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Further Studies on the Protein Chemistry and Property of Glutathione-Added Rice Bread : Evidence of Glutathionylation of Batter Protein as well as Crumb Structure/Sensory Evaluation

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Abstract

Rice is one of the major staple crops in many developing countries, so commercializing rice-based processed foods should help boost their economy. We have recently reported that the addition of glutathione, a natural ubiquitous tri-peptide, to rice batter allows it to retain the carbon dioxide gas in fermentation, whereupon subsequent baking swells the rice bread without the need for added gluten. Although we have just detected this phenomenon, further mechanical and qualitative investigation should accelerate studies for its practical application. We speculate that the swelling is due to the “barrier theory”. In short, disulfide-linked protein polymers hinder the adsorption of water by the starch granules. Glutathione may cleave or hinder the formation of the disulfide bonds of the polymers, which boosts starch hydration, resulting in increased viscosity and continuity of the rice batter. In this paper, we have obtained evidence that glutathione modifies the sulfhydryl groups of the barrier proteins (glutathionylation) and increases their solubility, thus fraying the barrier. Although further studies are needed to reveal the complete swelling mechanism, these observations support and extend the hypothesized barrier-linked swelling mechanism of glutathione bread. Moreover, from a bread quality perspective, the fine/coarse property of crumbs is found to be controllable, and the sensory assessment demonstrated that the bread smell is unimpaired by glutathione. All these data will accelerate the practical use of the glutathione bread.

Discipline: Food
Additional key words: disulfide, gluten-free

Introduction

Rice is the leading staple food in many developing countries, so commercializing rice-based products should help accelerate their economic growth. We have recently found that adding glutathione to rice batter boosts its retention of carbon dioxide in yeast fermentation[8,9]. The bread can be made by oven-baking the batter or using a home bakery. The basic ingredients are rice flour, water, yeast, sugar and glutathione. Glutathione is a natural tri-peptide which is used as a food ingredient in South-East Asia as well as in the United States, meaning no special appliances or ingredients are needed to make it. Also, it should be safe for people with wheat allergies or celiac disease.

Other than its practical value, the bread is also of scientific interest in food research. Since rice protein lacks the viscoelastic property typically found in gluten, commercially available rice bread is made by adding gluten or a semi-synthetic viscosity improver such as hydroxypropyl methylcellulose (HPMC). Meanwhile, the glutathione bread swells without needing these viscoelastic polymers. While the swelling mechanism has not been clarified, it should be new and elucidation of the mechanism is thought to be critical to improve bread quality, and further develop other rice-based foods.

We speculate that the swelling of the bread is explained by the “barrier theory” which was proposed earlier[3]. This unproven theory hypothesizes that water absorption by the starch molecule is interfered with by a network comprising disulfide-linked proteins attached to the starch granule[4]. The main component of the barrier is supposed to be glutelin polymers, while other low molecular proteins such as albumins, globulins and prolamins are entrapped in the large

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macromolecular complexes[6]. Accordingly, if the protein structure were disrupted, the starches would swell up further, thereby increasing viscosity[3]. Adding glutathione should cleave or hinder the formation of the disulfide bonds between the proteins, which would weaken the barrier. Consequently, both the continuity and viscosity of the batter are increased in fermentation and in the early stage of the baking process, which will ultimately improve gas retention and bread swelling[9].

However, from a mechanistic perspective, some important knowledge to support the hypothesis is missing. For example, while the cleavage or hindrance of the formation of disulfide bonds by glutathione is a key reaction in the theory, we have not yet obtained sufficient experimental data to support it. Also, the effect of glutathione on the disulfide-linked barrier, such as to demonstrate the fray of the barrier, has not been well investigated. Moreover, from a practical application perspective, the key quality of the bread, such as fine/coarse of the crumbs or a sensory assessment of smell has not been well studied. This short paper further investigates these critical subjects. Also, as there are two types of glutathione, namely reduced (GSH) and oxidized (GSSG), the differences in their action are also discussed.

