by analysis with a Hitachi 835 amino acid analyzer.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the discontinuous buffer system of Laemmli (1970). Analytical SDS-PAGE was performed on a slab gel (1 × 160 × 120 mm) at 20 mA for 4–5 hr. Preparative electrophoresis was performed on a slab gel (5 × 210 × 150 mm) at 20 mA for 16–20 hr. Gel that had been stained with 0.1% Coomassie Brilliant Blue R-250 in a solution of methanol, water, and acetic acid (50:10:40, v/v) for 20 min was destained in a solution of methanol, water, and acetic acid (10:83:7, v/v), with continuous shaking. The gel of analytical SDS-PAGE was dried on a filter paper. The gel of preparative SDS-PAGE was used for the electric extraction of protein.

Electric Extraction of Subunit Polypeptides

The main bands in preparative SDS-PAGE gel were cut out with a razor blade and crushed to small pieces. The crushed gels were stirred in 0.125 *M* Tris-HCl buffer, pH 6.8, containing 4% SDS and 20% glycerol for 12 hr, and were heated to 100° C. Protein was electrically extracted from the gel with a glass tube (20-mm diameter) according to the method of electrophoretic elution (Stephens 1975). The extraction was performed at 40 mA for 16–20 hr at 20° C using the buffer system of Laemmli (1970). The solution containing extracted subunit polypeptides was dialyzed against 0.1 *M* acetic acid and lyophilized. SDS bound to subunit polypeptides was removed according to the method of Henderson et al (1979).

RESULTS AND DISCUSSION

Liberation of SH Groups from Glutenin by Partial Reduction

Liberation of SH groups in glutenin was shown as a function of the concentration of 2-ME (Fig. 2). The amount of liberated SH groups first increased steeply with the increase of 2-ME concentration to 32 m*M* and reached about 30% of total SH groups at 32 m*M*. At concentrations of 2-ME above 32 mM, the liberation of SH groups was very low and attained only about 50% of total SH groups in 400 m*M* 2-ME.

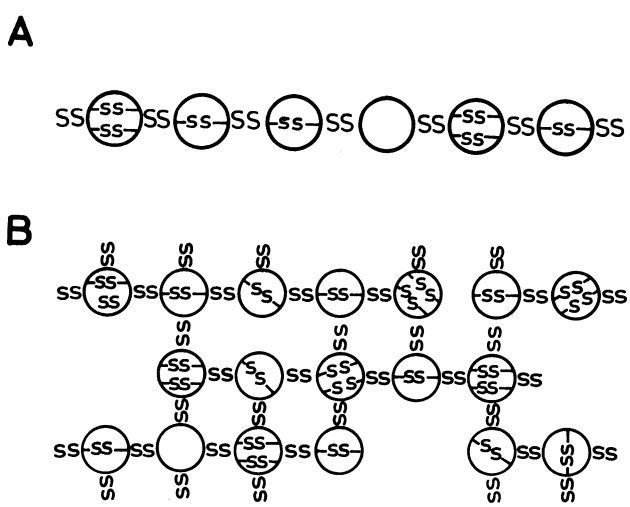

Fig. 1. Models of glutenin molecules. **A,** Linear Structure, in which each subunit has two interpolypeptide SS bonds. **B,** Three-dimensional network structure, in which each subunit has more than three interpolypeptide SS bonds.

