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ARTICLE

Relationship between Endogenous Protein Disulfide Isomerase Family Proteins and Glutenin Macropolymer

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The effects of endogenous protein disulfide isomerase (PDI) family proteins on the properties of gluten proteins in dough during breadmaking were determined using bacitracin, an inhibitor of PDI. Bread loaf volume in the presence of bacitracin was increased to 118% of that in the absence of bacitracin. The addition of bacitracin caused a decrease in the extension tolerance of the dough. The amount of sodium dodecyl sulfate (SDS)-insoluble glutenin macropolymer (GMP) in dough decreased to approximately 70% of that in flour during the 20 min of mixing for doughmaking. The addition of bacitracin to dough caused a dramatic GMP decrease, corresponding to ~20−30% of that in flour during the 20 min of mixing. The decrease in GMP was compensated by an increase in SDS-soluble glutenin polymer. Taken together, these results suggest that the endogenous PDI family proteins in flour suppress the depolymerization of GMP during dough mixing.

KEYWORDS: Protein disulfide isomerase; breadmaking; gluten; glutenin macropolymer

INTRODUCTION

In wheat endosperm, the major storage proteins, glutenin and gliadin, are synthesized and accumulated during seed development. The α/β and γ forms of gliadin are cysteine-rich prolamines, and their intrachain disulfide bonds are formed in the endoplasmic reticulum. Glutenin is composed of low molecular weight subunits (LMW-GS) that contain six conserved cysteine residues and one or more additional cysteine residues and high molecular weight subunits (HMW-GS) that contain between four and seven cysteine residues. Most of these cysteine residues form intrachain disulfide bonds, whereas some of these residues form interchain disulfide bonds to yield glutenin polymers (*1*−*4*). The formation of these intra- and interchain disulfide bonds is thought to be catalyzed by protein disulfide isomerase (PDI) family members, which are localized to the endoplasmic reticulum (*5*−*8*). After maturation and drying of seed, PDI remains in the endosperm, as the protein thiol oxidoreductase activity has been detected in wheat flour. Endogenous PDI activity has been suggested to be involved in the dehydroascorbic acid improver mechanism (*9*). Watanabe et al. have reported that the addition of exogenous PDI to dough resulted in a more elastic product, and this effect was basically the same albeit weaker as that produced by the addition of potassium bromate (*10*); however, the function of endogenous PDI activity in flour for breadmaking is still unclear.

The amount and composition of glutenin subunits that are included in wheat flour are the most important factors for breadmaking (*11*−*14*). During the mixing of dough, the interchain disulfide bonds in glutenin are interchanged with thiol groups from other proteins and small thiol compounds, such as glutathione (GSH) and cysteine (*15*−*19*). As a result, the amount of glutenin macropolymer (GMP), which is insoluble in a sodium dodecyl sulfate (SDS) solution, decreases (*20*). GMP is one of the important factors for the rheological properties of dough (*21*). Therefore, factors relating to interchain disulfide exchange reactions, such as the amounts of thiol compounds and enzymes in wheat flour, may also have important effects on breadmaking. As an example, GSH covalently binds to glutenin via a disulfide exchange reaction during dough mixing, resulting in depolymerization of glutenin (*18*, *19*). Because PDI and probably PDI-related proteins react with not only the thiols of proteins but also both GSH and glutathione disulfide (GSSG) (*22*), these proteins may exert some effect on breadmaking. Thus, we tried to clarify the relationship between GMP depolymerization and the activity of endogenous PDI family proteins. Bacitracin is known to inhibit mammalian PDI (*23*); it is a mixture of related cyclic polypeptides produced by organisms of the licheniformis group of *Bacillus subtilis* var. Tracy and is used medicinally to prevent or treat infection caused by Gram-positive bacteria. Its antibiotic effect is based on the inhibition of bacterial cell wall synthesis (*24*). In this study, bacitracin was used to inhibit the activity of endogenous wheat PDI family proteins, and its effects on GMP depolymerization during dough mixing were determined.

