

Molecular cloning, recombinant expression and IgE-binding epitope of ω -5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis

Hiroaki Matsuo, Kunie Kohno and Eishin Morita

Department of Dermatology, Shimane University School of Medicine, Izumo, Japan

Keywords

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Correspondence

H. Matsuo, Department of Dermatology, Shimane University School of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan

Fax: +81 853 21 8317

Tel: +81 853 20 2210

E-mail: hmatsuo@med.shimane-u.ac.jp

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Wheat ω -5 gliadin has been identified as a major allergen in wheat-dependent exercise-induced anaphylaxis. We have detected seven IgE-binding epitopes in primary sequence of the protein. We newly identified four additional IgE-binding epitope sequences, QQFHQQQ, QSPEQQQ, YQQYPQQ and QQQPQQ, in three patients with wheat-dependent exercise-induced anaphylaxis in this study. Diagnosis and therapy of food allergy would benefit from the availability of defined recombinant allergens. However, because ω -5 gliadin gene has not been cloned, recombinant protein is currently unavailable. We sought to clone the ω -5 gliadin gene and produce the homogeneous recombinant protein for use in an *in vitro* diagnostic tool. Using a PCR-based strategy we isolated two full-length ω -5 gliadin genes, designated ω -5 and ω -5b, from wheat genomic DNA and determined the nucleotide sequences. The protein encoded by ω -5a was predicted to be 439 amino acids long with a calculated mass of 53 kDa; the ω -5b gene would encode a 393 amino acid, but it contains two stop codons indicating that ω -5b is pseudogene. The C-terminal half (178 amino acids) of the ω -5a gliadin protein, including all 11 IgE-binding epitope sequences, was expressed in *Escherichia coli* by means of the pET system and purified using RP-HPLC. Western blot analysis and dot blot inhibition assay of recombinant and native ω -5 gliadin purified from wheat flour demonstrated that recombinant protein had IgE-binding ability. Our results suggest that the recombinant protein can be a useful tool for identifying patients with wheat-dependent exercise-induced anaphylaxis *in vitro*.

Wheat is one of the most widely cultivated staple foods for western people. Patient with wheat allergy, especially wheat-dependent exercise-induced anaphylaxis (WDEIA) has increased recently, as there is now a higher consumption of western style food in Japan [1,2]. WDEIA is a distinct form of wheat allergy in which the patient experiences a very severe allergic reaction in response to intense exercise after ingestion of wheat [3,4]. Our previous study demonstrated that exercise and aspirin intake facilitate absorption of the wheat allergens from the gastrointestinal tract in patients with WDEIA [5]. It follows that the allergens transferred into circulating blood cross-link receptor-

bound IgE on mast cells and cause degranulation followed by release of chemical mediators such as histamine. They induce immediate inflammatory reactions similar to those of common food allergies such as urticaria, angioedema, hypotension, and shock.

To diagnose WDEIA, we typically perform an exercise challenge test combined with wheat ingestion for patients who have episodes of anaphylaxis after wheat intake. However, the challenge test is unsafe for patients because an anaphylactic shock is sometimes provoked in the test. An radioallergosorbent test to wheat protein or wheat gluten is commercially available for diagnosis of wheat allergy, but this test is not

Abbreviations

WDEIA, wheat-dependent exercise-induced anaphylaxis.

always satisfactory to diagnose WDEIA because of low sensitivity or the occurrence of false-positive results [6]. The heterogeneity of antigens used in the test is considered to be a major cause of these problems. It has been reported that ω -5 gliadin is a major allergen in patients with WDEIA; the skin prick test and radioallergosorbent test with ω -5 gliadin is considered to be useful to diagnose WDEIA [6–8].

Common wheat (*Triticum aestivum*) is a hexaploid species, in which each cell contains six sets of chromosomes and is estimated to have several copies of the ω -5 gliadin gene [9]. In wheat there are at least six different ω -5 gliadin proteins; the primary structures of these is very similar but the contents vary according to growing districts or cultivated variety [10]. Hence it is difficult to prepare a homogeneous ω -5 gliadin protein from wheat flour.