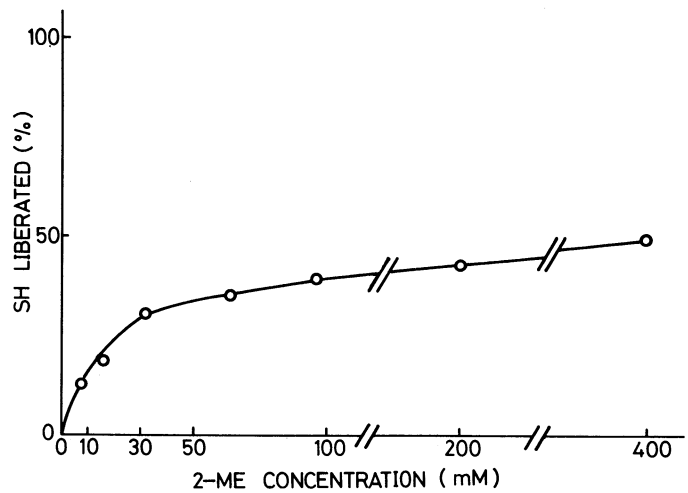

Fig. 2. Liberation of sulfhydryl (SH) groups from glutenin by partial reduction with various concentrations of 2-mercaptoethanol (2-ME). SH groups, liberated by the partial reduction, were carboxymethylated with iodoacetamide and determined as S-carboxymethylated cysteine by amino acid analysis. Amounts of SH groups are given as a percentage of the total SH groups liberated from glutenin by complete reduction.

Of the total SS bonds, only 50% was susceptible to a high concentration of 2-ME, such as 400 m*M*, indicating that reduction occurred under relatively mild conditions, because reactivity of 2-ME was fairly low at pH 4.0 where the conformation of glutenin subunits was still compact. Among SS bonds cleaved under this condition, about 60% (30% of total SS bonds), which easily cleaved with 2-ME below 32 m*M*, seemed to be highly reactive bonds. Ewart (1972) also reported that 40% of total SS bonds are highly susceptible to reducing agents.

Liberation of Glutenin Subunits by Partial Reduction

If SS bonds that are easily cleaved below 32 m*M* correspond to interpolypeptide SS bonds, some subunits with uncleaved intrapolypeptide SS bonds should be liberated. The mode of liberation of subunits by partial reduction with 2-ME was analyzed by SDS-PAGE to examine this possibility.

In the electrophoretic profiles (Fig. 3), the bands that migrate more slowly than the high molecular weight (HMW) subunits may be dimers and trimers of the HMW subunits, as shown by Lawrence and Payne (1983). The bands at the positions of molecular weight (104, 91, 81, 60, 44, 42, and 35 kD) represent glutenin subunits and are designated as B-1 through B-7, respectively. When profiles of glutenin reduced with 8–400 m*M* 2-ME were compared with those of completely reduced glutenin, HMW bands (i.e., B-1, B-2, and B-3) were liberated at low concentrations of 2-ME and increased with the increase of 2-ME concentration. Several low molecular weight (LMW) bands other than main subunits (B-5, B-6, and B-7) were newly observed by reduction with a low concentration of 2-ME. Molecular weights of these new bands were 39, 37, 36, and 32 kD, which were less than those of the main subunits (B-5, B-6, and B-7). With the increase of 2-ME concentration, these new bands (named B-5′, B-6′, and B-7′) decreased and disappeared, particularly above 64 m*M*; the main subunits (B-5, B-6, and B-7) appeared and increased to the intensity similar to that of completely reduced and carboxymethylated glutenin, with the increase of 2-ME concentration.

As suggested earlier (Matsumura et al 1984b), we interpreted the appearance of B-5′, B-6′, and B-7′ at a low concentration of 2-ME and the shift of these bands to B-5, B-6, and B-7, respectively, with the increase of 2-ME concentration according to an operational definition based on accessibility to reducing agents. Thus disulfide bonds cleaved at 2-ME concentrations below 32 m*M* are considered to be intermolecular, whereas those requiring 2-ME concentrations above 32 m*M* for cleavage are considered to be intramolecular. By this definition, B-5′, B-6′, and B-7′, which were liberated by partial reduction of glutenin with a low concentration of 2-ME such as 8–32 m*M* at acidic pH, must retain intrapolypeptide SS bonds although the interpolypeptide SS bonds of them were cleaved. With the increase of 2-ME above 64 m*M*, not only interpolypeptide SS bonds but also intrapolypeptide SS bonds were cleaved. Intrapolypeptide SS bonds may maintain the compact conformation of glutenin subunits even in the presence of SDS. Hence, the electrophoretic mobilities of subunits holding uncleaved intrapolypeptide SS bonds may be greater than the same subunits whose intrapolypeptide SS bonds have already been cleaved. If this interpretation is correct, B-5′, B-6′, and B-7′ must contain uncleaved intrapolypeptide SS bonds. From the results

