



Impact of ω -5 Gliadin on Wheat-Dependent Exercise-Induced Anaphylaxis in Mice

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The effects of ω -5 gliadin on wheat-dependent exercise-induced anaphylaxis (WDEIA) were investigated by using a mouse model. The gliadin fraction was prepared as a 70% ethanol-soluble solution, and ω -5 gliadin was purified by chromatography. Purified ω -5 gliadin was run on SDS-PAGE gel to reveal three bands with a molecular mass of 53–60 kDa and had the characteristic N-terminal sequence of ω -5 gliadin. The mice were sensitized to the gliadin fraction, and the anaphylactic response was assessed by measuring the body temperature and voluntary physical activity. An oral administration of ω -5 gliadin evoked a significant drop in both the body temperature and voluntary physical activity, similar to the effects of the whole gliadin fraction. ELISA and immunoblotting analyses revealed that the IgE expression from sensitized mice reacted most strongly to ω -5 gliadin. Taken together, these results indicate ω -5 gliadin to be a major allergen responsible for stimulating WDEIA in mice, with the characteristic potential for stimulating IgE production.

Key words: wheat; ω -5 gliadin; food allergy; wheat-dependent exercise-induced anaphylaxis; mouse model

Wheat is well recognized as a valuable and staple food source worldwide. Interestingly, however, wheat is also one of the foods most commonly responsible for causing allergic reactions,¹⁾ and has reported to be the most frequent cause of food-dependent exercise-induced anaphylaxis (FDEIA).^{2–8)}

Wheat endosperm proteins consist of water-soluble albumins, salt-soluble globulins, and water-insoluble gliadins and glutenins. Gliadins and glutenins are the two main protein groups of gluten comprising up to 80% of total wheat protein.⁹⁾ Gliadins are heterogeneous and

subdivided into α , β , γ and ω fractions according to their mobility by polyacrylamide gel electrophoresis in the acidic pH region.^{10–13)} Among the wheat proteins, gliadins have been found to be the major allergen in patients exhibiting wheat-dependent exercise-induced anaphylaxis (WDEIA) by using skin tests and immunoblotting studies.^{14–16)} In addition, ω -5 gliadin has been detected as a major allergen among the entire gliadin fractions.^{17–20)} Palosuo *et al.*¹⁷⁾ first demonstrated that a purified protein with the N-terminal sequence SRLI reacted strongly with IgE from WDEIA patients. Matsuo *et al.*²⁰⁾ have reported that IgE antibodies from 80% of patients with WDEIA strongly reacted to ω -5 gliadin and that IgE antibodies from the remaining patients reacted to high-molecular-mass glutenin subunits. However, the mechanism by which ω -5 gliadin causes WDEIA is currently poorly understood.

Studies on the mechanism responsible for anaphylaxis have significantly increased in recent years. Much of this effort has been directed toward the development and analysis of animal models, especially mice. Such an approach has tremendous advantages, with genetically modified strains now more readily available and economical.^{21–23)} Our group has previously reported the development of a murine model for FDEIA.²⁴⁾ Investigations with our animal model found that physical exercise by sensitized mice affected the absorption of allergens due to increased damage to the villi within the small intestine. We also investigated the effect of each specific wheat protein (*i.e.*, the salt-soluble, gliadin and glutenin fractions) on WDEIA, and found that the gliadin- and glutenin-sensitized mice had a greater elevation of serum IgE level, a markedly shorter running time, and a higher level of intestinal erosion than the salt-soluble fraction-sensitized mice or control animals.²⁵⁾

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Abbreviations: WDEIA, wheat-dependent exercise-induced anaphylaxis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay

The effects of purified ω -5 gliadin on WDEIA were investigated in this present study by using a mouse model. In addition, the reactivity of IgE antibodies to ω -5 gliadin in the serum from sensitized mice was measured by using ELISA and IgE immunoblotting. We found that challenging mice with ω -5 gliadin stimulated WDEIA in a similar manner to that produced by the entire gliadin fraction. Moreover, IgE expressed by the sensitized mice reacted most strongly to ω -5 gliadin.

