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Disaggregation and Reaggregation of Gluten Proteins by Sodium Chloride

TOMOYO UKAI,[†,‡] YASUKI MATSUMURA,[§] AND REIKO URADE*[,†]

Division of Food Science and Biotechnology and Division of Agronomy and Horticultural Science, Graduate School of Agriculture, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

This study showed that gluten proteins were extracted with distilled water from dough prepared in the presence of NaCl. To elucidate the interrelationship of NaCl and gluten proteins in dough, the extracted proteins were characterized. These proteins were primarily found to be soluble gliadin monomers by N-terminal amino acid sequencing and analytical ultracentrifugation. Extracted proteins were aggregated by the addition of NaCl at concentrations of >10 mM. A decrease in β-turn structures, which expose tryptophan residues to an aqueous environment in the presence of NaCl, was revealed by Fourier transform infrared analysis and scanning of fluorescence spectra. In addition, cross-linking experiments with disuccinimidyl tartrate showed that a large amount of protein was cross-linked in the dough only in the presence of NaCl. These results suggest that both interactions and distances between proteins were altered by the addition of NaCl.

KEYWORDS: Gluten; gliadins; protein aggregation; salt; cross-linking

INTRODUCTION

The addition of NaCl to dough influences not only the taste of bread but also loaf volume and crumb structure. These effects may result from the action of salt on both yeast and wheat flour components. NaCl increases gas retention time, which is responsible for optimal dough development and dough stability (*1–5*). These changes are assumed to be the results of changes in the interactions between gluten proteins. Gluten is formed with glutenins, which are polymerized via intermolecular disulfide bonds (*6–8*), and gliadins, which are non-covalently associated with each other (*9, 10*). NaCl is presumed to cause changes in the non-covalent interactions of these proteins. Several studies have shown that the extractability of proteins is modified by the addition of salts (*11–13*). Preston demonstrated that in salt concentrations between 500 mM and 4 M, gluten protein extractability is dependent upon anion type and follows the chaotropic anion series in increasing order (*11*). Antichaotropic salts, such as NaCl from 50 mM to 4 M, have been shown to reduce protein extractability compared to extractability in water. On the other hand, gluten strength is increased by the addition of metal chloride salts in accordance with the charge density of the metal ions (*4*). However, the details of the mechanisms that underlie the interactions between salt and gluten proteins remain unknown.

Gliadins, which are the most abundant proteins in wheat flour, are thought to have extremely low solubility in water and neutral buffers (*9*). Because gliadins are soluble in alcohol−water mixtures and acidic solutions such as diluted acetic acid and HCl, most studies on extracted gliadins have been performed in such solutions. However, it is unclear whether alcohol- or acid-solubilized gliadins have the same properties as those in dough. Clements discovered that approximately 60% of gluten proteins can be extracted from gluten homogenized in a NaCl solution by washing with water (*14*). Fu et al. and Sapirstein et al. also demonstrated that gliadins and glutenins, extracted from gluten prepared using a NaCl solution by washing with water, and the amount of extractable proteins were different between cultivars depending on the molecular size of the glutenins (*15, 16*). The proteins extracted into water from the dough prepared using a NaCl solution are predicted to have properties similar to those in dough containing NaCl. However, a detailed analysis has not been performed. In this study, we analyzed the salt sensitivity of extracted proteins as to their solubilities and secondary structures. In addition, we performed cross-linking experiments to examine differences in intermolecular interactions of gluten proteins in dough in both the presence and absence of NaCl.

MATERIALS AND METHODS

Materials. Spring wheat flour (Super King) (13.8% protein, 0.42% ash, and 14% water) was obtained from Nisshin Flour Milling, Inc. (Tokyo, Japan). Dithiobis[succinimidyl propionate] [DSP (12.0)], disuccinimidyl glutarate [DSG (7.7)], and disuccinimidyl tartrate [DST (6.4)] were purchased from Pierce Biotechnology, Inc. (Rockford, IL). All other reagents were of analytical grade.

* Author to whom correspondence should be addressed (telephone +81-774-38-3757; fax +81-774-38-3758; e-mail urade@kais.kyoto-u.ac.jp).

† Division of Food Science and Biotechnology.

‡ Present address: Department of Food Science and Nutrition, Doshisha Women's College of Liberal Arts.

§ Division of Agronomy and Horticultural Science.

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Preparation of Dough. Dough was prepared from 100 g of wheat flour and 67 mL of distilled water with or without salt (0.255 or 0.51 M). The ingredients were mixed and kneaded for 20 min with a mixer, KN-200 (Taisho Denki, Tokyo, Japan).

Extraction of Gluten Protein from Dough. The dough was subjected to six or eight sequential extractions with 500 mL of distilled water or 0.51 M NaCl for 10 min at room temperature (25 °C) with the mixer. After each extraction, the supernatant obtained by centrifugation (18000*g*, 10 min) was decanted. The concentrations of proteins in the supernatant were measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) using γ-immunoglobulin as an internal standard. The concentrations of NaCl in the insoluble residue obtained after each extraction step were calculated from the sodium value obtained by atomic absorption spectrophotometry analysis of the insoluble residue. For analytical ultracentrifugation, far-UV circular dichroism (CD) spectroscopy, salt sensitivity experiments, Fourier transform infrared (FT-IR) measurements, and fluorescence spectroscopy, 50 mL of the third distilled water extract from the dough prepared in the presence of NaCl was centrifuged for 10 min at 18000*g*. The protein solution was dialyzed twice against 3 L of distilled water overnight at 4 °C. The proteins appeared to be completely soluble.

Electrophoretic Analysis. Proteins in dough or proteins extracted in either distilled water or 0.51 M NaCl were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (*17*) and stained with Coomassie Brilliant Blue R-250. Prior to N-terminal amino acid sequencing, proteins were blotted onto a poly(vinylidene difluoride) membrane after SDS-PAGE and stained with Ponceau S. N-Terminal amino acid sequencing of each peptide was carried out using a Protein Sequencer model 492 (Applied Biosystems, Foster City, CA).

Analytical Ultracentrifugation. The molecular masses of the extracted proteins were determined by sedimentation equilibrium using an ultracentrifuge CP100a (Hitachi Koki, Tokyo, Japan). The sample was subjected to analysis unit ABS8 and centrifuged in a rotor A60 M for 28 h at 10000 rpm at 20 °C. Log absorbance at 280 nm versus r^2 was plotted to obtain a line with a slope that equals $M(1 - \bar{v}\rho)$ in the equation $d\ln(Ar)/dr^2 = M(1 - \bar{v}\rho)w^2/2RT$, where Ar = absorbance of the protein solution at 280 nm at any radial distance, r = radial distance, M = molecular weight of solute, $\bar{v}$ = partial specific volume of solute, ρ = density of solution, w = rotational velocity in radians/s, R = gas constant, and T = absolute temperature (*18*). The molecular mass was calculated using 0.69–0.75 cm^3/g as the $\bar{v}$ value for the protein (*19*).