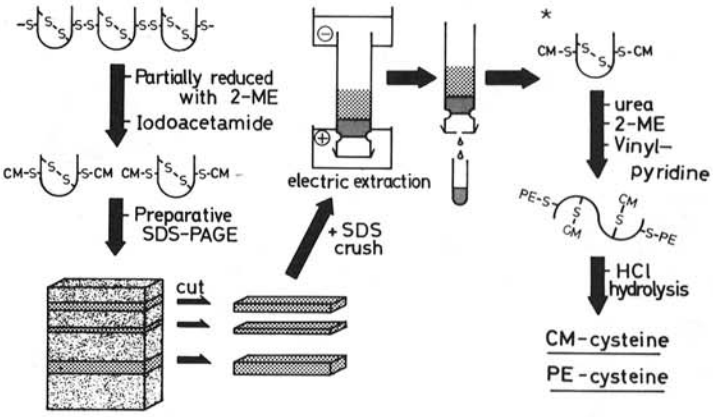

Fig. 4. Procedure for separating subunit polypeptides retaining intrapolypeptide SS bonds and determining interpolypeptide and intrapolypeptide SS bonds of each subunit. Main subunit polypeptides (B-1, B-2, B-3, B-5′, B-6′, and B-7′) were separated from glutenin that was partially reduced with 32 mM 2-mercaptoethanol. * = separated subunit polypeptides.

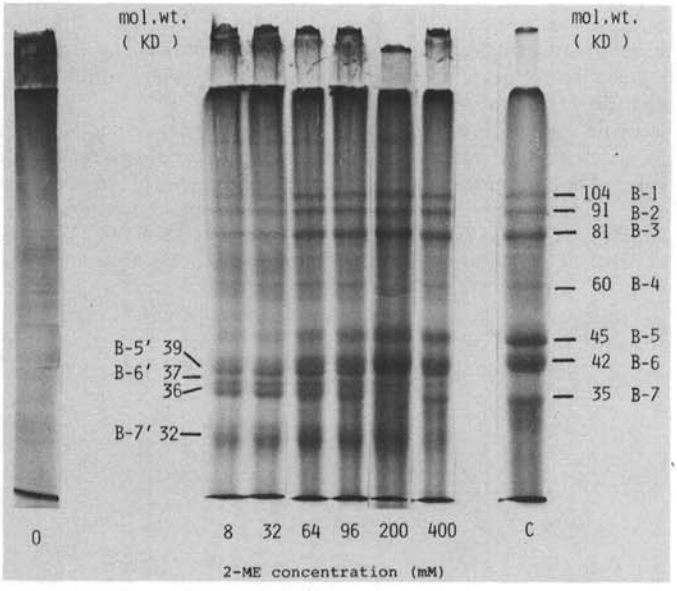

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of glutenin partially reduced with various concentrations of 2-mercaptoethanol (2-ME). Carboxymethylated glutenin, obtained by partial reduction with concentrations of 8–400 mM of 2-ME, was solubilized in SDS buffer and applied to SDS-PAGE without 2-ME. C = pattern of completely reduced carboxymethylated glutenin. 0 = 2-ME sample subjected to same conditions as others but without 2-ME.