Materials and Methods

Materials. Soft wheat flour was kindly presented by Nisshin Flour Milling, Tokyo, Japan. Female B10.A mice (5 weeks old, weighing 15–20 g) were purchased from Japan SLC, Shizuoka, Japan. Aluminum hydroxide (Imject Alum), Coomassie brilliant blue G-250 and amido black 10B were respectively purchased from Pierce (Rockford, IL, USA), Nacalai Tesque (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA). A Sequi-Blot PVDF membrane was obtained from Bio-Rad (Hercules, CA, USA), and the horseradish peroxidase (HRP)-conjugated goat IgG fraction to mouse IgE, Fc specific was purchased from Nordic Immunological Laboratories (Tilburg, Netherlands). Tween 20 and bovine serum albumin (BSA) were respectively purchased from National Diagnostics (Atlanta, GA, USA) and Wako Pure Chemical Industries (Osaka, Japan). Hybond-P and the ECL advance western blotting detection kit were purchased from GE Healthcare Japan (Tokyo, Japan). All other chemicals used in this study were of reagent grade and were used without further purification.

Fractionation of gliadin and purification of ω -5 gliadin. The gliadin fraction was prepared as described previously²⁵⁾ with some modifications. All fractionation procedures were performed at room temperature. Wheat flour (50 g) was stirred for 2 h in 0.5 M NaCl (500 mL), and the suspension centrifuged (1700 g, 15 min). The supernatant was discarded and the resulting pellet was resuspended in distilled water (500 mL), before being stirred for another 2 h and again centrifuged (1700 g, 15 min). This procedure with distilled water was repeated once more (twice in total). The pellet was collected, resuspended in ethanol (500 mL, 70%, v/v) and the suspension stirred for 2 h before being centrifuged (1700 g, 15 min). The resulting supernatant was dialyzed against 1% acetic acid for 60 h and then freeze-dried. ω -5 Gliadin was performed according to the method reported by Sandiford *et al.*²⁶⁾ The freeze-dried gliadin fraction was dissolved in a 10 mM glycine-acetate buffer (pH 4.6) containing 3 M urea. The gliadin fraction solution was separated by strong cation-exchange chromatography, using a Macro-Prep High S column (5 $\times$ 20 cm; Bio-Rad, Hercules, CA, USA) and eluting with a linear gradient of 0–0.25 M NaCl. The peak containing ω -5 gliadin determined by SDS-PAGE was concentrated to 10 mL with a Vivaspin 20 centrifugal concentrator (Sartorius Stedim Japan, Tokyo, Japan) and separated by size-exclusion chromatography, using a Sephacryl S-300 HR column (2.5 $\times$ 75 cm; GE Healthcare Japan, Tokyo, Japan) in 6 M urea and 1% acetic acid. The eluted peak of ω -5 gliadin was dialyzed against 1% (v/v) acetic acid for 60 h and then freeze-dried.

Animals and sensitization. B10.A mice ($n = 21$) were housed in a room with a 12-h light and dark cycle and maintained at $20 \pm 2^\circ\text{C}$. The mice had free access to standard feed and tap water. The experimental design was in accordance with the guidelines for animal experimentation and was approved by the Animal Experimental Committee of Kawasaki University of Medical Welfare (authorization number 09-004). The mice (5 weeks old) were randomly assigned to sensitized ($n = 15$) and unsensitized ($n = 6$) groups. The sensitized mice were initially injected intraperitoneally with 100 μg of the gliadin fraction and 4 mg of aluminum hydroxide in 0.2 mL of phosphate-buffered saline at pH 7.0 (PBS). The gliadin fraction was then reduced to 50 μg and injected intraperitoneally with 4 mg of aluminum hydroxide in 0.2 mL of PBS for the remaining injections. Each animal was given one injection every week for 6 weeks. The unsensitized animals received the vehicle alone. To assess the

sensitization, blood was obtained from the orbital veins of mice under light anesthesia 37 d after the initial injection and subjected to an enzyme-linked immunosorbent assay (ELISA) specific for the IgE to gliadin.