Far-UV CD Spectra Analysis. CD spectra were measured using a J-720 spectropolarimeter (JASCO Corp., Tokyo, Japan) in a 1 mm path length cell at 23 °C.

Turbidity. Salt was added and dissolved in the 0.27 mg/mL protein solution at the indicated concentrations. The turbidity of the solution was determined from its absorbance at 600 nm.

FT-IR Measurements and Data Treatment. The FT-IR transmission spectra were recorded on a FT-IR-480 Plus spectrophotometer (JASCO Corp.). The spectra of the protein solution without salt (37 mg/mL) or the protein suspension (37 mg/mL) with 0.51 M NaCl were measured using a cell equipped with two CaF_2 windows. For each spectrum, 50 scans were collected at a resolution of 4 cm^{-1}.

For determination of the secondary structure of the proteins, deconvolution of each spectrum was performed using JASCO FT-IR software according to the methods of Fourier self-deconvolution (*20*) and the finite impulse response operator (*21*). The spectra were analyzed by second derivatization (*22, 23*) and Gaussian curve fitting (*24, 25*) in the amide I region (1600–1700 cm^{-1}). The secondary structure was determined from the relative areas of the individual assigned bands in the amide I region. The assignment of individual components to the secondary structure was done as previously described (*26*). The results were expressed as means ± SEM. The statistical significance of difference was determined by the unpaired Student's t test.

Fluorescence Spectroscopy. Fluorescence measurements were performed on a Hitachi F-3000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with front-fluorescence equipment. The emission spectra of the protein solution (1 mg/10 mL) with or without 0.51 M NaCl were measured in the 300–400 nm wavelength region, with excitation at 280 nm.

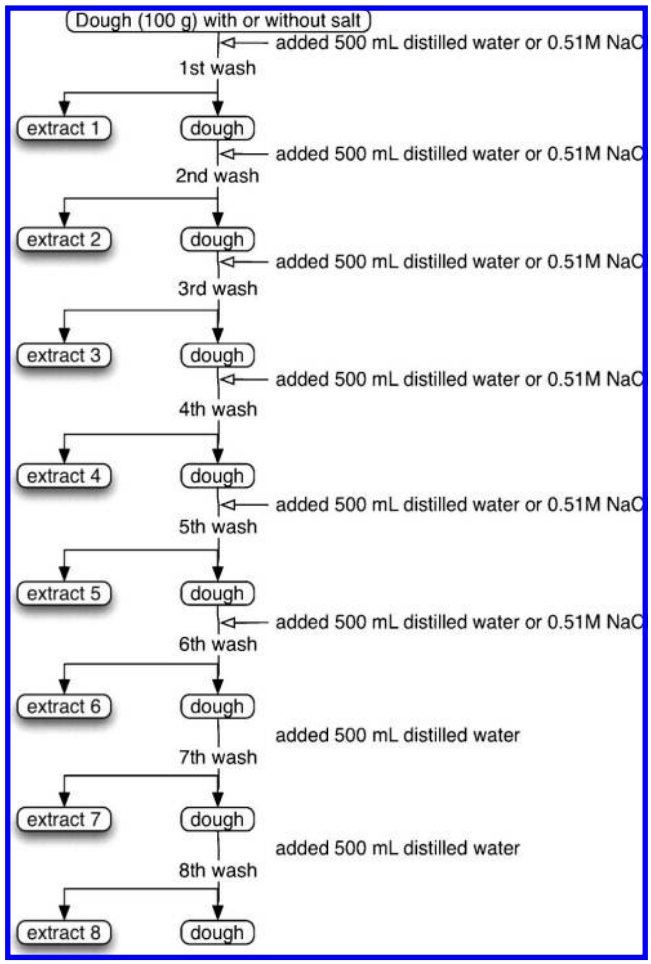
Cross-Linking Experiments. Dough was prepared using 100 g of wheat flour with either distilled water or 0.51 M NaCl. A piece of dough without or with NaCl (25 mg) was removed into 0.5 mL of 2.5 mM DSP (12.0), DSG (7.7), or DST (6.4) in either distilled water or 0.51 M NaCl and incubated for 24 h at room temperature. SDS sample buffer (175 μL of a 4× stock) (*17*) with or without β-mercaptoethanol was added to the reaction mixture. The piece of dough was resuspended by sonication and boiled for 5 min. The suspension was centrifuged at 18000*g* for 5 min at room temperature. To cleave the spacer arm of DST (6.4), the suspension (50 μL) was centrifuged again at 100000*g* at 20 °C for 1 h. The pellet was resuspended in 37.5 μL of 5 mg/mL sodium *m*-periodate in 0.1 M sodium phosphate buffer, pH 7.0, and then incubated for 30 min at room temperature in the dark. After the reaction, 12.5 μL of 4× SDS sample buffer with β-mercaptoethanol was added. The sample (10 μL/well) was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). SE-HPLC was performed according to the modified method described by Skerritt et al. (*27*). Briefly, 25 mg of dough prepared using 0.51 M NaCl was treated with DST (6.4) as described above. The dough was resuspended in 675 μL of SDS sample buffer with or without β-mercaptoethanol and boiled for 5 min. The solution was centrifuged for 7 min at 18000*g* at 4 °C. The obtained supernatant (50 μL) was subjected to a TSK G4000 gel filtration column (Tosoh, Tokyo, Japan) equilibrated with 0.1% (v/v) trifluoroacetic acid and 50% (v/v) acetonitrile in water. The exclusion size of the gel filtration column is $M_r = 7 \times 10^6$. Chromatography was performed at a flow rate of 0.4 mL/min at 4 °C. The detection wavelength was 214 nm. Fractions (each 0.4 mL) were collected, freeze-dried, and subjected to SDS-PAGE under reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250.

