

Evaluation of Reduced Allergenicity of Deamidated Gliadin in a Mouse Model of Wheat-Gliadin Allergy Using an Antibody Prepared by a Peptide Containing Three Epitopes

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ABSTRACT: Gliadin is the principal allergen of wheat-dependent exercise-induced anaphylaxis (WDEIA). The primary structure of IgE-binding epitopes in wheat gliadin includes tandem sequencing sites of glutamine residues. Therefore, deamidation would be an effective approach to reduce the allergenicity of wheat proteins. In our previous study, we deamidated wheat gliadin without causing peptide-bond hydrolysis or polymerization by use of carboxylated cation-exchange resins, and we found that the deamidated gliadin scarcely reacted with the sera of patients radioallergosorbent test (RAST)-positive to wheat. In this study, we examined the allergenicity of deamidated gliadin in a mouse model of wheat-gliadin allergy. Oral administration of deamidated gliadin to gliadin-sensitized mice suppressed enhancement in intestinal permeability, serum allergen level, serum allergen-specific IgE level, mast-cell-surface expression of FcεRI, and serum and intestinal histamine levels. Our results indicate that gliadin deamidated with no peptide-bond hydrolysis by cation-exchange resins has low allergenicity even under in vivo conditions.

KEYWORDS: wheat gliadin, deamidation, wheat allergy

INTRODUCTION

Wheat is one of the major food items that can induce serious allergic reactions in susceptible individuals, and the number of patients suffering from wheat allergy has continuously increased over the past decade.^{1–3} The associated clinical symptoms for wheat allergy differ between adults and children. Atopic eczema/dermatitis syndrome (AEDS) often occurs in children,^{4–6} whereas wheat-dependent exercise-induced anaphylaxis (WDEIA) mainly occurs in adults.^{7–9} The major allergens in wheat are gliadins and glutenins,¹⁰ which account for almost 86% of the total wheat proteins.^{11–13} In particular, wheat ω-5 gliadin and a high-molecular-weight glutenin subunit (HMW glutenin) are the most potent antigens for WDEIA, and certain tandem sequencing sites with glutamine residues have been identified as the primary structure of IgE-binding epitopes.^{9,14,15} Thus, deamidation, which converts glutamine residues to glutamic acid residues, could be an effective approach to reduce the allergenicity of wheat proteins.

Deamidation has been applied to increase the hydrophilicity of proteins and the binding capacity of calcium ions by use of acid,^{16–20} enzymes,^{21–23} and cation exchangers.^{24–30} Among the three, acid treatment is the most common, although it causes unfavorable peptide-bond hydrolysis, which results in the production of bitter-tasting peptides and a reduction in processing properties of the protein. The use of proteases is not recommended for the same reason.³¹ Glutaminases are available for protein deamidation, but they do not act on asparagine residues.²³ Moreover, these enzymes cannot be used for ethanol-soluble proteins (prolamines) because they are

inactivated in an ethanol solution. We have developed a technique for effective deamidation of proteins by using cation-exchange resins of the carboxylate type without causing any detectable peptide-bond hydrolysis.^{25–30} Since cation-exchange resins are resistant to ethanol, this method is applicable to the deamidation of ethanol-soluble proteins such as gliadins.^{25,29} The deamidated gliadin obtained by this technique showed higher foaming property than egg-white albumin and globulin; this property may improve the texture of bakery products such as bread and cakes. Deamidation increased the solubility of gliadin in water and salt solutions, which led to improved digestibility.²⁹ In addition, in vitro reactivity of gliadins with IgE and IgG antibodies in the sera of patients radioallergosorbent test (RAST)-positive to wheat was reduced by deamidation.²⁹ Therefore, the deamidated gliadins may show further reduction in allergenicity during digestion in the gut and could thus be used as a hypoallergenic food material for patients suffering from wheat allergy.

The allergic reaction mostly occurs after allergens are absorbed from the small intestine into the blood. Cross-linking of allergens with IgE antibodies on the surface of mast cells triggers the release of bioactive chemical mediators such as histamine, which is known to promote intestinal permeability.^{31,32} Allergen intake also promotes the production of