In the present study we analyzed IgE-binding epitopes in an extra three patients with WDEIA and cloned the ω -5 gliadin gene to obtain the IgE-reactive homogeneous recombinant ω -5 gliadin protein that can be used for diagnosis and possibly treatment of WDEIA.

Results

Identification of IgE-binding epitopes in ω -5 gliadin

The IgE-binding epitopes of ω -5 gliadin were analysed in three patients with WDEIA. The detected amino acid sequences of IgE-binding epitopes are summarized in Table 1. The serum IgE antibodies of patient one reacted to QQIPQQQ, QQLPQQQ, QQFPQQQ, QQSPEQQ, QQSPQQQ, QQYPQQQ and QQPPQQ.

Table 1. IgE-binding epitope sequences for patients with WDEIA. Unfilled circles indicate the IgE-binding epitopes detected in this study.

Epitope sequence		Patient		
		1	2	3
1	QQIPQQQ ^a	○	○	
2	QQLPQQQ ^a	○		
3	QQFPQQQ ^a	○	○	○
4	QQSPQQQ ^a	○	○	
5	QQSPEQQ ^a	○	○	
6	QQYPQQQ ^a	○		
7	PYPP ^a			
8	QSPEQQQ			○
9	YQQYPQQ		○	○
10	QQPPQQ	○		
11	QQFHQQQ			○

^a Epitope sequence reported previously [6].

The serum of patient two had specific IgE antibodies to QQIPQQQ, QQFPQQQ, QQSPEQQ, QQSPQQQ and YQQYPQQ. The serum of patient three had specific IgE antibodies to QQFPQQQ, QSPEQQQ, YQQYPQQ and QQFHQQQ. Among these IgE-binding epitope sequences, QQPPQQ, YQQYPQQ, QSPEQQQ and QQFHQQQ, were newly detected in this study.

Molecular cloning and sequence of ω -5 gliadin gene

Many kinds of genes encoding gliadins such as α -gliadin, γ -gliadin and ω -gliadin, have been cloned from common wheat (*T. aestivum*) and sequenced. The sequence data of gliadin genes showed that the gliadin gene contains no introns [11–16]. It was therefore decided to clone genomic gene encoding ω -5 gliadin using PCR method. To amplify the coding region of the ω -5 gliadin gene, PCR primers were designed at the position of the initiation and termination codons of the gene based on the nucleotide sequences extracted from a database of wheat expressed sequence tags (ESTs). A high-fidelity DNA polymerase was used to reduce the risk of introducing errors into the sequence.

Amplification of genes from wheat genomic DNA produced two products designated ω -5a and ω -5b, of 1.4 and 1.2 kb, respectively (Fig. 1). Both genes were cloned into *Escherichia coli* XL-10 Gold and the nucleotide sequence was determined. The ω -5a gene consists of 1413 bp and has an ORF throughout the

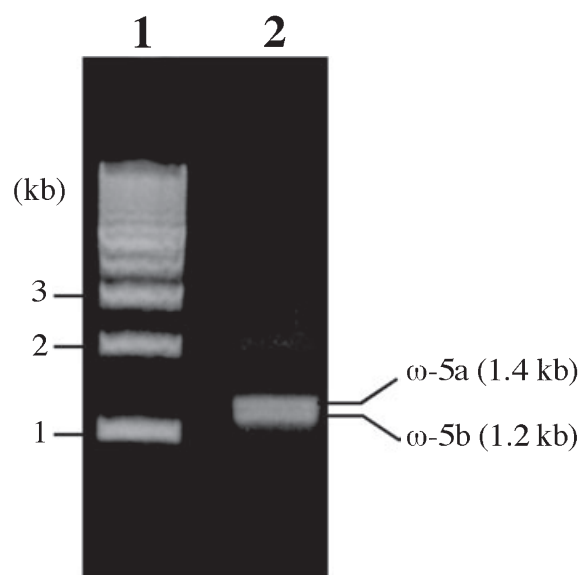


Fig. 1. Agarose gel electrophoresis of PCR product. The analysis of PCR products was performed on a 1% agarose gel. Lane 1, size marker; lane 2, PCR product.