Materials and methods

1. Baking Procedure

Breads were baked in a commercial bread-maker SPM-KP1 (Sanyo Electric Co., Ltd, Osaka, Japan) as described in a previous article[9]. In summary, 280 g each of distilled water and rice flour (Namisato Corporation, Tochigi, Japan), and 0.75 g GSH or 2 g GSSG (both Sigma-Aldrich, St. Louis, Mo., U.S.A.) were mixed by kneading paddles for 20 min in the bread-bin of the bread-maker, whereupon the batter was left overnight at room temperature. Subsequently, 15 g of sugar and 4 g of bakery yeast (Nisshin Flour Milling Inc., Tokyo, Japan) were added to the batter, which was mixed for 20 min. Subsequent fermentation was allowed to occur for 50 min, increasing the temperature to 38 °C, followed by baking at 140 °C for 35 min. Rice batter, which contained no GSH or GSSG, was also subject to yeast fermentation and baking (control bread). The increase in bread volume was calculated using a ratio of the height of glutathione-added breads to that of the control bread; the base area of the breads was identical.

2. Investigation of the effect of glutathione on the sulfhydryl groups of rice protein

Fluorescence labeling of the protein was conducted according to the previous report[10]. The SDS-soluble protein was extracted from the batters (1 g) at the early stage of baking with 10mL of a reductant-free Laemmli sample buffer containing 2 mM monobromobimane, which was then incubated for 20 min at room temperature. Subsequently, the extract was centrifuged at 10,000g for 10 min, and the supernatant was filtered through a 0.45 μm centrifugal filter (Ultrafree CL; Millipore, Bedford, MA) at 5,000g for 30 min. The filtrate was desalted with a centrifugal filter (Microcon YM-10; Millipore) at 14,000g for 30min. Finally, the concentrate was dissolved in a reductant-free Laemmli sample buffer, and 10 μL of the solution was subject to SDS-PAGE. The resultant gel was stored in a 30% methanol/5% acetic acid solution and examined under an FAS2513 365 nm UV light (Toyobo, Tokyo, Japan) to detect monobromobimane-labeled proteins. The gel was then incubated overnight at room temperature in 20% methanol containing 5% acetic acid and 0.025% Coomassie brilliant blue R-250 (CBB). Finally, it was destained with a solution of 20% methanol and 5% acetic acid until the protein bands were visible.

3. Quantitative analysis of glutathione

Rice flour, 5.6 g, was suspended in the 10 mg/ 5.6 g GSH or 20 mg/5.6g GSSG water solution at room temperature. After 24 h, the suspending solution was measured up to 100 mL (*crude solution*). A portion of the solution was centrifuged at 3,000 × *g* for 10 min, and the supernatant was obtained. The quantitative measurements of free GSH and total GSH (namely, the sum of GSSG, PSSG and free GSH) were conducted by the DTNB-HPLC method[7].

4. Effect of glutathione on the crumb properties of bread

The baking procedure was as above, except that a mixture of GSH and GSSG, the total amount of which was 0.9 g, was used.

5. Sensory evaluation of the bread smell

After baking, the loaves were allowed to cool at room temperature for 3 h and then sealed in 10-L polyethylene terephthalate (PET) bags designed specifically for headspace gas analysis (Omi Odor-Air Service Corporation, Shiga, Japan). The headspace gas was expanded to exactly 10 L by synthetic air, and the bread was broken into pieces in the sealed bag. Subsequently, the obtained gas was subject to an odor hedonics test conducted in a sensory panel room. The test panel, consisting of 6 experienced assessors (licensed smell examiners), sniffed the headspace of the control, GSH and GSSG bread and evaluated the breads on a 9-point hedonic scale (-4, hate; -3, strongly dislike; -2, dislike; -1, slightly dislike; 0, ambivalent; 1, like a bit; 2, like; 3, strongly like; 4, love).

