



Improvement of Digestibility, Reduction in Allergenicity, and Induction of Oral Tolerance of Wheat Gliadin by Deamidation

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Wheat gliadin was deamidated by using a cation-exchange resin in the presence or absence of added cysteine, with the change in digestibility being measured. The allergenicity of the gliadin was evaluated by using sera from patients RAST-positive to wheat. Gliadin-specific IgE was measured after the gliadin had been orally administered to rats. The addition of cysteine before the treatment with a cation exchanger effectively increased the deamidation level of gliadin. Deamidated gliadin showed higher solubility than the undeamidated form. There was no difference in the peptic digestibility of the gliadin, whereas deamidation enhanced the pancreatic digestibility *in vitro* and the digestibility in the mouse stomach *in vivo*. Deamidation of gliadin reduced its reactivity toward the sera of patients with wheat allergy. Rats administered with deamidated gliadin showed suppressed elevation of the gliadin-specific IgE level.

Key words: deamidation; wheat gliadin; digestibility; allergenicity; oral tolerance

Deamidating proteins results in enhancing their glutamate and aspartate residues with a change in their properties. Firstly, the carboxyl group can bind to cations such as Ca²⁺ or Na⁺. Secondly, the solubility of the protein in water increases. Thirdly, the change in the hydrophilic-hydrophobic balance of the protein often modifies its foaming and emulsifying properties.

We have shown that the calcium-binding property of soybean glycinin was improved by deamidation^{1,2)} and contributed to enhancing the calcium absorption in the

small intestine.^{3,4)} We have also shown that deamidating ethanol-soluble wheat gliadin and gluten was effective for increasing their solubility in water and an NaCl solution,^{5,6)} as well as their foaming capacity.⁶⁾ Such changes in the solubility and surface properties of these proteins improve their food processing qualities. Moreover, the increase in water solubility may contribute to improving their *in vivo* digestibility, because they would become more susceptible to the action of digestive enzymes.

Wheat is known to induce food-associated allergic reactions in children and adults. The main symptom of food allergy to wheat in children is atopic eczema/dermatitis syndrome (AEDS),^{7,8)} whereas in adults, the most frequent and severe symptom is wheat-dependent exercise-induced anaphylaxis (WDEIA).^{7,9,10)} Gliadins and glutenins, the major proteins of wheat flour, are involved in the development of food allergy to wheat.¹¹⁻¹⁵⁾ In particular, wheat ω -5 gliadin and a high-molecular-weight-glutenin subunit (HMW-glutenin) have been reported as the major allergens in WDEIA. Furthermore, IgE binding to α β-, γ - and ω -gliadins has been demonstrated in children and adults allergic to wheat.¹⁵⁾ Deamidation could be used to reduce wheat allergenicity, because some tandem sequencing sites involving glutamine residues in wheat gliadin and glutenin have been reported to constitute the primary structure of IgE-binding epitopes.¹⁶⁻¹⁸⁾

There are several methods that can be used to deaminate proteins, including the use of acids,¹⁹⁻²⁴⁾ enzymes,^{1,25-27)} and cation exchangers.^{2-6,28)} Among them, an acid treatment is the most commonly used,

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although this causes unfavorable peptide-bond hydrolysis resulting in the production of bitter-tasting peptides and a reduction in the processing properties of the protein. The use of proteases is not recommended for the same reason.²⁵⁾ Glutaminases are available for protein deamidation, but they do not act on asparagine residues.^{26,27)} Moreover, these enzymes cannot be used in an ethanol solution, because they are inactivated. We have developed a method that uses a cation-exchange resin of the carboxylate type for effectively deamidating proteins, without causing any detectable peptide-bond hydrolysis.²⁻⁶⁾ Since a cation-exchange resin is resistant to an ethanol solution, this method is applicable to alcohol-soluble proteins such as prolamines. We have found that the use of an ion exchanger resulted in 28% deamidation of gliadin⁵⁾ and 30% deamidation of gluten.⁶⁾ It is necessary to loosen the tight structure of the proteins to expose their buried glutamine and asparagine residues for efficient deamidation.

In this study, we prepared deamidated wheat gliadin in the presence of cysteine to loosen the structure of the protein by exchanging disulfide bonds in order to improve its solubility in water, salt solutions and buffered solutions at various pH values, as well as to increase its digestibility and decrease its allergenicity. The study also examines the oral tolerance of deamidated gliadin in rats.

Materials and Methods

Deamidation of wheat gliadin. Wheat gliadin was extracted from gluten (Ishizu Seiyaku, Osaka, Japan) with 60% ethanol. After being concentrated under vacuum, the extract was lyophilized. The undeamidated gliadin thus obtained was dissolved in 60% ethanol to a concentration of 0.1% [w/v] with and without the addition of cysteine (0.0145% [w/v]). For deamidation, the solution was mixed with 0.3 g/ml of a cation-exchange resin, carboxylate type (IRC50, Organo Co., Tokyo, Japan) and gently stirred at 25 °C for 24 h. The resin was then removed by filtration. Deamidated gliadin was obtained after concentration and lyophilization. The cysteine-added undeamidated gliadin and the cysteine-added deamidated gliadin were each mixed with 0.5 g/ml of a cation-exchange resin (XT1006, Organo Co., Tokyo, Japan) to remove the excess cysteine, and the resin was removed by filtration.