Animal protocol. All mice were exercised during a 3-d acclimatization period before any experimental treatment. The sensitized mice (12 weeks old) were orally administered with 10 mg of either the gliadin fraction ($n = 8$) or purified ω -5 gliadin ($n = 7$) in 0.5 mL of a 0.1 M acetic acid solution. The unsensitized mice ($n = 6$) were also orally administered with 0.5 mL of 0.1 M acetic acid alone serving as a control. After the oral administration, the mice were forced to run on a treadmill set to 15 m/min and a 20% gradient for 30 min. The rectal temperature was measured with an SK-1250MC digital thermocouple thermometer (Sato Keiryoki, Tokyo, Japan) to evaluate the body temperature. To determine the voluntary physical activity, each mouse was housed in a cage with a running wheel, and the number of revolutions per hour was monitored for 15 h from 8 p.m. to 11 a.m. by using a counting and accumulating printer (Natsume Seisakusho, Tokyo, Japan).

SDS-PAGE. SDS-PAGE was performed with 10% polyacrylamide gel according to the method of Laemmli.²⁷⁾ The protein bands were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250.

N-Terminal sequencing. The proteins separated by SDS-PAGE were electrophoretically transferred to a Sequi-Blot PVDF membrane. The proteins were stained with 0.1% (w/v) amido black 10B, and each individual band was cut off from the PVDF membrane. The N-terminal sequences were determined by a PPSQ21A protein sequencer (Shimadzu, Kyoto, Japan).

ELISA for specific IgE expression. The specific IgE levels in mouse serum were evaluated by ELISA as described previously.²⁵⁾ Flat-bottomed microtiter plates were precoated with 100 μL of either the gliadin fraction or purified ω -5 gliadin (10 $\mu\text{g}/\text{mL}$) in a 0.1 M carbonate buffer (pH 9.6) containing 3 M urea, and incubated overnight at 4°C . After the wells had been washed with PBS containing 0.05% Tween 20 (PBST), a 1% BSA/PBST solution was added to each well, and the solution incubated for 1 h at 37°C . A 100- μL amount of each serum sample diluted 50 $\times$ with 1% BSA/PBST was then applied to each well, and the mixture incubated for 1 h at 37°C . After washing each well five times with PBST, 100 μL of HRP-conjugated IgG anti-mouse IgE (diluted 1:1000 in 1% BSA/PBST) was added to each well, and the solution incubated for 1 h at 37°C . Each well was washed five times with PBST, and 100 μL of o-phenylenediamine (0.4 mg/mL) in a citrate-phosphate buffer (pH 5.0) containing 0.006% H_2O_2 was added. The reaction was quenched with 50 μL of 2.5 M H_2SO_4 (aq.) after 5 min at room temperature, and color development was measured by colorimetric photometry at 492 nm.

IgE immunoblotting. Immunoblotting was performed according to the method of Towbin *et al.*²⁸⁾ The proteins were separated by SDS-PAGE and electrophoretically transferred to a Hybond P membrane. The membrane was blocked with 2% ECL advance blocking reagent/tris-buffered saline containing 0.05% Tween 20 (TBST) at pH 7.5 and then incubated with mouse serum. After the membrane had been washed three times with TBST, HRP-conjugated IgG anti-mouse IgE was added, and the solution incubated. The protein bands were visualized with an ECL advance western blotting detection kit combined with a LAS-1000UVmini CCD camera system (Fujifilm, Tokyo, Japan).

