ORIGINAL ARTICLE

Characterization of Causative Allergens for Wheat-Dependent Exercise-Induced Anaphylaxis Sensitized with Hydrolyzed Wheat Proteins in Facial Soap

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ABSTRACT

Background: In Japan, hydrolyzed wheat proteins (HWP) have been reported to cause wheat-dependent exercise-induced anaphylaxis (WDEIA) by transcutaneous sensitization using HWP-containing soap. Patients develop allergic reactions not only with soap use, but also with exercise after the intake of wheat protein (WP). ω5-Gliadin and HMW-glutenin were identified as major allergens in conventional WP-WDEIA patients. However, the allergens in HWP-WDEIA have yet to be elucidated.

Methods: Sera were obtained from 22 patients with HWP-sensitized WDEIA. The allergenic activities of HWP and six recombinant wheat gluten proteins, including α/β-, γ-, ω1,2- and ω5-gliadin and low- and high molecular weight (HMW)-glutenins, were characterized by immunoblot analysis and histamine releasing test. IgE-binding epitopes were identified using arrays of overlapping peptides synthesized on SPOTs membrane.

Results: Immunoblot analysis showed that IgE antibodies (Abs) from HWP-WDEIA bound to α/β-, γ- and ω1,2-gliadin. Recombinant γ-gliadin induced significant histamine release from basophils in eight of 11 patients with HWP-WDEIA. An IgE-binding epitope "QPQQPFPQ" was identified within the primary sequence of γ-gliadin, and the deamidated peptide containing the "PEEPFP" sequence bound with IgE Abs more strongly compared to the native epitope-peptide. The epitope-peptide inhibited IgE-binding to HWP, indicating that the specific IgE to HWP cross-reacts with γ-gliadin.

Conclusions: HWP-WDEIA patients could be sensitized to HWP containing a PEEPFP sequence, and WDEIA symptoms after WP ingestion could partly be induced by γ-gliadin. These findings could be useful to help develop tools for diagnosis and desensitization therapy for HWP-WDEIA.

KEY WORDS

allergen, epitope, gliadin, hydrolysis, wheat

INTRODUCTION

Wheat proteins, especially gluten, are used as functional ingredients in processed foods and cosmetics. However, the industrial applications of native gluten are often limited because of its low solubility in water. To improve its physicochemical properties, native gluten is often modified by deamidation, which converts glutamine into glutamic acid residues, by hydrolysis using acids and proteolytic enzymes.[1,2] The

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hydrolyzed wheat gluten has better solubility, emulsifying and foaming properties, and is widely used as an emulsifier and stabilizer in foods, cosmetics and soaps.[3-5]

Gluten is composed of two proteins, gliadin and glutenin families.[6] Each gliadin and glutenin family can be separated into several fractions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and/or high performance liquid chromatography (HPLC), indicating that gliadin and glutenin proteins are produced by multiple genes.[6,7] Gliadins are monomeric proteins, and genetically classified into α/β-, γ-, ω1,2- and ω5-gliadin families. Glutenins are divided into low molecular weight (LMW-) and high molecular weight (HMW)-glutenins.[6]

Many previous reports have shown that wheat proteins, including gliadins and glutenins, are involved in various wheat allergies.[8-12] Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a peculiar form of food allergy, which is induced by exercise following the ingestion of wheat products.[13] High concentrations of IgE antibodies (Abs) specific to wheat proteins are usually observed in sera from patients with WDEIA, and patients typically present with generalized urticaria and systemic allergic reactions such as anaphylactic shock induced by a type I allergic reaction.[14] Conventionally, WDEIA is thought to result from oral sensitization by the ingestion of foods containing native wheat proteins (WP)[14,15] However, recent reports suggest that immediate food-allergic reactions including WDEIA may also be induced by intake of foods containing WP and/or HWP following transcutaneous sensitization with HWP.[4,5,16-20] Laurière et al. previously described that patients were sensitized transcutaneously with HWP and presented with contact urticaria to cosmetics containing HWP.[4] These patients also developed allergic symptoms such as generalized urticaria and anaphylaxis when they ate HWP-containing foods, although they could eat normal wheat foods without allergic symptoms. A recent social problem in Japan has been the sensitization of WDEIA patients with HWP (HWP-WDEIA) by using the same brand of facial soap.[5,18-20] Interestingly, Japanese patients with HWP-WDEIA developed allergic symptoms not only with the use of HWP-containing soap, but also with exercise after the intake of native WP, unlike European cases as described by Laurière et al..[4] This indicated that specific IgE Abs to HWP in Japanese HWP-WDEIA patients could cross-react with native WP.[5,18-20] ω5-Gliadin and HMW-glutenin were identified as major allergens in conventional WP-WDEIA patients, whereas the allergens and their IgE Ab binding epitopes in HWP-WDEIA patients have not been identified.[8-11,20] In the present study, we identified the causative allergen(s) and the primary IgE-binding epitopes of WP in Japanese HWP-WDEIA patients by immunoblot analysis using six recombinant wheat proteins, including α/β-,

γ-, ω1,2- and ω5-gliadins and LMW- and HMW-glutenins, and synthetic peptides. The allergenic activities of HWP and recombinant WPs were also characterized by histamine releasing test (HRT).