The degree of deamidation is expressed as the amount of removed acid amide to the total acid amide present before the treatment with the cation-exchange resin.^{2,5)} The amount of removed acid amide was obtained from the difference between the amount of acid amide in the gliadin before and after the ion-exchange resin treatment, which was determined from the amount of ammonia released from the gliadin during the HCl treatment. Each gliadin was individually dissolved in 2 N HCl, completely deamidated at 100 °C for 3 h, and then neutralized with 1 N NaOH before and after the

ion-exchange resin treatment. The amount of ammonia in a completely deamidated sample was measured according to Conway's micro-diffusion method²⁹⁾ and the indophenol method.³⁰⁾ The ammonia released from 0.5 ml of a sample solution by 2 ml of a strong alkali was absorbed into 0.5 ml of 0.01 N H₂SO₄ in a sealed vessel. After incubating at 37 °C for 2 h, 0.5 ml of the ammonium sulfate solution thus obtained was mixed with 0.05 ml of 0.003 M MnSO₄, 1 ml of an alkaline phenol solution and 0.5 ml of NaClO in ice-cold water. After being sealed, the solution was heated at 100 °C for 5 min, and then diluted with 10 ml of distilled water. The absorbance of indophenol produced by the reaction was measured at 625 nm with a spectrophotometer (UV-240, Shimadzu Corp., Tokyo, Japan). A calibration curve was obtained using a known amount of an ammonium sulfate solution.

Solubility of the gliadin. Each gliadin was mixed with water, a salt solution or buffered solution in the range of pH 2.0–10.0 to a final concentration of 1 mg/ml, and stirred for 1 h. After filtration, the protein concentration in the filtrate was determined from its absorbance at 280 nm. As salt solutions, 0.1 M NaCl and 1.0 M NaCl were used. The following buffered solutions were used for the pH-dependence test: 0.05 M KCl–HCl buffer at pH 2.0; 0.05 M sodium citrate buffer at pH 3.0, 4.0, and 5.0; 0.05 M sodium phosphate buffer at pH 6.0; 0.05 M Tris–HCl buffer at pH 7.0, 7.4, and 8.0; and 0.05 M sodium bicarbonate–NaOH buffer at pH 10.0.

Preparation of the rabbit anti-gliadin antibody. Prior to the digestibility test, an anti-gliadin antibody was prepared to detect any unhydrolyzed proteins or partially-hydrolyzed peptides remaining in the enzymatic digest. Japanese white female rabbits (Nippon Bio-Supply Center, Tokyo, Japan) were subcutaneously immunized with 2 mg of gliadin that had been emulsified with Freund's complete adjuvant for the first and second immunizations and with Freund's incomplete adjuvant thereafter. Blood was collected from the central artery in the ear 10 d after each immunization to check the antibody titer. Sera containing the anti-gliadin antibody were collected 10 d after the sixth immunization.

In vitro digestibility of gliadin by digestive enzymes. Each gliadin was hydrolyzed by digestive enzymes *in vitro*, and the degree of hydrolysis and antigenicity of the hydrolysate against the rabbit anti-gliadin antibody were measured. Pepsin from porcine stomach mucosa (3400 units/mg, Wako Pure Chemical Industries, Osaka, Japan) and pancreatin from hog pancreas (protein digestibility of 28 units/mg, Wako Pure Chemical Industries, Tokyo, Japan) were used as digestive enzymes. A gliadin was mixed with each enzyme (0.1 mg/ml) at a concentration of 0.1% and an enzyme-substrate ratio of 1:100. Digestion with pepsin and with

pancreatin used 0.01 N HCl and a Tris-HCl buffer at pH 7.4, respectively, to dissolve the proteins. After incubating at 37 °C for up to 24 h, the enzymatic hydrolysis was stopped by heating the sample at 100 °C for 10 min.

The degree of hydrolysis is expressed as the proportion of nitrogen contained in the 0.6 M trichloroacetic acid-soluble fraction to the total nitrogen content as described previously.^{1,2)}

The antigenicity of each hydrolysate was evaluated by ELISA, using the rabbit anti-gliadin antibody. Each hydrolysate was dissolved in a 0.1 M Tris-HCl buffer at pH 8.6 containing 4 M urea to a concentration of 5 µg/ml, and a 100-µl aliquot was added to each well of a microtiter plate, which was then allowed to stand at 4 °C overnight. Non-specific binding was blocked by incubating with 200 µl of 2% bovine serum albumin (BSA) at 37 °C for 2 h, and then 100 µl of the anti-gliadin antibody at different dilutions was added to each well. After a 1-h incubation at room temperature, the biotinylated goat anti-rabbit IgG antibody (EY Laboratories, San Mateo, CA, USA) diluted 6,000 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (TPBS) was added as the secondary antibody, and incubation was continued for 2 h at room temperature. A 100-µl amount of streptavidin-horseradish peroxidase (Funakoshi Co., Tokyo, Japan) was then added to each well, and the plate was incubated for another 20 min at room temperature. The wells used in every step were washed 5 times with TPBS. As the substrate for peroxidase, 100 µl of 0.04% *o*-phenylenediamine containing H₂O₂ was added. The reaction was allowed to proceed for 20 min at room temperature, prior to being stopped by adding 100 µl of 2 N H₂SO₄. The optical density of the content of each well was measured at 490 nm with a microtiter plate reader (Bio-Rad Laboratories, Tokyo, Japan).

