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Letter to the Editor

Quantification of the ω 5- and γ -gliadin content in wheat flour and rat plasma with an enzyme-linked immunosorbent assay using antibodies specific to their IgE-binding epitopes

Dear Editor,

ω 5-Gliadin and γ -gliadin are known as causative allergens for conventional and hydrolyzed wheat protein-type of wheat-dependent exercise-induced anaphylaxis (WDEIA), respectively.¹ As a wheat-eliminating diet is crucial for patients with WDEIA, measuring the amounts of ω 5- and γ -gliadins, in processed foods or hypoallergenic wheat is helpful to ensure the safety of patients.^{2–4} WDEIA is typically diagnosed based on the results of a positive-oral challenge test to exercise and/or aspirin intake in combination with wheat ingestion. Previous reports have shown that serum gliadin monitoring in combination with oral challenge tests was useful to exclude the false-negative results from diagnosis for WDEIA.^{5,6} Thus, monitoring of ω 5- and γ -gliadins content in plasma can further improve the precision of diagnosis for WDEIA using oral challenge tests. We previously produced rabbit polyclonal antibodies (Abs) specifically recognizing the IgE-binding epitope sequences (KQQSPQQFPQQIPQQQ) of ω 5-gliadin.⁷ In this study, we also produced rabbit polyclonal Abs specifically recognizing the IgE-binding epitope sequences (QFLQPQQFPQQPQ) of γ -gliadin.^{4,8} Furthermore, we determined the content of ω 5- and γ -gliadin in wheat cultivars and rat plasma after gluten ingestion using enzyme-linked immunosorbent assay (ELISA) with the derived Abs.

Polyclonal Abs (IgGs) against IgE-epitope peptides of γ -gliadin were produced using New Zealand white rabbits and affinity purified using allergen peptide. IgGs against the ω 5-gliadin epitope peptide produced in a previous study was used.⁷ To determine the contents of total ω 5- and γ -gliadin in samples, sandwich ELISA were performed using each anti-gliadin Ab. This study was approved by the animal ethics committee of Hiroshima University (approval No. A16-138). All details are shown in Supplementary Methods.

We first confirmed the specific detection of wheat proteins with sandwich ELISA using the IgE-binding epitope Abs. The ELISA using the anti-gliadin Ab could detect total gliadin (Tokyo Chemical Industry, Tokyo, Japan) but not each recombinant gliadin component produced in *Escherichia coli* (Supplementary Fig. 1(A)).⁴ The ELISA using anti- ω 5-gliadin Ab specifically detected ω 5-gliadin alone whereas the ELISA using anti- γ -gliadin Ab reacted with ω 1,2-gliadin more strongly rather than γ -gliadins (Supplementary Figs. 1(B) and 1(C)). This might be reasonable because the repeating numbers of the QPQQFPQ sequence in the epitope domains on ω 1,2-gliadin (14 sites) were reportedly more than those on

γ -gliadin (two sites) (Supplementary Fig. 2).⁴ Therefore, detection of ω 1,2-gliadin in wheat products can result in the overestimation of γ -gliadin content though the γ -gliadin content in wheat gluten is $\approx$ 5-fold higher than that of ω 1,2-gliadin.⁹

Next, we determined the contents of total, ω 5- and γ -gliadins in glutes from Hokushin, ω 5-gliadin-deficient Hokushin presented by Kohno *et al.*⁷ and Norin 61 as well as reagent gluten (Tokyo Chemical Industry) and hydrolyzed gluten (Glupearl 19S[®], Katayama Chemical, Osaka, Japan). The concentrations of total, ω 5-, and γ -gliadins in each sample were calculated using reagent, recombinant ω 5- and γ -gliadins as a standard, respectively. The cut-off value was defined as the two-fold mean value of the standard deviation for each gliadin. The calibration curves for absorbance against gliadin content each fit the four-parameter logistic analysis. The detection limit of total, ω 5-, and γ -gliadin using the ELISA with each Ab were obtained as 1.6, 0.2 and 3.1 ng/ml, respectively. The total gliadin content in gluten from Hokushin and ω 5-gliadin-deficient Hokushin and the γ -gliadin content in all types of gluten exceeded 1 g/g gluten (Table 1). Wheats are polyploids with four or six sets of chromosomes and gliadin is encoded by plural genes, indicating that amino-acid sequences of native gliadin components can be slightly different among wheat cultivars. Thus, the results might be explained by differences in the affinities of anti-gliadin and anti- γ -gliadin Abs against each gliadin component between each sample and the recombinant protein used as standards. The total gliadin content in gluten from Norin 61 was slightly lower than those of Hokushin and ω 5-gliadin-deficient Hokushin. Additionally, total gliadin from reagent gluten was significantly lower than those from all Japanese cultivars. The content of ω 5-gliadin from ω 5-gliadin-deficient Hokushin decreased to 20% of that from Hokushin, though no significant difference was observed between Hokushin and Norin 61. Our preliminary immunoblot assay showed that the anti- ω 5-gliadin Abs slightly cross-reacted with other gliadin components except for ω 5-gliadin. Thus, the slight detection by anti- ω 5-gliadin Ab in the ω 5-gliadin-deficient wheat may be due to the cross-reactivity to gliadin components except for ω 5-gliadin. These results indicate that the ELISA using the anti- ω 5-gliadin Ab is suitable to detect ω 5-gliadin in wheat cultivars to a similar extent as using it to detect recombinant ω 5-gliadin dissolved in solution. The γ -gliadin amount did not significantly differ among the three Japanese cultivars and reagent gluten. In hydrolyzed gluten, total gliadin was minimally detected but ω 5- and γ -gliadins were not detected due to its degradation and deamidation.

