

# Low Molecular Weight Glutenins in Wheat-Dependant, Exercise-Induced Anaphylaxis: Allergenicity and Antigenic Relationships with Omega 5-Gliadins

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## Key Words

IgE binding • Immunoblot-inhibition method • Low molecular weight glutenins • Recombinant proteins • Skin prick test • Wheat-dependant, exercise-induced anaphylaxis • Wheat allergy •  $\omega$ 5-gliadins

## Abstract

**Background:** Adults suffering from wheat-dependant, exercise-induced anaphylaxis (WDEIA) develop IgE directed against wheat  $\omega$ 5-gliadins (major allergens for this allergy) and against wheat low-molecular weight glutenin subunits (LMW-GS). However, the ability of LMW-GS to trigger an inflammatory response is still unknown. It also remains to be determined if IgE from these patients bind the same epitopes on LMW-GS and  $\omega$ 5-gliadins or if the epitopes are independent. **Methods:** WDEIA patients were selected and skin prick tests (SPTs) were performed on them using commercial gluten, wheat flour extracts, prolamin fractions and a purified natural LMW-GS P42. The IgE-binding ability of natural and recombinant wheat prolamins was verified by immunoblot experiments. Cross-reactivity between LMW-GS and  $\omega$ 5-gliadins was studied by immunoblot inhibition experiments, using purified natural  $\omega$ 5-gliadin as an inhibitor. **Results:** Patients developed positive SPTs with natural

LMW-GS fractions and/or with the purified LMW-GS P42. Natural and recombinant LMW-GS were highly reactive with patient IgE in immunoblot experiments, as was  $\omega$ 5-gliadin. However, differences in reactivity were evident within the LMW-GS group. Except for one recombinant LMW-GS (P73), IgE cross-reactivity between LMW-GS and natural  $\omega$ 5-gliadin was only partial. **Conclusion:** LMW-GS are able to promote local inflammation and they share common epitopes with  $\omega$ 5-gliadins. The nature of these epitopes is discussed. LMW-GS also carried specific epitopes, completely independent from the  $\omega$ 5-gliadin epitopes. Thus, LMW-GS behaved partly as independent allergens.

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## Introduction

Wheat-dependant, exercise-induced anaphylaxis (WDEIA) in adults [1] is a severe form of allergy in which the ingestion of wheat products induces anaphylactic symptoms when associated with a physical effort. Wheat proteins are divided in two main classes: water/salt soluble proteins and prolamins. The latter are storage proteins and are further divided in two groups according to their solubility and degree of polymerization. The first

group of prolamins is composed of gliadins, which are monomeric proteins soluble in alcoholic solutions. They are themselves divided in three groups according to their acid-polyacrylamide gel electrophoresis (PAGE) decreasing mobility: they are named  $\alpha/\beta$ -gliadins,  $\gamma$ -gliadins and  $\omega$ -gliadins. In this latter group,  $\omega$ 1,2-gliadins and  $\omega$ 5-gliadins designate slow and fast moving  $\omega$ -gliadins [2], respectively. The amino acid composition of  $\omega$ -gliadins differs from other gliadins and is characterized by a high level of glutamine and proline with a low level of sulfur-containing residues. Their sequences consist mainly of stretches of repetitive motifs, with very short, nonrepetitive N- and C-termini.

The second group of prolamins is composed of glutenins, which are polymeric proteins that are soluble, like gliadins, in alcoholic solutions, but contain a reducing agent. They are themselves divided in two groups according to the molecular weight of their subunits: high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). The amino acid sequences of LMW-GS are characterized by a proline-rich repetitive domain made of interspersed repeats, based on two consensus heptapeptide motifs: PQQPPFS and QQQQPVL. Three different types of LMW-GS have been described, depending on their N-terminus, s-LMW-GS (SHIPGL-), m-LMW-GS (METRCIP-) and i-LMW-GS (ISQQQQ-) [3]. They all have a similar basic structure, with an N-terminal half containing repeats rich in proline and glutamine residues and a C-terminal half containing most, or all, the cysteine residues [4].

Wheat allergens belong to both water/salt soluble proteins and prolamins. As such many prolamins have been shown to be major IgE-binding proteins in various wheat food allergies [5], including WDEIA [1]. Among gliadins,  $\omega$ 1,2-gliadins have been identified as IgE reactive in immediate hypersensitivity to hydrolyzed wheat proteins through skin contact and food ingestion (c/f-IHHWP) [6], while  $\omega$ 5-gliadins have been identified as major allergens for children with immediate allergy to ingested wheat [7] and for adults with WDEIA [1, 8, 9]. Among glutenins, HMW-GS have been identified as major allergens in the Japanese population suffering from WDEIA [10]. LMW-GS have been identified as IgE-reacting proteins for food allergies to wheat [11–13], including WDEIA [14, 15]. It is interesting to note that, while  $\omega$ 5-gliadins and LMW-GS were both detected by ELISA and immunoblotting as major IgE-reacting proteins in WDEIA, this was not the case when an in vitro mast cell degranulation test was used. LMW-GS generated a de-

granulation process but  $\omega$ 5-gliadins did not [16]. This result emphasizes the differences of reactivity of these two kinds of prolamins with cells involved in the immune response.

In this context, our objective was to study the intrinsic ability of undigested LMW-GS to trigger an inflammatory response and the antigenic relationships between LMW-GS and  $\omega$ 5-gliadins. In the first step, we tested the ability of WDEIA patients to develop a positive skin prick test (SPT) with natural wheat prolamins fractions and a purified natural LMW-GS. In the second step, we studied the in vitro reactivity of IgE with natural wheat prolamins and recombinant proteins by immunoblot assays with sera from patients suffering from WDEIA. We used natural wheat prolamins to identify IgE-reacting wheat proteins present in the grain. We also used fully characterized recombinant wheat proteins to confirm the identity of these IgE-reacting proteins and to access structural data. In the third step, we studied cross-reactivity between LMW-GS and  $\omega$ 5-gliadins by immunoblot inhibition experiments, using highly purified natural  $\omega$ 5-gliadin as an inhibitor. The inhibition of IgE binding would mean that the same IgE recognize the same epitopes on both inhibitor and blotted proteins.

## Materials and Methods

### Reagents and Material

Analytical grade reagents were from Sigma-Aldrich Chimie (St. Louis, Mo., USA). Precast 12% Bis-Tris polyacrylamide gels and 3-(N-morpholino) propane sulfonic acid buffer were supplied by Novex (San Diego, Calif., USA). Polyvinylidene difluoride (PVDF) blotting membranes were from Millipore (St-Quentin-en-Yveline, France). The SuperSignal West Dura Extended duration substrate was from Thermo Scientific, Pierce Biotechnology (Rockford, Ill., USA). The rabbit anti-human IgE conjugated to horseradish peroxidase was from Dako S.A. (Trappes, France).

The MALDI-TOF apparatus was a Voyager DE super STR, and the sequencer apparatus was a Procise sequencer both supplied by Applied Biosystems (Foster City, Calif., USA). Wheat *Triticum aestivum* cv. Soissons, Capitot, Élysée, Thésée, Neepawa and Prinqual were grown at INRA, in Clermont-Ferrand, France (except the latter in Thiverval-Grignon, France).