METHODS

CLONING OF cDNA AND GENOMIC DNAs ENCODING WHEAT GLIADINS AND GLUTENINS

Total RNA and genomic DNA were extracted from Japanese soft wheat cultivar, Norin 61 (Shimane Agricultural Experiment Station, Shimane, Japan) as reported previously.[21] To clone α/β-, γ- and ω1,2-gliadins and LMW-glutenin cDNAs, total RNA was isolated from 0.1 g frozen wheat seeds (2 or 5 weeks after pollination) with the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). Genomic DNA was isolated from 0.1 g frozen leaves with the Isoplant DNA extraction kit (Takara Bio, Shiga, Japan) to clone HMW-glutenin genes. cDNA was synthesized by First Stand cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) using total RNA and PCR was performed by iCycler (Bio-Rad Laboratories, Hercules, CA, USA) using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). The primers, for α/β-gliadin, 5'-CCGG ATCCGCAGTTAGAGTTCCAGTGCCACAATTG-3 ', 5'-TTGAATTCTCAGTTAGTACCGAAGATGCCAAAT G-3'; for γ-gliadin, 5'-GCGGATCCAATATGCAGGTCG ACCCTAGCAGCC-3', 5'-TGGAATTCTCATTGGCCA CCAATGCCGGCGAC-3'; for ω1,2-gliadin, 5'-CCGGAT CCGCTAGGGAGTTAAACCCTAGCAACAAAGAG-3', 5'-TGGAATTCTCATTGGCCACCGATGCTTGTAAG ACTACTCCCAT-3'; for LMW-glutenin, 5'-TTGGATC CATGGAGAATAGCCACATCCCTGGTTTGGAGAG ACCAT-3', 5'-TTGAATTCTCAGTAGCCACCAACTC CGGTGCCAACGCCGA-3' and for HMW-glutenin, 5'-CCCATATGGAAGGTGAGGCCTCTGAGCAACTAC AGTGT-3', 5'-GAGTCGACTCACTGGCTGGCCGACA ATGCGTCGCCGC-3', were synthesized based on nucleotide sequences deposited at GenBank with the following accession numbers: U08287 (α/β-gliadin), AF120267 (γ-gliadin), AF280605 (ω1,2-gliadin), AB 181300 (ω5-gliadin), AB062853 (LMW-glutenin) and X12928 (HMW-glutenin). The PCR products were cloned into a pGEX-6P-1 vector (GE Healthcare, Little Chalfont, UK) or pET-21a (+) vector (Novagen, Madison, WI, USA). All cloning products were sequenced by the dideoxy chain termination method using a Big-Dye terminator v3.1 cycle sequencing kit and the ABI PRIZM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences have been deposited in the DNA Data Bank of Japan (DDBJ) database as follows: AB766205 for α/β-gliadin, AB766206 for γ-gliadin, AB766207 for ω1,2-gliadin, AB766208 for LMW-glutenin, and AB766209 for HMW-gliadin.

Table 1 Oligonucleotide sequences for the amplification of genes encoding wheat proteins

Gene		Sequence
α/β-gliadin		
	Forward	5'-TGCATATGGCAGTTAGAGTTCCAGTGCCACAAT-3'
	Reverse	5'-TTGAATTCTCAGTTAGTACCGAAGATGCCAAATG-3'
γ-gliadin		
	Forward	5'-TGCATATGAATATGCAGGTCGACCCTAGCAGCC-3'
	Reverse	5'-TGGAATTCTCATTGGCCACCAATGCCGGCGAC-3'
ω1,2-gliadin		
	Forward	5'-CCGAGCTCGCTAGGGAGTTAAACCCTAGCAACAAAGAG-3'
	Reverse	5'-GGGTACCTCATTGGCCACCGATGCTTGTAAGACTACTCCC-3'
ω5-gliadin		
	Forward	5'-CCATATGAGTAGGCTGCTAAGCCCTAGAGGCAAGGAATTG-3'
	Reverse	5'-TCGTCGACTCATAGGCCACTGATACTTATAACGTCGCTCC-3'
HMW-glutenin		
	Forward	5'-CCCATATGGAAGGTGAGGCCTCTGAGCAACTACAGTGT-3'
	Reverse	5'-GAGTCGACTCACTGGCTGGCCGACAATGCGTCGCCGC-3'
LMW-glutenin		
	Forward	5'-TGCATATGGAGAATAGCCACATCCCTGGTTTGGAGA-3'
	Reverse	5'-TTGAATTCTCAGTAGCCACCAACTCCGGTGCCAACGCCGA-3'

Each primer was constructed based on sequences deposited at GenBank with the following accession numbers: U08287 (α/β-gliadin), AF120267 (γ-gliadin), AF280605 (ω1,2-gliadin), AB181300 (ω5-gliadin), X12928 (HMW-glutenin) and AB062853 (LMW-glutenin). LMW, low molecular weight; HMW, high molecular weight.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

PCR was performed using plasmid DNA containing cloned genes. The primers used are shown in Table 1. The PCR products of α/β- and γ-gliadin and LMW- and HMW-glutenin genes were inserted into pET-21a (+). The PCR product of ω5-gliadin digested with *Nde*I and *Sal*I was ligated to pCold III vector (Takara Bio). The ω1,2-gliadin gene was also ligated to pCold III vector using *Sac*I and *Kpn*I sites. The ligated plasmids of ω5-gliadin and the others were transformed into *E. coli* JM109 (ECOS™ JM109, Nippon Gene, Tokyo, Japan) and *E. coli* Rosetta DE3 (Novagen) competent cells, respectively. For the expression of ω5- and ω1,2-gliadins, *E. coli* JM109 or *E. coli* Rosetta cells harboring pCold III inserted genes were cultured in LB medium containing 100 μg/mL ampicillin or 100 μg/mL ampicillin-34 μg/mL chloramphenicol, respectively. Then, isopropyl thio-β-D-galactoside (IPTG) was added at a final concentration of 1 mM for the expression of ω5-gliadin or 0.1 mM for ω1,2-gliadin, respectively, and the bacterial broth was cultured at 15℃ for 24 h. Other recombinant proteins were also induced by the same method used for ω1,2-gliadin except cells were cultured at 37℃ instead of 15℃. To extract ω1,2- and ω5-gliadins, the pellet was suspended in 99.5% ethanol and sonicated using an Ultrasonic processor (Sonics & Materials, Newtown, CT, USA). The other proteins were extracted with 50% 1-propanol containing 1% dithiothreitol. The suspensions were incubated at 37℃ for γ-gliadins or 50℃
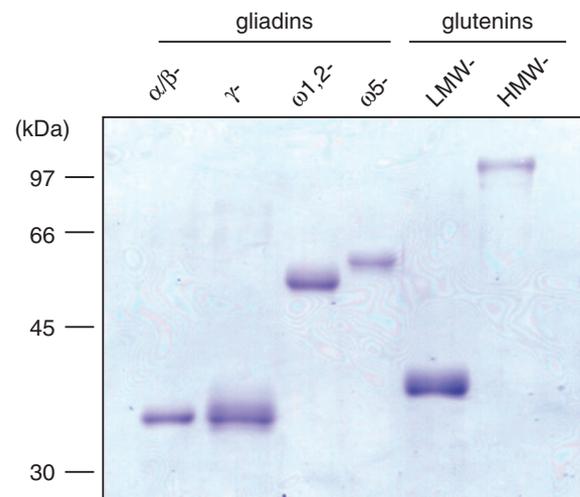