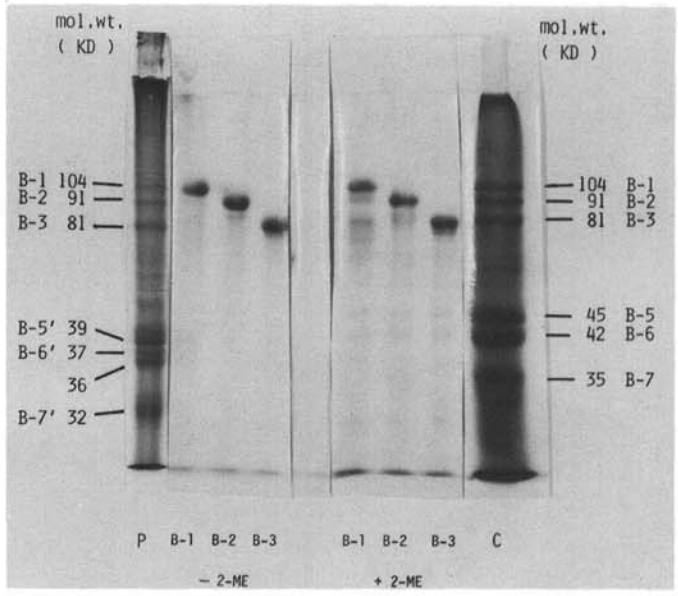

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of separated subunit polypeptides in high molecular weight region. Separated subunit polypeptides B-1, B-2, and B-3 were solubilized in SDS buffer and applied to SDS-PAGE. P = partially reduced carboxymethylated glutenin. C = completely reduced carboxymethylated glutenin. +2-ME and −2-ME = presence and absence of 2-mercaptoethanol, respectively.

(Figs. 2 and 3), we considered that partial reduction by 32 m*M* 2-ME attained selective cleavage of only interpolypeptide SS bonds of glutenin, because at 32 m*M* 2-ME, only B-5′, B-6′, and B-7′ were liberated with trace amounts of B-5, B-6, and B-7, but above 64 m*M* 2-ME, B-5′, B-6′, and B-7′ began to decrease with the increase in B-5, B-6, and B-7.

Separation of Glutenin Subunits Retaining Intrapolypeptide SS Bonds

Glutenin subunits retaining intrapolypeptide SS bonds were separated according to the procedure shown in Figure 4. Glutenin was partially reduced with 32 m*M* 2-ME under the conditions described. After carboxymethylation of liberated SH groups, carboxymethylated glutenin was applied to a preparative SDS-PAGE to separate subunits. Each band of subunit (B-1, B-2, B-3, B-5′, B-6′, and B-7′) was cut from the preparative gel, and subunit polypeptides were extracted from the gels by electric extraction. The homogeneity of each extracted polypeptide (Fig. 4) was checked by SDS-PAGE.

HMW bands B-1, B-2, and B-3 were well separated and did not change their mobilities on SDS-PAGE after complete reduction (Fig. 5). The separation of LMW bands (B-5′, B-6′, and B-7′) was well attained (Fig. 6). By complete reduction, B-5′, B-6′, and B-7′ decreased their mobilities and shifted to the positions of B-5, B-6, and B-7, respectively (Fig. 6). These results support our previous interpretation that B-5′, B-6′, and B-7′ have uncleaved intrapolypeptide SS bonds and are the same subunits as B-5, B-6, and B-7, respectively.

In contrast to LMW subunits, HMW subunits B-1, B-2, and B-3 did not change their mobilities after complete reduction. We think that B-1, B-2, and B-3, liberated at 32 m*M* 2-ME, contain intrapolypeptide SS bonds, but the contribution of intrapolypeptide SS bonds to their conformation is not as great as that in LMW subunits. Accordingly, the shape of the SDS (HMW subunits) complex must be similar among subunits with and without intrapolypeptide SS bonds, resulting in the same electrophoretic mobilities regardless of the absence or presence of 2-ME. A small amount of bands other than the subunit bands was observed in the presence of 2-ME (Figs. 5 and 6). This indicates that each band liberated by partial reduction contained a small amount of other subunits polymerized through SS bonds. However, densitometric determination showed that the content of the other contaminated subunits was usually below 5% (data not shown).

TABLE I
Amino Acid Compositions of Subunit Polypeptides of Carboxymethylated Glutenin and S-Pyridylethyl Glutenin[a]