### Patients

Seven patients (No. 1–7) were adults suffering from characterized WDEIA triggered by ingestion of wheat products. One control patient (No. 8) was an adult suffering from c/f-IHHWP. They were identified at Hospital Tenon, AP-HP, Paris, France. All of these patients developed a positive response in SPT with the wheat flour extracts from Stallergene S.A. (Antony, France), the wheat flour 55-type from Les Grands Moulins de Paris (Paris, France) and/or the gluten from ALK (Varenes en Argonne, France) (table 1). In addition, patients No. 5–8 were evaluated by SPT with natural

**Table 1.** SPT results in patients with WDEIA (No. 1–7) and c/f-IHHWP (No. 8)

| Patient No. | Age years | SPT (wheal diameter in mm – erythema diameter in mm) |                          |                           |                      |                   |                                   |                                |
|-------------|-----------|------------------------------------------------------|--------------------------|---------------------------|----------------------|-------------------|-----------------------------------|--------------------------------|
|             |           | Histamine                                            | Gliadin fraction 4 mg/ml | LMW-GS fraction 2.5 mg/ml | LMW-GS P42 0.3 mg/ml | Gluten (Allerbio) | Wheat flour extract (Stallergène) | 55-type wheat flour, 100 mg/ml |
| 1           | 42        | 8–30                                                 | ND                       | ND                        | ND                   | ND                | 0–0                               | 6–30 (pure)                    |
| 2           | 63        | 5–30                                                 | ND                       | ND                        | ND                   | ND                | 5–25                              | 4–25 (pure)                    |
| 3           | 32        | 8–40                                                 | ND                       | ND                        | ND                   | 4–45              | 4–40                              | 10–40 (pure)                   |
| 4           | 58        | 7–25                                                 | ND                       | ND                        | ND                   | 10–30             | 9–45                              | 10–35 (pure)                   |
| 5           | 22        | 6–54                                                 | 5–36 ( $10^{-3}$ )       | 7–34 ( $10^{-1}$ )        | 5–19 (pure)          | ND                | 0–0                               | 28–84 ( $10^{-1}$ )            |
| 6           | 60        | 13–40                                                | 11–40 (pure)             | 5–35 (pure)               | ND                   | 14–50             | 5–20                              | 6–25 ( $10^{-1}$ )             |
| 7           | 41        | 6–45                                                 | 5–20 ( $10^{-2}$ )       | 3–25 (pure)               | 5–23 (pure)          | 11–41             | 0–0                               | 5–35 ( $10^{-2}$ )             |
| 8           | 26        | 8–30                                                 | 0–0                      | 0–0                       | 0–0                  | 20–45             | 0–0                               | ND                             |

The results show the wheal and the erythema diameters induced by the highest dilution of the extract that gave a positive response. Numbers in brackets indicate the dilution of the extract giving the response. ND = Not determined.

**Table 2.** Summary of PCR conditions for A12

|            | Template         | Primers                                                                                                                                            |
|------------|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| First PCR  | cDNA             | forward: 5'-CACC ATG AGT AGG CTG CTA AGC CCT AGA-3'<br>M S R L L S P R<br>reverse: oligodT-based primer (Invitrogen 3'RACE kit)                    |
| Nested PCR | First PCR medium | forward: 5'-ATACC ATG GGT AGG CTG CTA AGC CCT AGA-3'<br>M G R L L S P R<br>reverse: 3'-CAA TAT TCA TAG ACA CCG GAT GAG CTC GTG-5'<br>V I S I C G L |

wheat prolamin fractions. Among these patients, two were further evaluated by SPT with the purified natural LMW-GS P42. All proteins were solubilized in 50% (w/w) ethanol and diluted in glycerol-saline solution before testing. Increasing concentration, from dilution of  $10^{-3}$  to pure mother solution (4 mg gliadins/ml, 2.5 mg LMW-GS/ml and 0.3 mg LMW-GS P42/ml) were applied to the skin, depending on the patient's reactivity. Histamine and the glycerol-saline solution were used as positive and negative control, respectively. The absence of reactivity of 50% (w/w) ethanol was verified systematically. Two control sera, a normal human serum (No. 9) and the serum from an atopic patient allergic to peanuts (No. 10), were purchased from PlasmaLab Int. (Everett, WA, USA). All sera were used for immunoblot experiments with natural wheat prolamin fractions. Sera from patients 2, 3 and 4 were also used for immunoblot experiments with recombinant proteins.

This study was approved by the committee for protection of human subjects in biomedical research of Cochin Hospital (AP-HP, Paris, France), and each patient gave informed consent before giving blood.

#### PCR Cloning of Recombinant Proteins

In order to get recombinant  $\omega$ 5-gliadin, immature grains from wheat (*T. aestivum*, cv. Prinqual) were harvested 14 days after an-

thesis, and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted using a midi spin columns kit (Macherey-Nagel GmbH, Düren, Germany). Then, cDNA was synthesized using Superscript II<sup>TM</sup> reverse transcriptase (Invitrogen, Carlsbad, Calif., USA). The first PCR amplification was done using Accuprime PFX<sup>TM</sup> (Invitrogen) and primers (table 2). After sequencing, a second PCR was done using reverse primers designed from the obtained sequence, and the coding DNA was inserted into the pET28b plasmid (Novagen, Merck Biosciences, Darmstadt, Germany) and sequenced. One gene called A12 was obtained. The cloning of the two recombinant LMW-GS, called LMW-GS B16 and P73, has already been described [15].

#### Expression and Partial Purification of Recombinant Proteins

The Rosetta2<sup>TM</sup> *Escherichia coli* strain from Novagen, Merck Biosciences was transformed with recombinant plasmid. This strain contained the plasmid pRare2LysS providing genes for tRNA specific for rare *E. coli* codons.

Bacteria pelleted from a one-night preculture were resuspended in LB medium with kanamycin and chloramphenicol and grown for about 1 h before 1 mM IPTG induction. Bacteria were collected after 4 h, and the pellet was frozen until use.

Bacteria were resuspended in 50 mM Tris-HCl, pH 8.5 buffer and lysed by incubation with B-PER™ (Thermo Scientific, Pierce Biotechnology) containing 0.25 mg/ml lysozyme and 0.0125 µg/ml Dnase I for 30 min at 37°C. After an additional 1 h of incubation with 1 M NaCl, the cells were centrifuged, and the pellet was resuspended in 25 mM Tris-HCl, pH 8.5 containing 8 M urea. Ethanol extraction was done by incubating the suspension with 2 volumes of absolute ethanol. The LMW-GS B16 and P73 in the ethanolic extract were reduced by incubation for 30 min with 100 mM DTT and alkylated by incubation for 30 min with 0.5 M iodoacetamide. After centrifugation, the recombinant proteins were recovered from the supernatant by ethanol evaporation (for A12) or overnight precipitation with 7.5% (w/v) NaCl and centrifugation (for LMW-GS B16 and P73).

#### *Preparation of the Proteins Used for SPTs*

Purified natural LMW-GS (named LMW-GS P42) from *T. aestivum* cv. Neepawa, gliadin and LMW-GS fractions from a pool of 4 wheat flours (*T. aestivum* cv. Soissons, Capitot, Elysée, Thésée) were prepared.