entire 1317 bp coding region. The nucleotide and deduced amino acid sequences are shown in Fig. 2. The nucleotide sequence of the ω -5b gene – which

has 1275 bp – is almost identical to that of ω -5a gene except that the repetitive domain is 138 bp shorter. It has a 1179-bp ORF, but there are stop codons

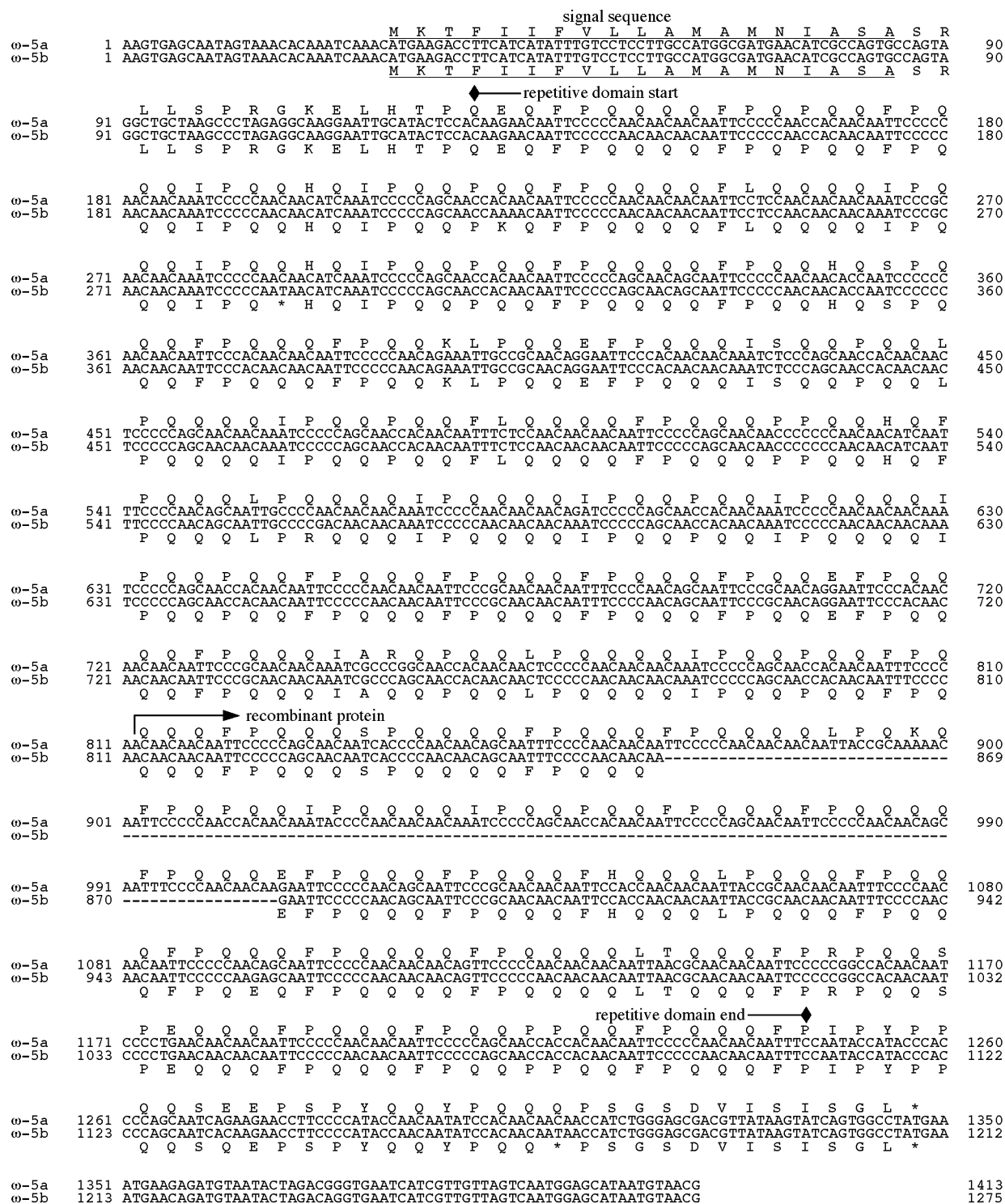


Fig. 2. Nucleotide and deduced amino-acid sequences of ω -5a and ω -5b gliadin genes. Stop codons are indicated by asterisks. Dashes indicate gaps in the alignment. The signal sequences are indicated by underlining. The arrow indicates the region of recombinantly expressed protein.

