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Immunoglobulin-E Reactivity and Structural Analysis of Wheat Low-Molecular-Weight Glutenin Subunits and Their Repetitive and Nonrepetitive Halves

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ABSTRACT: The IgE reactivity of the recombinant glutenin subunits P73 and B16, and of their repetitive N-terminal and nonrepetitive C-terminal halves, was analyzed using dot-blot with sera from patients diagnosed with baker's asthma, wheat-dependent exercise-induced anaphylaxis, or allergy to hydrolyzed wheat proteins. The linear epitopes of B16 were identified using the Pepscan method. Except for one common epitope, the IgE binding domains of glutenins differ from those of ω5-gliadins. Secondary structure content of the proteins was determined using synchrotron radiation circular dichroism (SRCD): while α structures were predominant in all glutenin subunits, fragments, or chimeras, a high IgE reactivity was associated with proteins rich in β structures. Mixing B16 halves induced conformational interaction, as evidenced by dynamic light scattering and SRCD. IgE reactivity was correlatively increased, as when the halves were associated in the B16−P73 chimera. These results suggest that structural interaction between N- and C-terminal halves may promote epitope presentation.

KEYWORDS: *wheat allergy, low-molecular-weight glutenin subunits, IgE reactivity, epitope mapping, synchrotron radiation circular dichroism, dynamic light scattering*

INTRODUCTION

Wheat has represented the main source of food in Western civilizations since their origin. Unfortunately, wheat-derived products are also responsible for adverse reactions such as intolerances and allergies. Wheat seed proteins are classified according to their solubility profiles. While the salino-soluble fraction comprises albumins and globulins, the water insoluble fraction, which is the main component of gluten, is composed of prolamins, so-called because of their high content in proline and glutamine residues. Wheat prolamins are divided into two groups based on their solubility in aqueous alcohols: gliadins and glutenins. Gliadins are monomeric and extractable with alcohol solutions, while glutenin subunits, which are polymerized through intermolecular disulfide bridges, are extractable with alcohol solutions under reducing conditions. The glutenin subunits are divided into two classes: the high-molecular-weight glutenin subunits are in the 80−160 kDa range, while the low-molecular-weight glutenin subunits (LMW-GSs) range from 20 to 45 kDa. The sequences of the most typical LMW-GSs are of the B-type[1] as their N-terminal region contains one cysteine residue and a repetitive domain with short repeated sequences rich in glutamine and proline, while their C-terminal region contains seven cysteine residues and a glutamine-rich domain. LMW-GSs of the C-type type are modified α- and γ-gliadins, and those of the D-type are modified ω-gliadins.[1]

As glutenins are known to be insoluble in aqueous solutions, it has proven very difficult to obtain structural information on such proteins using nuclear magnetic resonance or crystallography techniques, both of which require several milligrams of pure and concentrated protein solutions. As such, high resolution structure of gluten proteins is not yet available. Indications on size and overall shape (rodlike) were obtained from scanning tunneling microscopy,[2] and secondary structures were analyzed via circular dichroism and infrared spectroscopy.[3,4]

Wheat seed proteins may induce allergic reactions that differ according to the route of sensitization. In adult patients, food ingestion may trigger classical symptoms of dietary allergy, or the symptoms may occur only upon physical effort, as in wheat-dependent exercise-induced anaphylaxis (WDEIA).[5] Flour inhalation may result in respiratory allergies (rhinitis and baker's asthma).[6] The use of hydrolyzed wheat proteins as an additive in cosmetics and food products led to the emergence of contact or dietary allergies (immediate hypersensitivity to hydrolyzed wheat proteins, IHHWP).[7]

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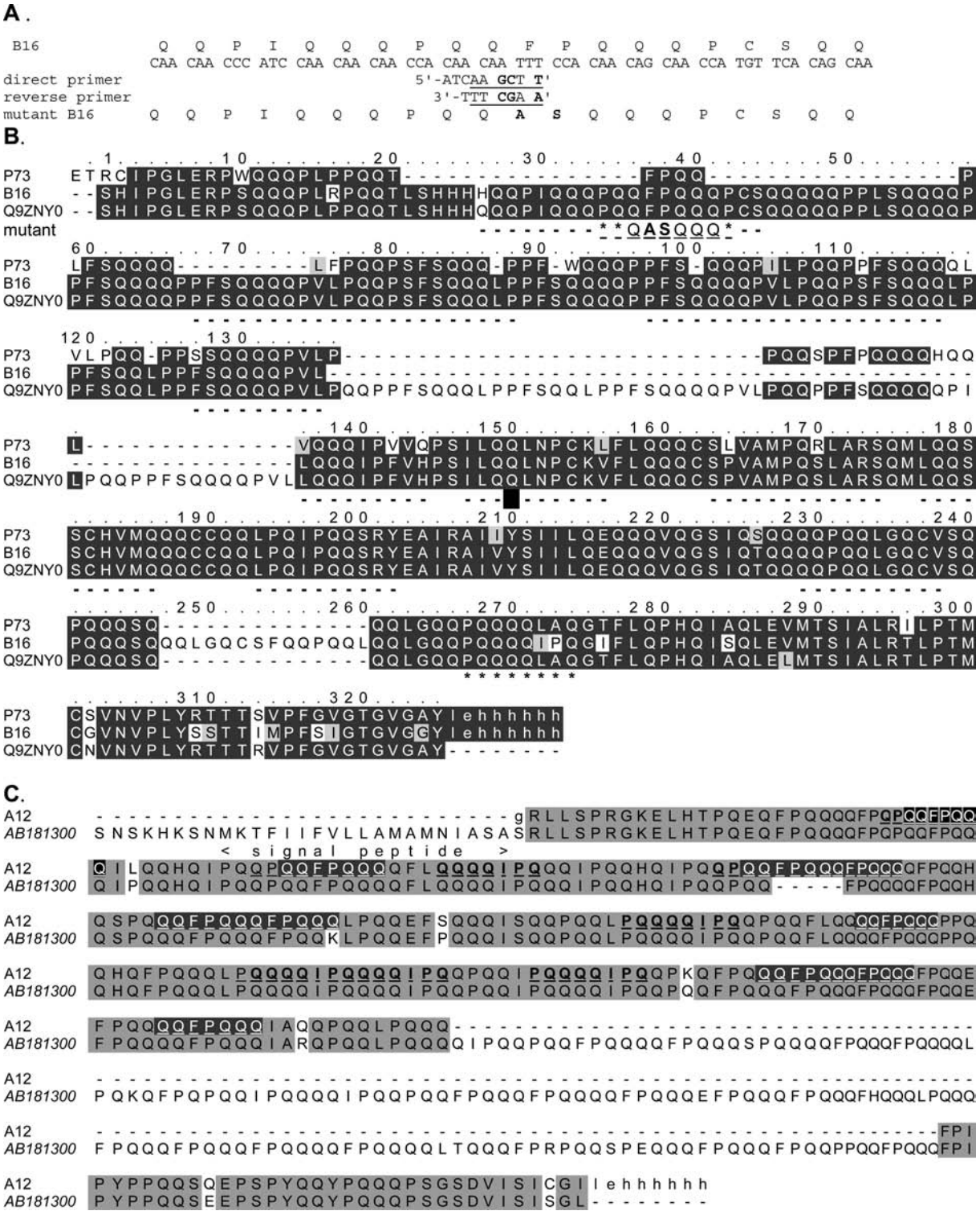