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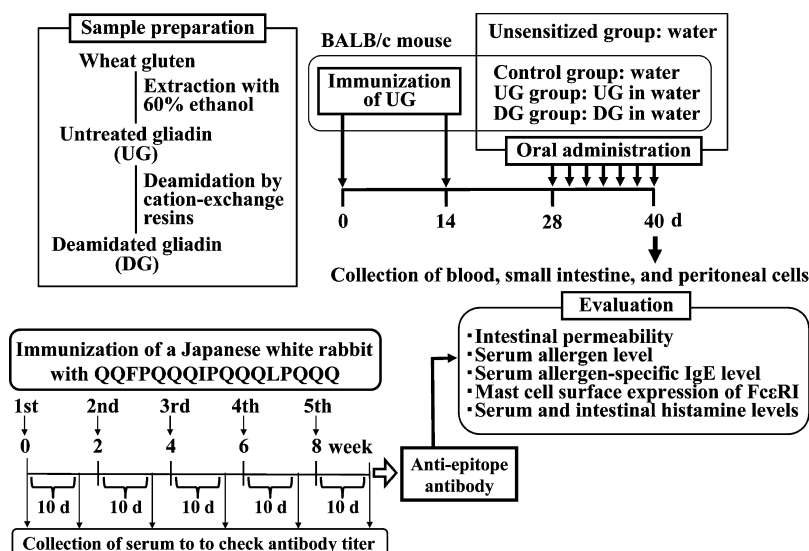


Figure 1. Preparation of deamidated gliadin and evaluation of its in vivo allergenicity by using anti-epitope antibody prepared by immunization with a peptide containing three epitopes.

IgE, and an increase in the level of IgE potentially enhances the expression of FcεRI, the high-affinity IgE receptor, on the surface of mast cells.^{33–38} Although the deamidated gliadin obtained by cation-exchange treatment has low reactivity with the sera of wheat-allergy patients, retention of its low allergenicity after digestion needs to be confirmed. Therefore, prior to an oral challenge to wheat-allergy patients, we evaluated the allergenicity of deamidated gliadin by measuring the intestinal permeability, serum allergen level, serum allergen-specific IgE level, mast-cell-surface expression of FcεRI, and serum and intestinal histamine levels of gliadin-sensitized mice.

MATERIALS AND METHODS

Deamidation of Wheat Gliadin. Wheat gliadin was extracted from gluten (Nakari Tesque, Kyoto, Japan) with 60% ethanol. After being concentrated under vacuum, the extract was lyophilized. A cation-exchange resin of the carboxylate type (WK-11, Mitsubishi Chemical Corp., Tokyo, Japan) was washed with 1 N NaOH and then packed into a glass column (5750 × 50 mm, Nippon Flex Co., Aichi, Japan), after which the column was equilibrated with 60% (v/v) ethanol. Then 30 mL of 10 mg/mL undeamidated gliadin (UG) in 60% (v/v) ethanol was loaded onto the column, and the column was eluted with 60% (v/v) ethanol at 5 mL/min. Fractions were collected over 220 min. Deamidated gliadin (DG) obtained as the eluate was concentrated under vacuum and lyophilized.

Degree of Deamidation. The degree of deamidation was obtained from the ratio of amount of acid amide removed by treatment with cation-exchange resin to total acid amide in UG, as described previously.^{24,27} Briefly, the acid amides in UG and DG were both completely deamidated by heating in a HCl solution, and the amount of ammonia produced was measured by Conway's micro-diffusion method³⁹ and the indophenol method.⁴⁰ Then the amount of acid amide deamidated was calculated by subtracting the amount of ammonia produced by DG from that produced by UG.

Preparation of Rabbit Anti-Gliadin-Epitope Antibody. As QQFPQQQ, QQIPQQQ, and QQLPQQQ are the major epitope sequences of gliadin, a peptide (QQFPQQIPQQQLPQQQ) that comprised these three major epitope sequences was synthesized and conjugated to keyhole limpet hemocyanin (KLH). We used the hydrophobic tag-assisted liquid-phase method^{41,42} in combination with linker strategy^{43,44} to prepare the desired sequence, followed by addition of a cysteine residue to its N-terminus. After deprotection of all protective groups, including the tag, the peptide was conjugated to KLH through the maleimide–thiol reaction.

As shown in Figure 1, Japanese white female rabbits (Nippon Bio-Supply Center, Tokyo, Japan) were subcutaneously immunized with 2 mg of the three-epitope peptide in phosphate-buffered saline (PBS) emulsified with the same volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) for the first and second immunization, and Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI) was used for subsequent immunization. Blood was collected from the central artery in the ear 10 days after each immunization to check the antibody titer. Sera containing the anti-gliadin-epitope antibody were collected 10 days after the fifth immunization. This experiment was performed in accordance with the Guidelines for Animal Experiments of the College of Bioresource Sciences of Nihon University (approval no. 09-12).

Binding Capacity of Rabbit Anti-Gliadin-Epitope Antibody.

For the identification of IgG-binding sequence in the 17-mer three-epitope peptide, 11 7-mer overlapping peptides with an offset of one amino acid were synthesized. The hydrophobic tag-assisted liquid-phase method was also used to produce these peptides.