at positions 288 and 1170. The nucleotide sequences obtained from this study have been deposited in the DDBJ database under accession numbers AB181300 and AB181301. The protein encoded by the ω -5a gene is found to have 439 amino acid residues with a putative signal peptide of 19 amino acids. The molecular mass of the protein without the signal sequence was calculated to be 50 900.

Expression in *E. coli* and purification of recombinant ω -5 gliadin

As DNA encoding the full-length ω -5a gliadin protein could not subcloned into the *E. coli* expression vector because of plasmid instability we tried to produce half of the protein: the C-terminal 178 amino acids, at position 813–1346 in ω -5a gene in Fig. 2, includes all of the detected IgE-binding epitope sequences. After amplification of the DNA encoding this half of the ω -5a gliadin protein by PCR, the DNA fragment was inserted into the expression vector pET-21a. *E. coli* Rosetta (DE3) was used as a host strain as the ω -5a gene has a lot of rare *E. coli* codons. As shown in Fig. 3 lane 2 a high level of expression of recombinant protein, designated rOG5C, was obtained. The ω -5 gliadin purified from wheat flour is slightly soluble in water and soluble in 70% ethanol, whereas the rOG5C

protein is soluble in both water and 70% ethanol. Therefore the recombinant protein was extracted with TBS buffer and then 70% ethanol, and was separated to homogeneity by RP-HPLC (Fig. 3, lane 4). The apparent molecular mass (27.2 kDa) of the rOG5C determined by SDS/PAGE was higher than the molecular mass (21.7 kDa) calculated from the amino acid sequence. It was confirmed that the first 10 amino acids from the N terminus was MQQQFPQQQS-identical to that deduced from the nucleotide sequence of ω -5a gene except the first methionine. Approximately 2.4 mg recombinant protein was purified from 1 L bacterial culture.

IgE-binding reactivity of native and recombinant ω -5 gliadin

The native ω -5 gliadin (nOG5) was purified from a gliadin mixture by RP-HPLC and we confirmed that the N-terminal sequence (S/GRMLSPRG) was identical to that of ω -5 gliadin reported previously [9]. Immunoblot analysis was performed on serum from each of the three patients with WDEIA and who had been diagnosed by provocation test, to compare the IgE-binding ability of nOG5 and rOG5C. The IgE antibodies in the sera of all three patients recognized both nOG5 and rOG5C whereas no IgE reactivity was observed in normal controls (Fig. 4).

In a further step we investigated whether rOG5C shares the IgE-binding epitopes in nOG5 by dot blot inhibition experiments with sera of the three patients. The binding of IgE to nOG5 was completely inhibited by increasing amounts of rOG5C in all patients. At an inhibitor concentration of 0.1 and 1 $\mu\text{g mL}^{-1}$, nOG5 inhibited IgE binding more effectively than rOG5C (Fig. 5).

Discussion

In this study we identified new linear IgE-binding epitopes in ω -5 gliadin and described the gene cloning, expression in *E. coli*, purification, and immunological characterization of the recombinant ω -5 gliadin.