To purify LMW-GS P42, proteins were extracted from 1 g of flour with 25 ml of 1 M Tris pH 7.1 buffer under gentle mixing for 2 h at 4°C. After centrifugation for 20 min at 20,000 g, 25 ml of 65% (w/w) n-propanol containing 1% (v/v) 2-mercaptoethanol were added to the pellet. After gentle mixing for 2 h at room temperature, tubes were centrifuged for 10 min at 12,000 g, and 100 ml of 7.5% (w/v) NaCl was added to the supernatant to precipitate LMW-GS overnight at 4°C. After centrifugation for 30 min at 15,000 g, 3 ml of Laemmli's sample buffer [17] containing 5% (v/v) 2-mercaptoethanol was added to solubilize the LMW-GS present in the pellet (extract of total natural LMW-GS). In a second step, sodium dodecyl sulfate-PAGE (SDS-PAGE) was carried out with 3 mg of total natural LMW-GS loaded onto a 12% preparative acrylamide gel. The gel was then transferred onto a PVDF membrane under semidry conditions using the protocol of Laurière [18]. The membrane was dried and then soaked with 20% (v/v) ethanol to visualize the bands corresponding to LMW-GS. The LMW-GS P42 band was excised, and the proteins were eluted from the membrane with 60% (v/v) acetonitrile containing 0.1% (v/v) TFA and lyophilized. The LMW-GS P42 was solubilized: either in Laemmli's sample buffer containing 5% (v/v) 2-mercaptoethanol and boiled for 5 min for SDS-PAGE analysis or in 50% (w/v) ethanol for SPTs.

To prepare gliadin and LMW-GS fractions, proteins were sequentially extracted with 1 M Tris pH 7.1 buffer (gliadins) and then 65% (w/w) n-propanol containing 1% (v/v) 2-mercaptoethanol (LMW-GS). All solubilized prolamins were precipitated with 3 volumes of 7.5% (w/v) NaCl overnight. The pellets were resolubilized as previously described for SDS-PAGE analysis or for SPTs.

#### *Preparation of Proteins for Immunoblot Experiments*

Highly purified natural ω5-gliadin, gliadin and glutenin fractions were prepared from *T. aestivum* cv. Soissons.

To purify the ω5-gliadin, total gliadins were extracted in a first step from 0.5 g of flour with 6.25 ml of 1 M pH 7.1 Tris buffer under gentle mixing for 30 min at 4°C. After centrifugation for 20 min at 20,000 g, 18.75 ml of 7.5% (w/v) NaCl was added to the supernatant to precipitate the gliadins overnight at 4°C. After centrifugation for 30 min at 15,000 g, 3 ml of Laemmli's sample

buffer containing 5% (v/v) 2-mercaptoethanol was added to solubilize the gliadins present in the pellet. The second step was the same as above for LMW-GS P42, except that 2 mg of total gliadins were loaded onto the gel.

To prepare the gliadin and glutenin fractions, the same protocol as previously described was used, except that proteins were extracted with, respectively, 1 M Tris pH 7.1 buffer (gliadins) and then 35% (w/w) n-propanol, 0.1 M acetic acid containing 5% (v/v) 2-mercaptoethanol (glutenins).

#### *SDS-PAGE and Western Blotting*

For the analysis of the sensitization pattern of patients, SDS-PAGE was carried out on large gels according to Singh et al. [19]. For the other analyses, entailing the characterization of the prolamins used for SPT, the analysis of the IgE reactivity of sera towards recombinant proteins, and all immunoblot inhibition experiments, SDS-PAGE was performed on precast 12% Bis-Tris polyacrylamide gels, using 3-(N-morpholino)propane sulfonic acid SDS running buffer with anti-oxidant according to the manufacturer's instructions. Gels were blotted onto PVDF membranes under semidry conditions using the protocol of Laurière [18]. Natural wheat prolamins (20 µg) and purified and recombinant proteins (5 µg) were loaded onto gels.

#### *Immunoblot Experiments*

According to Snegaroff et al. [20], PVDF membranes were first saturated for 2 h in 50 mM phosphate pH 7.4 buffer, 150 mM NaCl, 0.1% (w/v) Tween 20 (PBST) and 2% (w/v) polyvinylpyrrolidone-40 (PVP-40). They were then incubated overnight at 4°C with patient serum diluted 1:10 in PBST containing 2% (w/v) PVP-40. After 3 washing steps, the rabbit anti-human IgE-horseradish peroxidase conjugate was added, at 1:25,000 in PBST containing 3% (w/v) dried cow milk, for 2 h. After 7 washing steps, the membranes were incubated for 5 min with the SuperSignal West Dura Extended duration substrate. All washing steps lasted 10 min each in PBST containing 2% (w/v) PVP-40, except the two latter which were in PBS. All steps were performed under gentle rocking at room temperature, except for the incubation with the patient serum. For all experiments, a control membrane without patient serum was processed in the same way as the assays.

#### *Immunoblot Inhibition Experiments*

According to Snegaroff et al. [20], immunoblots were performed as described above, except that highly purified natural ω5-gliadin or bovine serum albumin [solubilized in 0.02% (w/v) SDS and 0.1% (w/v) Tween 20] was added at 25 µg/ml in the solution containing the patient serum diluted 1:10, gently mixed for 2 h and then incubated with the membrane overnight. The assay without inhibition was performed in the same way but without inhibitor.

For all experiments, a control membrane without patient serum was processed in the same way as the assays.

#### *In-Gel Protein Staining*

Proteins were stained by incubating the gel overnight with 2 volumes of 0.4% (w/v) Coomassie Brilliant Blue G-250 in ethanol and 8 volumes of 2% (w/v) orthophosphoric acid, 10% (w/v) ammonium sulfate according to Neuhoof et al. [21].

### Chemiluminescence Detection and Quantification

Chemiluminescence was recorded using the luminescent image analyzer LAS-3000 supplied by Fujifilm (Valhalla, N.Y., USA). Quantification was done using Bio 1D software (Vilbert-Lourmat, Torcy, France) by calculating the volume of the chemiluminescent responses, in arbitrary units. The volume was the sum of the intensity of each pixel contained in the delimited area. For immunoblot inhibition experiments, calculation of the inhibition was:  $100 - (\text{volume assay with inhibition} \times 100 / \text{volume assay without inhibition})$ .

## Results

### Characterization of the Prolamins Used for SPTs

Figure 1 shows the SDS-PAGE analysis of the prolamins used for SPTs. LMW-GS P42 (lane 1) gave only one major band (indicated by  $\Delta$ ). The weakly observable additional upper bands were identified using specific rabbit anti-LMW-GS antibodies. They corresponded to aggregates of LMW-GS P42 (results not shown). The determination of the N-terminal sequence of the LMW-GS P42 over 13 cycles gave the single sequence SHIPGLEEPSQQQ, which showed that the protein was very pure and belonged to the s-LMW-GS. Its average molecular weight, evaluated by MALDI-TOF, was 42,649.7 Da.

Gliadins and LMW-GS fractions were obtained from sequential extractions. The protocol used was designed for its ability to produce typical fractions with very limited cross-contamination. Prolamins displayed a characteristic pattern. The gliadin fraction (fig. 1, lane 2) contained all types of gliadins and especially  $\omega$ 5-gliadins (indicated by an asterisk). The LMW-GS fraction (fig. 1, lane 3) was clearly devoid of HMW-GS and  $\omega$ 5-gliadins contaminants.