Figure 1. Sequences of primers and proteins used in this study: (A) Primers used to mutate the B16 glutenin subunit in order to suppress a WDEIA epitope from ω5-gliadin. (B) Sequence alignment of P73, B16, and Q9ZNY0 glutenin subunits. Letters in black background indicate identical residues between the three protein subunits. Light gray background indicates similar residues. The black rectangle indicates the ends of N-terminal and C-terminal moieties (NP73, NB16, P73C, B16C) and the location of recombination in chimeras. The B16 mutant is indicated only by the mutated ω5-gliadin epitope, the other parts of its sequence being identical to B16. *: B16 sequence identity with A12 ω5-gliadin, using a seven amino acid word. Broken lines: peptides binding IgE using the Pepscan technique. The histidine-tag sequence is indicated using small letters. (C) Sequence alignment of ω5-gliadins A12 (used in this work) and of the reference ω5-gliadin corresponding to GenBank ID *AB181300*. Gray background indicates identical residues. Bold and underlined: identities with B16. White letters on dark background correspond to the WDEIA epitope regions common with the B16 subunit, as previously described,[13] and mutated in the B16 mutant.

Of the many wheat seed proteins, LMW-GSs were found to exhibit IgE-binding activities in the allergies described above.[8,9] Their role in allergy was also substantiated through skin prick test,[10] or in vitro mast cell degranulation assays.[11] They were described as major allergens in WDEIA, but the molecular basis of their recognition by patient IgE was not studied in detail, as is the case for ω5-gliadins.

The present work aimed at analyzing IgE binding toward two recombinant glutenin subunits, P73 and B16, displaying strong homologies, but relatively different by their IgE reactivities. Their N-terminal and C-terminal halves, separately produced, or recombined in chimeras, were also studied. As the B16 glutenin subunit contained a major ω5-gliadin epitope involved in WDEIA,[13] a mutant modified on this epitope and an ω5-gliadin were used for comparison. Three groups of patients suffering from different wheat allergies were selected. The first group comprised patients suffering from IHHWP to highly modified wheat proteins after dietary sensitization, and sometimes after contact sensitization. In the second group, patients were suffering from WDEIA, after both dietary sensitization and exercise. In the third group, inhalation of wheat proteins induced respiratory allergy, a major professional allergy. A dot-blot technique was used with sera from these patients to determine the IgE binding toward the proteins and their fragments. In parallel, sera were used for linear epitope mapping with synthetic peptides. In order to investigate the relationship between protein structure and IgE reactivity, information on the secondary structures of recombinant LMW-GS subunits and their N- and C-terminal halves was obtained using synchrotron radiation circular dichroism (SRCD).

MATERIALS AND METHODS

Expression and Partial Purification of Recombinant Proteins and Fragments. The two LMW-GSs, known as P73 and B16, recorded in the EMBL database under numbers *TAE16835* and *AJ937920*, respectively, were cloned as previously described.[12] Their respective N-terminal halves, named NP73 and NB16, as well as their C-terminal halves, coined P73C and B16C, and chimeras made of the N-terminal half of one glutenin subunit and the C-terminal half of the other, were cloned in pET32b or pET28b (Novagen, Darmstadt, Germany). The plasmids were introduced in the *Escherichia coli* T7 Express strain (New England Biolabs, Ipswich, MA). The B16 sequence contains the $_{35}$QQFPQQQ$_{41}$ epitope described for wheat ω5-gliadin.[13,14] A mutant of this epitope was obtained as follows: the plasmid containing the B16 gene was amplified by PCR using the divergent primers shown in Figure 1A. After digestion of the template with *Dpn*1 and of the amplified plasmid with *Hin*dIII, it was recircularized by ligation and introduced into bacteria. The resulting mutant protein contained the peptide sequence $_{35}$QQ**AS**QQQ$_{41}$ in lieu of $_{35}$QQ**FP**QQQ$_{41}$. A recombinant ω5-gliadin called A12[10] (Figure 1C) was also used as a reference in order to compare its IgE-binding activity to those of the B16 protein and of its mutant.