Reactivity of the 7-mer overlapping peptides, 17-mer three-epitope peptide, UG, and DG with the anti-gliadin-epitope antibody was measured by use of an inhibition enzyme-linked immunosorbent assay (ELISA) conducted as follows: Either 0.3 mg of peptide (7-mer or 17-mer) in 30 μL of dimethyl sulfoxide or 3 μg of gliadin (UG or DG) in 30 μL of 60% ethanol (v/v) was mixed with 270 μL of rabbit anti-gliadin-epitope antibody or pooled serum from patients RAST-positive to wheat in 0.2% bovine serum albumin (BSA)/phosphate-buffered saline containing 5% Tween20 (PBST), and the samples were incubated for 1 h at 37 °C. Sera were donated from wheat-allergy outpatients (8 females and 12 males, mean age 24.2 years, range 0–53 years, mean total IgE 21 076 IU/mL, range 1030–84 616 IU/mL) who were under treatment at Yokohama City University Hospital after a full explanation of contents of the study. Flat-bottomed microtiter plates were coated with 100 μL of 10 μg/mL gliadin in 60% ethanol at 4 °C overnight and blocked with a 2% BSA/PBST solution for 2 h at 37 °C. The mixture of peptide/gliadin and antibody was added to the wells, and the plates were further incubated for 1 h at 37 °C. Then horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was reacted for 1 h at 37 °C with the rabbit anti-gliadin-epitope antibody bound to the gliadin that had been coated onto the wells. The wells were washed five times with PBST at every step. As a substrate for HRP, 100 μL of 0.04% *o*-phenylenediamine containing H₂O₂ was added. The reaction was allowed to proceed for 20 min at room temperature and then stopped by adding 100 μL of 2 N H₂SO₄. The optical density of the formazan at 490 nm was measured with a

microtiter plate reader. The inhibition percent was determined according to the following formula:

$$\text{inhibition (\%)} = \frac{\text{OD}_{490\text{nm}} \text{ without peptide} - \text{OD}_{490\text{nm}} \text{ with peptide}}{\text{OD}_{490\text{nm}} \text{ without peptide}} \times 100$$

Animals. Male BALB/c mice, 5 weeks old, were purchased from Japan SLC Inc. (Shizuoka, Japan). All mouse experiments were performed in accordance with the Guidelines for Animal Experiments of the College of Bioresource Sciences of Nihon University (approval no. AP11B088). Water was made available continuously through automatic ports, and a commercial mouse diet was provided ad libitum.

Sensitization and Intra gastric Challenge. As illustrated in Figure 1, mice were sensitized twice, 2 weeks apart, with 50 μg of UG in the presence of 1 mg of aluminum hydroxide (Imject Alum; Thermo Scientific, Kanagawa, Japan) as an adjuvant by intraperitoneal injection. Two weeks after the second sensitization, the mice were orally administered 10 mg of UG or DG in 500 μL of water via an intragastric feeding needle (S202; Fuchigami Instrument Co., Kyoto, Japan) every other day for a total of seven times. Before each intragastric challenge, the animals were deprived of their diet for 4 h. Then 40 min after the final administration, the mice were anesthetized by barbital, and thereafter peritoneal cells, blood samples, and each part of the small intestines were collected. For collection of peritoneal cells, 3 mL of a Tyrode buffer ([10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-ethanesulfonic acid (HEPES), pH 7.4, containing 130 mM NaCl, 5.6 mM glucose, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5 mM KCl, and 0.1% (w/v) BSA] was injected into the peritoneal cavity, after which the abdomen was gently massaged for about 60 s. The peritoneal cavity was then carefully opened, and the peritoneal cell-containing fluid was aspirated by use of a Pasteur pipet. Blood samples were withdrawn from the postcaval vein, and the sera were separated by centrifugation at 1300g and 20 $^{\circ}\text{C}$ for 15 min after clotting. The duodenum, jejunum, and ileum were excised and rinsed with PBS.

Intestinal Permeability. Intestinal permeability was evaluated by the amount of HRP (molecular mass 40 kDa; Wako Pure Chemical Industries, Osaka, Japan) that passed through the wall of the jejunum according to the method of Isobe et al.⁴⁵ Briefly, 0.1 mL of 0.1 mg/mL HRP in PBS was injected into the lumen of a closed loop of a 5-cm length of jejunum, and the loop was then placed in 5 mL of PBS and incubated at 37 $^{\circ}\text{C}$ for 30 min. Then 50 μL of the outside solution was collected into the well of a microtiter plate, and the concentration of HRP that had permeated from the lumen through the intestinal wall was measured by adding 100 μL of 0.04% *o*-phenylenediamine with H_2O_2 to the outside solution.

