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報 文

Glutathione changes physical properties of rice batter without increasing its allergenicity

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Abstract

In our previous studies, glutathione, a biologically ubiquitous tripeptide, has been shown to improve swelling of gluten-free rice bread. To further characterize the effects of glutathione, we studied structural changes in batter protein in relation to gas retention properties of the batter. Glutathione (just as dithiothreitol and cysteine) cleaved the glutelin polymers into monomers, and concurrently improved gas retention capacity of the rice batter. Moreover, glutathione apparently did not promote formation of disulfide bonds among proteins. This was in contrast to wheat dough where extensive intermolecular disulfide bonds among proteins formed during kneading. Besides, an allergenicity test involving serum samples from dogs with wheat allergy suggested that the use of glutathione did not increase allergenicity of gluten-free rice bread. Our results suggest that glutathione modestly changes the structure of food protein while improving the physical properties of food without increasing its allergenicity. Our findings show usefulness of glutathione for research into the development of new foods.

Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

Key words: Bread; Glutathione; Gluten-free; Rice; Sorghum; Wheat allergy

Introduction

The demand for gluten-free foods has increased (Lamacchia et al. 2014) due to the high prevalence of celiac disease (Green and Cellier 2007) and wheat allergy (Mansueto et al. 2014). In our previous studies, addition of glutathione improved swelling of gluten-free rice bread (Yano 2010; Yano et al. 2013). Moreover, the use of oxidized glutathione (GSSG) rather than reduced glutathione improved the smell of the bread (Yano 2012). Although the glutathione-rice batter had less volume during fermentation in the presence

of salt, a key flavor of bread, addition of sorghum flour to the batter prevented the volume reduction even in the presence of salt (Yano and Fukui 2015). The specific volume of this bread is ~4 mL/g, which is comparable to wheat bread. Furthermore, purified glutathione, which is not allowed as food material in several European and Asian countries, has been successfully replaced with edible yeast extract that contains 18% (w/w) of GSSG (Yano and Fukui 2015). Thus, a feasible formulation of gluten-free rice bread has been accomplished because all ingredients—rice/sorghum flour, yeast extract, dry yeast (for fermentation), sugar, salt, butter,

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and water—are foodstuffs.

On the other hand, the effects of glutathione as well as the swelling mechanism have not been studied adequately. For example, although the intermolecular disulfide bond of glutelin polymers has been shown to be cleaved to produce monomers in the presence of glutathione, comparative studies with other relevant compounds such as cysteine and other amino acids have not been conducted. Besides, fermentation gas capacity of the batter before baking is not clear. Structural changes in proteins during the manufacturing process as well as their allergenicity are poorly understood. Research in this field should help to understand the mechanism of action of glutathione. The resulting knowledge will facilitate the use of glutathione in a wide variety of foods. Therefore, in this study, we sought to further characterize the effects of glutathione on rice batter/bread.

Materials and Methods

Materials

Rice flour (10.7% moisture, 0.3% ash, 6.2% protein, 0.9% lipids, and 81.9% starch) was obtained from Namisato, Co., Ltd. (Tochigi, Japan). Sorghum flour (12.4% moisture, 0.6% ash, 9.6% protein, 1.5% lipids, and 73.6% starch) was purchased from Nakano Sangyo, Co., Ltd. (Kagawa, Japan). Dried yeast (Nisshin Super Camellia) was obtained from Nisshin-foods Co., Ltd. (Tokyo, Japan). Purified glutathione was purchased from Nacalai Tesque (Kyoto, Japan). The GSSG-containing (18%, w/w) yeast extract, YH-D18, was a gift from Kohjin Life Sciences Co., Ltd. (Tokyo, Japan).

Gas retention properties of rice batter

Rice batter was prepared in a commercial bread maker SD-BH105 (Panasonic Corporation, Osaka, Japan) as described elsewhere (Yano 2010) with some modifications. In brief, 160 g of rice flour, 140 g of distilled water, and one of additives (dithiothreitol, glutathione, cysteine, or alanine) were mixed by kneading paddles for 20 min in a bread bin of the bread maker. The batter was then left overnight in a refrigerator (4°C). Next, 15 g of sugar and 2.5 g of baker's yeast (Nisshin Flour Milling Inc., Tokyo, Japan) were added to the batter, which was then mixed for 20 min. After that, 200 g of the batter was transferred to a 1-L beaker. Finally, each beaker was incubated at room temperature (25°C ± 1°C), and the volume of the swelling batter during the

fermentation process was recorded periodically.