RESULTS

Extraction of Proteins in Distilled Water from Dough Prepared with NaCl. When the dough, which was prepared with either distilled water or 0.51 M NaCl solution, was washed twice with distilled water, either 1 or 1.2 g of protein was eluted into the extract, respectively (extracts 1 + 2 in **Scheme 1**). It was shown that the number and intensity of protein bands on SDS-PAGE gel were almost the same between the doughs (**Figure 1**, lanes 1 and 7). Abundant proteins with molecular masses between 30 and 50 kDa were extracted from the dough, which was prepared with the NaCl solution, by the third washing (extract 3 in Scheme 1; **Figure 1**, lane 9), whereas little protein was extracted from the dough that was prepared without NaCl by additional washing (lane 3). Amounts of the proteins extracted by washing with distilled water depended on the concentration of NaCl, which had been used for the preparation of dough. Thus, 1.38 and 3.7 g of protein were extracted from the doughs, which were prepared with 0.255 M NaCl and 0.51 M NaCl, between the third and sixth washings (extracts 3 + 4 + 5 + 6 in **Scheme 1**), respectively. No additional protein was extracted from either dough by additional washes (data not shown). Similar results were obtained for dough prepared with delipidated wheat flour (data not shown) (*28*). If NaCl solution was used for washing, proteins were barely extracted, even from dough that was prepared with NaCl, between the third and sixth washings (**Figure 1**, lanes 15–18). When the dough was washed four times with distilled water after washing four times with the NaCl solution, abundant proteins were extracted (lanes 23–26). Taken together, the elution of proteins from the dough prepared with NaCl appeared to depend on the concentration of the residual NaCl in the dough. Subsequently, the amount of residual NaCl in the dough after each washing with distilled water was measured. After three washings, almost all NaCl was removed from the dough. Thus, NaCl content was decreased from 1220 to 5.3 mg of NaCl/100 g of dough by three washings.

Scheme 1. Extraction Procedures of Gluten Proteins from Dough



Extraction of proteins by washing with distilled water from dough supplemented with other monovalent salts was then determined. Among the counterions of sodium salt, the chaotropic ions I^- and Br^- showed much stronger effects than that of Cl^- (**Table 1** and Supporting Information Figure S1). Thus, 1.16- and 3.15-fold of proteins were extracted from the dough with Br^- and I^- by third and sixth washings, respectively. The addition of $NaCH_3COO$ showed no effect on the extraction of proteins between the third and sixth washings. In the case of the counterions of chloride salt, a consecutively higher proportion of proteins was extracted from the dough with each salt in the sequence, $Na^+ > K^+ > NH_4^+ > Li^+$. The sequence did not follow the chaotropic series ($Li^+ > Na^+ > K^+ > NH_4^+$). The compositions of proteins extracted from the dough with the respective salt between the third and sixth washings were almost the same (Supporting Information Figure S1).

Analysis of the Proteins Extracted from the Dough That Was Prepared with NaCl by Washing with Distilled Water. The proteins extracted from dough and the proteins residing in the dough after washing with distilled water were analyzed by reducing SDS-PAGE. The proteins with molecular masses between 30 and 50 kDa were predominantly extracted, and the high molecular weight glutenin subunits (HMW-GSs) with molecular masses between 76 and 120 kDa resided in the dough (**Figure 2a**, lanes 2 and 3). After nonreducing SDS-PAGE, proteins with molecular masses between 30 and 40 kDa were detected in the extract (lane 5); only small amounts of proteins were detected in the dough (lane 6). Because HMW-GSs and low molecular weight glutenin subunits (LMW-GSs) form polymers linked by inter-disulfide bonds (*6–8*), they were unable to enter into the resolving gel under nonreducing conditions. On the other hand, gliadins (30–40 kDa monomers) were resolved by nonreducing SDS-PAGE. Therefore, the major proteins eluted in distilled water are presumed to be gliadins. The N-terminal amino acid sequences of the major protein bands were then analyzed. All of the N-terminal sequences obtained corresponded to those of α/*β*-gliadin or *γ*-gliadin (*29–31*) (**Figure 2b**). We analyzed the N-terminal sequence of the 65 kDa band, which appears likely to be an *ω*-gliadin. However, the amino acid residue could not be detected. Perhaps the N terminus of the 65 kDa band was blocked.

Gliadins are known to be soluble in alcohol−water mixtures, but insoluble in water and neutral buffers. However, the proteins extracted with distilled water were seemingly soluble in water. The extract was subjected to ultracentrifugation to precipitate insoluble material. No proteins in the extract were precipitated by ultracentrifugation (**Figure 3a**). In addition, the proteins were not precipitated after dialysis against distilled water, which removed trace amounts of NaCl from the washing solution (data not shown). The molecular masses of the extracted proteins were determined to be between 30000 and 35000 Da by sedimentation equilibrium using an ultracentrifuge (**Figure 3b**). These results clearly show that major proteins in the extract are gliadin monomers, which become soluble in distilled water. Hereafter, these proteins were referred to as gliadin-rich proteins. The pH of the extract was 5.8 due to air dissolved in water. When the pH became 7 after degassing under vacuum, the proteins were still soluble in water (data not shown).

The CD spectra of gliadin-rich proteins in distilled water are shown in **Figure 3c**. The prediction of their secondary structure indicated that these proteins are composed of 14% α-helix. However, the assignments of *β*-structures, aperiodic structures, and polyproline II/random coil, etc., are difficult from the CD spectram from a mixture of gliadins (*32–34*). Gliadin-rich proteins were very thermostable proteins. No thermal denaturation was observed via differential thermal analysis between 30 and 110 °C (Supporting Information Figure S2). α/*β*-Type and *γ*-type gliadins have a few segments of α-helical secondary structure, but they apparently do not undergo a hydrophobic core collapse. This may explain why they do not show enthalpic peaks in DSC.

Aggregation of Gliadin-Rich Proteins by Salts. Gliadin-rich proteins were instantaneously aggregated by the addition of NaCl (**Figure 4**). Aggregation of the proteins was initiated between 5 and 10 mM NaCl and was dramatically increased between 10 and 30 mM NaCl. At NaCl concentrations between 30 mM and 1 M, aggregation gradually increased. Chaotropic salts at low concentrations induced aggregation more effectively than NaCl. Thus, either 20 mM NaI or 20 mM NaBr caused the aggregation of gliadin-rich proteins similar to 30–40 mM NaCl (**Figure 4a**). However, the aggregation of gliadin-rich proteins dramatically decreased at concentrations of NaI >200 mM and gradually decreased at concentrations of NaBr >200 mM (**Figure 4c**). $NaCH_3COO$, which had little effect on the extraction of gliadin-rich proteins from dough (**Table 1**), also showed a potent aggregation effect (**Figure 4a**). KCl, LiCl, and NH_4Cl showed aggregation effects similar to those of NaCl (**Figure 4b**,**d**). The pH value is known to affect protein solubility. However, no correlation between the pH of the salt solutions and the effects of salt on protein solubility was observed. It is possible that the addition of salt may induce secondary changes in gliadin-rich proteins that result in aggregation. To examine this possibility, the secondary structures of gliadin-rich proteins were determined in the absence or