Level of Epitope Absorbed into the Blood. The level of epitope absorbed from the intestinal lumen into the blood was evaluated by performing a competitive inhibition ELISA with the anti-gliadin-epitope antibody as described above. A serum sample in 0.3% BSA/PBST was mixed with the same amount of rabbit anti-gliadin-epitope antibody in 0.3% BSA/PBST, and the mixture was incubated for 1 h at 37 $^{\circ}\text{C}$ for the antigen–antibody reaction to be completed before its addition to gliadin-coated plates.

Level of Gliadin-Specific IgE in Blood. For determination of the level of gliadin-specific IgE in the serum, 100 μL of 5 $\mu\text{g}/\text{mL}$ rat anti-mouse IgE antibody (eBioscience, Inc., San Diego, CA) in a 0.05 M sodium carbonate buffer at pH 9.6 was added to a microtiter plate, and the plate was allowed to stand at 4 $^{\circ}\text{C}$ overnight. Nonspecific binding was blocked by with the addition of 200 μL of 2% BSA/PBST and incubation at 37 $^{\circ}\text{C}$ for 1 h. Then 100 μL of test serum diluted 10 $\times$ with 0.3% BSA/PBST was added to each well, and incubation was continued for 2 h at 37 $^{\circ}\text{C}$. Biotinylated gliadin, prepared by use of a biotin labeling kit (Dojindo Laboratories, Kumamoto, Japan), was diluted 500 $\times$ with 0.3% BSA/PBST, and a 100- μL aliquot of it was added to each well, followed by incubation for 2 h at 37 $^{\circ}\text{C}$. A 100- μL aliquot of HRP-conjugated streptavidin (Funakoshi Co., Tokyo, Japan) diluted 3000 $\times$ with 0.3% BSA/PBST was added to each well, and incubation was continued for another 1 h at 37 $^{\circ}\text{C}$. The wells were

washed five times with PBST at every step. As a substrate for HRP, 100 μL of 0.04% *o*-phenylenediamine containing H_2O_2 was added to the wells. The reaction was allowed to proceed for 20 min at room temperature and then stopped by adding 100 μL of 2 N H_2SO_4 . The optical density was measured at 490 nm with a microtiter plate reader.

Detection of Peritoneal Mast Cells. Mast cells were separated from macrophages and small lymphocytes in the peritoneal cell population by sedimentation at 150g for 10 min at room temperature, and the pellet was resuspended in Tyrode buffer. Mast cells, detected by their expression of the high-affinity IgE receptor (Fc ϵ RI) and c-kit, were stained with anti-Fc ϵ RI (fluorescein isothiocyanate-conjugated) antibody (diluted 1000 $\times$) and anti-c-kit (phycoerythrin-conjugated) antibody (diluted 2000 $\times$) at 4 $^{\circ}\text{C}$ for 30 min. These antibodies were purchased from eBioscience (San Diego, CA). Flow cytometry was performed with a LSRII flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed with FlowJo 6.4.2 software (Tree Star Inc., Ashland, OR).

Total Histamine in Gut and Free Histamine in Plasma. Total histamine level in the gut, including that in mast cells and basophils, was measured to estimate the inflammatory status of the small intestines. The first 5 cm of the duodenum and ileum, distal from the ligament of Treitz, was collected and flushed with 5 mL of cold 0.9% NaCl solution. After being weighed, the tissue was homogenized with 1 mL of 0.9% NaCl. Then 200 μL of 1.2 N perchloric acid was added to the homogenate, which was subsequently centrifuged for 10 min at 13000g. The supernatant was then collected. This procedure was repeated, and the supernatants were pooled and stored at -20°C until further analysis.

For measurement of the level of free histamine in the plasma, 500 μL of a mouse plasma sample was mixed with the same amount of 1 N perchloric acid, and then centrifuged for 10 min at 13000g. The supernatant was collected and stored at -20°C until further analysis.

The histamine level was measured by fluorometric analysis as described by Komatsu.⁴⁶ After 1 mL of each sample had been mixed with 0.135 mL of 1 N NaOH containing 0.75 mg of NaCl, 1.75 mL of a 3:2 (v/v) mixture of *n*-butanol and chloroform was added, and the mixture was stirred for 5 min. After centrifugation at 500g for 5 min, 1.5 mL of the organic solvent layer was recovered and mixed with 1.5 mL of *n*-heptane and 0.6 mL of 0.1 N HCl, and the mixture was stirred for 5 min. After further centrifugation at 500g for 5 min, 1.5 mL of the HCl layer recovered was mixed with 0.12 mL of 1 N NaOH and 0.1 mL of 0.2% *o*-phthalaldehyde (Wako Pure Chemical Industries, Osaka, Japan). The reaction was allowed to proceed for 40 min at 4 $^{\circ}\text{C}$ and terminated by adding 0.05 mL of 0.5 N H_2SO_4 . The fluorescence intensity (excitation at 360 nm and emission at 440 nm) was measured by use of a microtiter plate reader.