Statistical analysis. Data are expressed as the mean $\pm$ SEM. All statistical analyses were performed by using SPSS 14.0J for Windows (SPSS Japan, Tokyo, Japan). A between-group analysis was performed by using Student's *t*-test, and a within-group analysis was conducted by a one-way analysis of variance (ANOVA), with data concerning hourly changes in the number of revolutions run by the mice analyzed by two-way ANOVA with repeated measures. Tukey's tests were also performed *post hoc*. Data are considered to be significantly different with *p*-values of less than 0.05.

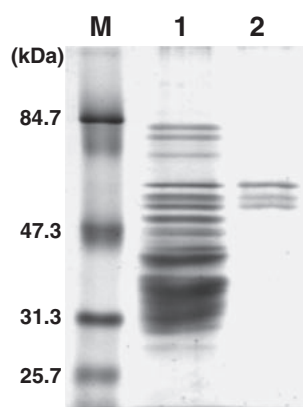


Fig. 1. SDS-PAGE Analyses of the Gliadin Fraction and Purified ω -5 Gliadin.

Lane M denotes the molecular mass marker; lane 1, the gliadin fraction; lane 2, purified ω -5 gliadin. The gliadin fraction was prepared as a 70% ethanol-soluble solution, and ω -5 gliadin was purified by using chromatography as described in the Materials and Methods section. The gel was stained with CBB.

Results and Discussion

Gliadin fraction and purified ω -5 gliadin

The gliadin fraction was prepared as a 70% ethanol-soluble component from soft wheat flour, and ω -5 gliadin was further purified by using chromatographic methods. Figure 1 reveals the protein ladders from an SDS-PAGE gel for the gliadin fraction and the purified ω -5 gliadin. Gliadins exhibit extreme molecular heterogeneity and are traditionally subdivided into α/β -, γ - and ω -gliadins.⁹⁾ The many individual proteins in the region of apparent molecular mass of 30–45 kDa are α/β - and γ -gliadins, with distinct bands of ω -gliadins apparent at a molecular mass of 45–65 kDa.¹⁾ The results of SDS-PAGE confirm the gliadin fraction to be composed of the α/β -, γ - and ω -gliadin fractions. The gliadin fraction also had bands at a molecular mass of around 80 kDa, indicative of high-molecular-mass glutenin subunits (HMM-GS), this being consistent with previous reports on alcohol-extracted gliadin from wheat.^{29,30)}

The results of SDS-PAGE revealed the purified ω -5 gliadin to comprise three bands of apparent molecular mass 53–60 kDa (Fig. 1). The amount of purified ω -5 gliadin was 50–70 mg from 4 g of the gliadin fraction. ω -Gliadins are sometimes referred to as ARE-, ARQ-, KEL- and SRL-type proteins on the basis of the N-terminal sequences. The ARE-, ARQ- and KEL-type proteins are ω -1,2 gliadin, whereas the SRL-type protein is ω -5 gliadin.²⁹⁾ Thus, the N-terminal sequences of our purified ω -5 gliadin were determined, and all three bands on SDS-PAGE showed 'SRLLSPRGKE,' this being the characteristic N-terminal sequence of ω -5 gliadin. Combining SDS-PAGE and N-terminal sequencing indicated that ω -5 gliadin had been successfully purified collectively as the three bands.

Assessment of WDEIA by using our mouse model

The effects of challenges on WDEIA of both the whole gliadin fraction and purified ω -5 gliadin were investigated by using the mouse model. Mice were sensitized with the gliadin fraction, using an alum adjuvant, by an interperitoneal injection. Specific IgE to

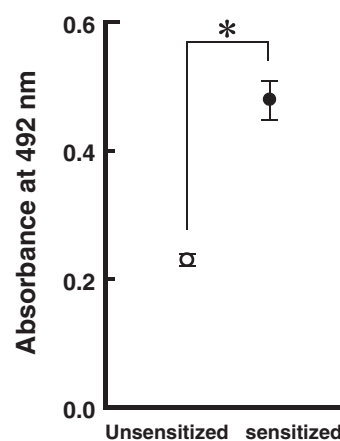


Fig. 2. IgE Levels Specific to the Gliadin Fraction in Sera from Mice Sensitized by the Gliadin Fraction or Unsensitized.