In our previous study we showed that QQIPQQQ, QQFPQQQ, QQLPQQQ, QQSPQQQ, QQSPEQQ, QQYPQQQ and PYPP sequences in ω -5 gliadin are IgE-binding epitopes in patients with WDEIA and that four of these sequences, QQIPQQQ, QQFPQQQ, QQSPQQQ and QQSPEQQ, are dominant [6]. In the present study we carried out an additional IgE epitope analysis in three patients with WDEIA. Two of the three patients have IgE antibodies that react with the four dominant epitope sequences but IgE antibodies in

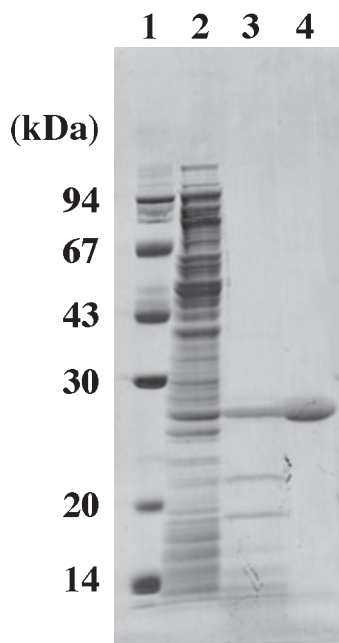


Fig. 3. SDS/PAGE analysis of the proteins at various purification steps. Lane 1, molecular mass size marker; lane 2, cell extract from *E. coli* (pETO5C) grown in the presence of isopropyl thio- β -D-galactoside; lane 3, crude proteins extracted by 70% ethanol; lane 4, purified recombinant protein.

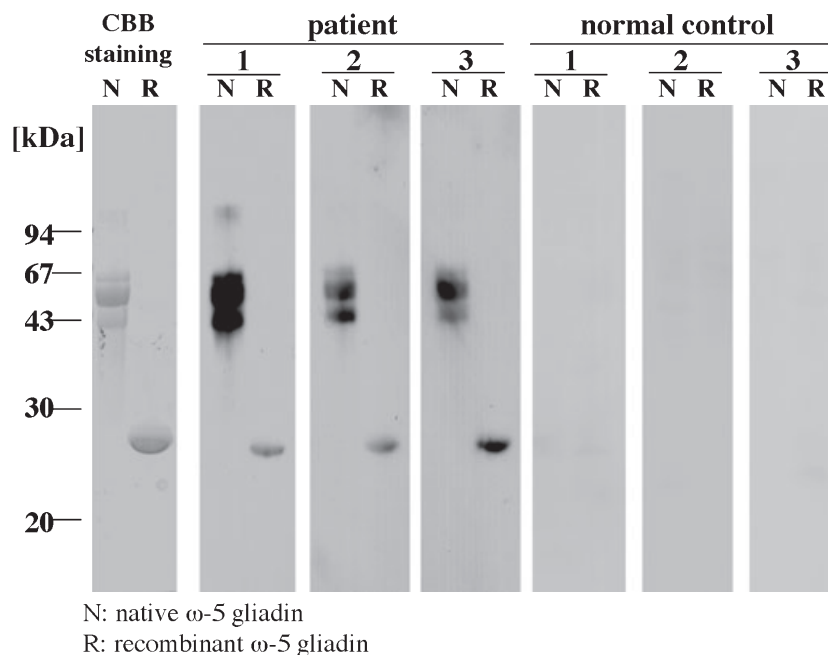


Fig. 4. Western blot analysis of native and recombinant ω -5 gliadin with IgE antibodies from patients with WDEIA and healthy controls. One microgram of each protein was separated by SDS/PAGE and blotted onto a polyvinylidene difluoride membrane. The membrane was probed with serum from subjects. The gel was stained by Coomassie brilliant blue (CBB).