	B-1	B-2	B-3	B-5′	B-6′	B-7′
Lysine	9.3	9.2	7.1	4.6	4.4	3.7
Histidine	10.1	9.9	11.2	4.4	4.5	4.2
Arginine	19.1	16.0	13.3	8.3	7.7	7.3
Aspartic acid	17.1	18.0	13.3	8.3	7.7	7.3
Threonine	25.4	22.7	19.1	9.7	8.7	8.0
Serine	64.7	66.3	51.9	28.4	26.0	17.9
Glutamic acid	253.9	208.8	205.9	108.2	100.1	76.3
Proline	112.0	87.5	53.7	55.6	50.7	43.7
Glycine	134.8	112.1	93.5	23.1	20.3	14.3
Alanine	31.4	28.3	23.0	11.7	12.0	9.1
Valine	25.3	20.7	19.7	17.6	17.5	14.2
Methionine	3.7	2.5	2.8	3.1	3.7	3.2
Isoleucine	17.9	15.6	14.4	13.2	14.6	12.9
Leucine	47.3	35.8	34.7	30.6	27.8	22.2
Tyrosine	33.2	28.5	23.5	6.1	6.2	6.4
Phenylalanine	17.4	15.4	15.2	12.4	11.4	9.8
CM-Cysteine[b]	1.8	1.6	1.9	1.7	2.2	2.0
PE-Cysteine	7.0	4.7	4.3	4.2	4.7	5.1

[a] Values are expressed as residual numbers per mole protein. Values are the averages of duplicate. Standard error is within 5%.
[b] CM-cysteine and PE-cysteine represent SH groups involved in interpolypeptide and intrapolypeptide SS bonds, respectively.

Determination of Interpolypeptide and Intrapolypeptide SS Bonds in Subunit Polypeptides

The preceding results guaranteed selective cleavage of interpolypeptide SS bonds and good separation of subunits retaining uncleaved intrapolypeptide SS bonds. Therefore, based on the operational definition of accessibility to reducing agents, we tried to determine the number of interpolypeptide and intrapolypeptide SS bonds in separated subunit polypeptides according to the procedure shown in Figure 4.

Separated subunits B-1, B-2, B-3, B-5′, B-6′, and B-7′ (represented with asterisks in Fig. 4) were completely reduced with 2-ME in the presence of 8*M* urea at pH 8.0 and liberated SH groups were pyridylethylated with vinylpyridine, followed by 6*N* HCl hydrolysis to determine CM-Cys and PE-Cys by amino acid analysis. Thus, in this procedure, CM-Cys and PE-Cys represent cysteine residue involved in SS bonds of interpolypeptide and intrapolypeptide, respectively. Amino acid composition of each subunit polypeptide is shown in Table I. Values are given as amino acid residual numbers per mole of protein subunit. All subunits had 2 moles of CM-Cys per mole subunit. In contrast, B-1 had 7.0 moles of PE-Cys and other bands had 4–5 moles of PE-Cys per mole subunit. Glutenin had no free SH groups (data not shown). Hence, the numbers of CM-Cys and PE-Cys (Table I) indicate that each subunit contains two interpolypeptide SS bonds in common and two to three intrapolypeptide SS bonds as shown in Figure 7.

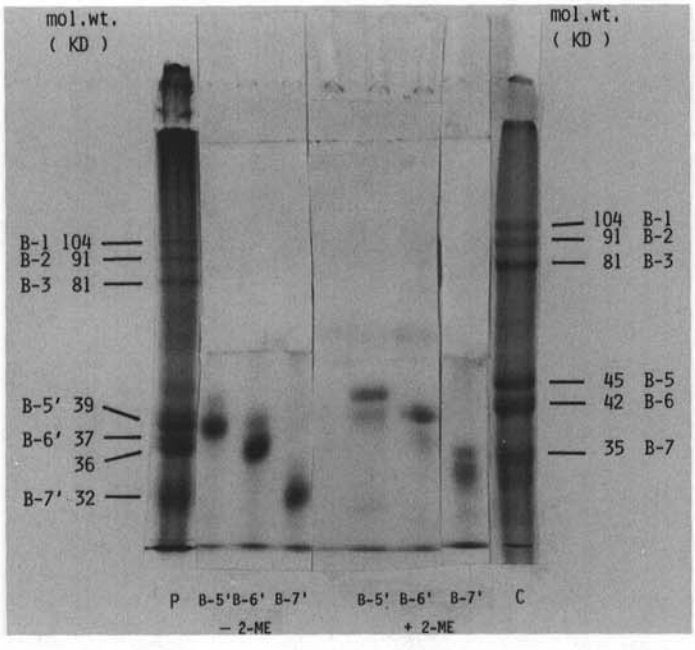

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of separated subunit polypeptides in low molecular weight region. Separated subunit polypeptides B-5′, B-6′, and B-7′ were solubilized in SDS buffer and applied to SDS-PAGE. P = partially reduced carboxymethylated glutenin. C = completely reduced carboxymethylated glutenin. +2-ME and −2-ME = presence and absence of 2-mercaptoethanol, respectively.