### Patient Testing Using SPTs

Table 1 shows the SPT results for the 8 patients. In order to identify the immunoreacting proteins, we performed SPTs with natural wheat prolamins (gliadins and LMW-GS) and with the purified natural LMW-GS P42. These additional tests were performed with 4 patients [3 suffering from WDEIA (No. 5–7), and 1 from c/f-IHHWP (No. 8) as a control]. All WDEIA patients developed positive SPT with the gliadin fraction, the LMW-GS fraction and the LMW-GS P42. Two of three (No. 5 and 7) developed a higher inflammatory response towards gliadins than towards LMW-GS. Patient No. 8, used as a control, did not develop positive SPT to prolamins. This is in agreement with previous results [6, 22] and showed that positive SPTs were not due to extra components present in the extract.

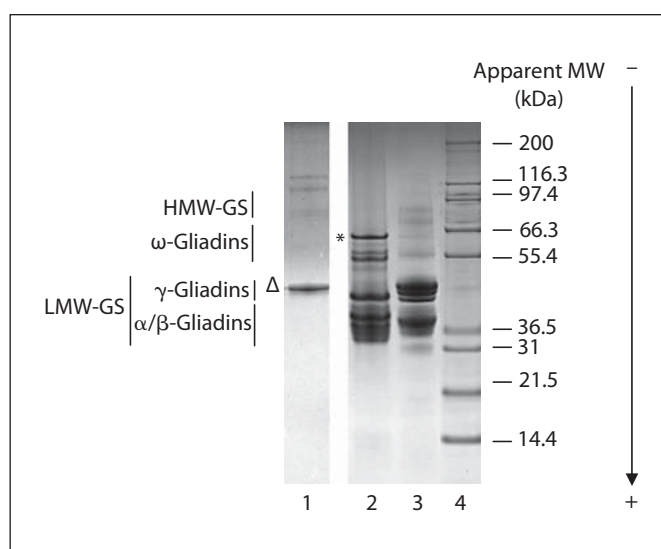


**Fig. 1.** SDS-PAGE analysis of natural prolamins used in SPT. Lane 1: purified natural LMW-GS P42 (5  $\mu$ g); lane 2: gliadin fraction (20  $\mu$ g); lane 3: LMW-GS fraction (20  $\mu$ g); lane 4: molecular weight standard Mark 12 from Novex. The asterisk denotes  $\omega$ 5-gliadin. The triangle denotes LMW-GS P42. Proteins were stained with Coomassie Brilliant Blue.

### Recombinant Wheat Proteins

LMW-GS B16 and P73 have been previously described [15]. They can be considered as models for LMW-GS coded by wheat genomes B and D, respectively. According to their N-terminal amino acid, methionine or serine, they belong to two different groups of LMW-GS. LMW-GS B16 belongs to the m-type, whereas LMW-GS P73 belongs to the s-type. The two proteins displayed 70% identity in calculations based on their full-length amino acid sequences. When the calculation was restricted to the repetitive domains or to their C-termini, the identities were 57 and 81%, respectively. The LMW-GS B16 sequence carries 3 copies of the peptide motif QQQPP, which has been described to be an IgE-binding epitope for wheat-associated allergies [23]. Conversely, LMW-GS P73 carries only 2 copies of this motif. The LMW-GS B16 sequence has 1 copy of the QQFPQQQ motif, which was identified as an IgE-dominant epitope for WDEIA patients [5, 24], but LMW-GS P73 has none.

The cloning of  $\omega$ 5-gliadin resulted in a gene named A12, which was recorded under the following accession number in the EMBL sequence databank: FM212917. Its sequence was very homologous to the  $\omega$ 5-gliadin already sequenced and recorded under number AB181300 in

**Table 3.** Comparison between the amino acid sequences of recombinant  $\omega$ 5-gliadin and A12

|          |                                                                                            |
|----------|--------------------------------------------------------------------------------------------|
| A12      | -----gRLLSPRGKELHTPQEQQFPQQQFPQPQQFPQQ                                                     |
| AB181300 | SNSKHKS NM KTFIIFVLLAMAMNIASASRLLSPRGKELHTPQEQQFPQQQFPQPQQFPQQ<br><----signal peptide----> |
| A12      | QILQQHQIP QQPQQFPQQQQLPQQEF SQQQISQQPQQLPQQQIPQQPQQFPQQQFPQQQFPQQH                         |
| AB181300 | QIPQQHQIP QQPQQFPQQQQLPQQEF PQQQISQQPQQLPQQQIPQQPQQFPQQQFPQQH                              |
| A12      | QSPQQQFPQ QQFPQQQLPQQEF SQQQISQQPQQLPQQQIPQQPQQFLQQQQFPQQQPPQ                              |
| AB181300 | QSPQQQFPQ QQFPQQQLPQQEF PQQQISQQPQQLPQQQIPQQPQQFLQQQQFPQQQPPQ                              |
| A12      | QHQPQQQLP PQQQIPQQQIPQQPQQIPQQQIPQQPQQKQFPQQQFPQQQFPQQQFPQQE                               |
| AB181300 | QHQPQQQLP PQQQIPQQQIPQQPQQIPQQQIPQQPQQKQFPQQQFPQQQFPQQQFPQQE                               |
| A12      | FPQQQFPQ QQIAQQPQQLPQQQ-----                                                               |
| AB181300 | FPQQQFPQ QQIARQPQQLPQQQQIPQQPQQFPQQQFPQQQSPQQQFPQQQFPQQQL                                  |
| A12      | -----                                                                                      |
| AB181300 | PQKQFPQPQ QIPQQQIPQQPQ QFPQQQFPQQQFPQQQEFPPQQQFPQQQFHQQQLPQQQ                              |
| A12      | -----FPI                                                                                   |
| AB181300 | FPQQQFPQ QFPQQQFPQQQLTQQQFPRPQQSPEQQQFPQQQFPQQPPQQFPQQQFPI                                 |
| A12      | PYPPQSQE PSPYQQYPQQQPSGSDVISICGLlehhhhh                                                    |
| AB181300 | PYPPQSQE PSPYQQYPQQQPSGSDVISISGL-----<br>His-tag                                           |

A12: recombinant protein described in this paper (FM212917). AB181300:  $\omega$ 5-gliadin [25]. Identical amino acids are in gray; amino acids modified by cloning are in lower case.

Genebank by Matsuo et al. [25] (table 3). The two proteins share a common part showing 95% identity. They differed mainly by a lower number of repetitive stretches resulting in a shorter length for A12. Just a few peptides of  $\omega$ 5-gliadin from Matsuo et al. [25] were missing in our A12. Another difference was the presence of a cysteine residue at the C-terminus of A12. However, all known types of  $\omega$ 5-gliadin epitopes described as IgE-reactive in WDEIA [5, 24] were present in A12.

The amino acid sequences of  $\omega$ 5-gliadin and LMW-GS B16 were compared: LMW-GS B16 shares only two peptides with  $\omega$ 5-gliadin. The first peptide corresponds to the sequence QQFPQQQ (LMW-GS B16, amino acids 36–42), which was identified as an IgE-dominant epitope for WDEIA patients [5, 24]; it appears 8 times in the  $\omega$ 5-gliadin sequence. The second peptide corresponded to the sequence QQPQQQIPQ (LMW-GS B16, amino acids 266–272); it appears 6 times in the  $\omega$ 5-gliadin sequence. The comparison between the amino acid sequences of  $\omega$ 5-gliadin and LMW-GS P73 showed no homology.

#### *Sensitization Pattern of Patients towards Natural Wheat Prolamins*

The sensitization pattern of all patients included in this study was analyzed in vitro using natural wheat prolamins.