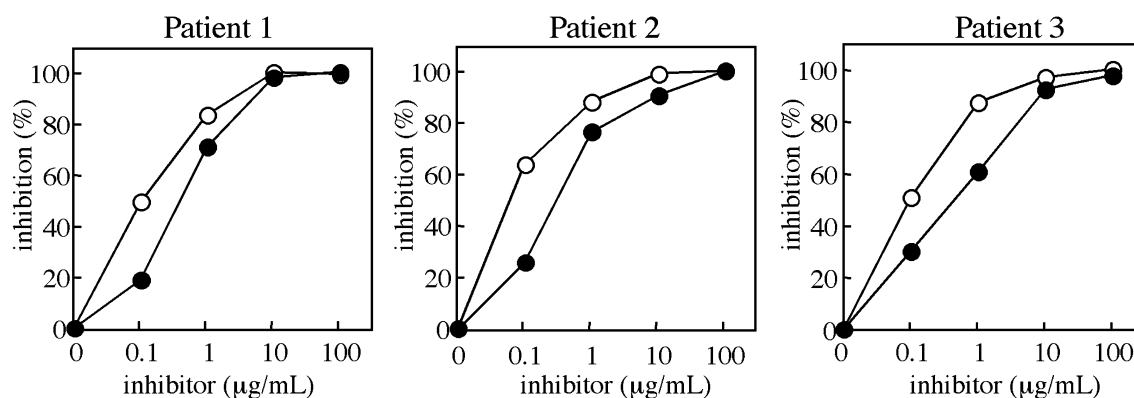


Fig. 5. Inhibition of IgE binding to native ω -5 gliadin with native (open circles) and recombinant (closed circles) ω -5 gliadin proteins as inhibitors in three patients with WDEIA. Dot blots were performed by applying 2 μg of the native ω -5 gliadin onto a polyvinylidene difluoride membrane. The membrane was blocked and incubated with 10% of the patient's serum previously incubated with different concentrations of purified native or recombinant ω -5 gliadin.

the serum of patient three reacted only with peptide QQFPQQQ (Table 1). In addition, IgE antibodies of patients two and three did not react with QQYPQQQ but did react with YQQYPQQ. The two epitopes, QSPEQQQ and QQFHQQQ, were detected only in patient three, and similarly QQPPQQ was detected only in patient one. These results indicate that the four newly detected IgE-binding epitopes, QQPPQQ, YQQYPQQ, QSPEQQQ and QQFHQQQ, are not common but might be important epitopes for the development of allergic symptoms in WDEIA.

We cloned and determined the nucleotide sequence of two ω -5 gliadin genes, ω -5a and ω -5b, from genomic DNA of wheat cultivar Norin 61, a Japanese soft

wheat variety. Neither of the isolated genes contains introns, like other genes encoding gliadins such as α -gliadin, γ -gliadin, ω -1,2 gliadin. The ω -5a gene has an ORF which may encode the protein but the ω -5b gene is assumed to be a pseudogene because it has two stop codons in the putative ORF (Fig. 2). Some gliadin genes are unstable in the *E. coli* vector and deletion of the repetitive domain usually occurred during DNA cloning [16]. The nucleotide sequences of ω -5a determined from five clones in this study were identical and the 1413 bp DNA of the sequenced ω -5a gene is the same length as the PCR products indicating that the cloned ω -5a gene has no artificial deletion. The existence of repeat sequences of QQXP, QQQXP and

QQQQXP where X is F, I or L and the lack of a cysteine residue in ω -5a gliadin are compatible with the structural features of ω -5 gliadin. Kasarda *et al.* reported that the N-terminal amino acid sequence of ω -5 gliadin from wheat (*T. aestivum* 'Justin') is SRLSPRGKELHTPQQQFPQQ [17]. DuPont *et al.* showed that ω -5 gliadin from wheat (*T. aestivum* 'Butte') was separated into two fractions, 1B1 and 1B2, and the N-terminal amino acid sequences are SRLSPRGKELHTPQEFQFPQQQ and SRLSPRGKELHTPQEQFPQQQ, respectively [9]. The deduced N-terminal amino acid sequence of ω -5a is identical with 1B2 ω -5 gliadin. The 1B2 ω -5 gliadin fraction from *T. aestivum* Butte was resolved into three peaks of molecular mass 49 085, 50 300, and 51 500 by MALDI-TOF MS [9]. However the calculated molecular mass (50 900) of ω -5a gliadin did not coincide with any of these molecular masses. The differences in mass between the three 1B2 ω -5 gliadins and ω -5a gliadin may be accounted for by a difference of the number of repeat sequences.