Analysis of disulfide-linked polymerization of protein

To investigate disulfide-linked polymerization among proteins in the rice batter, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out without dithiothreitol (DTT) (Yano et al. 2001) as follows. After overnight incubation of the batter as described above, a portion of the batter (1.0 g) was mixed with 10 mL of a DTT-free Laemmli sample buffer (Laemmli 1970). After homogenization, the sample was centrifuged at 10,000 × g for 10 min. Then, 10 μL of the supernatant was subjected to SDS-PAGE. The electrophoresis was run on a precast polyacrylamide gel with a linear 4% to 20% acrylamide gradient (Bio-Rad, Hercules, USA) by means of the Criterion precast gel system (Bio-Rad) with a constant current of 30 mA per gel. After the electrophoresis, the gel was incubated (with moderate shaking overnight at room temperature) in 20% methanol containing 5% acetic acid and 0.025% Coomassie brilliant blue R-250 (CBB). The gel was then destained with a solution of 20% methanol and 5% acetic acid until the protein bands became visible.

Bread making

After adding bakers' yeast and mixing the batter for 20 min as described above, we transferred 200 g of the batter to an 800-mL square oven case. After fermenting the batter at 40°C (for ~60 min) until the batter swelled to the top of the oven case, we baked the batter at 180°C for 25 min in an EMO-C16C electric oven (Sanyo Electric, Osaka, Japan).

Allergenicity testing of the batter/bread

Pooled serum from dogs with wheat allergy. The serum of three dogs with high IgE titers against wheat were used in this experiment. The wheat-specific IgE concentration in these serum samples was 699 ng/mL, 609 ng/mL, and 755 ng/mL. These three samples were mixed in equal amounts to obtain pooled serum with the concentration of wheat-specific IgE of 730 ng/mL. The pooled serum was used in the following experiments.

Protein extract from cereal flour or breads. The protein extract was prepared from the following five food samples by means of FASPEK ELISA II Kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan): wheat flour, mixed flour of rice/sorghum, wheat bread baked in our lab,

rice/sorghum bread baked in our lab, and wheat bread from a local store. The protein concentration in each extract was determined with the Bio-Rad Protein Assay Kit (Bio-Rad).

Quantitative enzyme-linked immunosorbent assay (ELISA). This assay was performed as follows (Okayama et al. 2011). In summary, microwells were coated with the above-mentioned protein extract (0.1, 1.0, or 10.0 $\mu\text{g}/\text{mL}$) overnight at 4°C. Next, the wells were washed three times with PBST (phosphate-buffered saline containing 0.2% Tween 20) followed by blocking with phosphate-buffered saline (PBS) containing 1% gelatin for 4 hours at 4°C. After removal of the blocking buffer, the pooled serum diluted 1:400 with dilution buffer (1% gelatin in PBST) was added at 100 $\mu\text{L}/\text{well}$ and incubated overnight at 4°C. After three washes with PBST, 100 μL of a 0.5 $\mu\text{g}/\text{mL}$ solution of a biotinylated anti-dog IgE antibody (Bethyl, USA) diluted with dilution buffer (1% gelatin in PBST) was added to each well. After 2-hour incubation at room temperature, the wells were washed three times with PBST. After that, 100 μL of 0.05 U/mL Streptavidin-beta-Gal Conjugate (Roche Diagnostics GmbH, Penzberg Germany) in dilution buffer was added to each well, and the mixture was incubated for 2 hours at room temperature. After the wells were washed three times with PBST, 100 μL of 0.1 mM 4-methylumbelliferyl-d-galactopyranoside (4MU, Sigma–Aldrich, Saint Louis, USA) in reaction buffer (0.1% gelatin, 0.1 M NaCl, and 1 mM MgCl_2 in phosphate buffer, pH 7.4) was added to each well. After 1-hour incubation at room temperature, 100 μL of the stop solution (0.25 M Na_2CO_3) was added, and the fluorescence was detected on the microplate reader Gemini XPS (Molecular Devices, Sunnyvale, USA). The data were analyzed in the Softmax Pro software (Molecular Devices).