Gliadin and glutenin fractions were analyzed using SDS-PAGE (fig. 2) and stained with Coomassie Brilliant Blue. Proteins analyzed on the gels displayed a characteristic pattern. In the glutenin fraction, HMW-GS gave several bands (from 66 to 97 kDa) and LMW-GS displayed bands from 25 to 50 kDa. In the gliadin fraction,  $\omega$ 5-gliadins (indicated by an asterisk) gave one band around 60 kDa, and  $\alpha/\beta$  and  $\gamma$ -gliadins displayed several bands from 25 to 45 kDa. Immunoblottings were performed using patient sera. The pattern of IgE-binding to gliadins and to glutenins showed that IgE from all WDEIA patient sera (lanes 1–7) strongly reacted with  $\omega$ 5-gliadins and with a cluster of LMW-GS (around 40 kDa). In some cases (patients 1 and 4),  $\alpha/\beta$ - and  $\gamma$ -gliadins were also IgE reactive. These patterns of IgE reactivity were characteristic of WDEIA and different from those obtained with Japanese patients [10]. The specificity of the reaction was assayed, using several controls. One serum from a patient allergic to wheat and suffering from c/f-IHHWP was

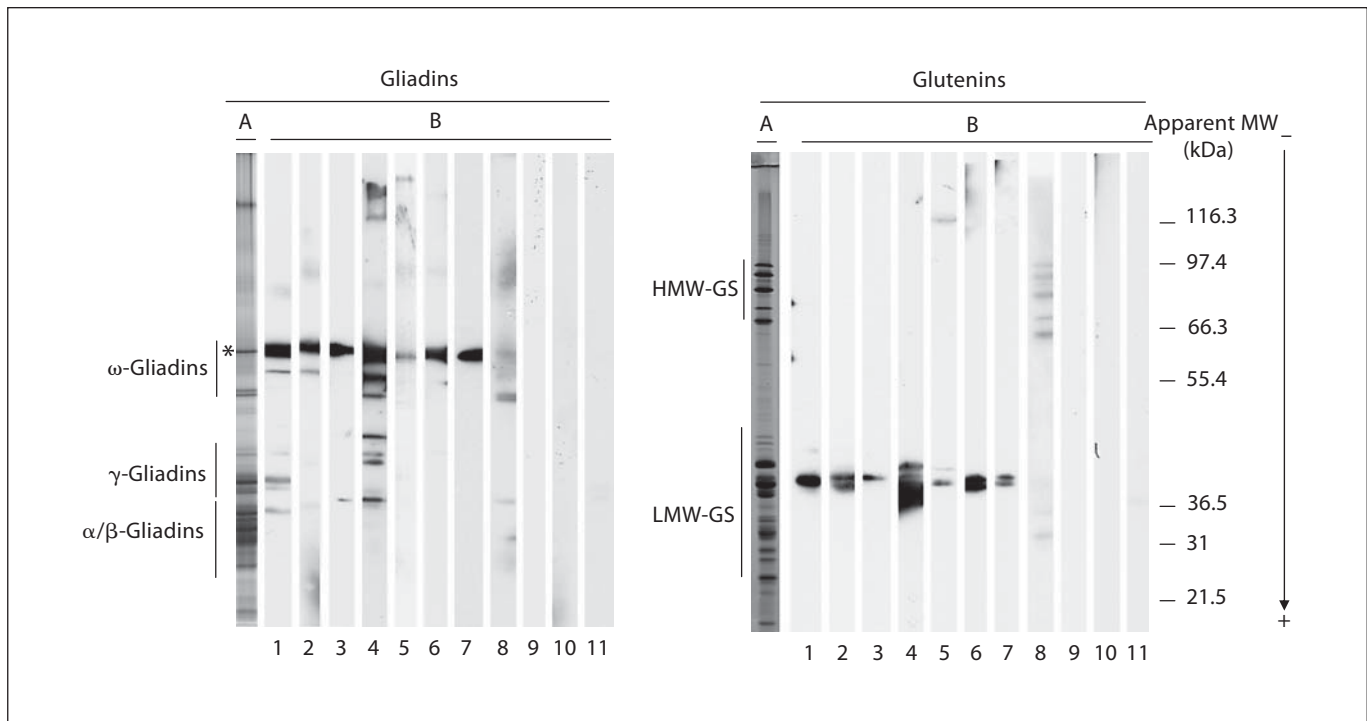


**Fig. 2.** Sensitization pattern of patients. SDS-PAGE analysis of gliadins and glutenins, either stained with Coomassie Brilliant Blue (panels A), or immunoblotted on PVDF membrane (panels B). Lanes 1–7: immunoprobings with serum from WDEIA patients 1–7, respectively. Lane 8: immunoprobings with the serum from a patient suffering from c/f-IHHWP. Lane 9: immunoprobings with

the normal human serum. Lane 10: Immunoprobings with the serum from an atopic patient allergic to peanuts. Lane 11: control membrane immunoprobed without serum. Twenty micrograms of protein were loaded on gels. The detection of binding was done using chemiluminescence.

used for comparison with WDEIA sera (lane 8). It displayed a pattern of IgE reactivity different from that observed with WDEIA and specific of c/f-IHHWP patients.  $\omega$ 1,2-Gliadins [6] were especially recognized. In addition, 3 other controls were carried out (lanes 9–11). Under these conditions, no reaction was observed with gliadins and glutenins. These controls showed the specificity of the IgE response.

#### *IgE Reactivity of Sera towards Recombinant Wheat Proteins*

The identity of IgE-reacting proteins described above was confirmed using recombinant wheat proteins. Recombinant LMW-GS B16, LMW-GS P73 and A12 were analyzed using SDS-PAGE (fig. 3), and stained with Coomassie Brilliant Blue. Each protein analyzed on the gel displayed one major band, with additional bands of lower size. The molecular weights deduced from the sequences were 37 kDa for LMW-GS B16, 32 kDa for LMW-GS P73 and 34 kDa for A12. The apparent molecular weights

visualized by SDS-PAGE were slightly larger than their actual molecular weights, but such an observation is not unusual with wheat prolamins. Immunoblottings were performed using patient sera. They showed that only the main band corresponding to the whole recombinant protein bound IgE. The additional bands did not, showing that extensive purification was not necessary. The IgE-binding ability of each recombinant protein showed that antigenicity was conserved throughout the expression and purification process. A12 was highly IgE reactive with each WDEIA patient serum, while LMW-GS P73 was weakly IgE reactive. Interestingly, the LMW-GS B16 was strongly IgE reactive for 2 of the 3 WDEIA patient sera. The identity of LMW-GS as IgE-reactive proteins in WDEIA was thus confirmed. It should also be noted that the same controls as above were performed. They showed no nonspecific reactivity and no IgE reactivity with the recombinant proteins.