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). Bacitracin and ribonuclease A (RNase A) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Some commercial bacitracin preparations

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have been reported to contain proteases, the presence of which could explain inhibitory effects against PDI (*25*). Then, bacitracin was dissolved in water and purified by size exclusion column chromatography with a C16/70 column packed with SephadexG-75 (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ). The bacitracin eluted from the column was lyophilized. When the denatured RNase was incubated with 5 mM purified bacitracin for 20 min at room temperature in 200 mM 4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$, 0.5 mM GSSG, and 2 mM GSH, no degradation of RNase and PDI family protein was detected. Horseradish peroxidase-conjugated anti-rabbit IgG goat serum was obtained from Promega Corp. (Madison, WI). Western Lightning Chemiluminescence Reagent was purchased from Perkin-Elmer Life Science (Boston, MA). A protein assay kit (RC DC protein assay) and ReadyStrip IPG Strip (pH 3−10) were purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents were of analytical grade.

Western Immunoblot Analysis. Wheat flour (10 mg) was suspended in sample buffer (100 μL) for SDS−polyacrylamide gel electrophoresis (PAGE) (*26*). Proteins were extracted by boiling for 5 min. The suspension was centrifuged at 18000*g* for 5 min, and the supernatant was subjected to SDS-PAGE (*26*). Proteins on the gel were blotted onto a polyvinylidene difluoride membrane. Blots were immunostained with antibodies against recombinant soybean PDI family proteins, PDIL-1, PDIL-2, PDIM, and PDIS-1 (*27*−*29*), and with horseradish peroxidase-conjugated IgG antiserum as a secondary antibody. Blots were developed with the Western Lightning Chemiluminescence Reagent.

Oxidative Refolding Assay with Reduced RNase A. The protein thiol oxidoreductase activity of PDI family proteins was assayed by measuring the oxidative refolding activity of reduced RNase A. Recombinant soybean PDIL-1, PDIL-2, PDIM, and PDIS-1 were prepared as described previously (*27*−*29*). To extract PDI family proteins, 30 g of wheat flour was suspended in 90 mL of 20 mM 4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (pH 7.5), 150 mM NaCl, and 2 mM $CaCl_2$ (buffer A) and then stirred for 10 min at 4 °C. The suspension was centrifuged at 10000*g* for 20 min. The proteins in the supernatant (65 mL) were precipitated by the addition of 33.5 g of ammonium sulfate (75% saturated ammonium sulfate). The precipitated proteins were collected by centrifugation at 10000*g* for 40 min and dissolved in 8 mL of buffer A. The protein solution was dialyzed once against 500 mL of buffer A at 4 °C for 19 h and twice against 500 mL of buffer A at 4 °C for 6 h and then centrifuged at 10000*g* for 10 min. Protein concentration in the supernatant was measured with the RC DC protein assay kit. PDI activity was assayed by measuring RNase activity produced through the regeneration of the active form from reduced and denatured RNase A. Reduced and denatured RNase A was prepared as described previously by Creighton (*30*). Each reaction mixture contained 200 mM 4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$, 0.5 mM GSSG, 2 mM GSH, 1 mg/mL reduced RNase A, and the protein extract from wheat flour or recombinant soybean PDI family proteins with or without 1 or 5 mM bacitracin. This reaction was incubated at 25 °C. An aliquot (16 μL) of the reaction mixture was removed, and the RNase A activity was measured spectrophotometrically at 284 nm with cytidine2′:3′-cyclic monophosphate as the substrate (*31*). Reactivation of reduced RNase A in the absence of PDI family protein or the extract from flour was subtracted from reactivation in the enzyme preparation.

Preparation of Bread. Dough was prepared from 200 g of wheat flour, 4 g of NaCl, 4 g of yeast, 10 g of sucrose, 10 g of shortening, and 134 mL of distilled water, 5 mM bacitracin solution, or 0.11 mg/mL GSH solution. The ingredients were mixed and kneaded for 20 min with a mixer (KN-200, Taisho Denki, Tokyo, Japan). The dough was initially fermented at 28 °C for 40 min (first fermentation). After kneading and resting, the dough was divided and shaped into five 60 g loaves, which were placed in baking pans. The loaves were fermented at 36 °C for 40 min (second fermentation) and then baked at 180 °C for 13 min. The bread was analyzed after cooling for 2 h. The volume of the bread was measured according to the rapeseed method (*32*).