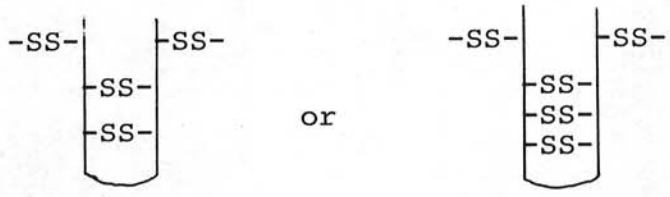

Fig. 7. Schematic drawings of disulfide bonds of glutenin subunit. **Left,** subunit with two intrapolypeptide disulfide bonds (4 moles cysteine residues per mole subunit protein). **Right,** subunit with three intrapolypeptide disulfide bonds (6 moles cysteine residues per mole subunit protein).

The results clearly demonstrate that glutenin is a polymer of several subunit polypeptides linked "linearly" through two interpolypeptide SS bonds as shown in Figure 1A. Each subunit has two to three intrapolypeptide SS bonds, namely 3.5 for B-1; 2.5 for B-2, B-6′, and B-7′; and 2.0 for B-3 and B-5′. Each subunit should have the integral number of intrapolypeptide SS bonds since no free SH groups are present in glutenin. Nevertheless, nonintegral numbers such as 3.5 and 2.5 were obtained in the separated subunits. Two possible explanations for such nonintegral numbers are: first, the bands (subunits) separated by SDS-PAGE may contain polypeptides with different numbers of intrapolypeptide SS bonds, though their molecular weights (mobility on SDS-PAGE) are the same. Second, fractions of separated subunits, especially HMW subunits, may contain not only monomer but also dimer or trimer subunits, which consist of other LMW subunits linked through SS bonds (Figs. 5 and 6), since partial reduction may produce dimer or trimer. For example, if 90% of the B-2 fraction is monomeric with 2 moles of intrapolypeptide SS bonds and 10% is dimeric, B-2 would have and average of 2.3 moles of intrapolypeptide SS bonds by the following equation:

$$\text{Avg. no. of intrapolypeptide SS bonds of B-2} = \frac{2 \text{ moles} \times 90\% + 5 \text{ moles} \times 10\%}{100\ (\%)} = 2.3 \text{ moles.}$$

Some amino acid compositions of the present subunits differ from our previous data (Matsumura et al 1984b). The previous subunits obtained at pH 6.0 probably were minor components or a mixture of dimeric or trimeric subunits with the same molecular weights on SDS-PAGE as those of the present subunits, because they were obtained under a heavily aggregated condition at pH 6.0. The subunits prepared by the reduction in a clear solution at pH 4.0 may represent the true subunits.

The present study demonstrates that glutenin consists of several kinds of subunits linked linearly through interpolypeptide SS bonds. However, this does not always imply that glutenin molecules take a linear stretched form in a hydrated state or in solution. Glutenin subunits are thought to interact strongly with others in the same molecule through noncovalent interaction (hydrophobic interaction and hydrogen and ionic bonds) and associate densely in glutenin molecules. Further such association is thought to occur also between glutenin molecules. Owing to such intramolecular and intermolecular associations, which may be a main cause for viscoelasticity of glutenin, glutenin is considered to take the complicated three-dimensional structure. The mode of association among glutenin subunits or among glutenin molecules is thought to be governed by the nature of the subunits. In this study, we attained selective cleavage of interpolypeptide SS bonds. We are now trying to separate glutenin subunits in a native state in the absence of SDS or other denaturants, to enable us to study the nature of glutenin molecules.

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