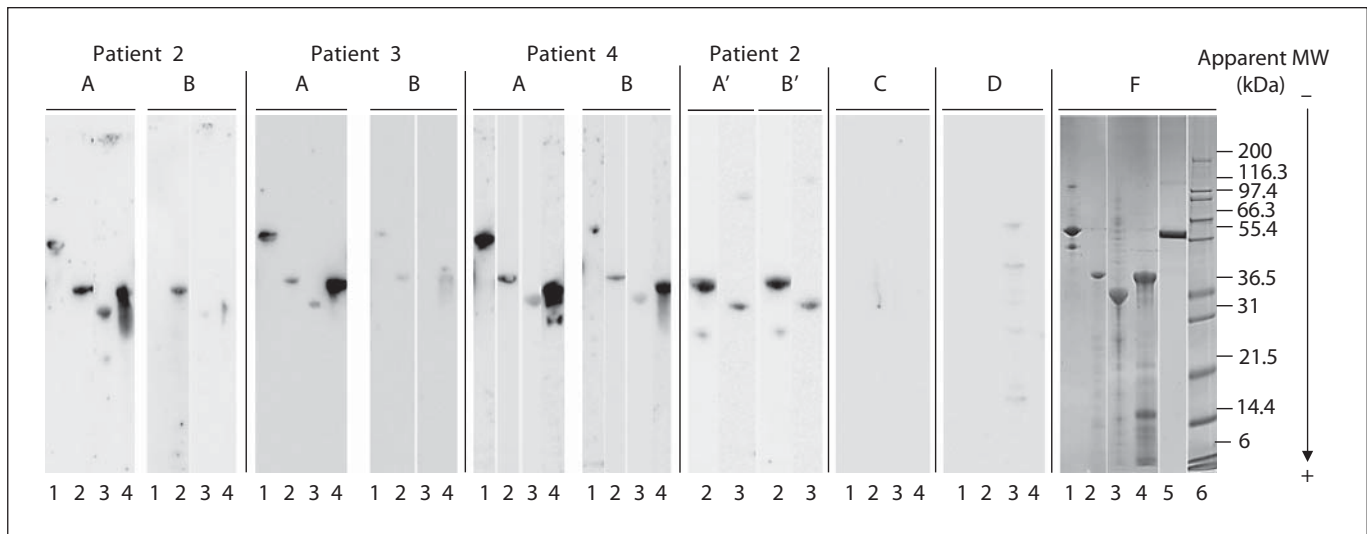


**Fig. 3.** IgE specificity towards purified natural  $\omega$ 5-gliadin and recombinant wheat proteins revealed using immunoblot and immunoblot inhibition experiments. SDS-PAGE analysis of proteins, either immunoprobed on PVDF membranes with patient sera (panels A–D) or stained with Coomassie Brilliant Blue (panel F). Lane 1: purified natural  $\omega$ 5-gliadin (5  $\mu$ g). Lane 2: recombinant LMW-GS B16 (5  $\mu$ g). Lane 3: recombinant LMW-GS P73 (5  $\mu$ g). Lane 4: recombinant A12 (5  $\mu$ g). Lane 5: highly purified

natural  $\omega$ 5-gliadin used as an inhibitor (5  $\mu$ g). Lane 6: molecular weight standard Mark 12 from Novex. Panels A and B: inhibition (B) or not (A) of the binding of IgE from WDEIA patients by the highly purified natural  $\omega$ 5-gliadin. Panels A' and B': inhibition (B') or not (A') of the binding of IgE from patient 2 by bovine serum albumin. Panel C: normal human serum. Panel D: atopic patient allergic to peanuts. The detection of binding was done using chemiluminescence.

**Table 4.** Study of the antigenic relationships between  $\omega$ 5-gliadins and LMW-GS using immunoblot inhibition experiments with highly purified natural  $\omega$ 5-gliadin as an inhibitor

| Type of proteins blotted on membrane | % of inhibition                     |                                               |                |            |            |     |
|--------------------------------------|-------------------------------------|-----------------------------------------------|----------------|------------|------------|-----|
|                                      | purified natural $\omega$ 5-gliadin | Natural gliadins (except $\omega$ 5-gliadins) | Natural LMW-GS | LMW-GS B16 | LMW-GS P73 | A12 |
| Patient 1                            | 84                                  | 70                                            | 30             | ND         | ND         | ND  |
| Patient 2                            | 100                                 | 18                                            | 52             | 60         | 98         | 95  |
| Patient 3                            | 82                                  | 32                                            | 47             | 58         | 92         | 100 |
| Patient 4                            | 86                                  | 26                                            | 21             | 42         | 72         | 62  |

Reactivity of wheat proteins blotted on membranes with IgE from WDEIA patient sera was inhibited (assay with inhibition) or not (assay without inhibition) by the highly purified natural  $\omega$ 5-gliadin. Calculation of the inhibition is:  $100 - (\text{volume assay with inhibition} \times 100 / \text{volume assay without inhibition})$ . ND = Not determined. Volumes were calculated using Bio 1D software from the intensity and the area of the chemiluminescent responses, in arbitrary units. The amount of natural gliadin and LMW-GS fractions loaded on the gel was 20  $\mu$ g protein/well, and the amount of purified and recombinant protein was 5  $\mu$ g/well.

#### Cross-Reactivity between LMW-GS and $\omega$ 5-Gliadins

Cross-reactivity between LMW-GS and  $\omega$ 5-gliadins in WDEIA was studied through immunoblot inhibition experiments.

We used a highly purified preparation of  $\omega$ 5-gliadin as an inhibitor to ensure that inhibition was not due to contaminants. This preparation showed only one band on a gel (fig. 3, panel F, lane 5). Another preparation of purified  $\omega$ 5-gliadins, slightly contaminated by an additional protein, was used for the control of inhibition on the blots. This  $\omega$ 5-gliadin preparation (fig. 3, panel F, lane 1) gave one major band around 60 kDa with a minor additional lower molecular weight band. The major band was IgE reactive for all WDEIA patient sera (fig. 3, panel A, lane 1).

We performed immunoblot inhibition experiments on recombinant and natural wheat proteins blotted onto PVDF after SDS-PAGE (fig. 3 and 4, respectively). For all, different levels of inhibition were observed according to the protein or group of proteins and also according to the patient. The IgE-reactive bands detected in figure 4 appeared different from those detected in figure 2 for the same patients, in the  $\gamma$ -gliadin zone. This was due to the types of gel, different in size and resolution. Tests of in-

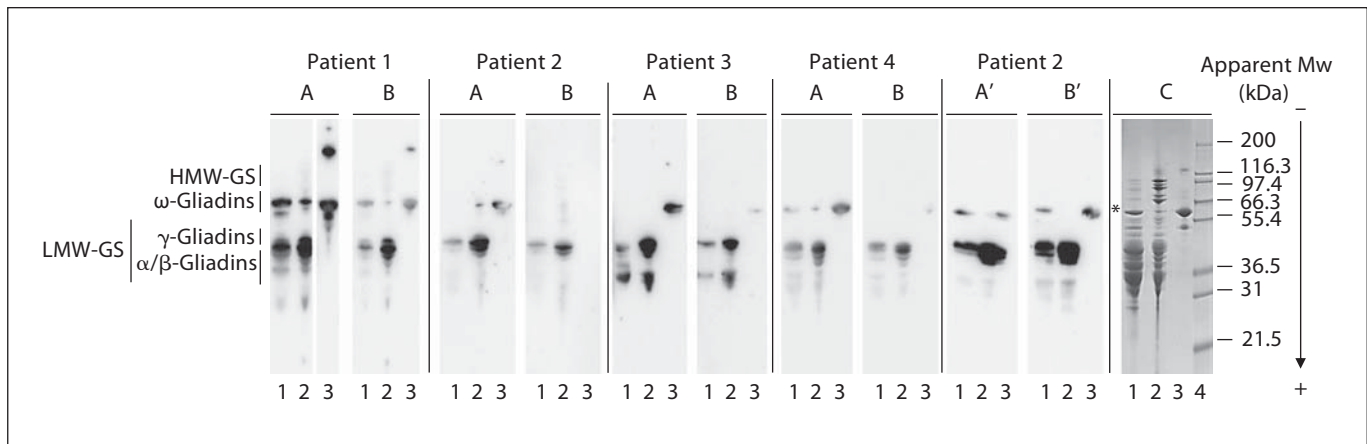


**Fig. 4.** IgE specificity towards natural wheat prolamins, revealed using immunoblot and immunoblot inhibition experiments. SDS-PAGE analysis of proteins, either immunoprobed on PVDF membranes with WDEIA patient sera (panels A and B) or stained with Coomassie Brilliant Blue (panel C). Lane 1: gliadin fraction (20  $\mu$ g). Lane 2: glutenin fraction (20  $\mu$ g). Lane 3: purified natural