Statistical Analysis. All data were expressed as the mean $\pm$ standard error of the mean (SEM), and the significance of differences (*P* values) between groups was evaluated by using Tukey–Kramer test.

RESULTS

Degree of Deamidation. Wheat gliadin was effectively deamidated with the cation-exchange resin of the carboxylate type. The acid amide deamidated amounted to 421.3 $\mu\text{mol}/\text{g}$ of protein. As the amount of acid amide in UG was about 1784.8 $\mu\text{mol}/\text{g}$ of protein, the degree of deamidation was 23.6%.

Reactivity of Rabbit Anti-Gliadin-Epitope Antibody with Synthetic Peptides. The inhibition rate of synthetic peptides with rabbit anti-QQFPQQQIPQQQLPQQQ IgG is shown in Table 1. The reactivity of QQIPQQQ, one of the major epitope peptides of gliadin, was the highest among the 11 7-mer overlapping peptides synthesized, its inhibition rate being approximately 42%. The reactivity of QFPQQQI and QQLPQQQ was also high, with inhibition rates of 32% and 15%, respectively. The inhibition rate of QQFPQQQ was about 7%. Therefore, rabbit anti-QQFPQQQIPQQQLPQQQ IgG was able to detect the major epitope peptides of wheat gliadin

Table 1. ELISA Inhibition Rate of Synthetic Peptides with Rabbit Anti-QQFPQQQIPQQQLPQQQ IgG

synthetic peptide	inhibition rate ^a (%)	synthetic peptide	inhibition rate ^a (%)
QQFPQQQ	6.94 ± 3.18	QIPQQQL	5.68 ± 3.14
QFPQQQI	32.30 ± 1.52	IPQQQLP	1.19 ± 0.94
FPQQQIP	5.54 ± 0.91	PQQQLPQ	4.95 ± 4.52
POOOIPQ	10.97 ± 0.71	QQQLPQQ	12.95 ± 1.60
QQQIPQQ	3.96 ± 1.32	QQLPQQQ	15.31 ± 2.05
QQIPQQQ	42.35 ± 1.59	full-length	76.07 ± 1.48

^aEach value is the mean ± SE of seven experiments.

and will be referred to as anti-gliadin-epitope antibody hereafter.

Reactivity of Rabbit Anti-Gliadin-Epitope Antibody and Patients' Serum with UG and DG. The reactivity of anti-gliadin-epitope antibody was compared with that of the patients' serum. Figure 2 shows the reactivity of anti-gliadin-

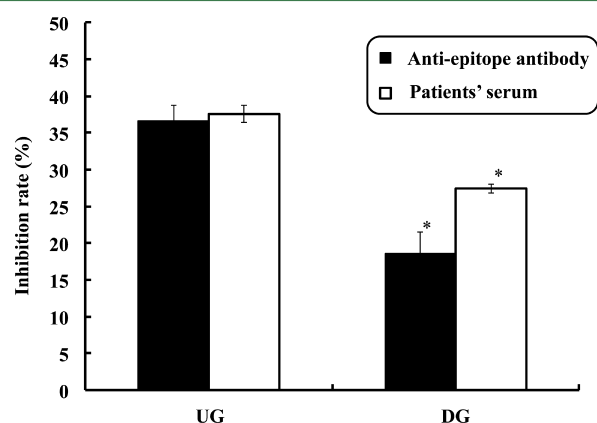


Figure 2. Reactivity of anti-gliadin-epitope antibody and wheat-allergy patients' serum with UG and DG. Asterisk-marked values of DG are significantly different from the corresponding UG value at $P < 0.05$. Each value is the mean of four experiments, with SE shown as a vertical bar.

epitope antibody and wheat-allergy patients' serum with UG or DG. The rabbit anti-gliadin-epitope antibody showed reactivity similar to that of the wheat-allergy patients' serum, and the inhibition rate by DG was lower than that by UG.