Fig. 1 SDS-PAGE analysis of purified recombinant wheat gliadins and glutenins. The gel was stained with Coomassie brilliant blue (CBB).

for the other proteins for 30 min and centrifuged at 15,800 × *g* for 20 min at 20℃ to obtain the supernatants. The recombinant proteins were purified by HPLC using the method described previously.[21] As shown in Figure 1, single bands corresponding to the molecular size of each WP were observed. The recombinant WP was identified by its N-terminal amino acid (aa) sequences by using a PPQS-10 auto protein sequencer (Shimadzu, Kyoto, Japan). The N-terminal

Table 2 Clinical characteristics of WP- and HWP-WDEIA patients

| No. | Age (years) | Gender | Total IgE (IU/mL) | Specific IgE (Ua/mL) | | | HMW-glutenin |
				Wheat	Gluten	ω5-Gliadin	
Patients with WP-WDEIA							
1	72	M	414	<0.35	<0.35	11.0	<0.35
2	43	F	105	<0.35	<0.35	2.14	<0.35
3	10	M	1,205	3.10	3.10	<0.35	24.4
4	19	M	602	1.34	0.52	13.1	<0.35
5	34	F	770	0.76	0.90	14.1	<0.35
6	51	M	245	<0.35	<0.35	11.0	0.38
7	46	F	119	5.02	4.90	<0.35	1.08
8	16	M	36	<0.35	<0.35	2.36	<0.35
9	19	M	110	<0.35	<0.35	<0.35	2.01
10	14	M	391	<0.35	<0.35	<0.35	10.50
Patients with HWP-WDEIA							
11	44	F	541	9.26	15.8	1.16	1.18
12	50	F	269	1.35	1.78	<0.35	<0.35
13	25	F	nd	3.81	3.60	<0.35	nd
14	47	F	1,820	1.66	4.90	1.69	nd
15	22	F	4,860	0.69	0.94	<0.35	nd
16	68	F	68	<0.35	<0.35	<0.35	nd
17	37	F	107	<0.35	<0.35	<0.35	nd
18	60	F	8	<0.35	0.41	<0.35	nd
19	39	F	350	1.23	1.41	<0.35	nd
20	60	F	136	0.59	1.08	<0.35	nd
21	23	F	62	<0.35	0.55	<0.35	nd
22	34	F	53	<0.35	0.45	<0.35	nd
23	62	F	nd	<0.35	<0.35	<0.35	nd
24	55	F	95	1.52	3.15	<0.35	nd
25	51	F	58	4.70	4.76	<0.35	1.14
26	49	F	77	2.27	4.56	<0.35	<0.35
27	52	F	20	0.46	0.65	<0.35	0.46
28	54	F	40	5.17	7.37	<0.35	0.37
29	46	F	246	1.18	1.40	<0.35	<0.35
30	27	F	213	1.01	1.41	<0.35	<0.35
31	34	F	225	0.63	1.05	<0.35	<0.35
32	38	F	253	3.15	3.46	<0.35	0.50

WP, wheat protein; HWP, hydrolyzed wheat protein; WDEIA, wheat-dependent exercise induced anaphylaxis; HMW, high molecular weight; nd, not determined.

sequences of α/β-gliadin (AVRVPVPQLQ), γ-gliadin (NMQVDPSSQV), LMW-glutenin (MENSHIPGLE) and HMW-glutenin (MEGEASEQLQ) were identical to the aa sequences deduced from the nucleotide sequences of these WP genes, whereas recombinant ω5- and ω1,2-gliadin contained TEE sequences (MNHKVHMXL) derived from the pCold III vector. In addition, ω1,2-gliadin was formed in a mixture with the protein that has 8 aa deletion in N-terminus.

SUBJECTS

Sera were collected from 32 patients with WDEIA who had recurrent episodes of anaphylaxis and were positive for a provocation test where they exercised after wheat ingestion as reported previously.[22] Sera from three healthy subjects without episodes of food allergy were used as controls. Among 32 WDEIA patients, 22 patients had used a similar facial soap containing HWP. The other patients were diagnosed as conventional WP-WDEIA. The sera were stored at -80℃ until use. Information regarding the sera from patients is described in Table 2. This study was approved by the ethics committee of Hiroshima University (approval No. 580).