After culture in LB medium and a 4 h induction period with IPTG (isopropyl-β-D-thiogalactopyranoside), bacteria were pelleted. For whole proteins, chimeras, and C-terminal moiety extractions, inclusion bodies obtained after enzymatic lysis were solubilized in a Tris buffer containing 2% w/v SDS (sodium dodecyl sulfate). The proteins were dithiothreitol-reduced and iodoacetamide-alkylated. After addition of 2 volumes of ethanol, the proteins were recovered from the supernatant by NaCl overnight precipitation. N-Terminal moieties were extracted by adding 2% SDS to crude lysates, then 3/4 volume of ethanol, and recovered using acetone precipitation. The protein content was quantified using TCA precipitation,[11] adapted to the microplate format. SDS–PAGE analysis was performed using bis-tris 12% acrylamide precast gels and MOPS (3-(*N*-morpholino)-propanesulfonic acid) electrophoresis buffer (Invitrogen, Grand Island, NY). Gels were Coomassie blue stained.

For dot-blot experiments, proteins were resuspended in a 50 mM Tris-HCl buffer pH 8.5 containing 2% SDS.[10] For SRCD and dynamic light scattering (DLS), proteins were resuspended in 50 mM sodium phosphate buffer pH 6.0 containing 2% SDS and 10 mM sodium sulfate.

Characteristics of Patients. Sera from adult patients (n = 6) allergic to hydrolyzed wheat proteins after dietary sensitization (IHHWP, sera 1 to 6) were tested for IgE reactivity. Records indicate that one patient reacted after effort (serum 5), and another after cutaneous contact (serum 6). These patients were not allergic to native wheat proteins.[7] Twelve patients suffering from WDEIA (sera 7 to 18) and six patients suffering from asthma or rhinitis (sera 19 to 24) were also selected and used in the present work. This study was approved by the Committee for protection of human subjects in biomedical research of the Cochin Hospital (Paris, France). Each patient gave informed consent before blood sampling.

Detection of IgE-Binding Activity (Dot-Blot). An amount of 15 μg was deposited for the glutenin subunit B16, and amounts resulting in the same molarity were deposited for the other proteins. The membranes were incubated with patient sera, washed, further incubated with rabbit HRP-conjugated antibody against human IgE (DakoCytomation, Copenhagen, Denmark), and washed again, according to a previously described procedure.[15] Bound IgE antibodies were detected using chemiluminescence with a Fuji Las3000 camera, and quantitative analysis was done with Multi-Gauge software, version 3.0 (Fujifilm, Tokyo, Japan).

Identification of IgE-Binding Epitopes Using Solid-Phase Bound Synthetic Peptides (Pepscan). Sera from 10 patients (4 patients with IHHWP, 5 with WDEIA, and 1 with baker's asthma) were used in epitope mapping experiments by Pepscan analysis. Three sera obtained from non-atopic subjects were used as negative control. Decapeptides overlapping by eight amino acid residues were used in this study. The used sequence was not that of B16, but that of a LMW glutenin subunit strongly homologous to B16 (333 amino acids: UniProtKB accession *Q9ZNY0*) and previously used with sera from patients suffering from dietary allergy to wheat.[16] The decapeptides were covalently immobilized by their C-terminus on a cellulose membrane (Abimed, Langenfeld, Germany). Membrane was carried out as previously described.[16] Chemiluminescence detection of the bound IgE was performed as described above. The spot with the highest intensity was taken as a reference, which provided the 100% value after subtraction of the background determined as the intensity of an empty spot.

Synchrotron Radiation Circular Dichroism. Samples were centrifuged for 30 min at 20800*g* at room temperature; then measurements were carried out on the DISCO beamline at Soleil synchrotron (Gif-sur-Yvette, France). Scans using wavelengths ranging from 170 to 280 nm with 1 nm steps were measured four times for each sample. Spectra of dialysis buffer were subtracted from those of the corresponding samples. The 263–270 nm region was set to zero, and resulting spectra were calibrated with d-10-camphorsulfonic acid using the CDtool software.[17] Spectra were normalized to protein concentrations. Ratios of secondary structures were determined using ContinLL program[18] in Dichroweb,[19] and using SP175 as the reference set. To date, SP175 database contains the largest set of spectra (72 soluble proteins).[20] For all measurements, spectra were analyzed down to 175 nm: this corresponded to a photomultiplier high tension remaining below half of its total variation. Normalized root-mean-square deviation (NRMSD) indicated the most accurate fit for each spectrum.

Dynamic Light Scattering. Measurements were performed using a HPPS Malvern apparatus with a 3.0 mW helium–neon laser emitting at 632.8 nm. Analysis was carried out at an angle of 173° with the incident beam, at 25 °C. Data were processed with DTS software, version 3.32 (Malvern Instruments, Ltd., Malvern, U.K.).

RESULTS

Production of Recombinant Proteins and Fragments. The P73 and B16 glutenin subunits used in this work (Figure 1) show the sequence features characteristic of B-type LMW-GS described above. They are examples of the polymorphism of this abundant protein group, P73 being a glutenin subunit encoded by the D genome, and B16 likely encoded by the B genome of hexaploid wheat.[1] For each glutenin subunit, the N-terminal halves ($_{1}$ETRCIP... ...VQPSILQ$_{121}$ for P73 and $_{1}$SHIP... ...HPSILQ$_{149}$ for B16), which comprised the repetitive domain, and the C-terminal halves ($_{122}$QLNPCKL... ...GTGVGAY$_{283}$ for P73, and $_{152}$NPCKV... ...GTGVGGY$_{325}$ for B16), which contained most of cysteine residues, were cloned and expressed separately. The N-terminal halves of the two proteins exhibit differences in the sequences and the number of the repeated motifs (57% sequence identity), whereas C-terminal halves are highly conserved (82% sequence identity). The whole glutenin subunits, the chimeras, the C-terminal halves, and the A12 ω5-gliadin expressed in *E. coli* were recovered as inclusion bodies, solubilized in SDS, and purified by alcohol addition and salt precipitation. This process led to proteins showing good purity (Figure 2). The N-terminal halves were more water-soluble, and therefore they were not present in inclusion bodies. So they were extracted with a lower alcohol concentration and were further recovered using acetone precipitation: this process led to a lower purity. Nevertheless, the contaminants were present at negligible concentrations. Immunoblots can be highly sensitive, a low amount of protein being able to produce a high IgE chemiluminescence signal. However, the protein impurities observed were from *E. coli* and, therefore, should not react with sera of patients allergic to wheat in dot-blot experiments. This was confirmed by Western blot experiments published previously.[12] Besides, SRCD displays a limited sensibility and uses concentrated protein solutions; the percent of impurity as assessed in SDS−PAGE was too low to significantly alter the calculated secondary structure content. In wheat seeds, glutenin subunits are characterized by their ability to polymerize through formation of disulfide bridges. Similarly, recombinant glutenins were not extractable without reducer. Therefore, alkylation with iodoacetamide was necessary to maintain them in solution, and to prevent any spontaneous polymerization which could induce changes in IgE reactivity and structural studies.