Rheological Measurements of Dough. Dough was prepared from 200 g of wheat flour, 4 g of sodium chloride, and 134 mL of distilled water or 5 mM bacitracin solution by mixing for 20 min. For assaying of large deformation properties, dough was sheeted to a 2 mm thickness and was then placed between sample holders with a round hole with a 10 mm diameter. Deformation was measured by pushing the dough sheet with a ball-shaped plunger (5 mm diameter) at 0.5 mm/s at 18 °C using a RheonerRE-3305 (Yamaden Co., Tokyo, Japan). The strength and stability of the dough were determined with a Mixograph (TMCO, Lincoln, NE). Flour (10 g) was mixed at optimum water absorption with water or 5 mM bacitracin solution, and the farinograph curve was centered on the 500 BU line.

Preparation of GMP and SDS-Soluble Proteins from Flour or Dough. Dough was prepared from 50 g of wheat flour, 1 g of NaCl, and 33 mL of distilled water or 5 mM bacitracin solution. The ingredients were mixed for 20 min with a mixer (KN-200). A portion of the dough (500 mg) was removed at 5, 10, or 20 min of the mixing time. Flour (300 mg) or dough (500 mg) was suspended in 5 mL of a chloroform/methanol mixture (2:1) to instantaneously stop the thiol- and disulfide-related reactions. The suspension was centrifuged at 1000*g* for 15 min. The supernatant was then removed. The pellet was re-extracted with 5 mL of the chloroform/methanol mixture. The suspension was centrifuged at 1000*g* for 15 min, and the supernatant was removed. The pellet was lyophilized, and the dried pellet (100 mg) was suspended in 1.5 mL of 1.5% SDS and shaken for 1 h at room temperature. The suspension was centrifuged at 18000*g* for 30 min, and the supernatant was removed. The pellet was resuspended in 1 mL of 1.5% SDS and centrifuged. The new supernatant was removed and combined with the previous supernatant. The supernatant was analyzed by size exclusion high-performance liquid chromatography (SE-HPLC). The pellet (GMP) was suspended in 0.5 mL of sample buffer containing 5% β-mercaptoethanol for SDS-PAGE and then boiled for 5 min. The suspension was centrifuged at 18000*g* for 5 min. The protein concentration of the supernatant was measured with the RC DC protein assay kit. GMP proteins were subjected to SDS- PAGE. Proteins on the gel were stained with Coomassie Brilliant Blue R-250. Detected protein bands on the gel were scanned with Image J (National Institutes of Health). For two-dimensional PAGE, GMP proteins were subjected to isoelectric focusing carried out on a Protean IEF Cell (Bio-Rad Laboratories) using the 7 cm ReadyStrip IPG strip (pH 3−10). IEF strips were then subjected to SDS-PAGE.

SE-HPLC. SE-HPLC was performed according to the modified method described previously (*33*). An SDS-soluble protein solution 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 was $M_r = 7 \times 10^6$. Chromatography was performed at a flow rate of 0.4 mL/min at 4 °C. The detection wavelength was 214 nm. Fractions, which were 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 AND DISCUSSION

Identification of PDI Family Proteins in Wheat Flour and Inhibition of Their Activity by Bacitracin. Phylogenic analysis of the genomic nucleotide sequence of *Arabidopsis thaliana* has revealed 10 groups of PDI-like protein genes (*34*). These genes are conserved between higher plant species. Among them, the quiescin-sulfhydryl oxidase-like and adenosine 5′-phosphosulfate reductase-like proteins should not be included as members of the plant PDI family due to their putative nonisomerase enzymatic activities encoded by an additional domain. Soybean PDI family proteins from groups I−V have been identified and characterized (*27*−*29*,*35*). Groups I (PDIL-1), II (PDIL-2), IV (PDIL-S), and V (PDILM) exhibit protein thiol oxidoreductase activity (*27*−*29*). d′Aloisio et al. reported the cloning, chromosomal assignment, sequencing, and expression analysis of the complete set of genes encoding the PDI and PDI-like proteins in bread wheat (*36*). Among these genes, only group II (i.e., TaPDIL2-1) has been identified and purified (*37*). After quantifying nine wheat PDI and PDI-like gene transcripts, the presence of *TaPDIL1-1*, *TaPDIL2-1*, *TaPDIL4-1*, *TaPDIL5-1*, *TaPDIL6-1*, *TaPDIL7-1*, and *TaPDIL8-1* transcripts was detected in 38 day postanthesis caryopses, when the deposition of seed storage reserves decreased and seeds began the process of desiccation (*36*). To confirm the existence of PDI family proteins in wheat flour, western immunoblot analysis of