In vivo digestibility of gliadin in the digestive tract of mice. Each gliadin was fed to mice and its *in vivo* digestibility evaluated by measuring the reactivity of the digestive tract contents with the rabbit anti-gliadin antibody. Five-week-old male Balb/c mice (Nippon Bio-Supply Center, Tokyo, Japan) were fed on a control diet containing 20% [w/w] casein for 5 d and acclimatized to the animal house environment (23 ± 2 °C with a light/dark cycle of 12 h). They were then divided into 5 groups, fasted for 48 h, and fed with 0.25 g of a diet containing 20% of each protein sample (casein, undeamidated gliadin, deamidated gliadin, cysteine-added undeamidated gliadin, and cysteine-added deamidated gliadin), 0.3% of DL-methionine, 15% of corn starch, 50% of sucrose, 5% of cellulose, 3.5% of an AIN-76 mineral mixture (Oriental Yeast Co., Tokyo, Japan), 1% of an AIN-76 vitamin mixture (Oriental Yeast Co., Tokyo, Japan), and 0.2% of choline bitartrate. At a certain interval after that, the mice were sacrificed by cervical dislocation, and the contents of the stomach,

and of the upper and lower parts of the small intestine were collected into 1 ml of a 0.1 M Tris-HCl buffer at pH 8.6 containing 4 M urea. After centrifuging the sample at 1,300 g and 4 °C for 15 min, the supernatant was collected, and then diluted 50,000 times with a 0.1 M Tris-HCl buffer at pH 8.6 containing 4 M urea. A 100-µl aliquot was added to each well of a microtiter plate, which was then allowed to stand at 4 °C overnight. The antigenicity of the mouse gastrointestinal contents against the rabbit anti-gliadin antibody was evaluated by ELISA as already described.

All animal experiments were performed in accordance with the Guidelines for Animal Experiments of the College of Bioresource Sciences, Nihon University.

In vitro allergenicity of gliadin against patients' sera. Sera RAST-positive to wheat were donated by 20 outpatients aged between 7 months and 50 years, who were under treatment at Yokohama City University Hospital, after a full explanation of the purpose of the study had been given to each patient or their guardian. Each gliadin was dissolved in 60% ethanol to a concentration of 50 µg/ml, and a 100-µl aliquot was added to each well of a microtiter plate which was then allowed to stand at 4 °C overnight. Non-specific binding was blocked by incubating with 200 µl of 2% bovine serum albumin (BSA) at 37 °C for 2 h. A 100-µl amount of serum from each patient RAST-positive to wheat was added to each well. After a 1-h incubation at room temperature, either the biotinylated goat anti-human IgE antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 3,000 times with TPBS or the biotinylated goat anti-human IgG antibody (EY Laboratories, San Mateo, CA, USA) diluted 6,000 times with TPBS was added as the secondary antibody, and incubation was continued for 2 h at room temperature. A 100-µl amount of streptavidin-horseradish peroxidase (Funakoshi Co., Tokyo, Japan) was then added to each well and developed with 100 µl of 0.04% *o*-phenylenediamine containing H₂O₂ as already described.

Induction of oral tolerance to gliadin in rats. Deamidated gliadin was fed to rats to examine if it could induce oral tolerance to gliadin. Five-week-old female Brown Norway rats (Nippon Bio-Supply Center, Tokyo, Japan) were fed on a standard diet containing 20% casein, 0.3% DL-methionine, 15% corn starch, 50% sucrose, 5% cellulose, 3.5% AIN-76 mineral mixture (Oriental Yeast Co., Tokyo, Japan), 1% AIN-76 vitamin mixture (Oriental Yeast Co., Tokyo, Japan), and 0.2% choline bitartrate for 7 d and acclimatized to the animal house environment (23 ± 2 °C with a light/dark cycle of 12 h). The rats were divided into 3 groups, and 0.2 ml of 37% ethanol alone (control), or 10 mg of undeamidated gliadin or deamidated gliadin in 0.2 ml of 37% ethanol was administered daily for 5 d *via* a stomach sonde needle designed for rats. Two days after the final administration, the rats were subcutaneously immunized

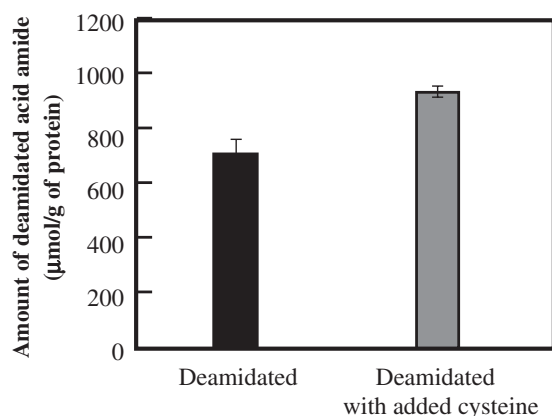


Fig. 1. Cation-Exchange Resin-Aided Deamidation of Wheat Gliadin Prepared with and without the Addition of Cysteine.