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Table 1

Contents of total, ω 5- and γ -gliadins in gluten from three wheat cultivars, reagent gluten and hydrolyzed wheat gluten.

Gliadin component	Total (g/g gluten)	ω 5 (mg/g gluten)	γ (g/g gluten)
Hokushin	1.15 $\pm$ 0.13	5.17 $\pm$ 0.51	1.66 $\pm$ 0.38
ω 5-gliadin-deficient Hokushin	1.25 $\pm$ 0.15	1.21 $\pm$ 0.10**	1.86 $\pm$ 0.62
Norin 61	0.61 $\pm$ 0.03	5.65 $\pm$ 0.90	1.51 $\pm$ 0.27
Reagent	0.17 $\pm$ 0.02**	3.49 $\pm$ 0.57	1.04 $\pm$ 0.13
Hydrolyzed wheat gluten	0.06 $\pm$ 0.00**	ND	ND

Reagent gluten was obtained from Tokyo Chemical Industry. Hydrolyzed wheat protein, glupearl 19S; ND, Not detected. Each value represents the mean $\pm$ SEM for three to six trials. ** $p < 0.01$; significantly different from Hokushin.

Table 2

Plasma concentrations of total and ω 5-gliadins 1 h after gluten ingestion.

Gliadin component	Total (ng/ml)	ω 5 (ng/ml)
Gluten (–)	ND	ND
Gluten (+)		
Rat #1	361 $\pm$ 43*	19 $\pm$ 12
Rat #2	869 $\pm$ 218**	87 $\pm$ 28*

ND, Not detected. Aspirin was administered at a dose of 30 mg/kg 20 min before oral administration of gluten (50 mg/body). Blood was collected via the abdominal aorta 1 h after vehicle [Gluten (–)] or gluten (Rat #1 and Rat #2) administration. Each value represents the mean $\pm$ SEM for three to five trials. * $p < 0.05$, ** $p < 0.01$; significantly different from control [Gluten (–)].

Finally, we attempted to measure the rat plasma concentrations of total, ω 5- and γ -gliadins after gluten ingestion. Rat plasma was collected 1 h after ingestion of vehicle alone or gluten (50 mg) following oral aspirin administration (30 mg/kg). Gliadins were extracted from rat plasma with 70% ethanol and dissolved with Can Get Signal® solution-1 for the ELISA assay. Calibration curves were produced using rat plasma containing standard gliadins. The detection limit of total gliadin and ω 5-gliadin were 100 and 3.1 ng/ml, respectively. However, we could not obtain the calibration curve for absorbance against γ -gliadin content because some impurities in plasma may interfere with the measurement of γ -gliadin by ELISA.

Total and ω 5-gliadins could not be detected in the plasma from control rats administered vehicle with aspirin. In contrast, increased plasma concentrations of both gliadins were detected 1 h after gluten ingestion (Table 2), indicating that the total and ω 5-gliadins that have multiple IgE-binding epitopes can be detected by ELISA in plasma using our epitope-specific Abs. According to the estimation suggested by Food and Drug Administration, 50 mg of gluten dose in rats corresponds to 1.3 g in humans.¹⁰ The challenge tests for patients with WDEIA were performed by ingestion of wheat products containing 60–135 g of wheat flour, which includes 5.1–11.5 g of gluten.^{5,9} Thus, total and ω 5-gliadins that have multiple IgE-binding epitopes in plasma can be measured by ELISA using our epitope-specific Abs even in clinical challenge tests.