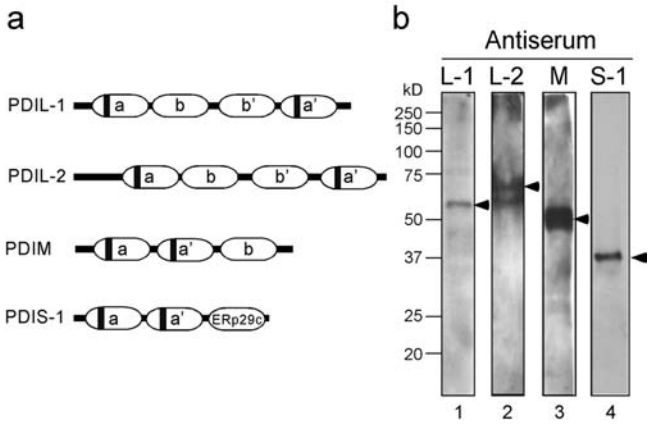

Figure 1. PDI family proteins in extracts from wheat flour. (**a**) Schematic structures of plant PDIL-1, PDIL-2, PDIM, and PDIS-1 as predicted by an NCBI conserved domain search and limited proteolysis of recombinant soybean PDI family proteins (*24*−*26*). Black boxes in domain a and a′ represent the active center CGHC. (**b**) Detection of PDI family proteins in wheat flour. Proteins (30 μg) extracted from wheat flour were separated by SDS-PAGE and analyzed by western immunoblot analysis with antiserum to PDIL-1 (L-1, lane 1), PDIL-2 (L-2, lane 2), PDIM (M, lane 3), and PDIS-1 (S-1, lane 4). Arrowheads indicate bands corresponding to PDI family proteins.

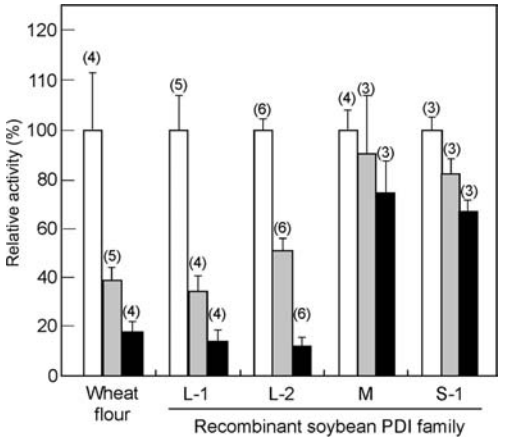

Figure 2. Inhibition of activities of PDI family proteins by bacitracin. The activity in the extract from wheat flour and the activities of recombinant soybean L-1, L-2, M, and S-1 were determined in the absence (white bar) or presence of 1 mM (gray bar) or 5 mM (black bar) bacitracin. The value is given as the percentage of the specific activity of each PDI in the absence of bacitracin as a mean ± SEM (n = 3−6 with the value indicated in parentheses). The mean specific activities of L-1, L-2, M, and S-1 in the absence of bacitracin were 0.472, 0.230, 0.067, and 0.071 mol of RNase/min/mol of PDI family protein. The mean specific activity of the extract from wheat flour was 0.0343 nmol of RNase/min/mg of protein.

wheat flour extracts was carried out using specific antibodies against the proteins encoded by orthologous soybean genes (**Figure 1**). Bands that coincided with the putative molecular weights of groups I (TaPDIL1-1, 56 kDa) (lane 1), II (TaPDIL2-1, 64 kDa) (lane 2), IV (TaPDIL4-1, 40 kDa) (lane 4), and V (TaPDIL5-1, 47 kDa) (lane 3) were detected by western immunoblot analysis using anti-PDI family protein antibodies from groups I, II, IV, and V, respectively. No band was detected with anti-PDI family protein from group III (data not shown). As expected, protein thiol oxidoreductase activity was also detected in the extract from wheat flour (**Figure 2**).