Cation-exchange resin, carboxylate type, was used at 0.3 g/ml–0.1% protein solution, and the treatment time was 24 hours. Each value is presented as the mean $\pm$ SE ($n = 3$).

with 1 mg of undeamidated gliadin in 0.5 ml of $\text{Al}(\text{OH})_3$ (Imject Alum, Pierce Biotechnology, Rockford, IL, USA) as an adjuvant for inducing a high IgE response, and also intraperitoneally injected with 0.2 ml of inactivated *Bordetella pertussis* cells (6×10^8 cells in PBS) as an additional adjuvant. Then, 14 d after the immunization, the rats were fasted for 18 h, prior to blood sampling from the jugular vein. Each blood sample was centrifuged at 1,300 g and 20 °C for 15 min to collect the serum.

To determine IgE specific to gliadin in the serum, the mouse anti-rat IgE antibody (Zymed Laboratories, San Francisco, CA, USA) was diluted with a 0.05 M sodium carbonate buffer at pH 9.6 to a final concentration of 5 $\mu\text{g}/\text{ml}$, and a 100- μl aliquot was added to each well of a microtiter plate which was then allowed to stand at 4 °C overnight. Non-specific binding was blocked by incubating with 200 μl of 2% BSA at 37 °C for 1 h. A 100- μl amount of the serum diluted 10 times with TPBS containing 0.3% BSA was then added to each well, and the plate was allowed to stand for 2 h at 37 °C. Digoxigenylated gliadin prepared by using a DIG protein labeling kit (Roche, Mannheim, Germany) was diluted 500 times with TPBS containing 0.3% BSA. A 100- μl aliquot was added to each well, and incubated for 2 h at 37 °C. Peroxidase-labeled anti-digoxigenin Fab fragments (Roche, Mannheim, Germany) were diluted 3,000 times with TPBS containing 0.3% BSA. A 100- μl aliquot was added to each well, and incubated for 1 h at 37 °C. The wells used in every step were washed 5 times with TPBS. As a substrate for peroxidase, 100 μl of 0.04% *o*-phenyldiamine containing H_2O_2 was added. 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 of the content of each well was measured at 490 nm with a microtiter plate reader.

To determine IgG specific to gliadin, a serum sample was diluted 50,000 times with TPBS containing 0.3%

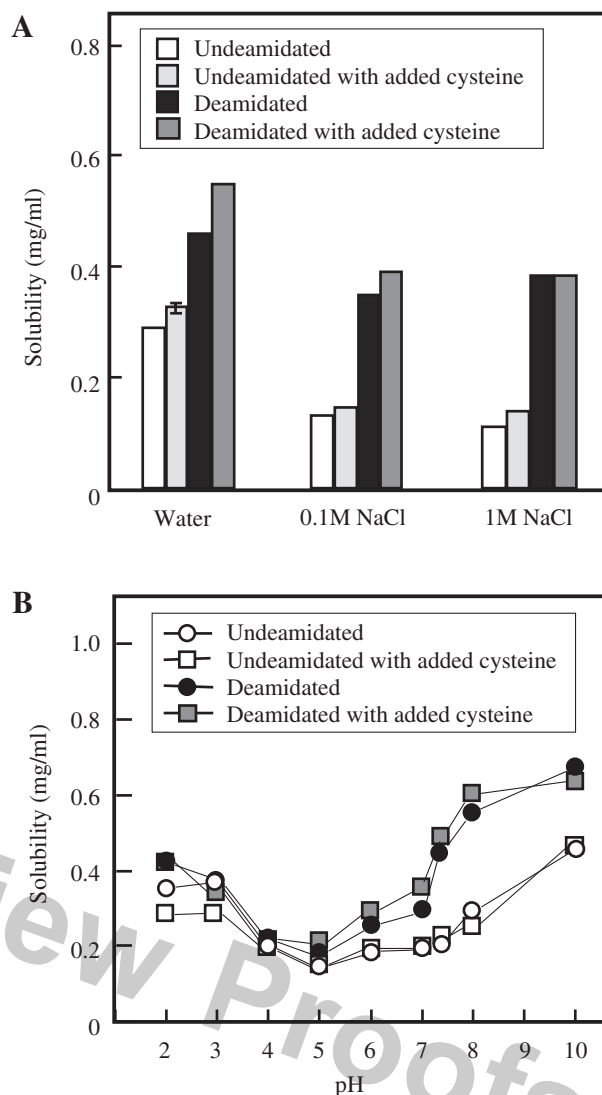


Fig. 2. Effects of Deamidation and the Addition of Cysteine on the Solubility of Wheat Gliadin in Water and a Salt Solution (A), and in a Buffered Solution at Various pH Values (B).

Each value is presented as the mean $\pm$ SE ($n = 3$).