Results and Discussion

Effects of glutathione-related additives on the gas retention properties of rice batter

First, to further characterize the effects of glutathione on gluten-free rice batter, we analyzed the structural changes in the batter protein in relation to the gas retention properties of the batter. Protein bands of glutelin polymers became thinner in the presence of DTT, cysteine, or glutathione (Fig. 1A; lanes 2, 3, and 4). On the other hand, the profile of glutelin molecules did not change significantly in the

presence of alanine (lane 5). In line with the cleavage of glutelin polymers, addition of glutathione, DTT, or cysteine enhanced the retention of gas by rice batter (Fig. 1B). The highest specific volumes, 4.5, 3.7, and 4.1 mL/g, were obtained respectively for glutathione, cysteine, and DTT. After maintaining the greatest volume for ~ 3 hours, the batter collapsed to a smaller volume. Because the greatest specific volume of glutathione bread is ~ 4 mL/g, our data point to moderate shrinkage of the batter during the baking process. This is a key difference between the rice bread studied here and typical wheat bread, which is characterized by “oven spring,” i.e., increased dough volume at the early stage of baking. By contrast, the control batter and the alanine-supplemented batter collapsed earlier (after 1 hour) with the greatest specific volume as low as ~ 2.5 mL/g. Furthermore, bubbles in the batter were larger in the case of alanine in comparison with glutathione (Fig. 1C) or other SH-containing additives (data not shown). These results suggest that rice batter without the gluten skeleton cannot maintain large bubbles during the fermentation process in contrast to the typical presence of large bubbles in wheat breads (such as French loaf). In addition, glutathione and other SH-containing additives may facilitate confinement of fermentation gas in small bubbles, thereby stabilizing the crumb structure even without a gluten skeleton. A comparison between our cysteine and alanine procedures confirmed that the sulfhydryl group (SH), not the basic structure of an amino acid, conferred the gas retention capacity to the batter.

Comparison of protein profiles between rice batter and wheat dough

Next, we studied the effects of glutathione on protein in the batter composed of the rice/sorghum flour mixture. In the case of wheat flour, the protein profile changed completely if we compare the profiles before and after the mixing of the dough (Fig. 2; lanes 1 and 2). This is because two major wheat seed proteins, i.e., gliadin and glutenin, interacted extensively during kneading into insoluble gluten. Therefore, the level of SDS-soluble protein decreased after kneading of the dough. The protein network of gluten helps the dough hold the fermentation gas during the baking process. On the other hand, in the case of sorghum/rice batter, no major change was observed after addition of GSSG and mixing of the batter (lanes 3 and 4), except for a decreased amount