IMMUNOBLOT ANALYSIS AGAINST WHEAT PROTEINS

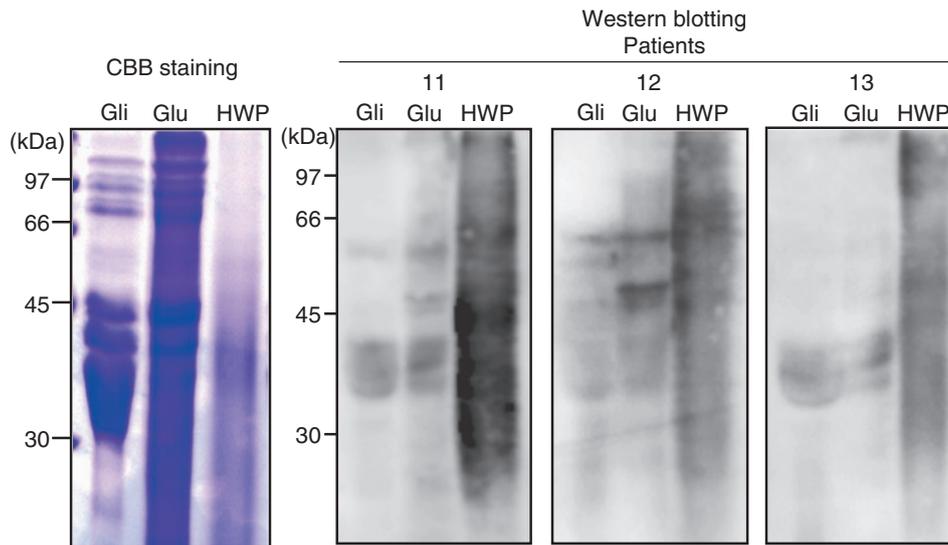Native gliadins and glutenins were isolated from

Fig. 2 Western blot analysis of native wheat gliadins, glutenins and HWP with IgE Abs from patients with HWP-WDEIA. Proteins (10 μg for WP and 20 μg for HWP) were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was incubated with 10% sera from HWP-WDEIA patients (Nos. 11, 12 and 13). For CBB staining, 10 μg of WP and 40 μg HWP proteins were loaded on the gel. Gli, gliadins; Glu, glutenins; HWP, hydrolyzed wheat protein.

bread wheat (Norin 61). Wheat flour was defatted and water-soluble proteins, albumin and globulin, were extracted in a 0.5 M NaCl solution. After centrifugation, gliadins were extracted with 70% ethanol. Then, glutenins were extracted from the pellet with 0.02 M NaOH solution. Native WP and HWP (glupearl 19S®, Katayama Chemical, Osaka, Japan) were applied to each lane of a 12.5% polyacrylamide gel, and SDS-PAGE was performed. The Coomassie brilliant blue (CBB) staining and western blot analysis were performed as reported previously using image analyzer LAS-4000mini (GE Healthcare).[23]

DOT BLOTTING AND INHIBITION STUDY FOR IgE-BINDING ASSAY
Dot blotting was performed according to a previous report with minor modifications.[23] For the inhibition study, the HWP, native or deamidated epitope-peptides were added to the patients' serum as an inhibitor at more than 15-fold excess concentration of dotted proteins. Bound IgE Abs were detected as described above, and the spot intensities were measured using Quantity One software (Bio-Rad Laboratories).

HISTAMINE RELEASE TEST (HRT)
HRT was performed with basophils obtained from the peripheral blood of HWP-WDEIA patients according to the method of Tanaka *et al.*.[24] The histamine release rate (%HR) was expressed by dividing the amounts of histamine released into the buffer by the total histamine amount. The net histamine release rate (Net %HR) was also estimated by subtracting the %HR in the absence of allergen from %HR in the presence of allergen. The positive rate was defined as Net %HR ≥5%.

PEPTIDE SYNTHESIS ON THE SPOTs MEMBRANE AND IgE-BINDING
Based on the amino acid (aa) sequence of γ-gliadin (GenBank accession no. AB766206), 14 individual peptides, overlapping by 7 aa, were synthesized on a SPOTs membrane (Sigma-Aldrich, St. Louis, MO, USA) as described previously.[10,23] To identify the core IgE-binding epitope sequences, peptides with deletion of the N- or C-terminal aa residues were also synthesized according to the IgE-binding study. The IgE-binding assay was performed as described previously.[23] To use the SPOTs membrane repeatedly, the membrane was washed with water, Regeneration buffer IIA [8 M urea, 1% SDS and 0.1% (v/v) 2-mercaptoethanol], Regeneration buffer IIB [10% (v/v) acetic acid and 50% (v/v) ethanol] and TBS-T.