BSA, and the biotinylated mouse anti-rat IgG antibody (Chemicon International, Temecula, CA, USA) was diluted 6,000 times with TPBS. To determine IgG1, a serum sample was diluted 3,000 times with TPBS containing 0.3% BSA, and the biotinylated mouse anti-rat IgG1 antibody (Zymed Laboratories, San Francisco, CA, USA) was diluted 6,000 times with TPBS. To determine IgG2a, the serum was diluted 1,000 times with TPBS containing 0.3% BSA, and the biotinylated mouse anti-rat IgG2a antibody (Zymed Laboratories, San Francisco, CA, USA) was diluted 6,000 times with TPBS. The other conditions were the same as those used to determine the antigenicity of each gliadin hydrolysate against the rabbit anti-gliadin antibody.

All animal experiments were performed in accordance with the Guidelines for Animal Experiments of the College of Bioresource Sciences, Nihon University.

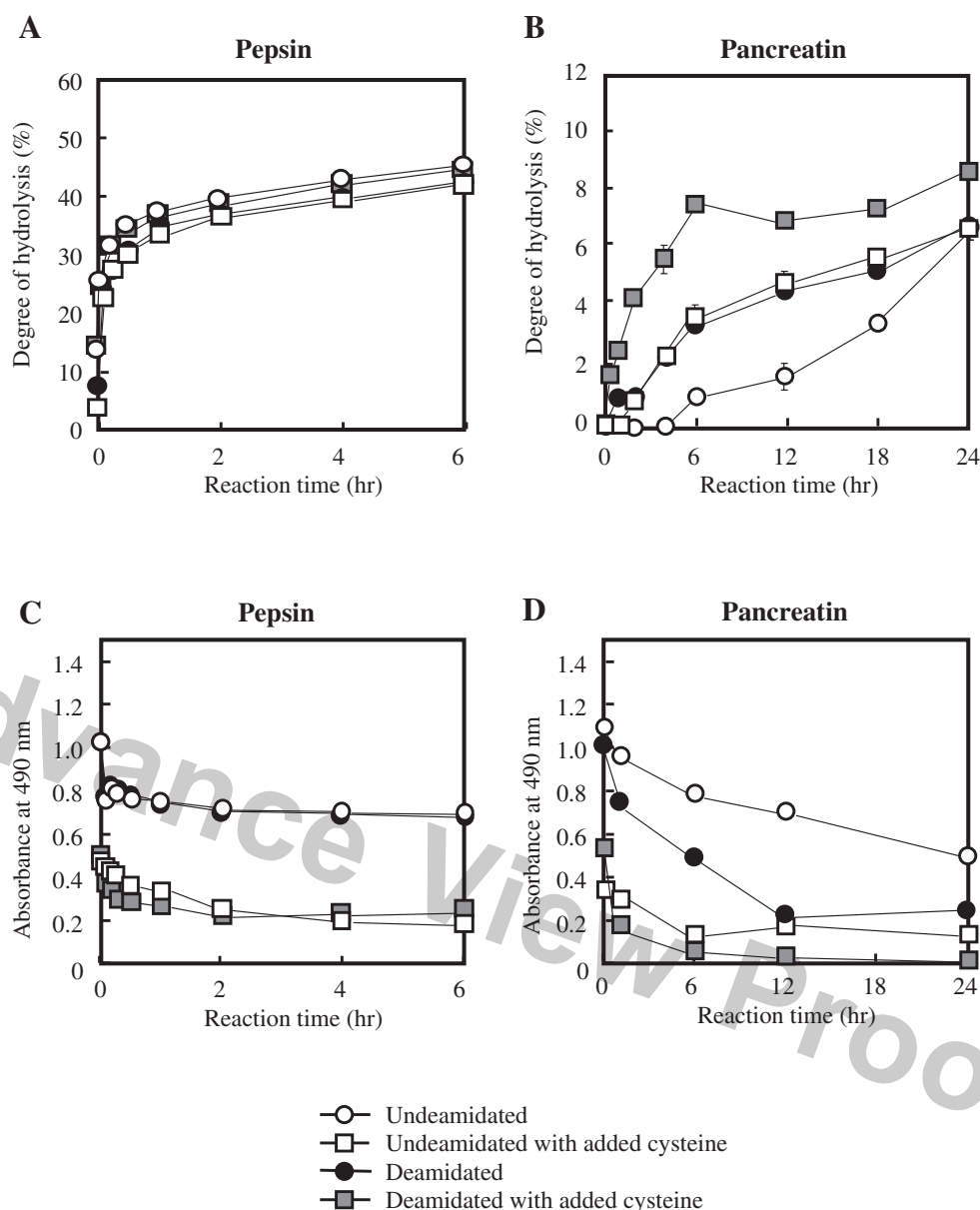


Fig. 3. Time-Course Changes in the Degree of Hydrolysis of Gliadins Digested with Pepsin (A) or Pancreatin (B) *in Vitro*, and in the Antigenicity of Gliadin Hydrolysates after the Digestion with Pepsin (C) or Pancreatin (D) *in Vitro*.

Each value is presented as the mean $\pm$ SE ($n = 3$). The degree of hydrolysis is presented as the proportion of nitrogen contained in the 0.6 M trichloroacetic acid-soluble fraction to the total nitrogen content. The antigenicity of each hydrolysate was evaluated by ELISA, using the rabbit anti-gliadin antibody.