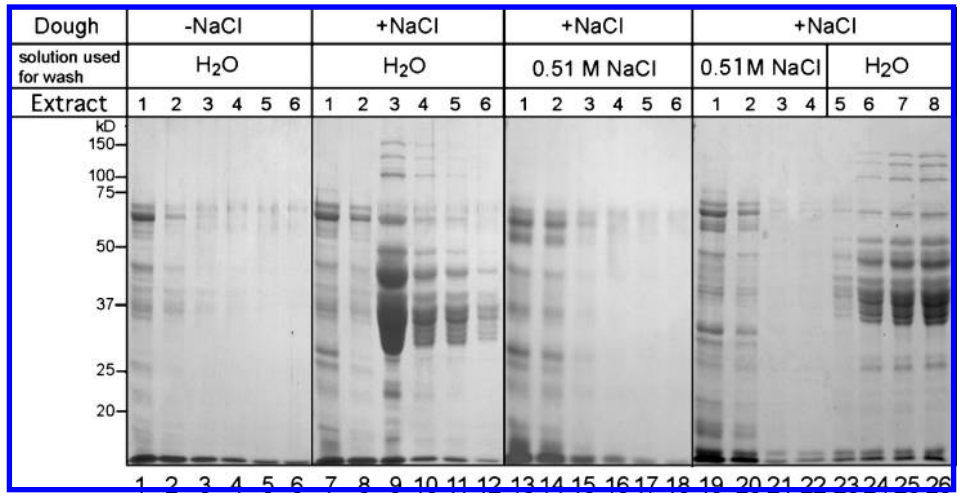


Figure 1. Extraction of gluten proteins from dough by washing. A flowchart of procedures is shown in **Scheme 1**. Dough was prepared without (−NaCl) (lanes 1–6) or with NaCl (+NaCl) (lanes 7–26) and washed six times with distilled water (lanes 1–12) or 0.51 M NaCl (lanes 13–18) or sequentially washed four times with 0.51 M NaCl (lanes 19–22) and four times with distilled water (lanes 23–26). Each extract (7.1 μL) was subjected to SDS-PAGE under reducing conditions. Proteins were stained with Coomassie.

Table 1. Amounts of Protein Extracted from Dough Prepared with or without Salt by Washing with Distilled Water[a]

	amount of protein eluted from dough (%)		
salt added to dough	extracts 1 and 2	extracts 3–6	total (extracts 1–6)
NaCl (0.255 M)	100 ± 1.4 [1.2 g]	100 ± 2.4 [1.38 g]	100 [2.58 g]
NaCl (0.51 M)	100 ± 0.0 [1.2 g]	270.9 ± 0.1 [3.7 g]	191.6
NaI (0.255 M)	97.6 ± 4.8	315.8 ± 5.2	214.4
NaBr (0.255 M)	93.2 ± 3.3	116.1 ± 1.6	105.5
$NaCH_3COO$ (0.51 M)	79.2 ± 1.5	4.2 ± 0.0	39.1
KCl (0.255 M)	100.0 ± 1.0	62.8 ± 1.5	80.1
NH_4Cl (0.255 M)	104.1 ± 2.2	56.9 ± 1.2	78.9
LiCl (0.255 M)	90.5 ± 1.2	33.2 ± 0.2	59.8
none	76.3 ± 0.9 [1 g]	15.9 ± 1.6	44.0

[a] The dough was prepared with water (none) or with either a 0.255 or 0.51 M salt solution. The dough was then washed six times with distilled water. The first and second extracts were combined as were the third, fourth, fifth, and sixth extracts. The amount of proteins in the combined extracts was analyzed. Values were calculated as a percentage of the total amount of proteins extracted from the dough prepared with the 0.255 M NaCl solution. The results are shown as the mean ± SEM for three experiments. Values in brackets are the amount of protein eluted from the dough after washing.

presence of NaCl by FT-IR. The spectra in the amide I region were deconvoluted. Band fitting with Gaussian band shapes was performed on the deconvoluted spectra to estimate secondary structure content. Fourier-deconvoluted amide I bands, curve-fitted with the sums of Gaussian bands of water-soluble gliadin-rich proteins in the absence or presence of 0.51 M NaCl, are shown in **Figure 5a**,**b**, respectively. **Figure 5c** shows the relative areas of the bands, which are assigned to the structural components as described previously (*26*). Differences in the compositions of the secondary structures of gliadin-rich proteins in the absence or presence of NaCl were insignificant. Only β-turns (1668–1671 cm^{-1}) were significantly decreased by the addition of NaCl.

The fluorescence emission spectra of gliadin-rich proteins were analyzed in the absence or presence of NaCl. The maximum emission wavelength for gliadin-rich proteins without salt was 338 nm, whereas the wavelength in the presence of 0.51 M NaCl shifted to 344 nm (**Figure 6**), indicating that the tryptophan residues were exposed to a polar aqueous environment in the presence of NaCl. In addition, the fluorescence intensity of the spectrum increased by the addition of NaCl.

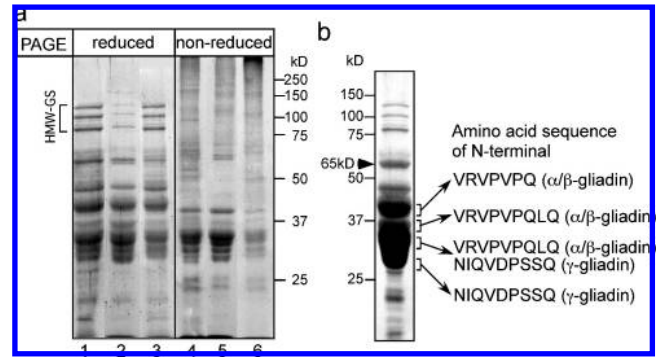


Figure 2. Major proteins extracted from dough prepared with NaCl by washing with distilled water. (**a**) Untreated dough (lanes 1, 4), washed dough [three times with distilled water (lanes 3 and 6)], and extract obtained from dough by third washing with distilled water (lanes 2 and 5) were subjected to SDS-PAGE under reducing (lanes 1–3) or nonreducing condition (lanes 4–6). Proteins were stained with Coomassie. (**b**) The proteins extracted by the third washing with distilled water were separated by SDS-PAGE under reducing conditions and blotted on a PVDF membrane. Amino-terminal amino acid sequences of the primary bands were analyzed using a protein sequencer. The amino acid sequences are shown on the right of the gel image. Proteins predicted from the amino acid sequence are indicated in parentheses.

The fluorescence spectra are likely quite strongly affected by the energy transfer from the tyrosine to the tryptophan residues, and this energy transfer process may be responsible for the apparent change in intensity of the tryptophan side chains.