RESULTS

IgE-BINDING PROFILES OF SERA FROM PATIENTS WITH WP- AND HWP-WDEIA
HWP appeared as smeared band on SDS-PAGE with CBB staining while separated bands were detected in WPs (Fig. 2). Immunoblot assays including western blotting and dot blotting were performed to compare the IgE-binding profiles to WP among patients with
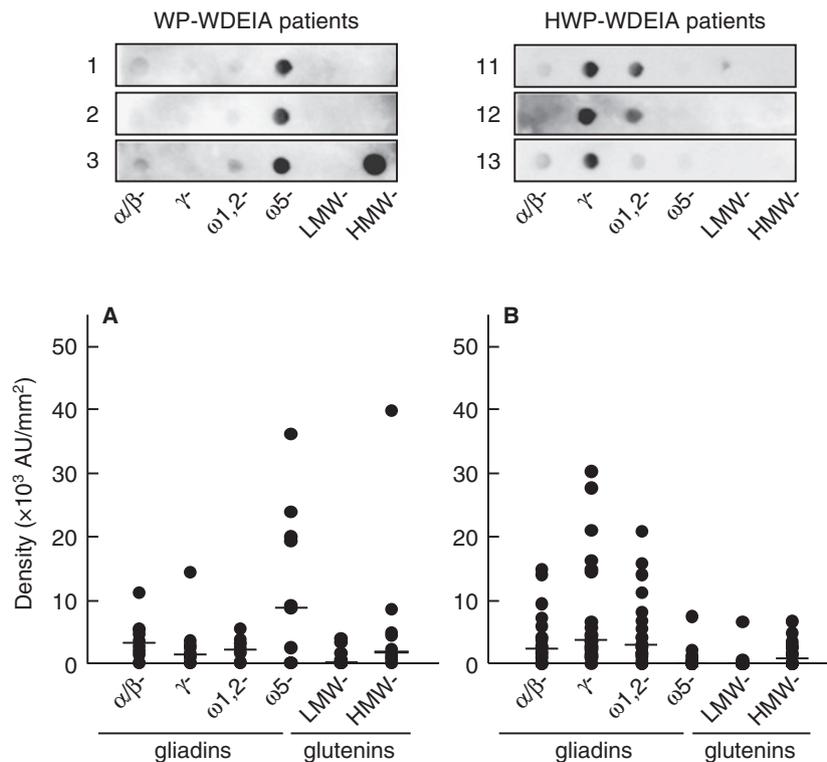
Fig. 3 IgE-binding profiles of sera from patients with WP- and HWP-WDEIA. Dot blots were performed by applying 0.5 μg of the recombinant WP onto a PVDF membrane. Upper, representative membranes of WP with sera from WP- (Nos. 1, 2 and 3) and HWP-WDEIA patients (Nos. 11, 12 and 13). Bottom, densitometry of IgE-binding WP with sera from 10 WP-WDEIA patients (Nos. 1-10) (**A**) and 22 HWP-WDEIA patients (Nos. 11-32) (**B**). HWP, hydrolyzed wheat protein. Bars represent the median values of densities.

WP- and HWP-WDEIA. Sera from all HWP-WDEIA patients showed the smear IgE-binding to HWP in wide molecular weight ranges by western blotting (Fig. 2). The specific IgE-binding to gliadins and glutenins were also observed, but the bands for gliadins did not correspond to the molecular size of ω5-gliadin. Furthermore, we characterized the IgE-binding profiles of patients with WP- and HWP-WDEIA by dot blotting using recombinant gliadins and glutenins (Fig. 3). IgE Abs from WP- and HWP-WDEIA patients exhibited strong binding to gliadins and glutenins, but the binding profiles of these proteins were different between the two WDEIA patient groups. Most patients with WP-WDEIA showed specific IgE-binding to ω5-gliadin and HMW-glutenin, confirming previous reports (Fig. 3A).[8] In contrast to WP-WDEIA patients, higher specific IgE-reactivity to α/β-, γ- and ω1,2-gliadin were observed in HWP-WDEIA patients (Fig. 3B).

HISTAMINE RELEASE FROM BASOPHILS OF HWP-WDEIA PATIENTS BY WP
HRT was performed among 11 patients with HWP-

WDEIA to investigate the functional allergenic activity of recombinant gliadins and glutenins. All basophils isolated from HWP-WDEIA patients exhibited degranulation induced by anti-IgE Abs as a positive control and HWP stimuli (Fig. 4). The histamine releases were observed by γ-gliadin in 8 of 11 HWP-WDEIA patients, and with α/β- and ω1,2-gliadins in one of 11 patients each. No histamine release was induced by ω5-gliadin.

DETERMINATION OF IgE-BINDING EPITOPES IN γ-GLIADIN
Several studies to determine the IgE-binding epitopes of wheat gliadins suggested that the length of the epitope sequence is <7 aa.[10,25,26] Therefore, we synthesized overlapping peptides with 14 aa offset by 7 aa based on the aa sequences of γ-gliadin, which reacted with IgE Abs of HWP-WDEIA patients most strongly as shown in Figure 3. Sera from three patients reacted with many peptides, in particular, the sequence "QFLQPQQPFPQQPQ" of peptide 9 was recognized most strongly by IgE Abs of 2 HWP-WDEIA patients (Fig. 5). To investigate the core epitope sequence for
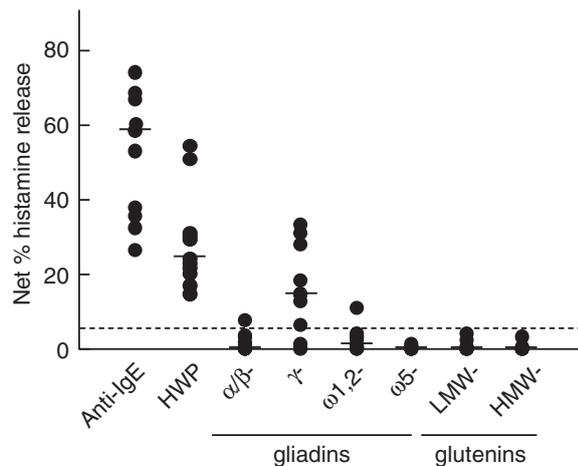
Fig. 4 Histamine release (HR) test in patients with HWP-WDEIA. Anti-IgE Abs and each wheat protein were added into the buffer at a concentration of 1 μg/mL and 100 ng/mL, respectively. The positive rate was defined as Net% HR ≥ 5% (dotted line). HWP, hydrolyzed wheat protein. Bars represent the median values of results from 11 patients (Nos. 14-24).