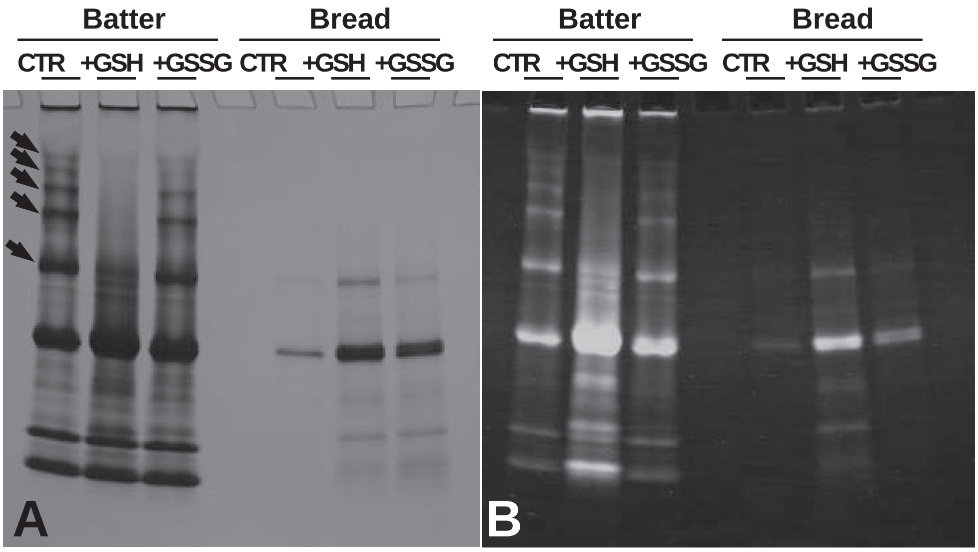

Fig. 1. Profile of the SDS-soluble protein in rice batter and bread
A, staining of protein by Coomassie blue; *B*, detection of monobromobimane-labeled fluorescent protein under UV light. *CTR*, control rice batter or bread made without the addition of glutathiones. *+GSH* and *+GSSG*, rice batter or bread made with reduced or oxidized glutathione, respectively. *Arrows*, glutelin polymers.

Results and discussions

1. Effect of glutathione on the rice batter/bread protein

First, SDS-soluble proteins were compared between the control (no addition of GSH or GSSG), GSH-added and GSSG-added batter/bread samples (Fig. 1). To observe the disulfide-linked polymerization of proteins, reductants such as DTT were not used in the experiment. Figure1 A shows the profile of CBB-stained proteins, while Fig. 1 B shows the fluorescent labeling of the free sulfhydryl (SH) groups of the protein observed under the UV light. As reported in our previous papers[8,9], disulfide-linked glutelin polymers (*arrows*) were observed in the control rice batter before baking. Conversely, in the case of GSH- or GSSG-added rice batters, these polymers decreased significantly, especially for the GSH-added variety. The results suggest that both GSH and GSSG have cleaved or hindered the formation of the disulfide bonds between the polymers.

In contrast, after baking, very little protein was recovered from the control bread (Fig. 1A). This may be because while heating, the protein barrier is strengthened by additional disulfide cross-linking[2]. In other words, the baking process has prompted further aggregation of glutelins, which made them less soluble to the SDS buffer[6]. Conversely, far more glutelin was recovered from GSH- or GSSG- bread, which suggests that they cleaved or hindered the formation of the disulfide bonds of the polymers and increased the solubility of the protein, which should result in fraying the barrier.

In this experiment, free sulfhydryl (SH) groups were labeled fluorescently by mBBr (Fig. 1B), so we attempted to determine whether there was a difference in the free SH/protein ratio between the GSH- and GSSG-added batter/bread. Densitometry analyses showed that the free SH/protein ratio of the GSH-bread was 1.67-fold compared to that of the GSSG-bread.

We hypothesized in the earlier paper[8] that GSH and GSSG, respectively, cleave the intermolecular disulfide bond between proteins in the following reactions:

PSSP+GSH → PSSG+PSH (1)
PSH+GSSG → PSSG+GSH (2)
PSSP+GSSG → 2PSSG (3)

Equation (1) increases one free SH group of protein per reaction, because the free SH group of GSH is converted to PSH. Conversely, in the case of equation (2), one free SH group of protein decreases per reaction. Also, equation (3) neither increases nor decreases the free SH group of protein, meaning the higher free SH/protein ratio in GSH-bread (compared to the SGGS-bread) supports the above reaction process. This is strong evidence to support the fact that glutathionylation of the SH group of protein, namely, generation of PSSG, occurs in rice protein. It also supports the hypothetical process that the disulfide-linked protein barrier is frayed by glutathionylation.