$\omega$ 5-gliadin (5  $\mu$ g). Lane 4: molecular weight standard Mark 12 from Novex. Panels A and B: inhibition (B) or not (A) of the IgE binding by the highly purified natural  $\omega$ 5-gliadin. Panels A' and B': inhibition (B') or not (A') of the IgE binding by bovine serum albumin. The detection of binding was done using chemiluminescence. The asterisk denotes  $\omega$ 5-gliadin.

inhibition by an unrelated protein, bovine serum albumin, were performed as negative controls. This protein, which displayed no reactivity with IgE from the patients (results not shown), did not inhibit IgE binding on blotted wheat prolamins. This proved the specificity of the inhibition observed when using  $\omega$ 5-gliadin as the inhibitor. The chemiluminescent signals were recorded: light emission is directly proportional to the amount of peroxidase conjugates fixed on the blot. The use of a specific luminescence recorder with a high dynamic linear range detection signal, specially designed for Western blot analysis, allowed quantitative evaluation of the IgE fixed on their antigens. The percentage of inhibition was calculated as explained in Materials and Methods, and table 4 summarizes the results obtained. Despite the interpatient variations, distinct features were observed with each studied fraction. The highly purified natural  $\omega$ 5-gliadin, used as inhibitor in the reaction medium, strongly or fully inhibited (82–100%) the binding of IgE to its natural counterpart fixed on the membrane. This showed that the protein had enough conserved antigenic structure after the purification process to retain its ability to bind IgE, allowing correct inhibition measurements. IgE binding to recombinant A12 was strongly inhibited (95 and 100% for sera No. 2 and 3, respectively). This showed that all IgE-reacting epitopes carried by A12 were also present on the highly purified natural  $\omega$ 5-gliadin. This validated the use of the latter as an inhibitor to study relationships between

$\omega$ 5-gliadins and LMW-GS. IgE binding to natural gliadins other than  $\omega$ 5-gliadin ( $\alpha/\beta$ - and  $\gamma$ -gliadins) were slightly inhibited (from 18 to 32%), except for serum No. 1. With all sera, IgE binding to natural LMW-GS was only partly inhibited (from 21 to 52%). The level of inhibition of IgE binding to LMW-GS B16 was roughly the same as natural LMW-GS. On the contrary, IgE binding to LMW-GS P73 was very highly inhibited (from 72 to 98%).

## Discussion

SPTs were carried out with well-characterized wheat prolamins fractions and with the purified natural LMW-GS P42 for 2 of 3 patients. These proteins elicited positive response in selected patients. The high reactivity of gliadin fraction was mainly due to the presence of  $\omega$ 5-gliadins in the fraction (fig. 1, lane 2). However, involvement of other gliadins, and especially  $\gamma$ -gliadins, cannot be fully excluded, as they were present in the gliadin extract and were IgE reactive on blot for some patients. Clearly, the LMW-GS fraction (fig. 1, lane 3) and the purified LMW-GS P42 (fig. 1, lane 1) did not contain  $\omega$ 5-gliadins. This demonstrates that LMW-GS intrinsically elicited positive SPTs in these WDEIA patients. To our knowledge, this is the first report of such results. The fact that 2 of 3 patients displayed a higher response to gliadins

than to LMW-GS could mean that gliadins, and especially  $\omega$ 5-gliadins, are more immunoreactive than LMW-GS. However, only further investigations with a higher number of patients will confirm these data. Regardless, the reactivity in SPT means that undigested LMW-GS has the intrinsic ability to promote the degranulation process of mastocytes and basophiles, thus triggering a local inflammation. However, one has to remember that WDEIA is a food allergy, wheat is eaten cooked and proteins are processed by the digestion tract. This may change the role of LMW-GS in the anaphylactic reaction. Either a decrease or an increase in their allergenic properties is possible. Additional experiments, namely a double blind food challenge, will be necessary to answer this question.

The IgE-reactivity of WDEIA patient sera towards natural and recombinant wheat prolamins was studied.  $\gamma$ -Gliadins were IgE reactive for some patient sera, as already described [9]. The main reactivity of IgE with  $\omega$ 5-gliadins and a cluster of LMW-GS around 40 kDa were characteristic of WDEIA. The ability of the recombinant LMW-GS B16 to bind IgE confirmed that LMW-GS were effective IgE-reacting proteins. The results described here are in agreement with other studies which showed the strong reactivity of IgE from WDEIA patients towards LMW-GS [13, 14]. Some studies have compared the allergenic status of LMW-GS and  $\omega$ 5-gliadins by using in vitro tests. For instance, Pastorello et al. [13] studied a European population (patients were from Italy, Denmark and Switzerland) and found that LMW-GS were more IgE reactive than  $\omega$ 5-gliadins for WDEIA patients. However, the clinical relevance of this reactivity was not clear because no wheat-allergic patients were monosensitized to these proteins. In the same way, mast cell degranulation assays [16] have detected LMW-GS as major reacting proteins for a group of patients suffering from WDEIA, urticaria and anaphylaxis, whereas  $\omega$ 5-gliadins were not. The authors hypothesized that  $\omega$ 5-gliadins would have to be modified by transglutaminase to develop their immunogenicity. Here, the reactivity of two recombinant LMW-GS with IgE from WDEIA patients was compared. Unlike LMW-GS B16, IgE-binding to LMW-GS P73 was weak and almost completely inhibited by  $\omega$ 5-gliadin. This demonstrates that the IgE-reacting epitopes carried by LMW-GS B16 and P73 are different. Thus, all LMW-GS do not have the same allergenic status in WDEIA.

Antigenic relationships between LMW-GS and  $\omega$ 5-gliadins were also studied. IgE binding to natural LMW-GS and recombinant LMW-GS B16 was inhibited by  $\omega$ 5-gliadin, but it is clear that this inhibition was only partial.

These results demonstrate that LMW-GS carry both epitopes common with  $\omega$ 5-gliadin, as well as specific epitopes not shared with  $\omega$ 5-gliadin. An investigation of the similarities between LMW-GS B16 and  $\omega$ 5-gliadin showed two short, common amino acid sequences, including one linear IgE-reacting epitope already described [5, 24, 25]. These sequences may be involved in IgE binding and in the observed inhibition. However, a similar analysis with LMW-GS P73, which was fully inhibited, showed no common amino-acid sequence. This absence of similarity at the primary structural level might suggest the participation of unknown epitopes, potentially conformational, common between  $\omega$ 5-gliadin and LMW-GS P73 and responsible for the observed inhibition. These results are in accordance with previous studies [15] suggesting the involvement of the three-dimensional structure in the reactivity of LMW-GS with IgE from WDEIA patients.