Effects of NaCl on Cross-Linking of Gluten Proteins. Gliadins may aggregate in dough because a concentration of NaCl in the dough prepared by the recipe used in this study was approximately 0.5 M. Generally, aggregated or associated proteins are cross-linked with a cross-linker using a spacer arm that is the approximate length of the intermolecular distance between the proteins. To examine the effects of NaCl on the distances between proteins aggregated in the dough, cross-linking experiments were carried out using three homobifunctional cross-linkers that have the same reactive group (*N*-hydroxysuccinimide, which reacts with primary amines) but different spacer arms.

DSP (12.0) is a cross-linker that possesses a 12.0 Å spacer arm that is cleavable by disulfide reducing agents such as

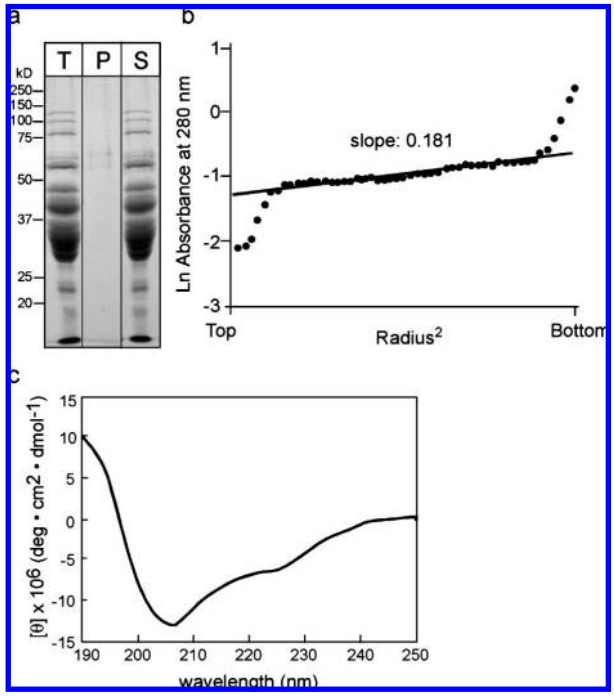


Figure 3. Extracted gliadins are monomers and soluble in water. (**a**) The extract obtained from dough prepared with 0.51 M NaCl after the third washing with distilled water was centrifuged at 100000*g* for 1 h at 4 °C. The extract (T), precipitate (P), and supernatant (S) obtained after centrifugation were all subjected to SDS-PAGE under reducing conditions. (**b**) Ultracentrifugation analysis of the extract obtained from the dough prepared with 0.51 M NaCl after the third washing with distilled water. Data were plotted as the natural log of the absorbance at 280 nm versus the radius^2. Molecular masses (30000−35000) were calculated from the slope of the plotted line (0.181) as described under Materials and Methods. (**c**) CD spectrum of the third extract obtained from the dough prepared with 0.51 M NaCl after the third washing with distilled water.

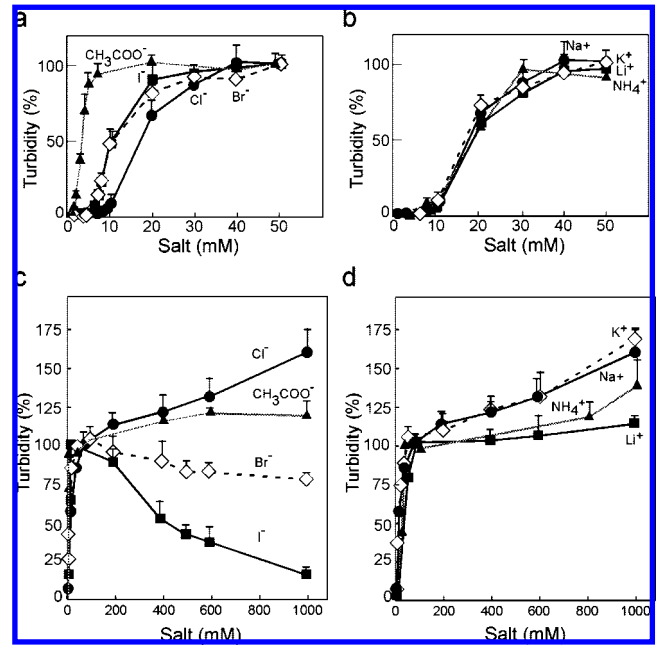


Figure 4. Effects of salts on the aggregation of water-soluble gliadin-rich proteins. (**a**, **c**) Effects of sodium salts of chloride (circle), iodine (square), bromine (rhomboid), and acetate (triangle). (**b**, **d**) Effects of chloride salts of sodium (circle), ammonium (triangle), lithium (square), and potassium (rhomboid). Salt was added to the gliadin-rich protein at concentrations of 0–50 mM (**a**, **b**) or 0–1000 mM (**c**, **d**). Data are expressed as the percent of the absorbance (0.613) obtained by the addition of 50 mM NaCl. The results are shown as the mean ± SEM for three experiments.

β-mercaptoethanol. After cross-linking, the proteins in the dough were analyzed by SDS-PAGE. Under conditions to reduce disulfide bonds, almost all of the protein bands were detected in the treated dough with or without DSP (12.0) (**Figure 7a**, lanes 1–4). Little difference in the intensity of the bands was observed between the dough prepared with or without NaCl. Only a 65 kDa band, which may be an ω-gliadin, disappeared in the dough treated with NaCl. After nonreducing SDS-PAGE, the intensities of all protein bands on the gel decreased after DSP (12.0) treatment (lanes 5–8). Decreases in the intensities of the protein bands in the dough treated with DSP (12.0) were interpreted as follows: The aggregated proteins were intermolecularly cross-linked to polymers, which could not enter the resolving gel under nonreducing conditions. The intensities of the protein bands were much more reduced in the dough with NaCl than without NaCl after treatment with DSP (12.0), suggesting that more proteins were cross-linked in the dough in the presence of NaCl.

DSG (7.7) is a cross-linker that possesses an uncleavable 7.7 Å spacer arm. Three HMW-GSs with the highest molecular masses in both doughs with and without NaCl as well as a 65 kDa band in the dough with NaCl disappeared after cross-linking with DSG (7.7) (**Figure 7b**, lanes 2 and 4). In addition, the intensities of all protein bands decreased after DSG (7.7) treatment under both reducing (**Figure 7b**, lanes 1–4) and nonreducing conditions (**Figure 7b**, lanes 5–8). Decreases in the intensities of the protein bands were much greater in the dough with NaCl than without NaCl, suggesting that more proteins were cross-linked in the dough with NaCl.