○, unsensitized group (n = 6); ●, sensitized group (n = 15). Data are expressed as the mean ± SEM. **p* < 0.05. B10.A mice were sensitized by means of intraperitoneally injecting the gliadin fraction with an alum adjuvant. Each animal was given one injection every week for 6 weeks. The unsensitized animals received the vehicle alone. Blood was obtained from the orbital veins of mice 37 d after the initial injection and subjected to ELISA specific for the IgE to the gliadin fraction.

the gliadin fraction in mouse serum was determined by ELISA (Fig. 2). The amount of IgE specific to gliadin produced by the mice injected with gliadin was significantly more than that with the unsensitized group, indicating that sensitization to the gliadin fraction had occurred.

Both the whole body temperature and voluntary physical activity are commonly reduced during systemic anaphylaxis.^{22,31)} The body temperature and voluntary physical activity were therefore determined to assess WDEIA in mice. The rectal temperature of mice from each group was examined to evaluate the change in body temperature with WDEIA. Figure 3 displays the rectal temperature of sensitized mice after challenging by either the gliadin fraction or ω -5 gliadin and exercise. The rectal temperature for both groups challenged by the gliadin fraction or ω -5 gliadin was significantly lower than the control group, although it did not differ between groups, indicating that exposure to ω -5 gliadin had a similar effect on body temperature to that of the whole gliadin fraction.

The number of revolutions run by each treatment group during a normal period of activity (8 p.m. to 11 a.m.) was studied (Fig. 4) to estimate the change in voluntary physical activity with WDEIA. The sensitized mice administered with either the gliadin fraction or ω -5 gliadin ran significantly less during the first 6 h than the control group, suggesting that the allergen reduced the physical activity of the sensitized mice for an acute time period.

Figure 5 displays the number of revolutions run by each treatment group monitored for 15 h after either the gliadin fraction or ω -5 gliadin challenge and exercise. The animals subjected to the gliadin fraction or ω -5 gliadin achieved a significantly lower number of revolutions than the control group. The result for the gliadin fraction group did not significantly differ from the ω -5 gliadin group, indicating both treatments to have a similar effect on voluntary physical activity.

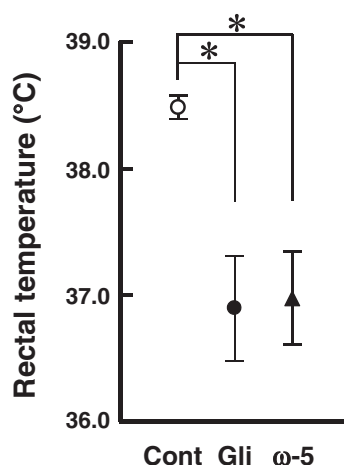


Fig. 3. Effect of the Gliadin Fraction and Purified ω -5 Gliadin on the Rectal Temperature of Sensitized Mice.

○, control group (n = 6); ●, gliadin fraction challenged group (n = 8); ▲, ω -5 gliadin challenged group (n = 7). Data are expressed as the mean $\pm$ SEM. * p < 0.05. Sensitized mice were orally administered with 10 mg of the gliadin fraction or ω -5 gliadin in 0.1 M acetic acid. The unsensitized mice were also orally administered with 0.1 M acetic acid alone, serving as the control. The mice were then forced to run on a treadmill set to 15 m/min and a 20% gradient for 30 min. The rectal temperature was measured as soon as the mice had exercised.