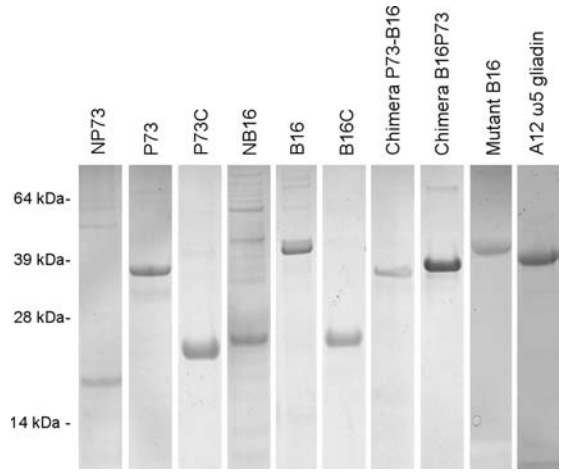

Figure 2. SDS−PAGE analysis of all recombinant proteins used. Amounts of protein loaded onto gels varied from 4 to 6 μg.

IgE Binding Analyses Using Dot-Blot and Epitope Mapping (Pepscan). The two recombinant glutenin subunits, their N-terminal and C-terminal halves, and chimeras were dot-blotted on membranes and incubated with sera from allergic patients. The respective halves of P73 and B16 were mixed in solution and incubated for 24 h, and the resulting mixture was also tested for IgE reactivity by dot-blot. The sequence of protein B16 contains the $_{35}$QQFPQQQ$_{41}$ epitope previously described in wheat ω5-gliadin,[13] a major allergen in WDEIA.[21] To assess the role of this epitope, we tested the IgE reactivity of a recombinant mutant protein and that of an ω5-gliadin containing several repeats of this epitope (Figure 1C). The results allowed for the comparison of the IgE reactivity of each serum with the various allergens. Among the sera obtained from patients suffering from IHHWP (Figure 3A), the IgE-binding patterns were homogeneous and exhibited weak interindividual variability. A significant IgE-binding activity was detected with the N-terminal halves of P73 and B16 (as an example, for serum 4 the chemiluminescence values were 641 and 1435 arbitrary units, respectively), while only weak signals were obtained with the C-terminal halves (chemiluminescence value about 400 au or less). A faint binding intensity to the B16 whole protein was detected in all sera (229 au or less). The strongest signals were obtained with the B16−P73 chimera (3803 au for serum 4), the B16 mutant (3650 au), and the A12 ω5-gliadin (3098 au). The mixtures of the two halves resulted in about the same binding intensity as that of the N-terminal half alone. Sera obtained from WDEIA patients showed a more heterogeneous IgE-binding pattern between patients (Figure 3B). Weak to faint binding signals were detected with P73, its halves, NB16, and the P73−B16 chimera (chemiluminescence values less than 400 au). Conversely, several patient sera resulted in strong IgE-binding activity to protein B16 (for example 2700 au for serum 12, and 3379 au for serum 14), and even higher intensities were recorded with the two mixed halves of B16 (3390 au for serum 12), and with the B16−P73 chimera (3394 au for serum 14). Weaker but still significant signals were detected with B16C (1734 au for serum 12) and B16 mutant protein. A12 ω5-gliadin was recognized by most sera (up to 2688 au in the case of serum 14), but often at a weak, although still significant, level (chemiluminescence values between 300 and 700 au). The six sera from patients suffering from respiratory allergy to wheat displayed a relatively homogeneous IgE binding pattern (Figure 3C). Overall, the IgE-binding intensities recorded in this group of sera were much lower than in the other two groups. The highest binding signals were obtained with the mixed P73 or B16 halves, and were most significant in the B16−P73 chimera (2239 au with serum 19) and the B16 mutant.

In order to map and characterize the linear epitopes in protein B16, which is the recombinant LMW-GS that yielded high IgE-binding responses in dot-blot, Pepscan analysis was carried out with ten of the patient sera used in the dot-blot experiment (Table 1). The decapeptides used were derived from the *Q9ZNY0* sequence, which spans 91% of the B16 sequence. The part of the B16 sequence absent from Q9ZNY0 is essentially the peptide QQLGQCSFQQPQQL (14-residues out of 326). No peptide was recognized either by non-atopic control sera or by the secondary antibody alone. Of the 10 patient sera selected for Pepscan, four sera did not give any binding signal: patients 1 and 2 from the IHHWP group,