Intestinal Permeability. Allergic inflammation may enhance intestinal permeability, permitting large molecules such as proteins to be absorbed from the small intestines. Figure 3 presents the permeability of the jejunum from mice orally administered water, UG, or DG after sensitization with UG. The oral administration of UG after sensitization with it greatly enhanced intestinal permeability of HRP. On the other hand, intestinal permeability for the UG-sensitized DG-administered group was almost the same as that of the unsensitized group or UG-sensitized water-administered group.

Level of Epitope Absorbed into the Blood. The low digestibility and high intestinal permeability of proteins may promote the absorption of antigens and epitope peptides into the blood. As shown in Figure 4, the level of epitope in the blood absorbed from the small intestine was much higher for mice orally administered UG after sensitization with it than for those orally administered DG or water. However, orally administered DG and/or its hydrolysates seem to have been

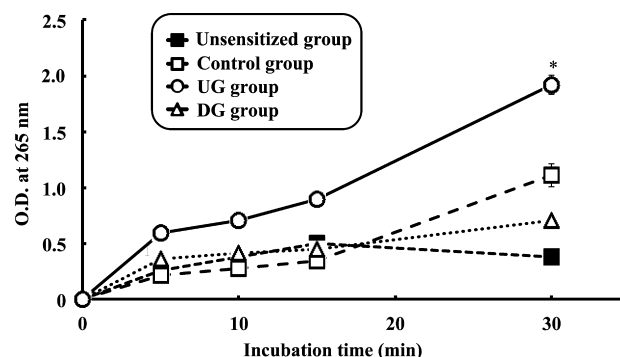


Figure 3. Permeability of small intestines from mice orally administered water, UG, or DG after sensitization with UG. Asterisk-marked values are significantly different from the corresponding values for unsensitized mice at $P < 0.05$. Each value is the mean of seven experiments, with SE shown as a vertical bar.

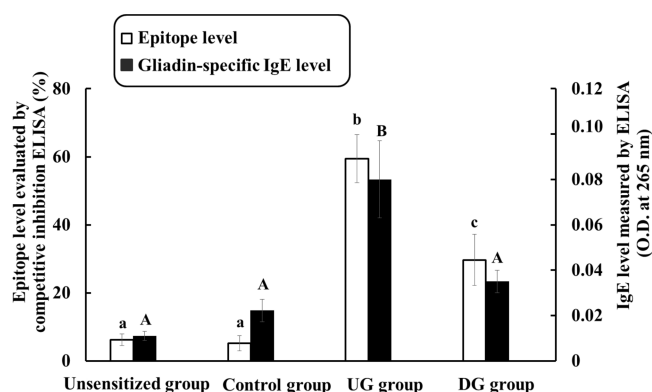


Figure 4. (Open bars) Epitope level and (solid bars) gliadin-specific IgE level in serum. Values with different letters are significantly different at $P < 0.01$. Each value is the mean of seven experiments, with SE shown as a vertical bar.

absorbed from the small intestines to some extent after 30 min of incubation.

Gliadin-Specific IgE Level in the Blood. Figure 4 shows the level of IgE specific for gliadin in the blood of mice orally administered UG, DG, or water after UG sensitization. Oral administration of UG after sensitization with it significantly enhanced the level of gliadin-specific IgE in the blood, whereas that of DG scarcely changed it.

Peritoneal Mast Cells. Mast cells, defined as FcεRI+ and c-kit+ cells, play a key role in allergic reactions, and these cells degranulate to release chemical mediators such as histamine when IgE binds to FcεRI on their surface. Figure 5 presents flow cytometric dot plots of FcεRI- and c-kit-expressing cells. The percentage of FcεRI+ and c-kit+ cells in the UG-sensitized orally administered UG group was much higher than in any of the other groups, being approximately 20% of total cells. On the other hand, the percentage of FcεRI+ and c-kit+ cells from UG-sensitized orally administered DG mice was only about 3%. The results are also shown graphically in Figure 5.

Total Histamine in Gut and Free Histamine in Plasma. Figure 6 shows the level of total histamine in the small intestines. This level in the UG-sensitized, orally administered UG group was almost double that in the other groups, whereas the level for the UG-sensitized, orally administered DG group was almost the same as for the unsensitized and control groups.