IgE-binding in HWP-WDEIA patients, we synthesized peptide 9 with a deletion in the N- or C-terminal aa residues (Fig. 6). The deletion of four or five aa residues in the N-terminal and of four residues in the C-terminal resulted in reduced IgE-binding, indicating that the common IgE-binding epitope in HWP-WDEIA was QPQQPFPQ (Fig. 6A). We further examined the effect of deamidation on the IgE-binding ability of epitope peptides using synthesized peptides where glutamine (Q) residues were substituted for glutamic acids (E). The substitutions generally increased IgE binding to peptides, and the common epitope was determined as PEEPFP (Fig. 6B). Finally, an IgE-binding inhibition study was performed to evaluate the specificity of IgE-binding ability to native or deamidated epitope peptides using sera from three HWP-WDEIA patients (Fig. 7). The deamidated peptide of EFLEPEEPFPEEPE was used in comparison with the native peptide of QFLQPQQPFPQQPQ. HWP in patients' sera completely suppressed the specific IgE-binding with HWP, native gliadins and recombinant γ-gliadin, suggesting that the HWP-specific IgE Abs appeared to cross-react with gliadins, especially γ-gliadins. Both native and deamidated epitope-peptides with sera partially suppressed IgE-binding to HWP and gliadins, and the inhibitory potency of the deamidated peptide appeared to be stronger than the native peptide. These results indicated that "specific" IgEs to HWP cross-react with the QFLQPQQPFPQQPQ aa sequence from γ-gliadin, and the IgE-binding ability could be enhanced by deamidation.

DISCUSSION

Many European HWP-WDEIA patients only suffer from immediate allergic reactions when they eat HWP-containing foods, while they can eat normal wheat products without problems. In contrast, many Japanese patients with HWP-WDEIA develop allergic reactions with exercise after the ingestion of natural wheat products.[4,5,16-20] These clinical case reports proposed that specific-IgE to HWP can cross-react with WP in Japanese HWP-WDEIA patients.[5,18-20] In this study, we identified the causative allergen(s) and the IgE-binding primary epitopes in WP by immunoblotting analysis among Japanese HWP-WDEIA patients sensitized transcutaneously. Furthermore, we identified an IgE-binding epitope in HWP.

The widely spread smear protein band of random degradation was observed in HWP by CBB staining and sera from HWP-WDEIA patients reacted with those proteins. It is notable that IgE-binding to HMW protein (>50 kDa) was observed although weak IgE-binding bands were detected in the lanes containing native gliadin and glutenin (Fig. 2), confirming previous reports.[4,16,22] IgE-binding entities with HMWs in HWP were not identified in this study; however, two possible hypotheses may explain this. First, pre-existing HMW allergen proteins might be modified by acidic hydrolysis such as deamidation. Gliadins and glutenins contain a large number of glutamine residues that can be easily deamidated to glutamic acid residues by acidic hydrolysis.[1,6,27] Denery-Papini *et al.* and Gourbeyre *et al.* reported that IgE Abs from HWP-allergy patients reacted with deamidated gliadins more strongly compared to native gliadins.[27,28] In this study, the substitution of glutamines to glutamic acid in epitope-peptides increased IgE-reactivity in the HWP-WDEIA patients (Fig. 6). Thus, pre-existing allergen with HMWs such as HMW-glutenin could be deamidated and strongly react with IgE Abs from HWP-WDEIA patients. Second, degraded smaller peptides could be reassembled and allergens with HMWs are newly produced. Bouchez-Maihiout *et al.* reported that HWP contained IgE-reacting HMW entities, resulting in a rearrangement of peptides generated from gluten, and polypeptides resistant to dissociating and reducing conditions observed by SDS-PAGE.[16] Our preliminary study also showed that sera from HWP-WDEIA patients responded to gliadins treated with acidic hydrolysis in the HMW range, similar to HWP, while IgE-binding patterns of gliadins deamidated by a cation-exchange resin were similar to those of native gliadins (data not shown). Thus, IgE-binding entities with HMWs can result from the rearrangement of degraded gliadin peptides due to the interchange of disulphide bonds and/or multiple non-covalent interactions formed through the acidic-hydrolysis process.

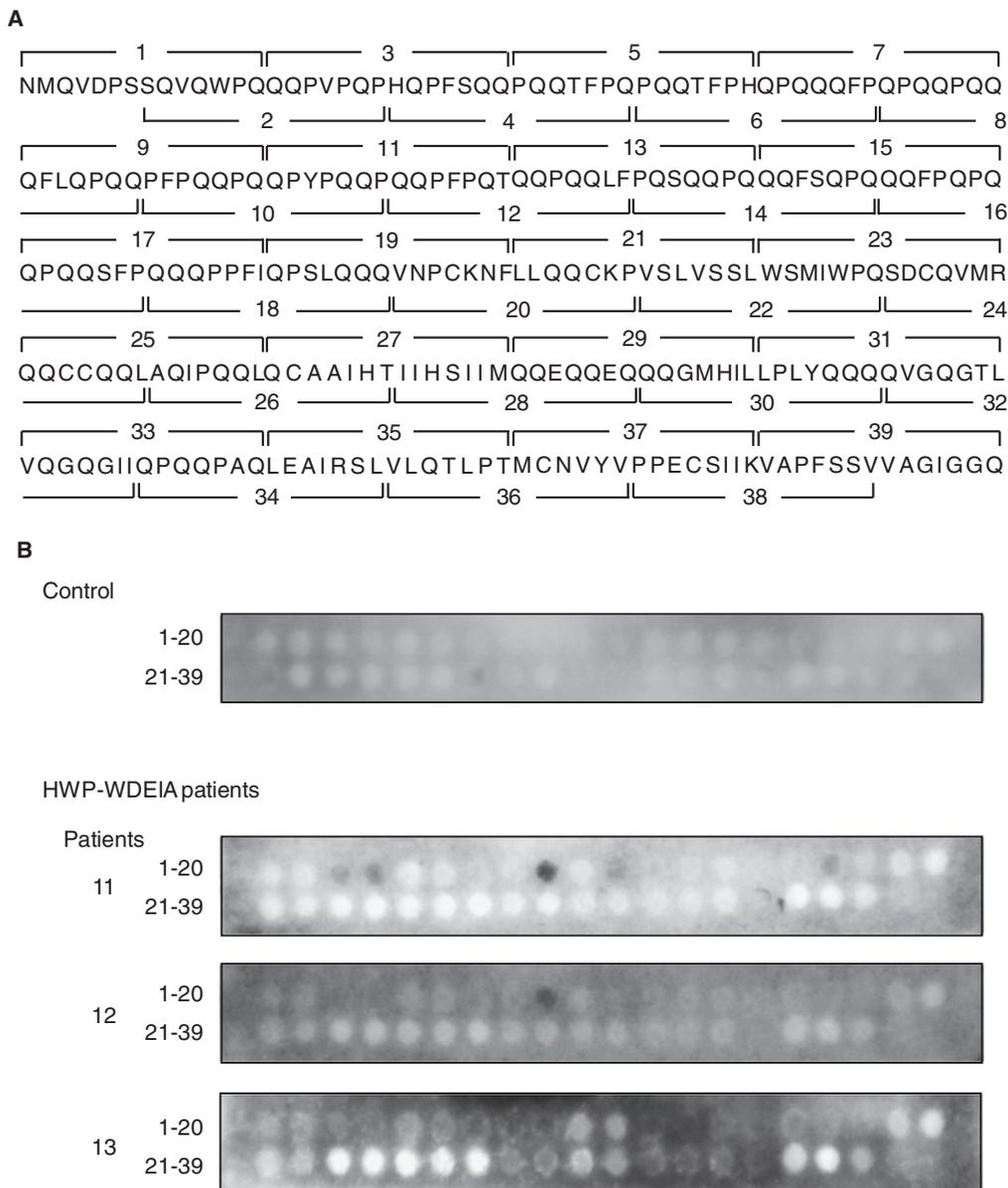Sera from HWP-WDEIA and WP-WDEIA patients