DST (6.4) is a cross-linker that possesses a 6.4 Å spacer arm that is cleavable after oxidation by sodium *m*-periodate. In dough without NaCl, the patterns and intensities of the protein bands under both reducing and nonreducing conditions were not influenced by DST (6.4) treatment (**Figure 7c**, lanes 1, 2, 5, and 6). In contrast, the intensities of all protein bands decreased after SDS-PAGE under both reducing and nonreducing conditions after DST (6.4) treatment in dough with NaCl (**Figure 7c**, lanes 3, 4, 7, and 8), suggesting that proteins were cross-linked only in the dough with NaCl.

These results suggest that cross-linking of proteins in dough may be dependent on the spacer arm length of cross-linkers used; that is, proteins are cross-linked in doughs to different extents with or without NaCl after either DSP (12.0) or DSG (7.7) treatment, whereas proteins are cross-linked in dough containing NaCl only after DST (6.4) treatment.

To identify which proteins were cross-linked by DST (6.4) treatment, the proteins were solubilized with SDS buffer in the absence or presence of reducing reagent from doughs with or without NaCl, which were treated with or without DST (6.4). Then, the solutions were subjected to SE-HPLC and each fraction was analyzed by SDS-PAGE. In dough without NaCl, both the chromatogram and eluted protein band profiles were nearly the same between the doughs treated with or without DST (data not shown). In the case of dough with NaCl, the levels of proteins solubilized with SDS buffer in the presence of reducing reagent decreased by treatment of dough with DST (6.4) (**Figure 8b**,**d**). No absorbance was detected in the void volume on the SE-HPLC chromatogram of cross-linked sample with DST (6.4) (**Figure 8c**). Hence, it was presumed that certain proteins cross-linked with DST (6.4) were insoluble in SDS and

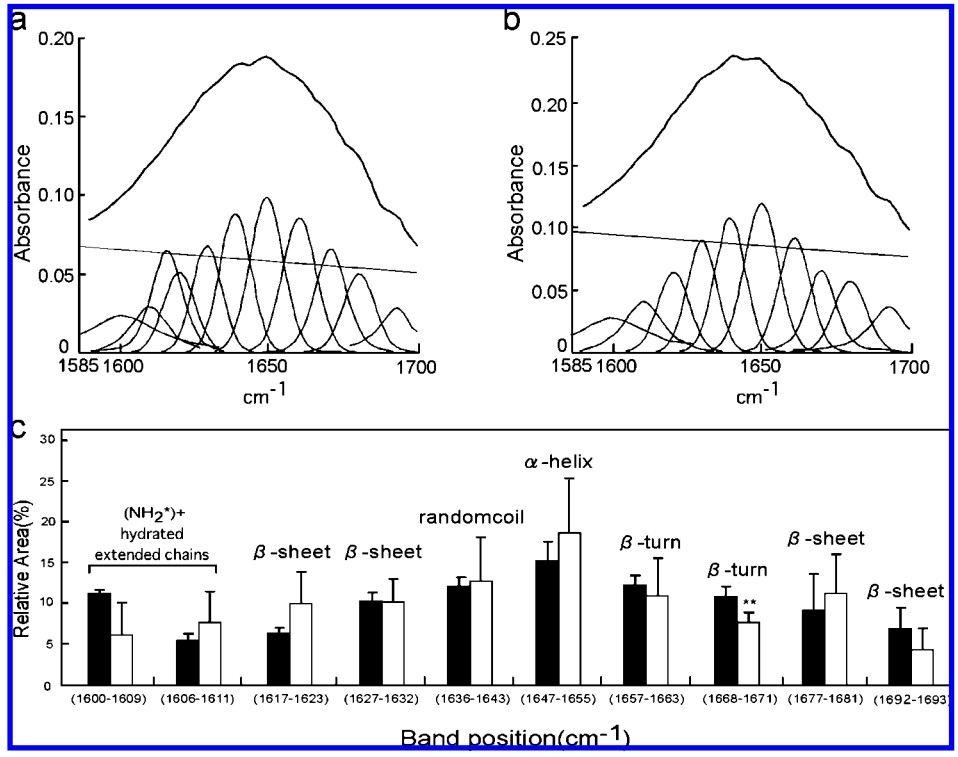


Figure 5. Representative Fourier-deconvoluted FT-IR spectra in the amide I region and reconstituted spectra after curve fitting of water-soluble gliadin-rich proteins: FT-IR analysis of water-soluble gliadin-rich proteins in the absence (**a**) or presence of 0.51 M NaCl (**b**). (**c**) Peak position and relative areas of bands fitted to the Fourier-deconvoluted spectra of water-soluble gliadin-rich proteins in the absence (black bars) or presence of NaCl (white bars). The results are shown as the mean ± SEM for four experiments.

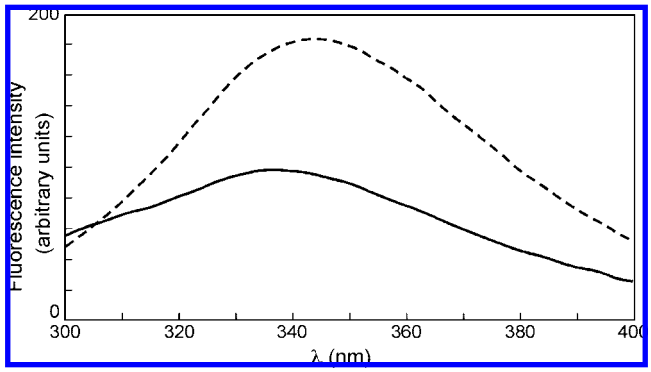


Figure 6. Fluorescence spectra of water-soluble gliadin-rich proteins in the absence (solid line) or presence (dotted line) of 0.51 M NaCl.

could not enter the SE-HPLC column. When the proteins were solubilized with SDS buffer in the absence of reducing reagent, glutenin polymers and gliadin monomers were separately eluted on SE-HPLC (**Figure 8f**). Both glutenin polymers and gliadin monomers eluted from the SE-HPLC column appeared to decrease by DST (6.4) treatment of dough (**Figure 8h**), suggesting that both gliadins and glutenins were cross-linked with DST (6.4) in dough with NaCl.

The extract with SDS in the presence of reducing reagent from dough treated with DST (6.4) was subjected to ultracentrifugation to precipitate the insoluble proteins. The precipitate was oxidized to cleave the spacer arms of DST (6.4) and then analyzed by SDS-PAGE (**Figure 7d**). Protein bands were detected only when the precipitate obtained from the dough with NaCl was oxidized, suggesting that certain proteins cross-linked with DST (6.4) in dough with NaCl became insoluble in SDS.