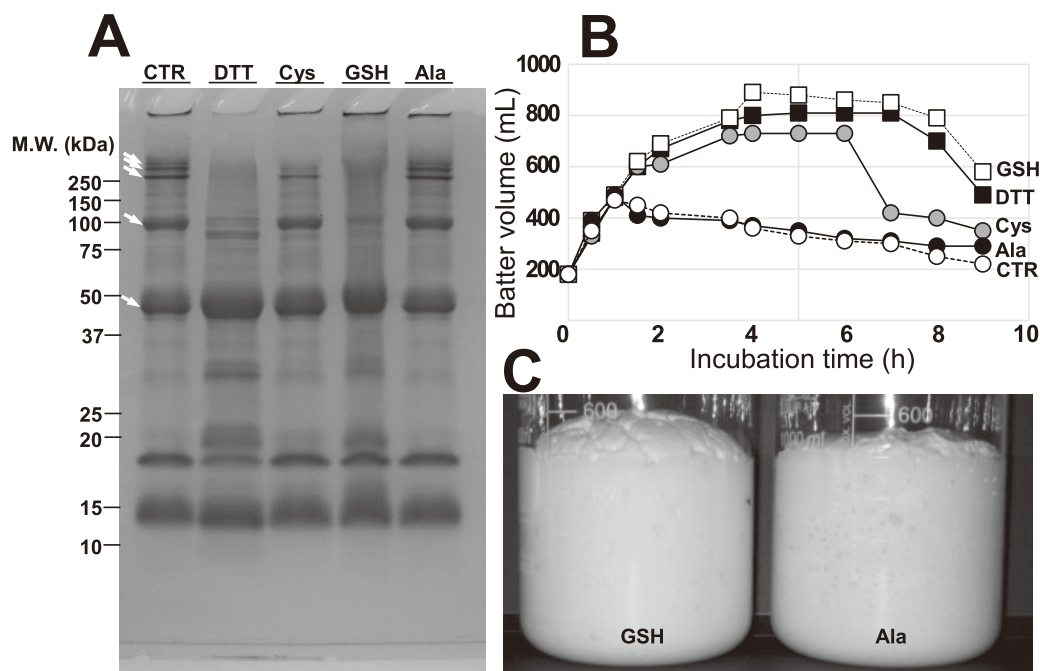


Fig. 1 Gas retention properties of gluten-free rice batter.

A, Profiles of the sodium dodecyl sulfate (SDS)-soluble protein in rice batter. B, Time course of the batter volume during fermentation. C, A close-up view of the glutathione-supplemented (left) and alanine-supplemented batter (right) after the first 1 hour of fermentation. Arrows: glutelin polymers. CTR: control rice batter (no additives); DTT, Cys, GSH, and Ala represent the types of batter to which dithiothreitol, cysteine, glutathione, or alanine was added, respectively. The batter was composed of rice flour (160 g), water (140 g), bakers' yeast (2.5 g), sugar (15 g), and one of the additives. The amount of cysteine, glutathione, and alanine that we added to batter was 1.3 mmol. In the case of dithiothreitol, the amount was 0.65 mmol because it has 2 SH groups in one molecule. After mixing the batter for 20 min, we transferred 200 g of the batter to a 1-L beaker for the fermentation test.

of glutelin polymers and concurrent upregulation of the monomer. Thus, the disulfide bond-mediated links among rice and sorghum proteins seemingly did not form even in the presence of glutathione. GSSG simply cleaved the intermolecular disulfide bonds of some glutelin polymers into monomers as shown in Fig. 1. These results support the theory (Yano and Fukui 2015) that swelling of batter is partially due to a physical effect, i.e., the "effect of mixing" of different types of flour as reported previously (Sanchez et al. 2002; Sciarini et al. 2010). GSSG made the crumbs of the rice/sorghum bread finer by facilitating confinement of the fermentation gas in small bubbles as mentioned in the previous section. These results confirm the complete difference in the swelling mechanism between wheat dough and rice batter.

Allergenicity of the batter/bread

Previously, we developed a formula for gluten-free

rice bread that involves yeast extract containing a high concentration of GSSG (18% w/w) (Yano and Fukui 2015). This is a feasible formulation because all the ingredients—rice/sorghum flour, yeast extract, dry yeast, butter, sugar, salt, and water—are foodstuffs. Accordingly, research in collaboration with bread makers for possible industrial production of the bread for patients with wheat allergy is currently in progress. In some cases, however, allergenicity of food proteins increases during the manufacturing process (Mills et al. 2009). Thus, to evaluate allergenicity of the bread of the new formula, a quantitative ELISA (Okayama et al. 2011) was performed on the serum from dogs with wheat allergy.

Allergenicity of wheat flour, rice/sorghum flour, wheat bread, and rice/sorghum bread was analyzed using pooled serum from three dogs allergic to wheat. The differences between this pooled serum and that from nonallergic dogs are shown in Fig. 3. Wheat flour and wheat breads showed