A

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        1              3              5              7
NMQVDPSSQVQWPQQQPVPQPHQPFSQQPQQTFPQPQQTFPHQPQQQFPQPQQPQQ
          2            4            6            8

      9          11          13          15
QFLQPQQPFPQQPQQPYPQQPQQPFPQTQQPQQLFPQSQQPQQQFSQPQQQFPQPQ
        10          12          14          16

    17          19          21          23
QPQQSFPQQQPPFIQPSLQQQVNPCKNFLLQQCKPVSLVSSLWSMIWPQSDCQVMR
        18          20          22          24

    25          27          29          31
QQCCQQLAQIPQQLQCAAIHTIIHSIIMQQEQQEQQQGMHILLPLYQQQQVGQGTL
        26          28          30          32

    33          35          37          39
VQGQGIIQPQQPAQLEAIRSLVLQTLPTMCNVYVPPECSIIKVAPFSSVVAGIGGQ
        34          36          38
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B

Fig. 5 Synthetic overlapping peptide sequence derived from γ-gliadin (**A**) and reactivity of overlapping peptides with sera from HWP-WDEIA patients (**B**). The membrane, where the peptides were synthesized, was incubated with 10% sera from three patients (Nos. 11, 12 and 13). Control: without sera from patients.

showed strong IgE-reactivity to gliadins (Fig. 2). Thus, we characterized the IgE-binding profiles between patients with WP- and HWP-WDEIA by dot blotting recombinant gliadins and glutenins. Sera from WP-WDEIA patients showed strong IgE-binding to ω5-gliadin and HMW-glutenin, the major allergens for WP-WDEIA, while those from HWP-WDEIA patients predominantly reacted with α/β-, γ- and ω1,2-gliadin. This indicated that the IgE-binding profiles were different between WP- and HWP-WDEIA patients (Fig. 3), although the study did not clarify differences in IgE-binding profiles between them. How-

ever, we obtained no entities responding to anti-ω5-gliadin Abs in HWP by western blotting in the preliminary study (data not shown). Thus, IgE-binding epitopes of ω5-gliadin that cause WP-WDEIA may disappear by the acid-hydrolysis process. In contrast to the immuno-binding ability evaluated by dot blotting, α/β- and ω1,2-gliadins induced histamine release from basophils in a few patients with HWP-WDEIA. However, γ-gliadin induced histamine release in eight of 11 patients (Fig. 4). The IgE-binding to γ- and ω1,2-gliadins in HWP-WDEIA is rational since the aa sequence of QPQQPFPQ in epitope domains exists in
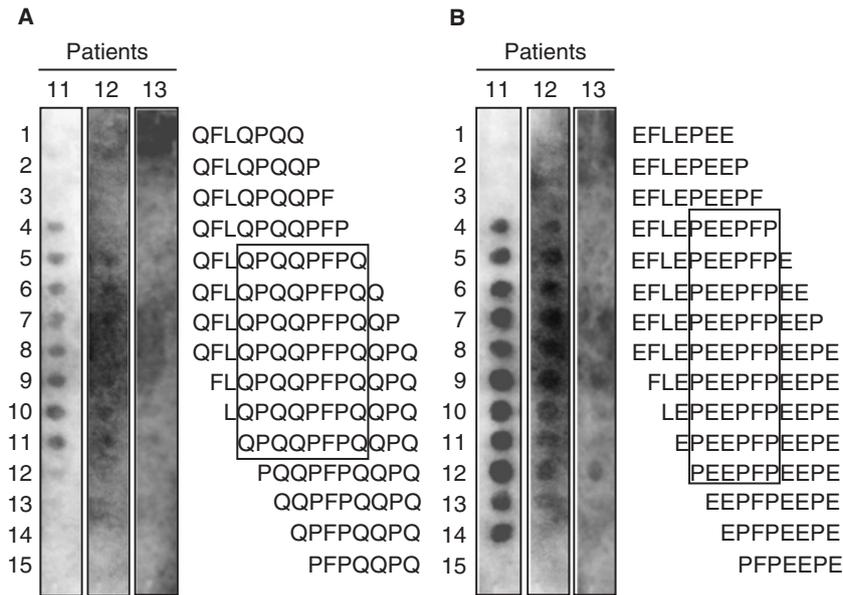
Fig. 6 Identification of core epitope sequences of native (**A**) or deamidated (**B**) synthetic peptides derived from γ-gliadin in HWP-WDIEA patients. The membrane, where the peptides were synthesized, was incubated with 10% sera from three patients (Nos. 11, 12 and 13). The boxed areas indicate the core epitope sequences.