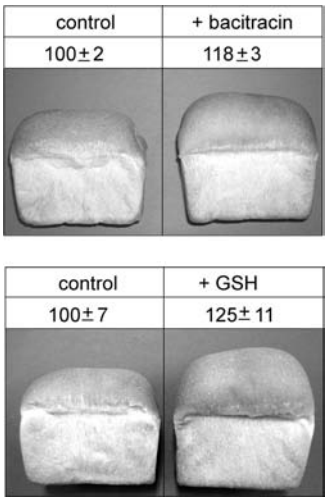

Figure 3. Effects of bacitracin on bread loaf volume. Bread was prepared with water (control), 5 mM bacitracin solution (+ bacitracin), or 0.11 mg/mL GSH solution (+ GSH). The pictures of representative bread loaves are presented. The value is given as the percentage of the specific volume of control bread (4.6 mL/g) as a mean ± SEM of five bread loaves.

The antibiotic bacitracin has been used to inhibit protein thiol oxidoreductase activity of mammalian PDI both in vivo and in vitro (*23*). To determine the sensitivity of plant PDI family proteins to bacitracin, the effects of bacitracin on the activity of the recombinant soybean PDI family proteins were examined (**Figure 2**). Bacitracin (5 mM) inhibited > 80% of the total activity of PDIL-1 or PDIL-2, whereas the activity of PDIS or PDIM in the presence of 5 mM bacitracin was decreased to only about 70−80% of the control activity. More than 80% of the total protein thiol oxidoreductase activity in the extract from wheat flour was also inhibited by 5 mM bacitracin. Karala and Ruddock studied the effect of bacitracin on PDI activity using a variety of in vitro assays (*38*). They concluded that bacitracin is not a specific inhibitor of PDI; however, the ability of bacitracin to inhibit PDI appears to occur via competition for substrate binding and, as such, the activities of the wheat PDI family proteins, included in flour, might be inhibited by bacitracin depending on their modular structures (i.e., the presence or absence of the substrate-binding b or b′ domain or the number and position of catalytic a domains).

Increase in Loaf Volume and Changes in Rheological Properties of Dough by the Addition of Bacitracin. The effect of bacitracin on bread loaf volume was determined. Loaf volume in the presence of bacitracin increased to 118% of that of bread in the absence of bacitracin (**Figure 3**).

In this study, superhard wheat flour was used. The dough prepared with such flour has a very high tolerance to extension due to a high content of GMP. The thiol−disulfide exchange reaction between a small molecule reductant such as GSH and cysteine and the intermolecular disulfide bond of GMP during mixing of dough caused depolymerization of GMP and resulted in a decrease in the extension tolerance of the dough (*15*). In addition, a decrease in extension tolerance of dough prepared with superhard flour by depolymerization of GMP after the addition of small molecule reductants caused an increase in loaf volume of the bread compared to that of bread without the addition of reductant (*16*). Addition of GSH (15 mg/200 g of flour) in this study also caused an increase in loaf volume to 125% that of control bread with no GSH (**Figure 3**). In addition, the loaf volume of the bread was increased by the addition of L-cysteine (data not shown). Then, the effects of bacitracin on the extension

Table 1. Effects of Bacitracin on the Rheological Properties of Dough[a]

	control	+ bacitracin
large-deformation property		
force at failure point (g)	29.3 ± 2.55 a	26.4 ± 0.96 a
failure point (cm)	1.60 ± 0.11 a	1.4 ± 0.08 a
mixography behavior		
development time (min)	6.0 ± 0.3 a	4.9 ± 0.2 a
mixing tolerance (min)	6.0 ± 0.1 a	5.0 ± 0.1 a

[a] The dough was prepared with water (control) or 5 mM bacitracin solution. The results are shown as the mean ± SEM for three experiments. The statistical significance of the differences was determined by unpaired Student's *t* test. "a" indicates $p < 0.01$.

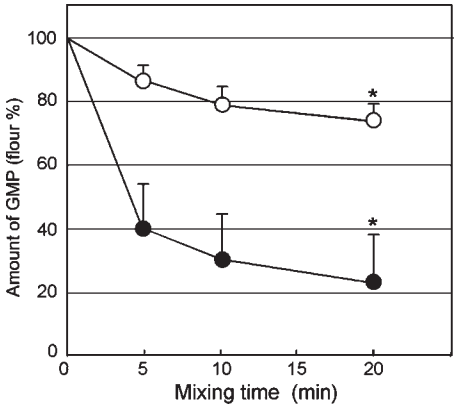

Figure 4. Bacitracin decreases the SDS-insoluble protein GMP in dough. The amount of GMP at time 0 was obtained by measuring the amount of GMP in flour. The amounts of GMP in dough with (black) or without (white) bacitracin were measured at 5, 10, and 20 min after mixing. The percents relative to the amount of GMP at time 0 are presented as mean ± SEM of three experiments. The statistical significance of the difference was determined using an unpaired Student's *t* test. ∗ indicates $p < 0.05$ for dough with bacitracin versus dough without bacitracin (control).