Results

Deamidation of wheat gliadin

Wheat gliadin was effectively deamidated when a cation-exchange resin of the carboxylate type was used. The acid amide deamidated amounted to 718.4 $\mu\text{mol/g}$ of protein in the case of gliadin treated without the addition of cysteine and to 942.3 $\mu\text{mol/g}$ of protein in the case of gliadin treated with the addition of cysteine (Fig. 1).

Solubility of gliadin

The solubility of gliadin in water and a salt solution

was enhanced by deamidation. In detail, the solubility of deamidated gliadin was about 1.6 times higher in water and 2.8 times higher in an NaCl solution than that of the undeamidated sample (Fig. 2A). The effect of deamidation on the solubility was more marked in the case of a solution at neutral or alkaline pH than in a solution at acidic pH (Fig. 2B).

In vitro digestibility of gliadin by digestive enzymes

There was no significant difference in the *in vitro* peptic digestibility of gliadin (Fig. 3A). On the other hand, deamidation as well as the addition of cysteine enhanced the *in vitro* pancreatic digestibility (Fig. 3B).

No difference in reactivity with the rabbit anti-gliadin antibody was found between an undeamidated and deamidated gliadin hydrolysate obtained by treating with pepsin, while the addition of cysteine lowered the reactivity (Fig. 3C). As for the hydrolysates obtained with pancreatin, deamidation reduced the reactivity with the rabbit anti-gliadin antibody, and the addition of cysteine further reduced it (Fig. 3D).

In vivo digestibility of gliadin in the digestive tract of mice

Deamidation was effective for enhancing the *in vivo* digestibility in the stomach of mice, whereas the addition of cysteine had little effect *in vivo* (Fig. 4). The anti-gliadin antibody failed to detect any undigested protein or peptide from any sample in the upper and lower parts of the small intestine (data not shown).

In vitro allergenicity of gliadin against patients' sera

The reactivity of gliadin to IgE and IgG in the sera from patients who were RAST-positive to wheat was markedly decreased by deamidation, and the cysteine treatment barely affected the reactivity to IgE and IgG (Fig. 5).

Induction of oral tolerance to gliadin in rats

In the oral tolerance study, the increase in gliadin-specific IgE caused by immunization with gliadin was suppressed when undeamidated or deamidated gliadin was administered to rats before the immunization (Fig. 6A). The levels of IgG, IgG1 and IgG2a specific to gliadin were elevated after immunization with gliadin, but the oral administration of undeamidated or deami-

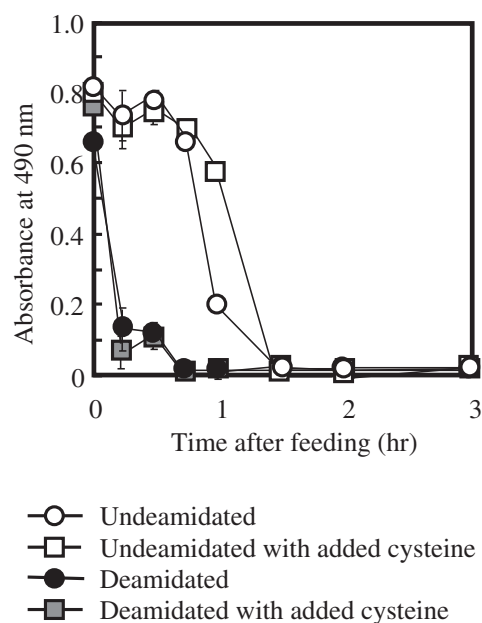


Fig. 4. Time-Course Changes in the Antigenicity of Each Gliadin Hydrolysate after Digestion in the Mouse Stomach *in Vivo*.

Each value is presented as the mean $\pm$ SE ($n = 3$). The antigenicity of the each hydrolysate was evaluated by ELISA, using the rabbit anti-gliadin antibody.

dated gliadin before the immunization had no significant effect on the elevation of IgG level (Fig. 6B, C and D).