In conclusion, the ELISA using our epitope-specific Abs is useful to determine the content of each gliadin component in wheat cultivars and plasma from WDEIA patients. Gliadins detected in our ELISA have multiple IgE-binding epitopes. However, all detected gliadin may not necessarily exhibit the allergenicity due to their reduced molecular sizes by intestinal digestion. Further study is necessary to clarify the allergenicity of gliadins detected in our ELISA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.alit.2018.04.012>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Methods

Production of polyclonal Abs against ω 5- and γ -gliadin epitope peptides

Polyclonal Abs (IgGs) against IgE-epitope peptides of γ -gliadin were produced according to a previous report with slight modifications.⁷ Briefly, the epitope peptide QFLQPQQPFPQQPQ including the IgE-binding epitope sequence of γ -gliadin (QPQQPFPQ) was synthesized.⁸ This peptide was conjugated with keyhole limpet hemocyanin (KLH), and independently subcutaneously injected into New Zealand white rabbits three times at 2-week intervals together with adjuvant. Production of Ab against the epitope peptide was confirmed by ELISA. IgG against the ω 5-gliadin epitope peptide (KQQSPEQQQFPQQQIPQQQ) produced in a previous report was purified using a peptide affinity column.⁷ IgG against the γ -gliadin epitope peptide was also purified using allergen peptide. Before using the anti- ω 5-gliadin epitope-peptide Ab (anti- ω 5-gliadin Ab) and anti- γ -gliadin epitope-peptide Ab (anti- γ -gliadin Ab) for detection of each gliadin by ELISA, these Abs were conjugated with horse radish peroxidase (HRP) using the Peroxidase Labeling Kit-NH₂ (Dojindo Molecular Technologies, Rockville, MD, USA). Conjugation of each Ab with HRP was confirmed by ELISA using each gliadin.

Preparation of gluten from wheat cultivars

Gluten was prepared from three wheat cultivars, Hokushin (Koshoku, Tokyo, Japan), ω 5-gliadin-deficient Hokushin (produced and presented by Kohno et al.⁷), and Norin 61 (Shimane Agricultural Experiment Station, Shimane, Japan). Each sample of wheat flour was kneaded by hand in water and left to rest for 30 min to form the gluten

network as a result of gliadin–glutenin interaction. Then, the gluten was washed with fresh water and kneaded in fresh water again. Washing and kneading were repeated 10 to 15 times and then the gluten was lyophilized. The gluten was ground into powder in a mortar and stored at -30°C until use. For the ELISA assay, each gluten powder was dissolved in 6 M urea containing 1% acetic acid and centrifuged at 20,000×g for 5 min at 20°C. The supernatant was appropriately diluted with Can Get Signal[®] immunoreaction enhancer solution-1 (Toyobo, Osaka, Japan), where Can Get Signal[®] solution showed higher sensitization to detect gliadin in our ELISA than phosphate-buffered saline (PBS, pH7.4) in our preliminary study.

Oral administration of gluten in rats

Male Sprague–Dawley (SD) rats (7–8 weeks old) were obtained from Japan SLC (Shizuoka, Japan). Rats were fed a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Rats were maintained in a temperature- and light-controlled environment for 1 week before the study. All experiments involving animals were carried out in accordance with the Guide for Animal Experimentation from the Committee of Research Facilities for Laboratory Animal Sciences of Hiroshima University (approval No. A16-138, Hiroshima, Japan). Rats were fasted overnight and anesthetized with pentobarbital (30 mg/kg, intraperitoneally). Reagent gluten (50 mg) was suspended in 400 µl of saline containing 50% ethanol. Vehicle alone or a gluten suspension was administered orally at a dose of 400 µl 20 min after oral aspirin treatment (30 mg/kg). Blood was collected via the abdominal aorta 1 h after vehicle or gluten administration and centrifuged to collect plasma. To extract gliadins from the plasma, 250 µl of plasma was mixed with 50 µl of distilled water and 700 µl of

ethanol (final concentration: 70%) as reported previously.⁶ After extraction by vortex mixing for 1 min, the mixture was centrifuged at 20,000×g for 5 min at 20°C. The supernatant was transferred to a new tube and evaporated. The residue was dissolved in Can Get Signal[®] immunoreaction enhancer solution-1 at appropriate dilutions for the ELISA assay.