DISCUSSION

In this study, NaCl was found to have at least two different effects on the structure of gluten proteins during the formation of gluten. One effect is to make monomeric α/β-gliadins and γ-gliadins soluble in distilled water. The extraction of gluten proteins from gluten treated with NaCl into distilled water was initially described by Clements (*14*). Later, Fu et al. reported that both gliadins and glutenins were extracted from gluten that had been prepared with NaCl by washing with water (*15*). Both gliadins and glutenins are thought to be largely unstructured proteins with flexible conformations in solution. In wheat flour, inter- and, perhaps, certain intramolecular interactions of gliadins and gluteinins may stabilize their conformations to varying degrees. Clements found that washing of the gluten which had been treated with NaCl with distilled water resulted in the swelling of the gluten into a voluminous gel, indicative of the highly hydrophilic nature of the residual proteins (most likely glutenins) (*14*). Such changes in glutenins may be caused by weakening of the hydrophobic or electrostatic interactions between glutenins and gliadins, as well as by the release of gliadins from the gluten network. However, the extracted gliadins were aggregated in the presence of NaCl, suggesting that the interactions of gliadins with other proteins were disrupted and that the interactions between the gliadin proteins themselves were facilitated by the addition of NaCl.

Another effect of NaCl on the structure of gluten proteins during the formation of gluten is to induce the aggregation of gliadins. The aggregation of gliadin-rich proteins was induced by various salts, but the effects were salt species-dependent. Therefore, gliadin aggregation may be induced by the interaction of a certain ion with the specific amino acid residues of a given protein, but not by an increase in ionic strength. Because there was little difference in aggregation among chloride salts and large differences among sodium salts, it is presumed that the

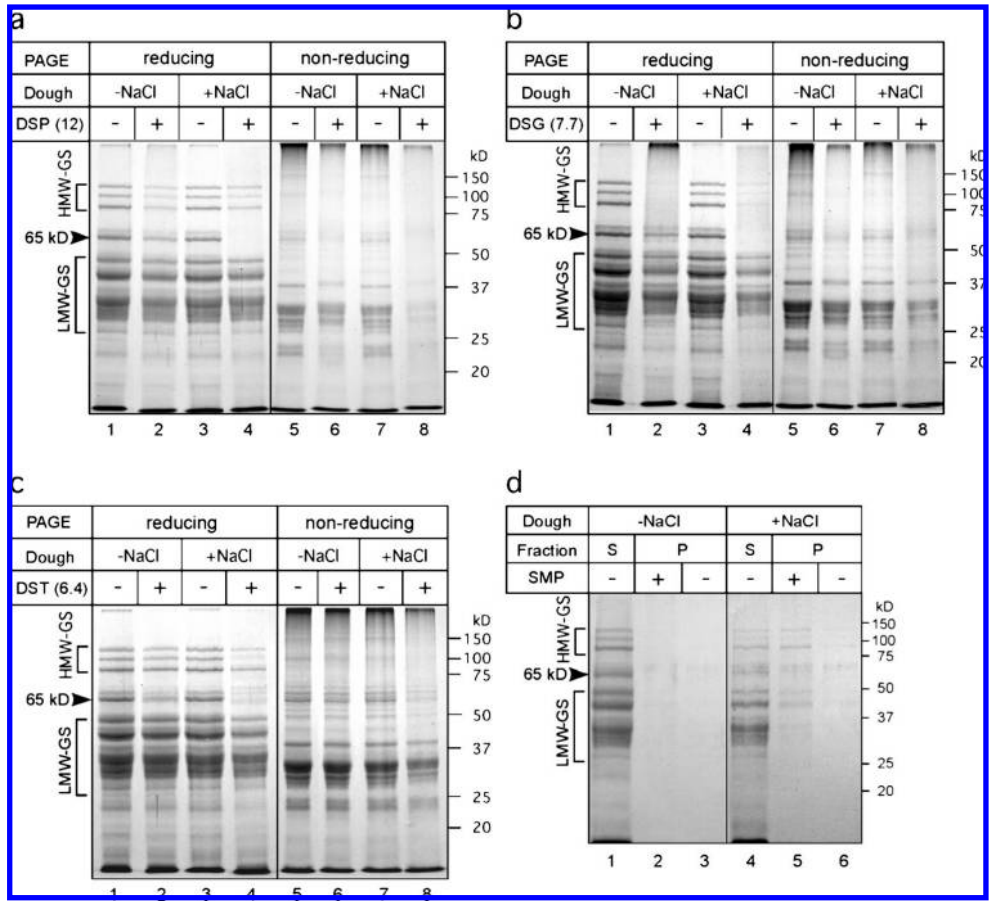


Figure 7. Effects of NaCl on the cross-linking of gluten proteins. (**a**−**c**) Dough prepared without (lanes 1, 2, 5, and 6) or with 0.51 M NaCl (lanes 3, 4, 7, and 8) was treated without (lanes 1, 3, 5, and 7) or with 2.5 mM DSP (12.0) (**a**, lanes 2, 4, 6, and 8), 2.5 mM DSG (7.7) (**b**, lanes 2, 4, 6, and 8), or 2.5 mM DST (6.4) (**c**, lanes 2, 4, 6, and 8). The treated dough (0.357 mg) was subjected to SDS-PAGE under reducing (lanes 1–4) or nonreducing conditions (lanes 5–8). (**d**) Dough prepared without (lanes 1–3) or with (lanes 4–6) 0.51 M NaCl was treated with 2.5 mM DST (6.4). After treatment, dough proteins were solubilized in SDS sample buffer containing β-mercaptoethanol. The SDS-insoluble proteins were separated from the soluble proteins (lanes 1 and 4) by ultracentrifugation and treated with sodium *m*-periodate (SMP) (lanes 2 and 5) or untreated (lanes 3 and 6). Each sample was subjected to SDS-PAGE under reducing conditions.