2. Quantitative analysis of glutathione

Next, quantitative analyses were conducted to determine whether glutathione was attached to the barrier proteins. Rice flour was suspended in the GSH- or GSSG-solutions and left overnight at room temperature, whereupon a portion of the solution ("crude") was centrifuged and the "supernatant" was obtained. Quantitative measurements of the free GSH and total GSH (namely, the sum of GSSG, PSSG and free GSH) were respectively performed for the crude solution and its supernatant (Table 1). The difference in the total GSH between crude and supernatant should reflect the amount of GSH bound to insoluble proteins. Because glutelin and polymers are water-insoluble without SDS[11], they should not be recovered in the supernatant. Table 1 summarizes the quantitative results. The difference in free GSH between the crude and supernatant was minimal in both GSH- and GSSG-suspensions, suggesting that non-covalent interactions between glutathione and non-soluble substances, such as the glutelin polymers, are negligible. However, the difference in the total GSH amount was 0.5 and 3.9 mg, respectively, for GSH- and GSSG-suspensions, suggesting that glutathione covalently binds to insoluble protein, namely, glutathionylation of the SH group of protein occurred. It is unclear why a larger amount of glutathione was consumed in the case of GSSG-suspension (3.9 mg) compared to GSH (0.5 mg).

At this point, it is also unclear how glutathione frays the barrier. The reduced and oxidized glutathione may work differently. The formation of glutelin polymers is likely to entail complex reactions involving SH/S-S exchanges, while reduced glutathione may suppress the SH/S-S exchange reaction between proteins. Conversely, GSSG may modify the reactive, free SH groups of protein, which results in terminating the SH/S-S exchange and the subsequent formation of protein polymers. Although further studies will elucidate the complete bread-swelling mechanism of glutathione, the experimental data, shown in Fig. 1 and Table 1, supports the theory that the glutathionylation of protein occurs in both GSH- and GSSG- batter/bread, thus confirming and extending the earlier results linking bread swelling with the barrier theory.

Table 1. Quantification of free and total glutathione in the GSH- or GSSG-suspension and its supernatant

		Free GSH (mg)	Total GSH (mg)
GSH-suspension	Crude	6.9	8.8
	Supernatant	7.1	8.3
GSSG-suspension	Crude	0.9	18.6
	Supernatant	0.8	14.7

Ten mg of GSH or 20 mg of GSSG was added against 5.6 g of rice flour.

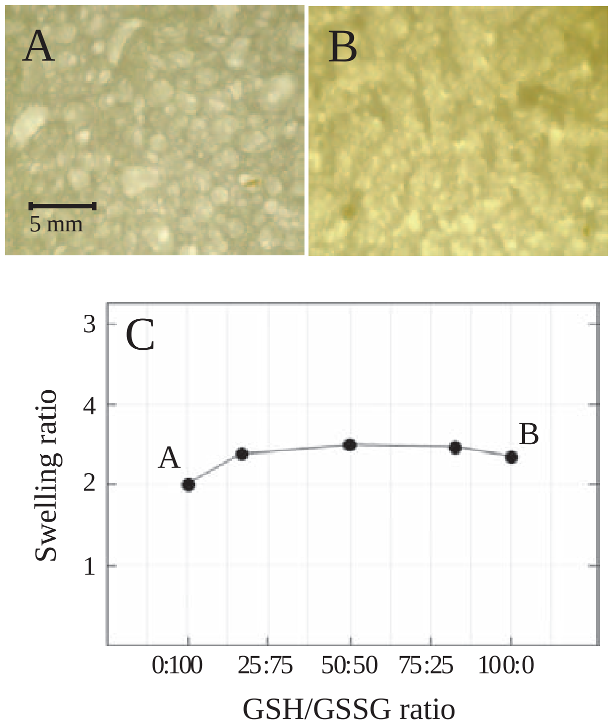

Fig. 2. Rice bread made using a mixture of reduced (GSH) or oxidized (GSSG) glutathione

A and *B*, cross section of GSSG- and GSH- rice bread, respectively. *C*, swelling ratio of rice breads made using multiple mixtures of GSH and GSSG in several proportions.