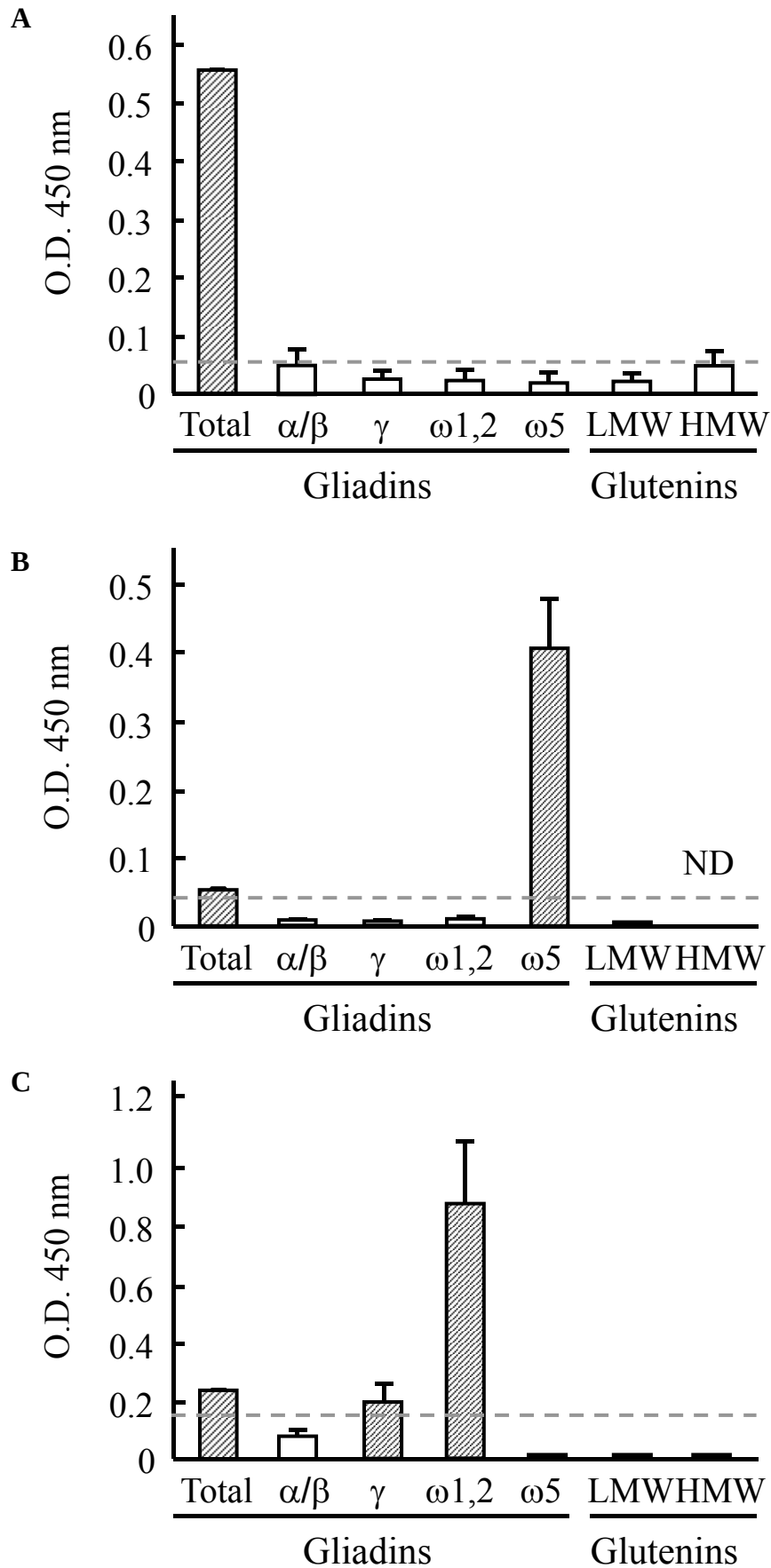
Quantification of gliadin content in wheat cultivars and plasma with ELISA

ELISA was performed using the IgE-binding epitope Abs produced in this study to confirm whether the ω 5- and γ -gliadins can be specifically detected. Briefly, microplate wells (F8 MaxiSorp loose Nunc-Immuno™ Modules, Thermo Fisher Scientific Inc., Yokohama, Japan) were coated with 100 μ l of anti-gliadin Ab (10 μ g/ml), anti- ω 5-gliadin Ab (5.0 μ g/ml) or anti- γ -gliadin Ab (2.5 μ g/ml) diluted with 50 mM carbonate buffer (pH 9.6) overnight at 4°C. After washing with PBS (pH 7.4) six times, plates were blocked with 1% blocking reagent (Block Ace[®], DS Pharma Biomedical, Osaka, Japan) for 1 h at 37°C. After washing with PBS containing 0.05% Tween-20 (PBST) six times, 100 μ l of each allergen sample (diluted appropriately with Can Get Signal[®] solution-1), including reagent gliadins (Tokyo Chemical Industry), recombinant α/β -, γ -, ω 1,2-, and ω 5-gliadins and LMW- and HMW-glutenins, was added to each well and incubated for 1 h at 37°C. After washing with PBST, wells were incubated with 100 μ l of HRP-conjugated anti-gliadin Ab (Abcam, Cambridge, UK), anti- ω 5-gliadin Ab or anti- γ -gliadin Ab (diluted 1 : 1000 with Can Get Signal[®] solution-2) for 1 h at 37°C. In general, two different Abs were used as the capture and detection Ab in sandwich ELISA. However, we used same anti- ω 5-gliadin Ab or anti- γ -gliadin Ab as a capture and detection Ab to detect each gliadin because higher sensitivity was obtained using