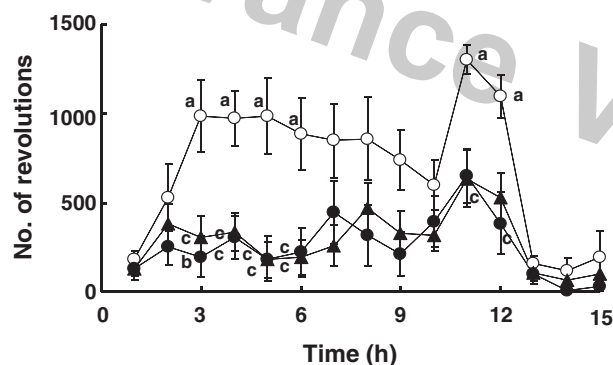


Fig. 4. Effect of the Gliadin Fraction and Purified ω -5 Gliadin on the Number of Revolutions Achieved Hourly by the Sensitized Mice.

○, control group (n = 6); ●, gliadin fraction challenged group (n = 8); ▲, ω -5 gliadin challenged group (n = 7). Data are expressed as the mean $\pm$ SEM. a-b, p < 0.01; a-c, p < 0.05. The sensitized and unsensitized mice were orally administered and then forced to run on a treadmill as described in Fig. 3. Each mouse was housed in a cage with a running wheel, and the number of revolutions per hour was monitored for 15 h from 8 p.m. to 11 a.m.

The body temperature and voluntary physical activity of sensitized mice were measured in the present study after either the gliadin fraction or ω -5 gliadin challenge and exercise. The ω -5 gliadin challenge reduced both the body temperature and voluntary physical activity of the sensitized mice in a similar manner to the gliadin fraction, despite these mice having been sensitized to the gliadin fraction, and not purified ω -5 gliadin. These results are consistent with recent findings indicating ω -5 gliadin to cause WDEIA.¹⁷⁻²⁰ The ω -gliadins are distinguished from the other gluten proteins by their repetitive sequences rich in glutamine and proline, with little or no methionine or cysteine.³² Moreover, an analysis of IgE-binding epitopes expressed by patients with WDEIA detected four predominant epitopes for ω -5 gliadin. These four epitopes were found to appear

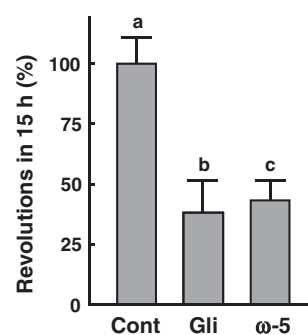


Fig. 5. Effect of the Gliadin Fraction and Purified ω -5 Gliadin Challenges on the Number of Revolutions Achieved in 15 h by the Sensitized Mice.

Cont, control group (n = 6); Gli, gliadin fraction challenged group (n = 8); ω -5, ω -5 gliadin challenged group (n = 7). Data are expressed as the mean $\pm$ SEM. a-b, p < 0.01; a-c, p < 0.05. The sensitized and unsensitized mice were orally administered and forced to run on a treadmill as described in Fig. 3. The number of revolutions was monitored over 15 h as described in Fig. 4.

repetitively in the primary sequence of ω -5 gliadin.⁸ These characteristics of ω -5 gliadin further support the hypothesis of ω -5 gliadin being the primary allergen responsible for WDEIA.

Reactivity to ω -5 gliadin of IgE antibodies in the serum of sensitized mice

Purified ω -5 gliadin stimulated WDEIA in a similar manner to the gliadin fraction. We therefore measured the reactivity of IgE antibodies to ω -5 gliadin in the serum from sensitized mice by using ELISA and IgE immunoblotting. Figure 6 displays the levels of specific IgE antibodies from the sera of sensitized mice determined by ELISA. Compared with IgE specific to the gliadin fraction, 75 $\pm$ 12% of IgE reacted to ω -5 gliadin, indicating that the IgE antibodies of sensitized mice strongly reacted to ω -5 gliadin. Figure 7 shows IgE immunoblots using the serum from sensitized mice. The bands of the gliadin fraction and the purified ω -5 gliadin were separated by SDS-PAGE gel electrophoresis. The IgE antibodies from sensitized mice reacted strongly to the bands of ω -5 gliadin in both the gliadin fraction and the purified ω -5 gliadin. Matsuo *et al.*²⁰ have reported that 80% of WDEIA patients' IgE strongly reacted to ω -5 gliadin. Our present results from IgE immunoblotting are consistent with these findings.²⁰