3. Effect of glutathione on the crumb properties of bread

Next, further investigation on the bread qualities of glutathione-rice bread was conducted. First, we sought to determine whether the crumb structure differed between the GSH- and GSSG- breads. Fig. 2 shows that when an equal amount (0.9 g of glutathione/280g of rice flour) was added, the crumbs of the GSH-bread were fine like cotton candy (B), while those of the GSSG-bread were rather coarse like French bread (A). Also, the swelling ratio of the bread was not reduced when GSH and GSSG were used in a mixture (C). These data suggest that the crumb property of glutathione bread, e.g. fine or coarse, can be controlled. Also shown was the fact that GSH and GSSG do not interfere with each other in the swelling of the bread, and can be used in a mixture to control bread quality. At present, it is unknown whether GSH and GSSG work cooperatively or independently. However, regulation of the SH/S-S exchange of the barrier protein and the consequent crumb property may be possible by changing the reductive/oxidative capability of the added glutathione.

4. Sensory evaluation of the bread smell

In the previous report, we conducted comparative studies between GSH- and GSSG- bread[8]. It has been reported that GSH in food causes a sulfurous odor when heated[1,5]. Analyses by a gas chromatography-flame photometric detector demonstrated the presence of hydrogen sulfide and methyl mercaptan in the headspace of GSH bread, and also their significant reduction in GSSG bread. Also, sensory tests revealed that GSSG bread had a significantly reduced sulfurous odor. Here, an odor hedonics test demonstrated that the overall odor of the GSH bread was disliked "slightly" (-1 ± 0.89). The odor of the GSSG bread was evaluated more favorably (0.17 ± 0.98), though the difference between them was only 1 point on a 9-point scale ($p < 0.05$) (Figure 3). Thus, the sensory test demonstrated a distinct difference in the aromatic effect caused by exogenously added GSH and GSSG. Also, because the preference of the smell of GSSG-bread was nearly identical to the control bread, and the GSH bread was rated as disliked "slightly", it was confirmed that both GSH and GSSG could be considered appropriate food ingredients.

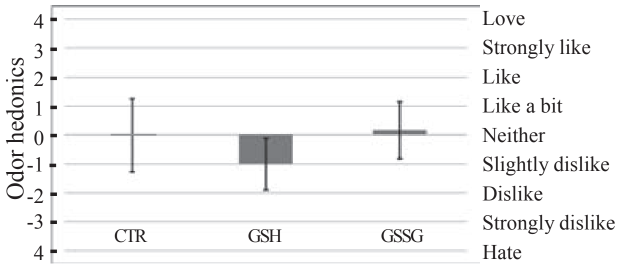

Fig. 3. Sensory evaluation of odor hedonics test

A test panel consisting of 6 experienced assessors evaluated the smell of breads on a 9-point hedonic scale (-4, hate; -3, strongly dislike; -2, dislike; -1, slightly dislike; 0, ambivalent; 1, like a bit; 2, like; 3, strongly like; 4, love). Results are expressed as means ± standard deviations (n=6). Reduced glutathione (GSH) or oxidized glutathione (GSSG) was added at 0.75g and 2g, respectively, to 280g of rice flour.

Concluding remarks

In this paper, further investigation of glutathione bread provided additional evidence for its swelling mechanism. It has been demonstrated that both GSH and GSSG directly modify the rice protein by glutathionylation. While the knowledge obtained to date from present and previous studies and literature data does not contradict the barrier theory, further studies are needed to elucidate the complete swelling mechanism.

Also, studies on bread qualities demonstrated that the cram structure can be controlled. The sensory assessment has also shown that the bread smell is acceptable as food. All the knowledge obtained here will accelerate its practical application.

Disulfide cross-linking of protein is one of the key factors controlling the physical and functional properties of food. As glutathione is safe and inexpensive, it can be used effectively in developing new processed food products.

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