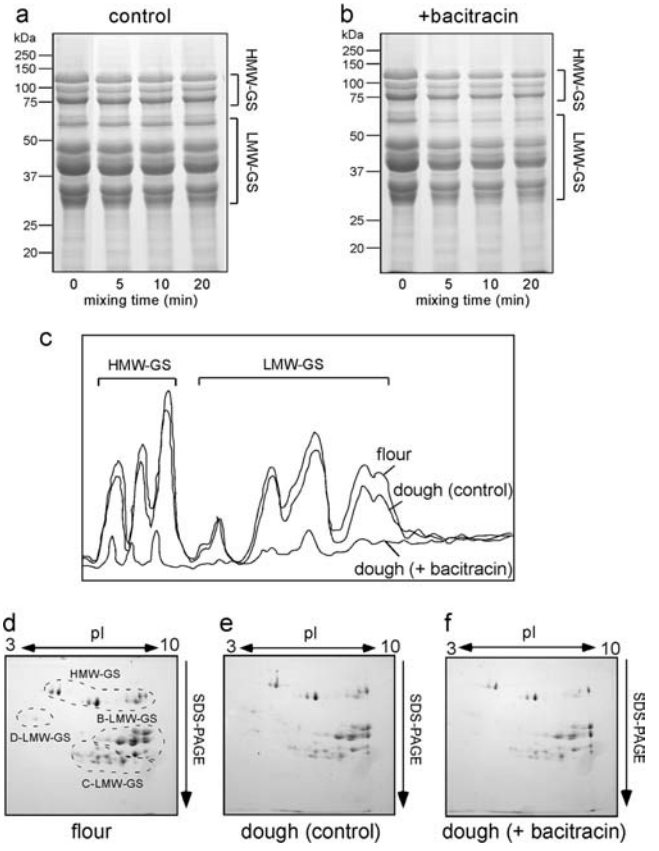

Figure 5. Effect of bacitracin on the protein composition of GMP in dough. SDS-PAGE gel of GMP from flour (mixing time is 0) or from dough made with water (**a**) or with 5 mM bacitracin solution (**b**) mixed for 5, 10, or 20 min. The gels from **a** or **b** were scanned (**c**). GMP from wheat flour (**d**), GMP from dough made with water (**e**), or GMP from dough made with 5 mM bacitracin solution (**f**) was subjected to two-dimensional PAGE. The proteins on the gel were stained with Coomassie Brilliant Blue R-250.

tolerance of dough were determined (**Table 1**). Both the failure point and the force at the failure point of the dough in an assay with a Rheoner were decreased by the addition of bacitracin. Upon mixography analysis, the development time and stability time were decreased by the addition of bacitracin (**Table 1**). These results suggested that the addition of bacitracin weakened the strength of the dough.

Increase in Depolymerization of GMP by Bacitracin during Mixing. To determine whether the protein thiol oxidoreductase activity of endogenous PDI family proteins involves depolymerization of GMP in dough during the mixing process, the amount of SDS-insoluble proteins in dough was measured (**Figure 4**). The amount of GMP in dough decreased to 75% of that in flour 20 min after mixing. The addition of bacitracin increased depolymerization of GMP during mixing. The amount of GMP in dough was decreased to 25% that in flour at 20 min after mixing.

GMP in flour is composed of HMW-GS as well as B-, C-, and D-type LMW-GS, which are linked with intermolecular disulfide bonds. The composition of GMP in dough after mixing for 20 min was almost the same as that of flour (**Figure 5**). The addition of bacitracin did not affect the composition of GMP in the dough. The SDS-soluble non-GMP proteins were separated by SE-HPLC. The absorbance of fractions 1−9, of which glutenin polymers were eluted on SE-HPLC, was increased by the addition of bacitracin (**Figure 6**), suggesting that the amount of SDS-soluble glutenin polymers was increased by the addition of bacitracin. This result suggests that depolymerization of GMP during mixing was increased in the presence of bacitracin. Hence, activities of PDI family proteins, such as bacitracin-sensitive PDIL-1 and PDIl-2, may suppress the depolymerization of GMP.