Discussion

Shih²⁸⁾ found that a cation-exchange resin of the

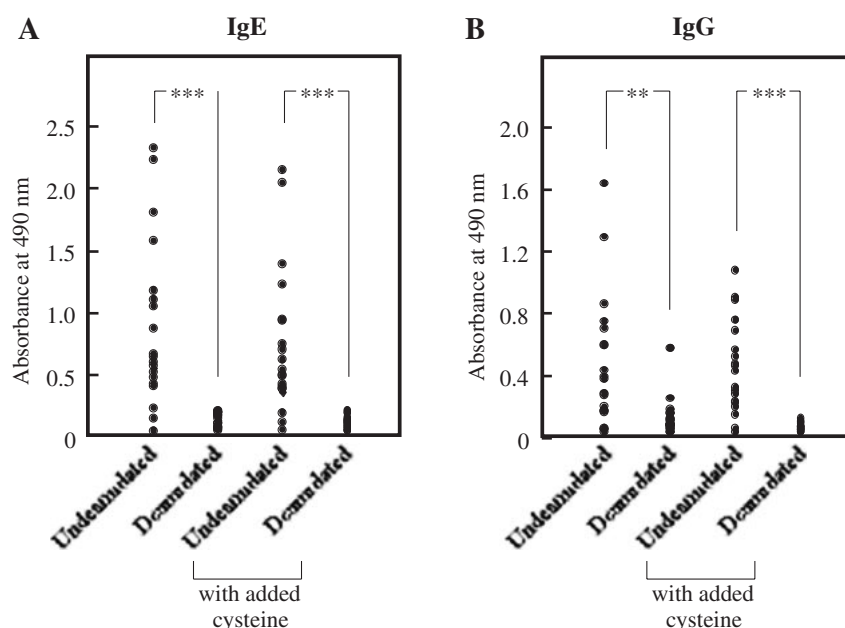


Fig. 5. Allergenicity of Deamidated and/or Cysteine-Added Gliadin.

The gliadin-specific IgE (A) and IgG (B) levels in sera from 20 patients RAST-positive to wheat were measured. ***Significant ($p < 0.001$) in a two-tailed paired Student's *t*-test. **Significant ($p < 0.01$) in a two-tailed paired Student's *t*-test.

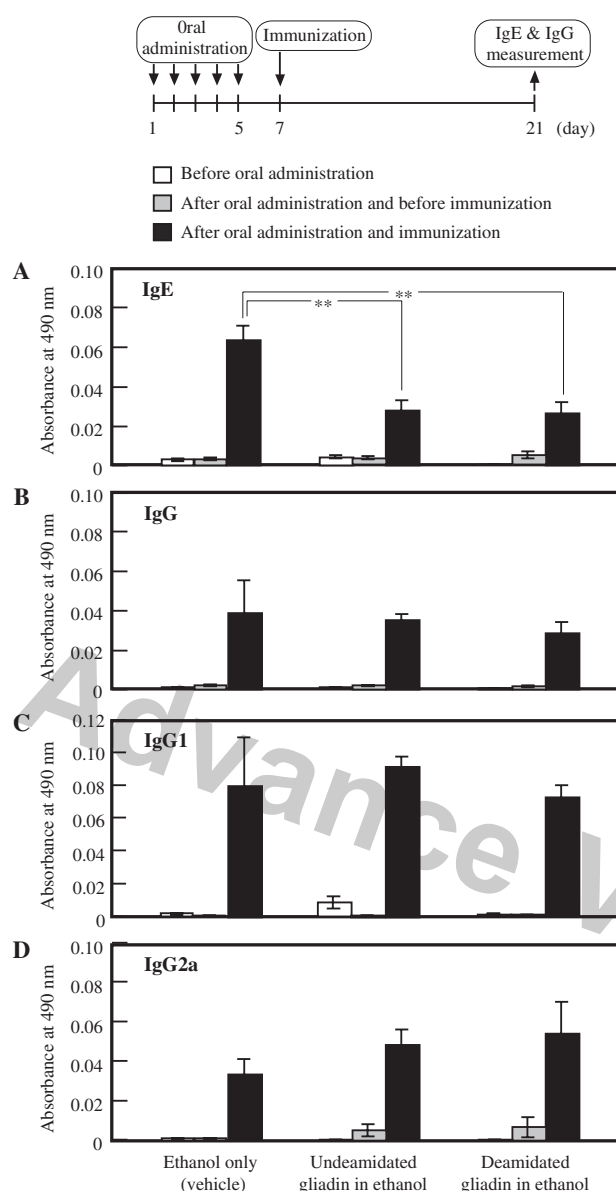


Fig. 6. Gliadin-Specific IgE (A), IgG (B), IgG1 (C) and IgG2a (D) Levels in the Sera of Rats before and after an Oral Administration of Each Sample and Immunization with Undeaminated Gliadin.

Brown Norway rats were orally administered with 0.2 ml of 37% ethanol alone, or 10 mg of undeaminated gliadin or deaminated gliadin in 0.2 ml of 37% ethanol for 5 d, and then immunized with 1 mg of undeaminated gliadin. **Significant ($p < 0.01$) in Tukey's multiple-comparison test. Each value is presented as the mean $\pm$ SE ($n = 5-6$).

sulfonate type had the ability to catalyze the deamidation of soybean proteins. We then examined the deamidation activity of various cation-exchange resins, and found that a cation-exchange resin of the carboxylate type was more effective than that of the sulfonate type.^{2,5)} As the reaction occurs by contact of the acid amide of a protein with the cation-exchange resin, catalysis of the deamidation reaction would be more significant if the protein structure were uncoiled with its inner functional groups exposed. Therefore, we added cysteine to gliadin to loosen its structure and deamidated

it by using a cation-exchange resin of the carboxylate type. As a result, the amount of deamidated acid amide increased from 718.4 to 942.3 $\mu\text{mol/g}$ of protein (Fig. 1), indicating that the cysteine treatment contributed to increasing the deamidation. Gliadin has a unique amino-acid composition, in that its glutamine accounts for about one third of the total content of amino acids. As the gliadin we used contained about 2500 $\mu\text{mol/g}$ of protein of the acid amide, the degree of deamidation was 28% for gliadin without the addition of cysteine and 38% for that with the addition of cysteine. Similar to our previous findings, no peptide-bond hydrolysis was apparent during the ion-exchange resin treatment (time 0 in Fig. 3B).