In wheat allergy, sensitization to inhaled wheat flour leads to baker's asthma [18], whereas sensitization to ingested wheat develops into a common food allergy or WDEIA. In addition, the causative allergen is different in various clinical manifestations, for instance the major allergen for baker's asthma is α -amylase inhibitor whereas that for WDEIA is ω -5 gliadin [19]. Recent studies have shown that ω -5 gliadin is a good candidate as a diagnostic tool not only for WDEIA but also for immediate allergy to wheat [20–22]. Accurate diagnosis of food allergy requires standardization of the food antigen used in the skin test and allergen specific-IgE RAST. However, it is difficult to prepare homogeneous allergen by direct extraction from food because the allergen content depends on the cultivated variety and place. Therefore identification and characterization of major allergens for each clinical manifestation is important and the use of standardized recombinant proteins might reduce inaccurate diagnosis. Some recombinant food allergens have been produced and the advantages of recombinant proteins have been clearly demonstrated for diagnosis [23,24]. One type of recombinant wheat gliadin has been produced in *E. coli* using a pET vector and applied to the identification of major allergens in patients with wheat allergy [25]. In the present study we tried to produce full-length ω -5a gliadin in *E. coli* but the entire DNA of ω -5a coding region could not be inserted into several types of *E. coli* expression vectors because of plasmid instability. Thus the C-terminal half of ω -5a gliadin, designated rOG5C and containing all detected IgE-binding epitopes, was overproduced using pET-

21a vector. The calculated mass (21.7 kDa) of the purified rOG5C was approximately 20% lower than the apparent molecular mass (27.2 kDa) determined by SDS/PAGE. This difference in mass is accounted for by the behaviour of native ω -5 gliadin as published previously [9].

It is vital to compare immunological properties of a recombinant protein with those of the native form before using the recombinant for diagnosis or treatment of food allergies. Western blot analysis of nOG5 and rOG5C showed that the IgE antibodies in sera of patients with WDEIA react to nOG5 rather than to rOG5C. Dot blot inhibition assays indicate that the IgE-binding capacity of nOG5 is larger than that of rOG5C due to a lack of N-terminal half of rOG5C. However, rOG5C had sufficient ability to detect the specific IgE to ω -5 gliadin because rOG5C completely inhibited the IgE binding to nOG5. Thus the recombinant ω -5 gliadin produced in this study provides reagent quantities of protein that would be useful in the serologic diagnosis of WDEIA.

Experimental procedures

Identification of IgE-binding epitope in ω -5 gliadin

Overlapping peptides of ω -5 gliadin were synthesized on SPOTs membranes (Sigma-Genosys, The Woodlands, TX, USA); sera from three patients with WDEIA and a positive provocation test result were used to probe the membrane as described previously [4].

Purification of ω -5 gliadin from wheat flour

Gliadin mixture purchased from Tokyo Kasei Kogyo (Tokyo, Japan), dissolved in 70% (v/v) ethanol and purified by HPLC on a Jasco model 880 (Tokyo, Japan) and a PREP-C8 column (20 $\times$ 250 mm; Shimadzu, Kyoto, Japan). The gradient of the elution solvents A [0.1% (v/v) trifluoroacetic acid] and B [99.9% acetonitrile, 0.1% trifluoroacetic acid, (v/v)] was linear from 24% B to 56%. The ω -5 gliadin peaks were collected and acetonitrile was removed using a rotary evaporator. After dialysis of the concentrated solution against 1% (v/v) acetic acid for 60 h, it was lyophilized.

N-terminal amino acid sequence

The N-terminal amino acid sequences of purified proteins were determined by Edman degradation method using PPQS-10 auto protein sequencer (Shimadzu, Kyoto, Japan).

DNA isolation and PCR amplification

The ω -5 gliadin gene was cloned from a Japanese soft wheat cultivar, Norin 61 (Shimane Agricultural Experiment Station). Total genomic DNA was isolated from 0.1 g frozen leaves by the Isoplant DNA extraction Kit (Takara Bio Inc., Shiga, Japan). PCR was performed using KOD DNA polymerase (Toyobo, Osaka, Japan) and DNA AMPLIFIER MIR-D40 (Sanyo, Osaka, Japan). To amplify the DNA fragments containing a complete ω -5 gliadin gene, oligonucleotides, 5'-AAGTGAGCAATAGTAAACACAAATCAAAC-3' and 5'-CGTTACATTATGCTCCATTGACTAACAACGATG-3', were constructed based on fragment DNA sequences of the ω -5 gliadin gene (GenBank accession numbers BE590673 and BQ245835). The following PCR profile was used: 94 °C, 1 min; 65 °C, 1 min; 68 °C, 1 min; 35 cycles.