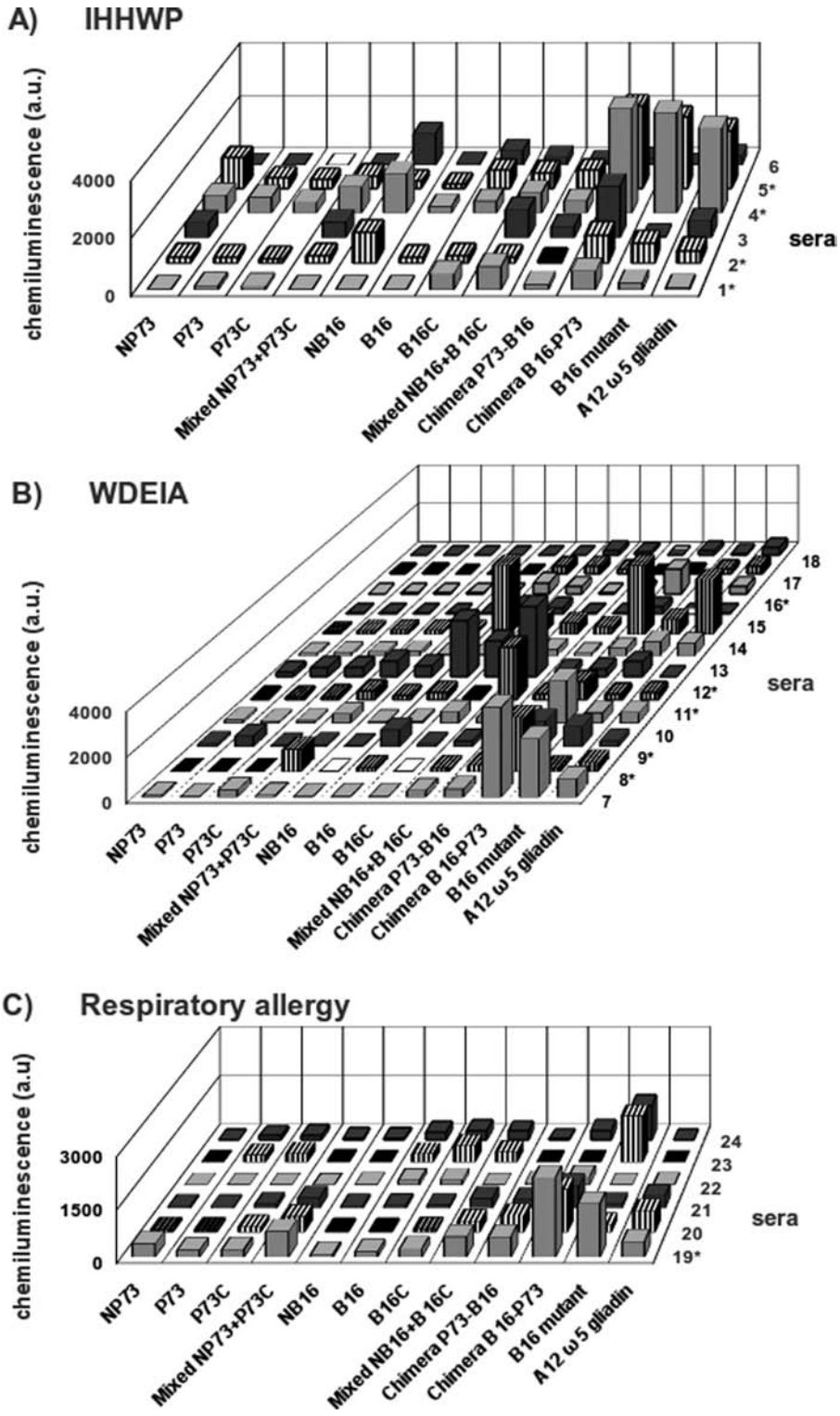

Figure 3. Dot-blot IgE reactivity of recombinant glutenins, their N-terminal halves, C-terminal halves, chimeras, and A12 ω5-gliadin. The IgE chemiluminescence signal quantified using the camera software is shown in arbitrary units. Asterisks indicate sera used in Pepscan experiment.

patient 8 from the WDEIA group, and the single patient (19) from the respiratory allergy group. The other six patient sera displayed binding activity to 16 different synthetic peptides, as detailed in Table 1. Overlapping decapeptides, such as $_{27}$QQPIQQQPQQ$_{36}$ and $_{29}$PIQQQPQQFP$_{38}$, were recognized by the same serum, thus, the sequence motif PIQQQPQQ may represent a consensus epitope. Ten IgE-binding peptides belonged to the N-terminal half of B16, five to its C-terminal half, and one (residues 147−156) overlapped the two expressed halves. In the N-terminal part, the peptides $_{27}$QQPIQQQPQQ$_{36}$, $_{29}$PIQQQPQQFP$_{38}$, and $_{129}$QQQQPVLLQQ$_{138}$ were recognized by IgE from three patients. Peptides $_{35}$QQFPQQQPCS$_{44}$ and $_{135}$LLQQQIPFVH$_{144}$ were detected by two sera. Other peptides

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Table 1. IgE Binding of Linear Epitopes from the B16 Sequence Identified by Pepscan[a]

IgE binding decapeptides	position in B16[b]	sera (IHHWP) 4	5	sera (WDEIA) 9	11	12	16
QQ**PIQQQPQQ***	27−36[A]	+++				+	++
PIQQQPQQFP	29−38[A]	+				+	++
QPQQFPQQQP	33−42[A]						+
QQFPQQQPCS*	35−44[A]		+++			+	
PFSQQQQPVL	67−76[A]				++		
	97−106[A]						
	126−135[A]						
PQ**QPSFSQQQ**	77−86[A]●		++				
	107−116[A]●						
QPSFSQQQLP	79−88[A]●		+				
	109−118[A]●						
QQQQPVLLQQ	129−138[A]●		+		+		+
LL**QQQIPFVH**	135−144[A]					+++	+
QQQIPFVHPS	137−146[A]					+	
ILQQLNPCKV	147−156[B]			++			
SP**VAMPQSLA**	163−172[C]					+	
VAMPQSLARS	165−174[C]					+	
LQQSSCHVMQ	177−186[C]●				+		
LPQIPQQSRY*	193−202[C]●					+++	
QQPQQLGQCV	229−238[C]●					+	

[a]No binding activity was detected in Pepscan analysis using patient sera 1, 2 (IHHWP), 8 (WDEIA), and 19 (respiratory allergy). In bold are indicated the consensus epitope peptides, as evidenced by positive consecutive spots. *Linear epitopes previously described.[16] Underlined residues designate the ω5-gliadin epitope,[13] which was mutated. +++: strongly positive IgE-Binding (more than 60% of the reference signal). ++: moderately positive (20% to 60% of the reference). +: weakly positive (less than 20% of the reference). [b]Superscripts defined: A, peptide belonging to NB16; B, peptide overlapping NB16 and B16C; C, peptide present in B16C; ●, peptide present in P73.

of the N-terminal half were recognized by single sera. The IgE-binding peptides from the C-terminal half of B16 were detected only by single sera from WDEIA patients. The Pepscan results obtained with B16 decapeptides could be extended to eight P73 linear epitopes.