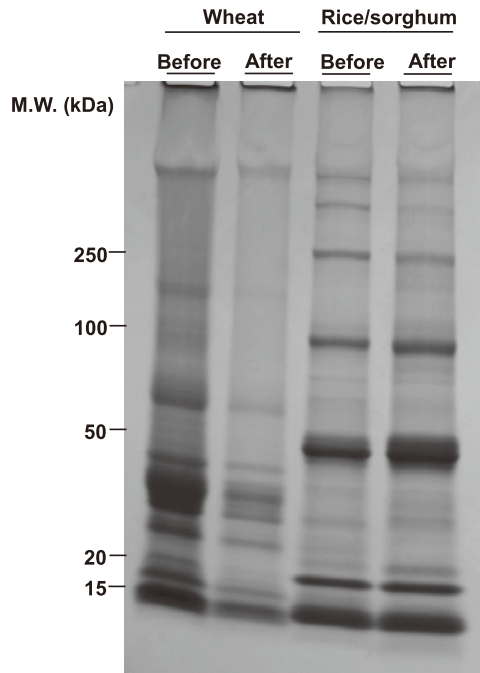


Fig. 2 The profile of sodium dodecyl sulfate (SDS)-soluble proteins before and after kneading/mixing of wheat dough/rice batter.

The rice/sorghum batter was made in the following proportions: rice flour (130 g), sorghum flour (30 g), water (140 g), and purified oxidized glutathione (1.0 g). Purified glutathione was absent in the sample in lane 3. Wheat dough was made in the following proportions: wheat flour (140 g), water (90 g), and salt (2.0 g). Before and after mixing/kneading of the batter/dough, 1.0 g of each sample was mixed with 10 mL of reductant-free Laemmli sample buffer. After centrifugation for 10 min at $10,000 \times g$, 10 μL of the supernatant was loaded into each well.

greater fluorescence as compared to rice/sorghum flour and bread when the protein concentration was 1.0 $\mu\text{g/mL}$ or higher. Moreover, there was no significant difference between rice/sorghum flour and bread. Thus, our results suggest that rice/sorghum bread with glutathione-rich yeast extract is less allergenic to dogs with wheat allergy. Besides, the allergenicity did not increase during the bread making process: there was no significant difference in allergenicity between the flour and bread. This preliminary study suggested that the glutathione-rice bread is safe for patients with wheat allergy.

A high degree of similarity has been reported between the amino acid sequence of the allergenic peptide of wheat and that of a yeast protein (Rasmussen 1994; Watanabe et al. 1995). Another report mentions that patients with wheat-associated allergy are so sensitive to bakers' yeast (Watanabe et al. 1994). Nevertheless, there is accumulating evidence that gluten-free breads that are fermented with commercially available bakers' yeast are generally safe for patients with wheat allergy. Additionally, in the present study, the pooled serum from dogs allergic to wheat did not react with glutathione rice/sorghum bread fermented with bakers' yeast. Although further confirmatory studies are needed, the use of commercially available bakers' yeast for production of breads for patients with wheat allergy seems to be safe.

In conclusion, empirical data from the previous studies and from the present one suggest that glutathione can change physical properties of food without increasing allergenicity.

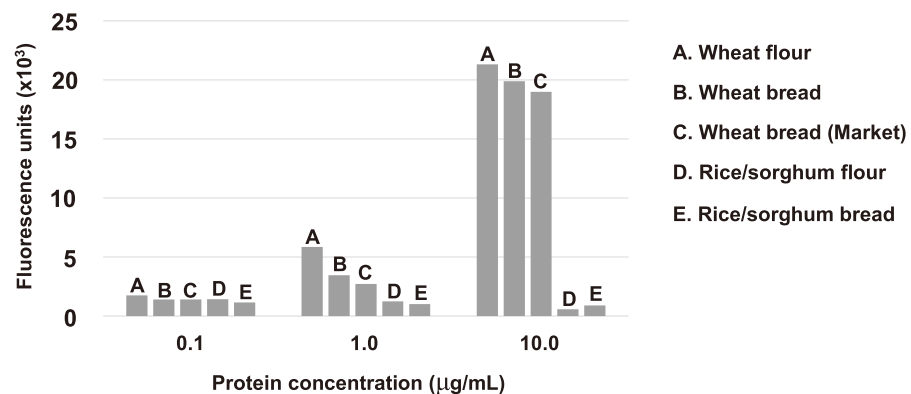


Fig. 3 Comparison of fluorescence intensity obtained by quantitative enzyme-linked immunosorbent assay (ELISA) among wheat bread, rice bread, and the various types of ingredient flour.

Accordingly, this compound is considered useful for the food industry (Yano 2014).

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