interactions of anions with specific amino acid residues may be necessary for aggregation. The properties of the salts obtained in this study may explain the results reported in a previous study (*11*) in which NaI had a potent effect on the extraction of gluten proteins at concentrations >500 mM, whereas NaCl had no effect over the same concentration range. Because NaI may disrupt the interactions of gliadins with other proteins and cause a very low aggregation effect at concentrations >300 mM, proteins were extracted with NaI solutions at concentrations >300 mM. On the other hand, because NaCl affected protein aggregation at high concentrations, proteins were not extracted even at a concentration of 1 M. Major intermolecular interactions that affect the aggregation of gliadin-rich proteins by NaCl may be electrostatic and not hydrophobic because the addition of a nonionic detergent, such as Triton X-100, could not prevent protein aggregation by NaCl (data not shown). Fu et al. hypothesized that salt neutralizes the repulsive forces that maintain the solubility of gluten proteins in distilled water (*15*). It is likely that the loss of these repulsive forces allow the proteins to form intermolecular hydrogen bonds that result in their aggregation. With the addition of NaCl, a slight increase in the intermolecular β-sheet structure (band position = 1617–1623 cm^{-1}) content, albeit not statistically significant, was observed. Such phenomena observed in gliadin-rich protein solutions agree with the effects of salts on the secondary structures of gluten proteins reported previously (*35*). FT-IR analysis of gluten showed that increases in NaCl concentrations caused a slight decrease in β-turn structure (band position = 1666 cm^{-1}) content, accompanied by an increase in intermolecular β-sheet (band position= 1620–1630 cm^{-1}) content. NaI caused a large increase in β-turn structure content, which was accompanied by a decrease in intermolecular β-sheet content. The increase in intermolecular β-sheet structure content may reflect an increase in the associations (i.e., aggregation) of the proteins. The changes in protein–protein interactions induced by NaCl in the dough were clearly indicated by the cross-linking experiments. More proteins were cross-linked in the dough in the presence of NaCl than in its absence. In particular, DST (6.4), with a spacer arm of 6.4 Å, cross-linked gluten proteins in dough only in the presence of NaCl, suggesting that the distances between the amino groups were shortened to <6.4 Å by NaCl. These results are consistent with the ultracentrifugation results that showed a decrease in the volume of gluten after NaCl treatment (*36*). Most kinds of gluten proteins are cross-linked to macro-polymers that are insoluble in SDS. Therefore, a model is proposed in which the interactions of gliadins and glutenins are altered by NaCl, coaggregating into protein complexes via intermolecular hydrogen bonds and/or ionic bonds. The formation of protein complexes may cause changes

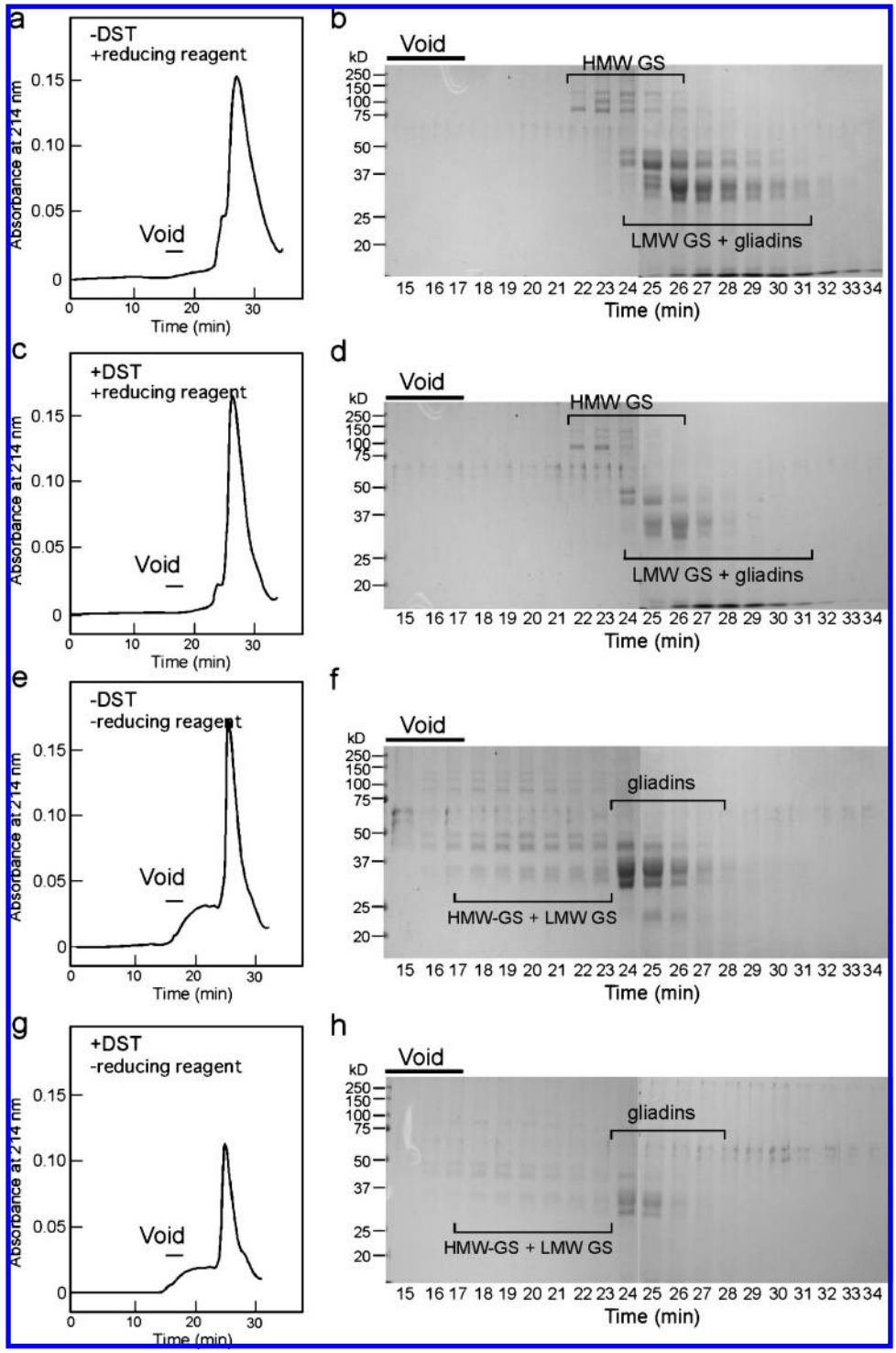


Figure 8. Analysis of gluten proteins from dough prepared with 0.51 M NaCl by SE-HPLC. SE-HPLC profiles for proteins solubilized with SDS buffer in the presence (**a**, **c**) or absence (**e**, **g**) of β-mercaptoethanol from dough after cross-linking with (**c**, **g**) or without (**a**, **e**) DST. Shown on the right (**b**, **d**, **f**, **h**) are the results for the SDS-PAGE of the fractions obtained from the chromatogram shown on the left.

in the rheological properties of dough induced by NaCl, such as dough stability and optimal dough development.

ABBREVIATIONS USED

DSP (12.0), dithiobis[succinimidyl propionate]; DSG (7.7), disuccinimidyl glutarate; DST (6.4), disuccinimidyl tartrate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; FT-IR, Fourier transform infrared; SE-HPLC, size exclusion high-performance liquid chromatography; HMW-GS, high molecular weight glutenin subunit; LMW-GS, low molecular weight glutenin subunit.

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Supporting Information Available: SDS-PAGE of the proteins eluted from dough prepared with various monovalent salts

and differential scanning calorimetry of the gliadin-rich soluble proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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