Serum from patient 9 exhibited IgE-binding activity to whole P73, B16, and chimeras, but not with the respective single halves. This may be explained by the presence of the peptide $_{147}$ILQQLNPCK$_{155}$ contained in the linear epitope $_{147}$ILQQLNPCKV$_{156}$. This sequence overlaps those of both halves and is effectively only partially present in each of the two halves of B16 protein. In Pepscan experiments, the IgE antibodies from three sera bound the $_{35}$QQFPQQQ$_{41}$ epitope previously reported in wheat ω5-gliadin.[13] Dot-blot experiments revealed a decreased binding intensity to B16 mutant protein as compared to B16 in the cases of 5 patient sera (patients 11, 12, 14, 16 and 22). In patient sera 12 and 16, the decrease may be related to the mutation of $_{35}$QQFPQQQ$_{41}$ (identified in Pepscan) to $_{35}$QQASQQQ$_{41}$. Interestingly, the same mutation led to an increased signal (even for patient serum 5 which strongly recognized the epitope in Pepscan), or had no effect, for the other patient sera. Other discrepancies were observed between the linear epitopes identified by Pepscan compared to the IgE reactivity characterized by dot-blot. Thus, sera from patients 1, 2, 8, and 19 did not recognize any of the synthetic peptides in the Pepscan analysis, but they exhibited significant binding activity in dot-blot experiments (for example serum 19 with the B16−P73 chimera and with the B16 mutant). Similarly, patient sera 4, 5, and 16 only bound to the linear peptides comprised in the N-terminal half during Pepscan analysis, but significantly reacted with the C-terminal half during the dot-blot.

The dot-blot experiments of IgE binding showed in several cases a surprisingly high binding level with whole proteins, with the simple mixtures of the two halves and with chimeras, as opposed to the individually produced halves. This is exemplified by sera 11 and 12 (Figure 3B), for which a large increase in IgE binding activity was observed with the mixed B16 halves (3390 au for serum 12, as compared to values of 1734 au or less for individual halves). This observation suggests that conformational changes occurred during the 24 h mixing period of the two protein halves that were separately prepared. This prompted us to investigate the possible structural differences.

Secondary Structure Content of the Recombinant Proteins and Their Respective Fragments. In order to assess structural changes between the whole proteins, their fragments alone or mixed, and chimeras, their secondary structure content was determined using SRCD (Figure 4). All low-molecular-weight glutenin subunits or fragments prepared in this work contained a significant majority of α structures to the detriment of β structures. The whole P73 protein contained ratios of α-helix, β-strand, and turns corresponding to the approximate mean of the secondary structure ratios of its N-terminal and C-terminal halves (Figure 4E). This means that no significant structural interaction between the two moieties was detectable through SRCD analysis, the whole P73 protein being probably a simple juxtaposition of its two halves. Conversely, whereas the B16C half showed a secondary structure content similar to that of the whole B16 protein, the NB16 half contained significantly more α structures and fewer β structures. In this case, the whole B16 protein does not seem to result from a simple juxtaposition of its N-terminal and C-terminal parts, as evidenced by SRCD spectra (Figure 4B). The P73−B16 chimera showed an overall secondary structure content similar to that of its NP73 half, whereas the B16−P73 chimera was similar to its P73C half (Figure 4D): here again, SRCD spectra strongly suggested that the fragment interactions in the chimeras induced conformational changes reflected by differences in secondary structure content.

Structural Interactions between the Two Halves of the B16 Glutenin Subunit. The mixture of NB16 and B16C halves led to a significant increase in IgE reactivity assessed by dot-blot (Figure 3), as compared to that of the halves alone, thus suggesting the occurrence of stable structural interactions. To get insight into such interactions, the B16 glutenin subunit and its two N-terminal and C-terminal halves, alone or mixed, were compared using DLS and SRCD analysis. The DLS results (Figure 5A) showed that the protein and fragments were essentially monodisperse in solution. The N-terminal and C-terminal halves had similar hydrodynamic diameters (around 4.8 nm). The whole protein showed a shifted peak corresponding to a greater diameter (∼5.6 nm). When the N- and C-terminal halves were mixed and incubated together, the resulting peak shifted to the same position as that of the whole protein. This was true after either 20 min (not shown) or overnight (Figure 5) incubation. This was a strong indication that the two halves formed an object the size of which was similar to that of the whole protein. A SRCD kinetics