both gliadins. The reason for the difference in IgE-binding abilities and allergenic activities of ω1,2-gliadin is not clear at present. However, the steric structure of ω1,2-gliadin may obstruct the accessibility of IgE Abs to multi-epitope domains and/or polymerization with multiepitopes, which are more suitable for bridging IgE Abs on basophils. Mills & Breiteneder suggested that multivalency is essential for allergens to trigger histamine release.[29] Palosuo *et al*. also showed that polymerization of ω5-gliadin enhanced IgE-binding in WP-WDEIA.[30] Some cysteine residues are present in the first structure of γ-gliadin, but not in ω1,2-gliadin.[6] Thus, γ-gliadins may form a polypeptide structure linked by interchain disulphide bonds that is more suitable for bridging IgE Abs on basophils, whereas ω1,2-gliadin cannot form the polypeptide structure.

Interestingly, the epitope of QPQQPFPQ identified in Japanese HWP-WDEIA was the identical to the epitope in European HWP-WDEIA reported by Denery-Papini *et al*. (Fig. 6).[27] Why the same epitope can cause different pathologies between Japanese and European patients with HWP-WDEIA sensitized transcutaneously is not clear. As shown in Figure 7, the native epitope peptide of QFLQPQQPFPQQPQ could not completely inhibit IgE-binding to HWP, suggesting that HWP contains other epitopes recognized by IgE Abs from Japanese HWP-WDEIA patients.

Sera from HWP-WDEIA patients also strongly reacted with deamidated peptide containing a PEEPFP sequence, suggesting that PEEPFP may be involved

in the sensitization of HWP in some Japanese patients with HWP-WDEIA (Fig. 6). One limitation of this study is the low number of patients; thus, further studies are necessary to clarify these details.

The curative therapy for WDEIA has not been established, so the reliable treatment for WDEIA is a strict elimination of wheat products from dietary foods. However, the elimination of wheat products causes the decline in quality of life for the WDEIA patients because wheat proteins are contained in various processed foods. Recently, desensitization immune therapy is being used for some food allergies. However, desensitization therapy using crude extracts from natural foods has often failed due to the limited efficacy and potential anaphylactic side effects. In mouse models, the desensitization therapy using hypoallergenic recombinant allergen that modified IgE-binding epitopes was found to be successful in treatment of food allergy.[31,32] Thus, the causative allergens and IgE-binding epitopes identified in this study can be useful to develop the safe and effective desensitization therapy for HWP-WDEIA patients.

In conclusion, HWP-WDEIA patients could be sensitized transcutaneously with HWP containing PEEPFP sequences, and after wheat ingestion WDEIA symptoms could be partly induced by cross-reactivity to QPQQPFPQ in γ-gliadin. These findings are useful for the diagnosis and development of desensitization therapy for HWP-WDEIA patients in Japan.
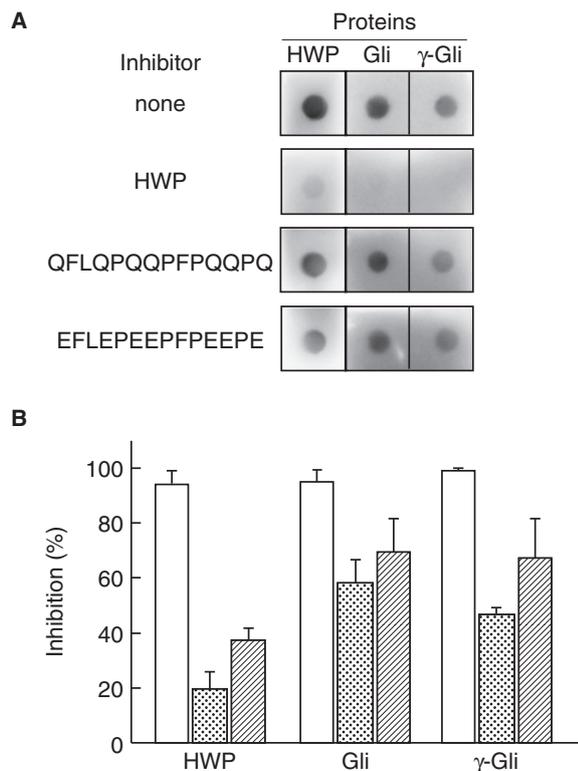
A

Proteins

Inhibitor	HWP	Gli	γ-Gli
none			
HWP			
QFLQPQQPFPQQPQ			
EFLEPEEPFPEEPE			

B

Fig. 7 Inhibition of IgE-binding to HWP and WPs by HWP and native or deamidated epitope-peptides as inhibitors in three HWP-WDEIA patients. Representative result of immunoblot analysis with serum from patient 11 (**A**) and Inhibitory rations estimated by densitometry of immunoblot analysis (**B**). Dot blots were performed by applying 0.38 μg of HWP, 1.0 μg of native gliadins and 3.0 μg of γ-gliadin onto a PVDF membrane. Inhibition ratios for IgE-binding to HWP and WPs by HWP (white columns) and native (dotted columns) or deamidated epitope peptides (diagonal line columns) at a concentration of 100 μg/mL. Inhibition ratios were estimated by measuring the spot intensities. HWP, hydrolyzed wheat protein; Gli, gliadins; γ-Gli, γ-gliadin. Each value represents the mean ± SE of results from three patients (Nos. 11, 12 and 14).

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