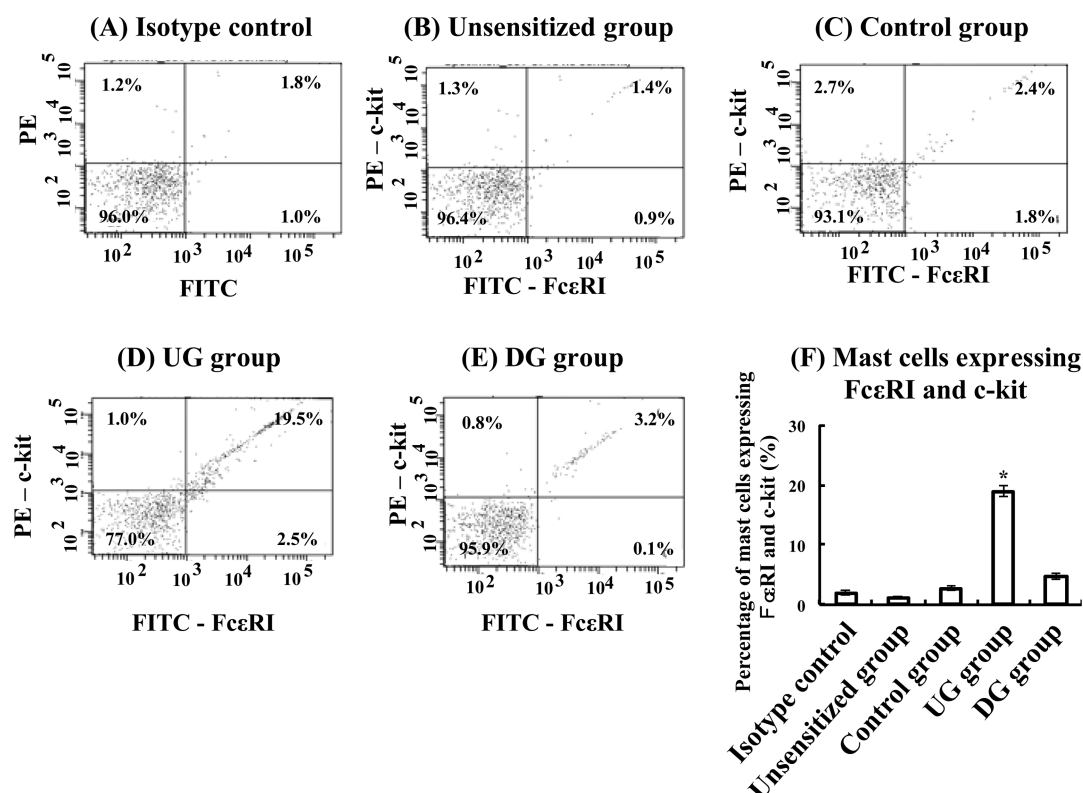


Figure 5. (A–E) Flow cytometric dot plots of FcεRI and c-kit expression on mast cells. (F) Percentage of mast cells expressing FcεRI and c-kit. Asterisk-marked values are significantly different from those of unsensitized mice at $P < 0.05$. Each value is the mean of seven experiments, with SE shown as a vertical bar.

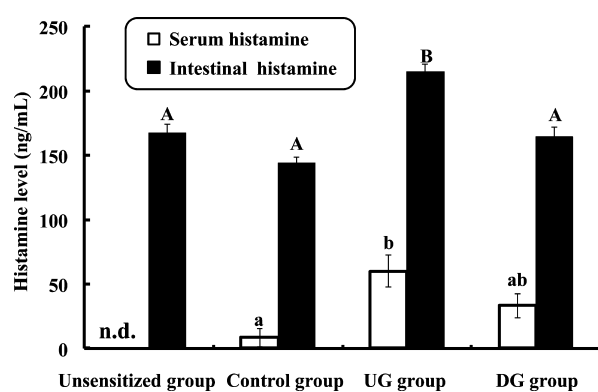


Figure 6. (Open bars) Free histamine level in the serum and (solid bars) total histamine level in the small intestine. Values with different letters are significantly different at $P < 0.01$. Each value is the mean of seven experiments, with SE shown as a vertical bar.

Similarly, oral administration of UG after sensitization with it enhanced the free histamine level in the serum, as shown in Figure 6. The free histamine level in the serum of mice orally administered DG was lower than that of the mice orally administered UG, though there was no significant difference between the two groups.

DISCUSSION

Certain tandem sequence motifs that have glutamine residues in wheat gliadin have been reported to constitute the primary structure of IgE-binding epitopes.^{9,14,15} Therefore, deamidation would be an effective means to reduce wheat allergenicity, although it may not be applicable to patients with celiac disease

or to those who are sensitive to deamidated proteins.^{47–49} Hydrochloric acid treatment has generally been used for deamidation of wheat protein.^{50,51,47} However, hydrochloric acid simultaneously causes hydrolysis of peptide bonds, which produces bitter-tasting peptides and reduces the processing properties of wheat such as extensibility and elasticity. The cation-exchange resin treatment used in this study does not cause peptide-bond hydrolysis and rather improves the processing properties.^{24,29} Therefore, deamidation by cation-exchange resins will provide proteins with health benefits and enhanced sensory properties.