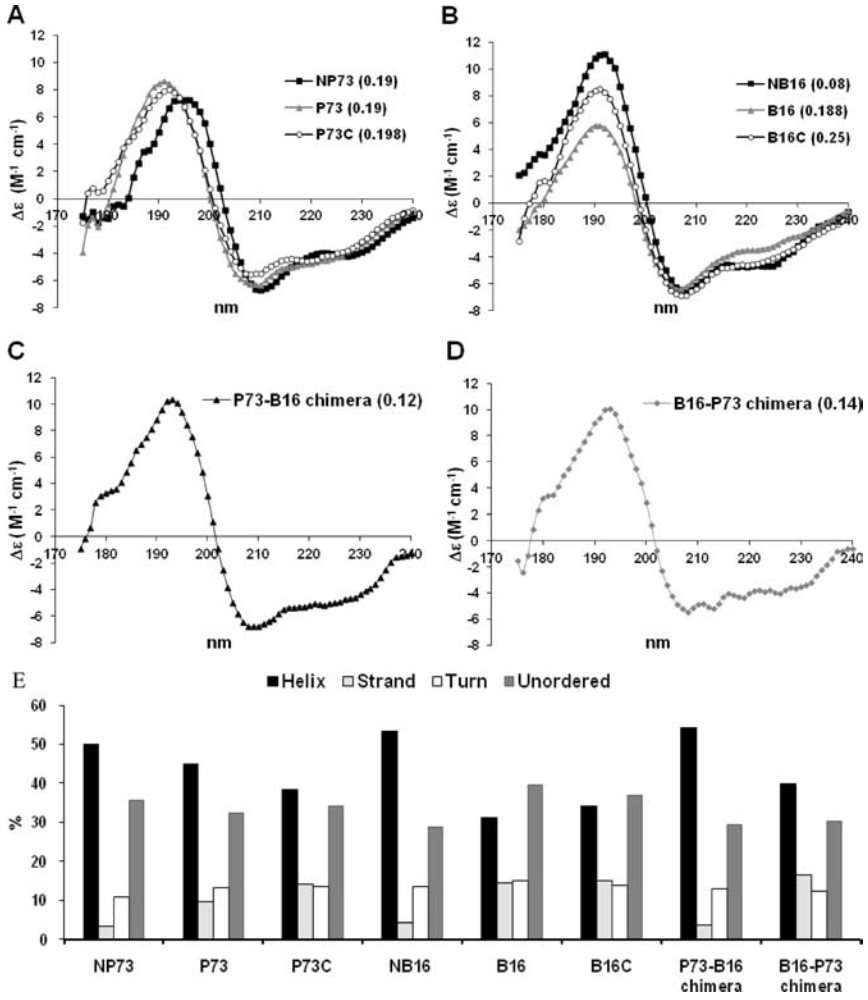

Figure 4. Synchrotron radiation circular dichroism on glutenins, halves, and chimeras. Spectra of the glutenin subunits P73 (A) and B16 (B), of their N-terminal and C-terminal fragments, and of their chimeras (C, D). NRMSDs are indicated in parentheses. (E) Graphs representing the percentages in secondary structure content.

experiment was performed on the mixture of the two B16 halves. The resulting spectra were compared to those of the B16 glutenin subunit and its N-terminal and C-terminal halves (Figure 5B). Six minutes after mixing, the spectrum was intermediate between those of the two halves. As time went by, a shift toward the spectrum of the whole protein occurred.

DISCUSSION

Wheat seed proteins are numerous and polymorphic. Their purification from wheat flour is a daunting task. Current literature suggests that proteins of the same family, or very similar in sequence, can differ significantly in their IgE reactivity. The use of recombinant proteins allows for the production of large amounts of well-characterized proteins. On the other hand, the disulfide bonds that contribute to the conformation of native glutenins are lost in recombinant proteins expressed in bacteria, so that some conformational epitopes could be lost. The absence of disulfide bridges could also modify the accessibility and presentation of epitopes. In this study, however, the alkylated glutenins kept significant IgE-binding properties.

We combined dot-blot and Pepscan analysis to identify the IgE-binding activities of recombinant glutenin subunits and their fragments toward patient sera obtained from wheat allergic individuals. Six out of ten sera were shown to bind 16 synthetic decapeptides derived from the native sequence of B16. Three of these linear epitopes ($_{27}$QQPIQQQPQQ$_{36}$, $_{35}$QQFPQQQPCS$_{44}$, and $_{193}$LPQIPQQSRY$_{202}$) were also reported in a previous study using sera from children and adult patients diagnosed with dietary allergy to wheat (including atopic eczema dermatitis syndrome, urticaria, or WDEIA).[16] The consensus epitope $_{29}$PIQQQPQQ$_{36}$ recognized by three sera in this work was also the most frequently bound and considered as immunodominant in ref 16. Here, we describe the existence of thirteen new linear epitopes (see Table 1), not found in this previous study. Overall, many linear epitopes were found using Pepscan, mainly in the N-terminal half, where most sequence differences between P73 and B16 are found. The absence of the epitopes PIQQQPQQ and QQFPQQQ in P73 may in part explain the different reaction levels observed in dot-blot between P73 and B16. The absence of the latter epitope $_{35}$QQFPQQQ$_{41}$ in the B16 mutant may explain that its reaction level was lower than that observed for B16 with a few sera. However, for most sera, the mutation did not affect, or increased, IgE-binding activity. Thus, the present study showed that IgE reactivity to LMW-GSs subunits was not essentially due to cross-reaction with ω5-gliadins, despite the common epitope $_{35}$QQFPQQQ$_{41}$. As such, LMW-GSs and ω5-

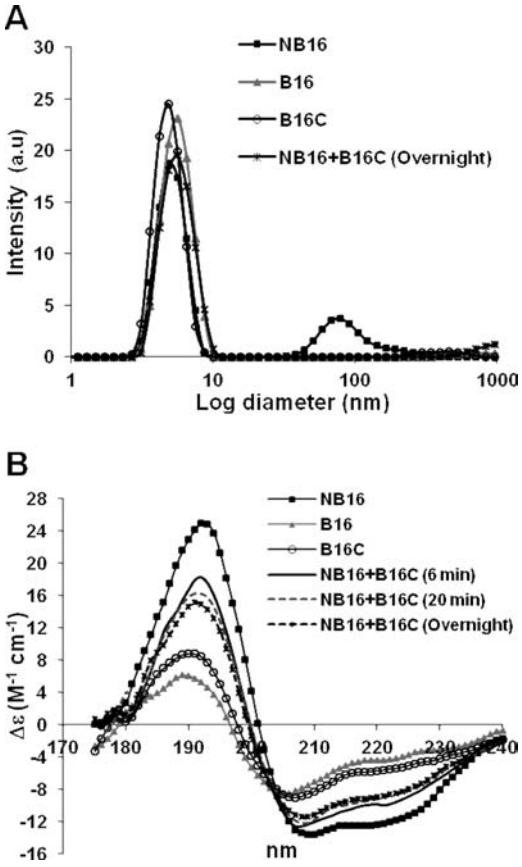

Figure 5. Structural interaction studies of the repetitive and nonrepetitive halves of the glutenin subunit B16. (A) DLS analysis; a.u., arbitrary units. (B) Kinetics analysis of NB16/B16C interaction assessed using SRCD.