The results of ELISA and immunoblotting (Figs. 6 and 7) indicate that IgE antibodies of the sensitized mice reacted to ω -5 gliadin with greater efficacy than other components of gliadin protein. Three differently sensitized mice were prepared in our previous study²⁵ with an alum adjuvant by an intraperitoneal injection of three fractionated wheat proteins: the salt-soluble, gliadin and glutenin fractions. It was found that the specific IgE levels in mouse serum were different among the differently sensitized groups, the greatest being in the gliadin-sensitized group. Deaman and Kimber²³ have developed a mouse model of food allergies by using intraperitoneal sensitization in the absence of an adjuvant. They revealed that proteins with the potential to cause allergic sensitization stimulated IgE antibody production, and non-allergic proteins were associated with IgG antibody responses in the absence of significant IgE production in

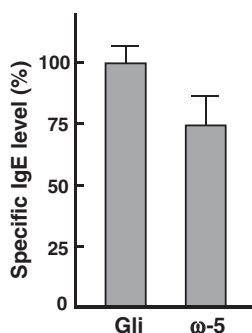


Fig. 6. IgE Levels Specific to the Gliadin Fraction and Purified ω -5 Gliadin in the Sera of Sensitized Mice.

Gli, IgE specific to the gliadin fraction ($n = 15$); ω -5, IgE specific to ω -5 gliadin ($n = 15$). Sensitized mice and their blood were obtained as described in Fig. 2. The IgE levels in the sera of sensitized mice were determined by ELISA.

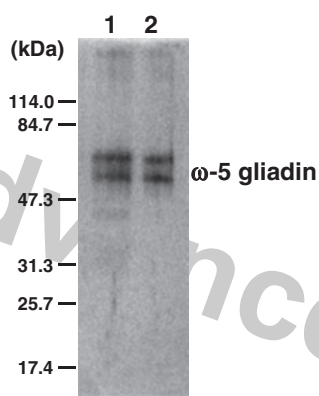


Fig. 7. IgE Immunoblotting Analyses of IgE Reactivity in the Sera from Mice Sensitized to Either the Gliadin Fraction or Purified ω -5 Gliadin.

Lane 1, gliadin fraction; lane 2, ω -5 gliadin. The gliadin fraction and ω -5 gliadin were subjected to SDS-PAGE and subsequent immunoblotting with IgE antibodies in the sera of sensitized mice as described in the Materials and Methods section.

mice. These results highlight the important characteristics of ω -5 gliadin as a food allergen as it possesses the capacity to provoke IgE antibody production.

The effects of ω -5 gliadin on WDEIA were investigated in this study by using a mouse model. WDEIA was caused by administering ω -5 gliadin, with the animals displaying reduced body temperature and lower voluntary physical activity, similar to the effects caused by the gliadin fraction. Furthermore, the IgE antibodies of mice sensitized to the gliadin fraction reacted strongly to ω -5 gliadin. Patients suffering from WDEIA express IgE that strongly reacts to ω -5 gliadin,²⁰⁾ consistent with the current findings and previous reports that hypothesized ω -5 gliadin to be a primary cause of WDEIA.^{17–20)} It is believed that the mouse model in this study will be of value in providing a better understanding of the mechanism for WDEIA in humans, as murine and human immune systems are highly similar and share anaphylaxis.

Acknowledgments

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