Deamidation is thus expected to reduce the allergenicity of wheat gliadin. However, gliadin in gluten deamidated to the degree of 36–51% by hydrochloric acid still reacted with the sera of patients suffering from wheat allergy to some extent.⁴⁹ Maruyama et al.⁵⁰ reported that gluten treated with hydrochloric acid showed reduced reactivity with the sera of patients allergic to wheat and that gluten with more than 50% deamidation showed markedly decreased IgE-binding capacity. Similarly, gliadin with 52% deamidation generated by treatment with hydrochloric acid showed low reactivity with IgE antibody.⁵¹ Yong et al.²³ reported that wheat gluten deamidated by protein glutaminase had low allergenicity when the deamidation degree reached 72%. These findings indicate that more than 50% deamidation is required to reduce the reactivity of gliadin deamidated by acid or enzyme, probably because of the exposure of inner epitope structure to the surface during acidic hydrolysis and enzymatic reaction. On the other hand, 28% deamidated wheat gliadin achieved with cation-exchange resin treatment scarcely reacted with IgE in the sera of patients allergic to wheat.²⁹ The low reactivity of this deamidated gliadin

with IgE could be due to the effective deamidation of glutamine residues on the surface of protein by cation-exchange resin.

As shown in our previous study, deamidation increased the solubility of gliadin in water and sodium chloride solutions²⁵ and enhanced its digestibility by pancreatin.²⁹ Thus, a further reduction in its allergenicity may occur during digestion. This study was therefore conducted to examine the *in vivo* allergenicity of DG by using wheat-allergy model mice. Although the degree of deamidation of DG used in this study was only 23.6%, oral administration of DG to mice did not induce allergic reactions. Therefore, the epitope sequences in the inner part of the gliadin structure were assumed to be effectively hydrolyzed during digestion.

When an allergic protein is orally administered to mice after immunization, the protein cross-links IgE molecules, thereby inducing an allergic reaction. Allergic inflammation in the small intestines may enhance intestinal permeability and increase the number of mucosal mast cells and eosinophils in the small intestines.^{32,52} Therefore, we examined the permeability of HRP through the jejunum of DG-administered mice. The administration of UG significantly enhanced the intestinal permeability, whereas that of DG scarcely altered it (Figure 3). The considerable rise in serum allergen level after UG administration may be attributed to the increase in intestinal permeability because of probable absorption of large molecules of UG from the small intestines. As the serum allergen level of DG-administered mice was lower than that of UG-administered mice (Figure 4), the epitope structure in DG would have been more extensively hydrolyzed by digestive enzymes in the gut than that in UG. However, the serum allergen level of DG-administered mice was still higher than that of water-administered mice. This result indicates absorption of DG to some extent. The serum IgE level, surface expression of FcεRI, and intestinal histamine level of DG-administered mice were almost the same as those of water-administered mice, indicating that administration of DG did not cause an allergic reaction.

The mechanism of antibody production after immunization with a peptide or protein is not yet completely understood. In this study, a 17-mer peptide (QQFPQQQIPQQQLPQQQ) having three major epitope sequences of gliadin (QQFPQQQ, QQIPQQQ, and QQLPQQQ) was synthesized, and rabbits were immunized with it. The obtained antibody notably recognized QQIPQQQ, one of the major epitopes. Its reactivity with QQLPQQQ was also high. Therefore, these two epitope sequences can be considered to promote antibody production. On the other hand, it recognized QFPQQQI much more than QQFPQQQ, which is another major epitope sequence. The reason for this might be due to differences in the mechanism of antibody recognition between rabbits and human or to the order of connected epitope peptide sequences. However, reactivity of the antibody from rabbits immunized with UG and DG was almost the same as that of patients' serum, indicating that the antibody from rabbit serum can be used for detecting epitope sequences *in vivo*.

In conclusion, the deamidated gliadin was confirmed to have low allergenicity even *in vivo*, suppressing the enhancement in intestinal permeability, serum allergen level, serum allergen-specific IgE level, mast-cell-surface expression of FcεRI, and serum and intestinal histamine levels. As deamidated gliadin exhibits a high foaming property,²⁵ deamidation by cation-exchange treatment with no peptide-bond hydrolysis is a promising technique for production of bread and cakes with low allergenicity and better expansibility.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AEDS, atopic eczema/dermatitis syndrome; DG, deamidated gliadin; FcεRI, high-affinity IgE receptor; HMW glutenin, high-molecular-weight glutenin; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; UG, undeamidated gliadin; WDEIA, wheat-dependent exercise-induced anaphylaxis

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