gliadins should be considered independent allergens, even in WDEIA. In this study the Pepscan results did not always help to explain the dot-blot responses. This emphasizes how different from the observed IgE binding on folded proteins the data from Pepscan can be.[22] It also suggests that epitope conformation is of great importance in LMW-GS binding by IgE.

In dot-blot experiments, the IgE-binding activity of IHHWP and WDEIA patient sera to the protein B16 was higher than the sum of the signals obtained with its two N- and C-terminal halves. Moreover, the B16−P73 chimera was often highly IgE-reactive, whereas the corresponding halves reacted weakly. This is in line with previous observations.[12] Such variation in IgE-binding activity suggested that the conformation of the whole protein or chimera does not merely result from the juxtaposition of their N- and C-terminal halves, and that the IgE recognition of the linear epitopes detected in Pepscan is somehow dependent on conformational event. Accordingly, we observed a difference in IgE binding to the halves exposed individually or after mixing: structural interaction occurred in vitro, when the halves were mixed overnight before blotting. This is reminiscent of previous observations[23] showing that industrially hydrolyzed wheat proteins could form aggregates which were highly IgE-reactive with sera from patients allergic to these hydrolysates. In the present mixing assays, the phenomenon was much simpler than aggregation in terms of the number of fragments mixed, but the resulting effects were similar.

In the absence of high resolution structure of gluten proteins, we resorted to secondary structures analysis to further elucidate the IgE-binding properties of this protein family. The glutenin subunits are not soluble in water, so we used a buffer containing 2% SDS to solubilize the proteins for our SRCD assays. As SDS can induce a transition toward α-helix formation for some proteins,[24] we assessed other buffers. The use of 1-propanol (40% v/v) resulted in comparable CD spectra. However, the far UV values could not be recorded (data not shown). Solubilization with acetic acid led to calculated ratios of α-helix and β-strand similar to those obtained with SDS (not shown), but the proteins were not as soluble as expected. Moreover, they could be spontaneously altered through glutamine deamidation by the acid.[25] We therefore selected 2% SDS as the optimum buffer, allowing comparison between all our different proteins. We assume that the interactions between N- and C-terminal halves should occur between protein zones not covered by SDS micelles.[26]

A number of structural studies have been published on wheat gliadins and high-molecular-weight glutenin subunits; however CD data related to LMW-GSs remain scarce. A first study[27] described complex mixtures of natural wheat gluten proteins solubilized in 50% v/v 1-propanol solution. The estimated secondary structure ratios obtained for the α content (about 35%) were similar to our results on recombinant LMW-GSs (45% for P73, 31% for B16). However, the β content (20%) was higher than the one we obtained (10% for P73, 14% for B16). This can be explained by glutenin heterogeneity. In addition, as compared to α structure, β structure determination is less accurate because CD spectra have weak amplitudes. The light sources of SRCD facilities provide a very high flux, making the UV region accessible down to 175 nm in our work, which increases the reliability of secondary structure content determination.

Recently, a recombinant LMW-GS, highly similar in sequence to the B16 protein, and in parallel its repetitive N-terminal domain, were analyzed using conventional CD and Fourier transform infrared spectroscopy.[4] The repetitive domain and whole protein formed polyproline II (PPII) helix structures in water or 1% v/v acetic acid. As glutenin sequences contain a remarkable proportion of glutamine and (to a lesser extent) proline residues, which are prone to PPII helix formation, we calculated the polyproline II helix content of our recombinant proteins using the ContinLL (set 5) program in Dichroweb. This resulted in percentage values of PPII helices inferior to 10%. As, in addition, CD spectra of PPII helices are difficult to distinguish from those of random coil polypeptides,[28] we did not focus on PPII helices here. In TFE (trifluoroethanol), the whole recombinant LMW-GS protein analyzed in ref 4 contained mainly α-helices, whereas the repetitive domain contained a large amount of β-sheets. The comparison of secondary structures in water vs TFE led the authors to suggest a structural interaction between the repetitive and nonrepetitive domains. Our SRCD results, using both repetitive and nonrepetitive halves of B16, showed such an interaction. This was observed when the fragments were mixed in vitro, and also when the two halves were bound together in the whole glutenin or B16−P73 chimera. It gives structural support to the observed variations in IgE reactivity on the same constructs.

Methods that predict epitopes from the sequence of protein allergens, based either on surface exposure, on flexibility, or on propensity for adopting a certain secondary structure, generally

lack precision.[29] The B16 protein contained slightly more β regions than P73, and showed an overall higher IgE reactivity. Accordingly, the B16−P73 chimera, which contains more β-strands than its P73−B16 counterpart, is more IgE-reactive. For the limited set of proteins studied here, β structures could favor the presentation of epitopes, either from β structures themselves or from neighboring α-helices or loops.

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ABBREVIATIONS USED

CD, circular dichroism; DLS, dynamic light scattering; IHHWP, immediate hypersensitivity to hydrolyzed wheat proteins; LMW-GS, low-molecular-weight glutenin subunit; NRMSD, normalized root-mean-square deviation; PPII, poly-proline II; SRCD, synchrotron radiation circular dichroism; TFE, trifluoroethanol; WDEIA, wheat-dependent exercise-induced anaphylaxis.

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