Together, these results suggest that endogenous PDI family proteins function to suppress depolymerization of GMP during mixing and to enhance extension tolerance of dough. The enhancement of extension tolerance by PDI family proteins may lead to a decrease in loaf volume when dough is made with superhard flour containing high amounts of GMP as used in this study.

For suppression of depolymerization of GMP by PDI family proteins, two possible mechanisms are postulated. The first possibility involves a decrease in endogenous GSH due to the oxidation of GSH to GSSG catalyzed by PDI family proteins (*22*), and the second possibility involves the formation of intermolecular disulfide bonds between glutenin subunits catalyzed by PDI family proteins. Wheat flour contains substantial amounts of GSH (*16*). Hüttner and Wieser reported that most of the GSH distributed in the glutenin fraction after mixing formed intermolecular disulfide bonds with cysteine residues of LMW-GS, which are important for polymerization of GMP (*19*). Hence, the thiol-exchange reaction between GMP and endogenous GSH is thought to play an important role in the depolymerization of GMP during mixing (*20*). If the GSH molecules are oxidized to GSSG by PDI family proteins, depolymerization of GMP may decrease; however, whether active PDI family proteins can oxidize endogenous GSH to GSSG in dough is unknown. Rather, the PDI family proteins likely repair the cleaved disulfide linkages

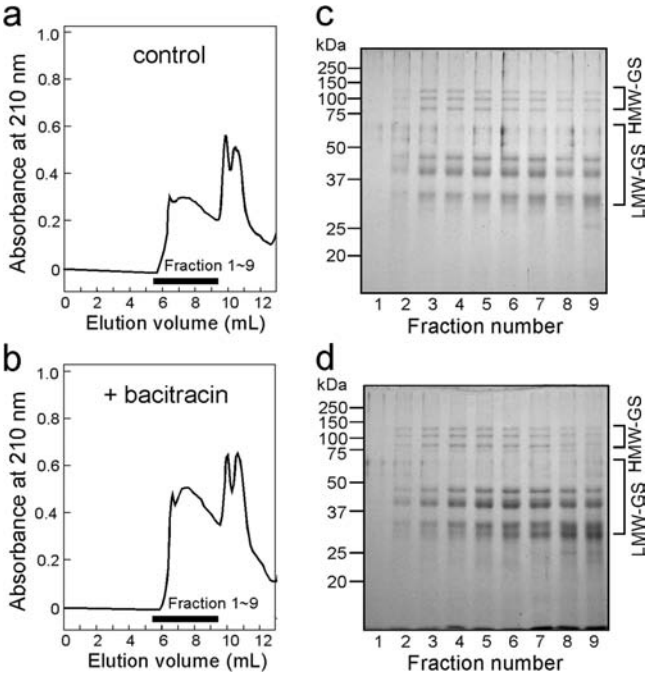

Figure 6. Bacitracin increases the amount of soluble glutenin polymers in dough. Dough was prepared with water (**a**, **c**) or with 5 mM bacitracin solution (**b**, **d**) by mixing for 20 min. Dough samples were dried by washing with a chloroform/methanol (2:1) mixture. Proteins extracted with 1.5% SDS from the dried dough were analyzed by gel filtration chromatography. Absorbance was monitored at 210 nm. Ten fractions (each 0.4 mL) were collected from the elution volume (5.4−9.4 mL). Glutenin polymers were eluted in these fractions (black line). Shown in **c** and **d** are the results for the SDS-PAGE of fractions 1−9 obtained from the chromatograms shown in **a** and **b**, respectively. The proteins on the gel were stained with Coomassie Brilliant Blue R-250.

or re-form new disulfide bonds between glutenin subunits produced from GMP. Further studies are necessary to elucidate the molecular mechanism of PDI function during breadmaking.

ABBREVIATIONS USED

LMW-GS, low molecular weight glutenin subunit; HMW-GS, high molecular weight glutenin subunit; PDI, protein disulfide isomerase; GSH, glutathione; GMP, glutenin macropolymer; SDS, sodium dodecyl sulfate; GSSG, glutathione disulfide; RNase A, ribonuclease A; PAGE, polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high-performance liquid chromatography.

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