Cloning and sequencing of PCR products

The PCR product was analysed by electrophoresis through 1% agarose gel and purified using MinElute™ Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified PCR product was cloned into a pPCR-Script Amp cloning vector (Stratagene, La Jolla, CA, USA) and then the ligated plasmid was transformed into *E. coli* XL10-Gold® Ultracompetent cells (Stratagene). Five clones containing the wheat DNA fragment were selected. Then the *Eco*RI digested DNA fragments were subcloned into pUC18 and sequenced by the dideoxy chain termination method using a BigDye termination sequencing kit and the ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Expression and purification of recombinant protein

Sense (5'-ATTTTCATATGCAACAACAATTCCCCAGCAACAATCA-3') and antisense (5'-TCTCGGATCCTCATAGGCCACTGATACTTATAACGTCGCTCCC-3') oligonucleotide primers having an initiation codon and an *Nde*I site at the 5'- and a *Bam*HI site at the 3'-adjacent region, were designed and synthesized based on the determined nucleotide sequences of ω -5a gliadin gene. PCR was performed using the conditions described above using plasmid DNA containing the cloned ω -5 gliadin gene as template. PCR product was digested with *Nde*I and *Bam*HI and ligated to an expression vector, pET-21a, digested with same enzymes to generate pETO5C. *E. coli* Rosetta (DE3) cells harbouring pETO5C was grown in terrific broth (Difco, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing 100 μ g·mL⁻¹ ampicillin. To induce the expression isopropyl thio- β -D-galactoside was added at a final concentration of 1 mM. The cells were grown at 25 °C for 24 h and harvested by centrifugation. The pellet was suspended in TBS (Tris-buffered saline pH 7.4) and

sonicated (Bioruptor, Cosmo Bio, Tokyo, Japan) using 15 s bursts for a total of 2 min with 30 s of incubation on ice between each burst. Ethanol was added to the supernatant at a final concentration of 70% and extracted by shaking for 30 min at room temperature. After centrifuging the mixture at 15 000 g for 15 min at room temperature, the supernatant was concentrated in a rotary evaporator. The solution then was dialysed against 1% acetic acid and lyophilized. The protein mixture was dissolved in 70% ethanol and subjected to HPLC using a reversed-phase C8 column as described above. The recombinant ω -5a gliadin peaks were collected.

SDS/PAGE and immunoblotting

SDS/PAGE was performed with 12.5% acrylamide gel and fractionated proteins were visualized by staining with Coomassie brilliant blue staining. For western blotting, the fractionated protein was transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA) and blocked with 5% skim milk in TBST (50 mM Tris-buffered, saline 1% Tween 20, pH 4.7). The membrane was washed three times with TBST and then probed with a 1 : 10 dilution of the patients' serum. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-human IgE (BioSource, Camarillo, CA, USA). To detect human IgE binding, ECL Plus Western blotting detection reagents (Amersham Biosciences, London, UK) was used. The resulting light was detected on autoradiography film.

Dot blot immunoassay for inhibition test

Dot blots were performed by applying 2 μ g of the native ω -5 gliadin onto a polyvinylidene difluoride membrane (Immobilon-P) using a dot-blot manifold. After blocking with 5% skim milk in TBST the blots were washed three times with TBST for 10 min. The membrane was then incubated with a 1 : 10 dilution of the patients' serum that had been previously incubated with different concentrations of purified recombinant or native ω -5 gliadin overnight at 4 °C. After washing with TBST, the bound IgE antibodies were detected as described above. After scanning the film, the spot intensities were measured using the Gel-Pro Analyzer software (Media Cybernetics Inc., Silver Spring, MD, USA).

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