same Ab compared with that using two different Abs in our preliminary study. Wells were washed with PBST and then incubated with 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) solution at room temperature. After incubation, the reaction was terminated with 100 μ l of 1 M phosphoric acid. Absorbance was measured at 450 nm using a Multiskan GO (Thermo Fisher Scientific, Waltham, MA, USA). Next, we also performed ELISA using Abs produced in this study to determine the contents of ω 5- and γ -gliadins in glutes from Hokushin, ω 5-gliadin-deficient Hokushin and Norin 61, hydrolyzed gluten (Glupearl 19S[®], Katayama Chemical, Osaka, Japan) and rat plasma after gluten ingestion according to the same method. The concentrations of total, ω 5-, and γ -gliadins in each sample were calculated by using reagent gliadin, recombinant ω 5- and γ -gliadins as a standard, respectively. The concentration of each recombinant protein was measured by Lowry method (RC DC Protein Assay, Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Curve fitting analysis was performed using Excel 2010 (Microsoft, Redmond, WA, USA) and the KaleidaGraph[™] program (Version 3.501, Synergy Software, PA, USA). The plots of calibration curves of each gliadin were analyzed using a four-parameter logistic equation. The cut-off value was defined as the two-fold mean value of the standard deviation for each gliadin.

Statistical analyses

Data are the mean $\pm$ standard error of the mean (SEM). Differences in mean values between groups were assessed by the Kruskal–Wallis test with Scheffe's F test. $P < 0.05$ was considered significant.



Supplementary Fig. 1. Detection of recombinant wheat proteins with ELISA using anti-gliadin Ab (A), anti- $\omega 5$ -gliadin Ab (B) and anti- γ -gliadin Ab (C). LMW; low molecular weight; HMW, high molecular weight; ND, not detected. Dashed lines represent cut-off values. Each value represents the mean $\pm$ SEM for three trials.

A

NMQVDPSSQVQWPQQQPVPQPHQPFSSQQPQQTFPQPQQTFPHQPQQQFPQPQQPQQQFLQ
PQQPFPQQPQQPYQPQQPFPQTQQPQQLFQSSQQPQQQFSQPQQQFPQPQQPQQSFPQ
QQPFIQPSLQQQVNPCKNFLQCKPVSLVSSLWSMIWPQSDCQVMRQQCCQQLAQIPQ
QLQCAAIHTIIHSIIMQQEQQEQQGMHILLPLYQQQQVGGTLVQGQGIIQPQQPAQLE
AIRSLVLQTLPTMCNVYVPPECSIIKAPFSSVVAGIGGQ

B

ARELNPSNKEQLSPQQSFSHQQQPFPQQPYQPQPYPSQQPYPSQQPFPPTPQQQFPQQSQQ
PFTQPQQPTPLQPPQFPQQPQQPFPQPQQPFPWQPPQFPQTQSSFPLQPPQFPQ
QPQQPFPQPQLPFPQQSEQIIPQQPQQPFPLQPPQFPQQPQQPFPQPQQPIPVQPQQSF
PQQSQSQQPFAQPQQLFPELQQPIPPQQPFPPLQPPQFPQQPQQPFPQQPQQSFPQQ
PQQPFPQQPQQPFPQQPQQPFPQQPQQPFPPLRPQQPFPQQPQQSQSQSFPQPQPQQPQQPS
ILQPQQPLPQQPQQPFQQPQQQLSQQPEQTISQQPQQPFPQQPHQPQQPYQPQQPYGSSL
TSIGGQ

Supplementary Fig. 2. Amino-acid sequences of γ -gliadin (A) and ω 1,2-gliadin (B). Amino-acid sequences of γ -gliadin and ω 1,2-gliadin are deposited in GenBank with the accession numbers of BAN29066 and BAN29067, respectively. The QPQQPFPQ sequence in the IgE-binding epitope domains are highlighted in gray.