## Conclusion

Despite the limited number of sera used, the present study highlights the importance of LMW-GS in WDEIA and gives new data about relationships between LMW-GS and  $\omega$ 5-gliadins. We have shown that at least some undigested LMW-GS gave positive SPTs in WDEIA patients and thus triggered a local inflammation. We have also shown that all LMW-GS did not have the same allergenic status in WDEIA. Only some of them reacted with the IgE from patients. Furthermore, reacting LMW-GS were shown to carry independent and common epitopes with  $\omega$ 5-gliadins. These common epitopes could be supported by primary structure but also, perhaps, by secondary or tertiary structures. Thus, our study has contributed to the identification of LMW-GS as potential allergens partly independent from  $\omega$ 5-gliadins. Their involvement in WDEIA should be evaluated by further clinical tests with a higher number of patients and, if confirmed, taken in consideration for the diagnosis of this allergy.

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## References

- Palosuo K, Alenius H, Varjonen E, Koivuluhta M, Mikkola J, Keskinen H, Kalkkinen N, Reunala T: A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol* 1999;103:912–917.
- Kasarda DD, Autran JC, Lew EJL, Nimmo C, Shewry PR: N-terminal amino acid sequences of omega-gliadins and omega-secalins. Implications for the evolution of prolamin genes. *Biochim Biophys Acta* 1983;747:138–150.
- Juhász A, Gianibelli M: Low molecular weight glutenin subunits: Insights into this abundant subunit group present in glutenin polymers; in Wrigley CW, Bekes F, Bushuk W (eds): *Gliadin and Glutenin: The Unique Balance of Wheat Quality*. St. Paul, AACC International, 2006, pp 171–212.
- Shewry PR, Tatham AS: The prolamin storage proteins of cereal seeds – structure and evolution. *Biochem J* 1990;267:1–12.
- Battais F, Mothes T, Moneret-Vautrin DA, Pineau F, Kanny G, Popineau Y, Bodinier M, Denery-Papini S: Identification of IgE-binding epitopes on gliadins for patients with food allergy to wheat. *Allergy* 2005;60:815–821.
- Lauriere M, Pecquet C, Boulenc E, Bouchez-Mahiout I, Snegaroff J, Choudat D, Raison-Peyron N, Vigan M, Branlard G: Genetic differences in omega-gliadins involved in two different immediate food hypersensitivities to wheat. *Allergy* 2007;62:890–896.
- Palosuo K, Varjonen E, Kekki OM, Klemola T, Kalkkinen N, Alenius H, Reunala T: Wheat  $\omega$ -5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *J Allergy Clin Immunol* 2001;108:634–638.
- Morita E, Matsuo H, Mihara S, Morimoto K, Savage AW, Tatham AS: Fast  $\omega$ -gliadin is a major allergen in wheat-dependent exercise-induced anaphylaxis. *J Dermatol Sci* 2003;33:99–104.
- Denery-Papini S, Lauriere M, Branlard G, Morisset M, Pecquet C, Choudat D, Merlino M, Pineau F, Popineau Y, Boulenc E, Bouchez-Mahiout I, Bodinier M, Moneret-Vautrin DA: Influence of the allelic variants encoded at the gli-B1 locus, responsible for a major allergen of wheat, on IgE reactivity for patients suffering from food allergy to wheat. *J Agric Food Chem* 2007;55:799–805.
- Matsuo H, Kohno K, Niihara H, Morita E: Specific IgE determination to epitope peptides of  $\omega$ -5 gliadin and high molecular weight glutenin subunit is a useful tool for diagnosis of wheat-dependent exercise-induced anaphylaxis. *J Immunol* 2005;175:8116–8122.
- Akagawa M, Handoyo T, Ishii T, Kumazawa S, Morita N, Suyama K: Proteomic analysis of wheat flour allergens. *J Agric Food Chem* 2007;55:6863–6870.
- Battais F, Pineau F, Popineau Y, Aparicio C, Kanny G, Guerin L, Moneret-Vautrin DA, Denery-Papini S: Food allergy to wheat: Identification of immunoglobulin E and immunoglobulin G-binding proteins with sequential extracts and purified proteins from wheat flour. *Clin Exp Allergy* 2003;33:962–970.
- Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi S, Iametti S, Fortunato D, Scibilia J, Bindslev-Jensen C, Ballmer-Weber B, Robino AM, Ortolani C: Wheat IgE-mediated food allergy in European patients:  $\alpha$ -amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int Arch Allergy Immunol* 2007;144:10–22.
- Mittag D, Niggemann B, Sander I, Reese I, Fiedler EM, Worm M, Vieths S, Reese G: Immunoglobulin E-reactivity of wheat-allergic subjects (baker's asthma, food allergy, wheat-dependent, exercise-induced anaphylaxis) to wheat protein fractions with different solubility and digestibility. *Mol Nutr Food Res* 2004;48:380–389.
- Snegaroff J, Branlard G, Bouchez-Mahiout I, Laudet B, Tylichova M, Chardot T, Pecquet C, Choudat D, Raison-Peyron N, Vigan M, Kerre S, Lauriere M: Recombinant proteins and peptides as tools for studying IgE reactivity with low-molecular-weight glutenin subunits in some wheat allergies. *J Agric Food Chem* 2007;55:9837–9845.
- Bodinier M, Brossard C, Triballeau S, Morisset M, Guerin-Marchand C, Pineau F, de Coppet P, Moneret-Vautrin DA, Blank U, Denery-Papini S: Evaluation of an in vitro mast cell degranulation test in the context of food allergy to wheat. *Int Arch Allergy Immunol* 2008;146:307–320.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature* 1970;227:680–685.
- Lauriere M: A semidry electroblotting system efficiently transfers both high- and low-molecular-weight proteins separated by SDS-PAGE. *Anal Biochem* 1993;212:206–211.
- Singh NK, Shepherd KW, Cornish GB: A simplified SDS-PAGE procedure for separating LMW subunits of glutenin. *J Cereal Sci* 1991;14:203–208.
- Snegaroff J, Bouchez-Mahiout I, Pecquet C, Branlard G, Lauriere M: Study of IgE antigenic relationships in hypersensitivity to hydrolyzed wheat proteins and wheat-dependent exercise-induced anaphylaxis. *Int Arch Allergy Immunol* 2006;139:201–208.
- Neuhoff V, Arold N, Taube D, Ehrhardt W: Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using coomassie brilliant blue g-250 and r-250. *Electrophoresis* 1988;9:255–262.
- Lauriere M, Pecquet C, Bouchez-Mahiout I, Snegaroff J, Bayrou O, Raison-Peyron N, Vigan M: Hydrolysed wheat proteins present in cosmetics can induce immediate hypersensitivities. *Contact Dermatitis* 2006;54:283–289.
- Tanabe S, Arai S, Yanagihara Y, Mita H, Takahashi K, Watanabe M: A major wheat allergen has a gln-gln-gln-pro-pro motif identified as an IgE-binding epitope. *Biochem Biophys Res Commun* 1996;219:290–293.
- Matsuo H, Morita E, Tatham AS, Morimoto K, Horikawa T, Osuna H, Ikezawa Z, Kaneko S, Kohno K, Dekio S: Identification of the IgE-binding epitope in omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. *J Biol Chem* 2004;279:12135–12140.
- Matsuo H, Kohno K, Morita E: Molecular cloning, recombinant expression and IgE-binding epitope of omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. *FEBS J* 2005;272:4431–4438.