The carboxyl group, when dissociated, is more hydrophilic than the acid-amide group, so the solubility of a protein can be expected to increase after deamidation. This effect of deamidation has already been verified with some proteins.^{5,6,19-21)} In agreement with those findings, each deaminated gliadin was more soluble in water, a salt solution, and buffered solution at neutral and alkaline pH than the undeaminated type (Fig. 2). However, there was little difference in solubility between the deaminated gliadin samples prepared with and without the addition of cysteine. This suggests that 28% deamidation would be enough to increase the solubility of gliadin.

There have been several studies on the digestibility of deaminated proteins,²²⁻²⁴⁾ but there is no information available on that of deaminated gliadin. Little difference in the *in vitro* peptic digestibility of gliadin (Fig. 3A) may be ascribed to the fact that deamidation barely increased the solubility of gliadin in an acidic pH range (Fig. 2B), and that proteins tend to aggregate in an acid solution. As a protein in the solubilized and/or uncoiled forms is more susceptible to enzymatic digestion, the increased solubility of gliadin above the neutral pH level and loosening of its structure by cysteine are presumed to be the reasons for the enhanced *in vitro* digestibility by pancreatin at pH 7.4 (Fig. 3B). According to our preliminary experiment, the digestibility by pancreatin was greater than that by trypsin or chymotrypsin alone, indicating that gliadin was hydrolyzed not only by trypsin and chymotrypsin, but also by some other minor enzymes such as carboxypeptidase and elastase.

After heating to stop the enzymatic reaction, the antigenicity of the cysteine-added gliadin against the rabbit anti-gliadin antibody was lower than that of gliadin without cysteine (Fig. 3C and D), although there was no significant difference in the antigenicity of gliadin without any heating (time 0 in Fig. 4). These results suggest that the rabbit anti-gliadin antibody recognized both the undeaminated and deaminated gliadins similarly, and that the added cysteine made gliadin more susceptible to heat denaturation by lowering its reactivity with the antibody.

Unexpectedly, deamidation enhanced the *in vivo* digestibility of gliadin in the stomach, although the

addition of cysteine had no effect on digestibility (Fig. 4). The reason for this discrepancy between the *in vitro* and *in vivo* results remains to be clarified, but it might have been due to the presence of some proteases other than pepsin such as gastricsin (pepsin C) and chymosin (rennin) in the stomach.^{31,32)} The dietary composition and gastric emptying effect in the *in vivo* experiment should also be considered. Details await further experimentation in our following study. Neither protein nor peptide was detectable by the anti-gliadin antibody in the small intestine. Therefore, it will be necessary to prepare an anti-gliadin-peptide antibody to examine the difference in digestibility of gliadin in the small intestine in a future study.

Similar to the IgE-binding epitopes in gluten,^{16,17)} those in gliadin contained the sequences of the glutamine residue tandem.¹⁸⁾ In this study, deamidation of gliadin markedly decreased its reactivity with both IgE and IgG in the sera from patients who were RAST-positive to wheat, although the addition of cysteine had little effect on its decreased reactivity with IgE. This finding of reduced allergenicity by deamidating a protein indicates that a cation-exchange resin can convert the glutamine residues in the epitopes (antigenic determinants) to glutamic-acid residues, and also that changing its primary structure is more effective for reducing allergenicity than an unfolded protein.

Orally-administered gliadin is known to induce oral tolerance.³³⁾ However, it is often difficult for patients to take intact antigens to induce oral tolerance, because oral administration of the causative allergen could cause a severe allergic response. Therefore, modified allergens, showing hypo-allergenicity while strongly inducing tolerance, are desirable for treating food allergy. Watanabe *et al.*³⁴⁾ have succeeded in reducing the allergenicity of gluten by an enzymatic treatment, suggesting that enzymatically-treated wheat flour induced oral tolerance and thereby suppressed the allergic reaction.³⁵⁾ In our present study, the oral administration of deamidated gliadin suppressed the production of gliadin-specific IgE, indicating that deamidated gliadin might have induced oral tolerance. This is apparently the first demonstration of the induction of oral tolerance by deamidated gliadin and of the possibility to use it for preventing wheat allergy. Oral immunotolerance is usually induced *via* antigen-specific T-cell deletion, anergy, and/or active suppression by regulatory T-cells. However, it remains to be clarified which one of these mechanisms was involved in the case of the production of gliadin-specific IgE being decreased. We will also consider the T-cell epitopes and the cytokine secretion to immunologically explain the phenomena we found in the present study. The induction of oral tolerance is influenced by the antigen dosage and frequency of administration. As no significant difference was apparent among the IgG levels of rats administered with each experimental protein, the dependence of antigen dose and administration period on the induction of the IgG

level needs to be investigated in our future study. The immunotherapeutic effect of deamidated gliadin will also be examined to expand its usage. Our findings indicate that deamidated gliadin may become a useful tool in immunoprophylaxis as well as a hypoallergenic food for patients with wheat allergy.

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