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Recombinant Proteins and Peptides as Tools for Studying IgE Reactivity with Low-Molecular-Weight Glutenin Subunits in Some Wheat Allergies

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Two genes of wheat low-molecular-weight glutenin subunits (LMW-GS), B16 and P73, were cloned and expressed in *E. coli*. They were homologous to proteins encoded respectively at *Glu-B3* and *Glu-D3* loci. The N-terminal and C-terminal halves of B16 (NB16 and B16C) and the two chimeras combining the halves of the two genes (B16-P73 and P73- B16) were also expressed. All these constructs were compared for their reactivity with IgE from 24 patients suffering from different forms of wheat allergies. The results confirmed that LMW-GSs bound IgE in all adult allergies tested. Strong differences in reactivity between all the constructs were observed. They were disease-dependent. In wheat-dependent exercise-induced anaphylaxis (WDEIA), the reactivity of the constructs depended partly on common epitopes with ω-5 gliadins but also on differences in molecule conformation. The presence of NB16 in the constructs greatly influenced their IgE reactivity.

KEYWORDS: Low molecular weight glutenin subunits; recombinant allergens; wheat allergy; IgE binding

INTRODUCTION

Wheat seed proteins may trigger various IgE-mediated allergies which differ according to sensitization route, food ingestion, flour inhalation, or cutaneous contact. Age of patients and symptoms are other elements to distinguish these diseases. They involve different wheat protein families. The observed pattern in IgE-binding is generally variable, but some features are specific to each disease. For example, salt-soluble proteins were the most reactive allergens in baker's asthma (*1, 2*) and in food allergies (α-amylase/trypsin inhibitor and LTP) (*3*). Among gliadins, ω-5 gliadin was found to be a major allergen in wheat-dependent exercise-induced anaphylaxis (WDEIA) (*4*) and in children's immediate allergy to ingested wheat (*5*). Proteins belonging to the glutenin fraction, particularly low-molecular-weight glutenin subunits (LMW-GSs), bound IgE at high rates in children and adult patients suffering from food allergies and in WDEIA patients (*3, 6, 7*).

Among wheat storage proteins, the glutenin group is characterized, as is the case for the other prolamins, by its high content of proline and glutamine residues and, in contrast with the case of gliadins, by its high polymerization due to intermolecular disulfide bridges. Its subunits are divided into two classes according to their molecular weights, low-molecular-weight glutenin subunits and high-molecular-weight glutenin subunits (HMW-GSs). They contain repetitive domains and show aggregating properties. They are soluble under reducing conditions in aqueous alcohol, but they are poorly soluble in water and insoluble in salt solutions (*8*).

The glutenin family is very polymorph and contains numerous proteins with similar physical properties, making their purification difficult. So, even fractions corresponding to one family often contain contaminants belonging to other families. To eliminate this problem, recombinant allergens were used for *in vitro* assays to study their allergenic properties (*9–11*). The

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Table 1. Summary of PCR Conditions[a]

	Template		Primers for PCR
	Genomic DNA	F	5′-CGA GCA TAT CCT AAC AGC CCA-3′ promoter area (not coding)
LMW-GS P73 (*16*)		R	3′-CG TGG CCT CAA CCA CGG ATG-5′ G T G V G A Y
	second PCR	F	5′-A CAT ATG GAG ACT AGA TGC ATC CCT-3′ M E T R C I P
		R	3′ CG TGG CCT CAA CCA CGG ATG ATT CTC GAG -5′ G T G V G A Y stop
		F	5'-G ACA AGT GGM ATT GCR CAR ATG GA-3′ T S A I A Q M E
	Genomic DNA		(signal peptide)
		R	3′- G TGG CCT CAA CCA CCG ATG ATT -5′ T G V G G Y stop
LMW-GS B16		F	5'-CACC ATG AGC CAC ATC CCT GGT TTG GAG-3'
	nested PCR		M S H I P G L E
		R	3′-G CCT CAA CCG TGG CCT CAA CCA CCG ATG ATT-5'
			G V G T G V G G Y stop
Peptide NB16	B16 plasmid	F	The same as for B16
		R	3′-CAA GTA GGT AGA TAG AAC GTC-5′ V H P S I L Q
Peptide B16C	B16 plasmid	F	5'-TCTCC ATG GTA AAC CCA TGC AAG GTA TTC-3′ M V N P C K V F
		R	3′-CG TGG CCT CAA CCA CCG ATG GAG CTC AGG_5′ G T G V G G Y
Chimeric glutenins (N-end half amplification)	B16 plasmid or P73 plasmid	F	5′-cgatcccgcgaaattaatacgac-3′ (T7 promoter)
		R	3′-CAC TAC GTC GTT GTT ACA ACG G-5′ V M Q Q Q C C Q

[a] F: forward primer. R: reverse primer.

expression of gluten proteins and peptides is not common. Tamás and Shewry (*12*) reviewed recently the papers about recombinant expression of gluten proteins. They found only nine papers about gliadin or LMW-GS expression in *E. coli*.

In this paper, we describe the cloning of the genes of two LMW-GSs. These proteins were expressed in *E. coli,* using a strain modified to adapt codon usage to wheat protein codons. Two recombinant peptides corresponding to the N-end, containing repetitive domains, and the C-end part of one of the recombinant glutenins were also obtained, along with chimeric constructs combining the N-end and the C-end of the two glutenins. These constructs were used to study IgE binding with sera from patients suffering from some different wheat allergies.

MATERIALS AND METHODS

Natural Protein Fractions. Flour from grains of wheat (*Triticum aestivum*), Neepawa or Soissons cultivars, was used to prepare the glutenin fraction, according to Singh et al. (*13*). Neepawa is a Canadian cultivar, and Soissons is a cultivar currently used in France. In the IgE-binding analysis, we chose this last cultivar because French patients have probably been exposed to its allelic proteins.

Human Sera. Twenty four sera from adult patients with different hypersensitivities to wheat were compared. Two atopic patients were polyallergic to cats, trees, grass and dust and showed a high level of IgE specific to wheat. One of them observed worsening of his allergic manifestation upon wheat consumption. Seven patients suffered from bakers' asthma, five from WDEIA, and ten from immediate hypersensitivity to hydrolyzed wheat proteins (IHHWPs). Four of this last group reacted to hydrolyzed wheat proteins only through skin contact (c IHHWP), and six reacted to hydrolyzed wheat proteins after skin contact or food ingestion (c/f IHHWP). These ten patients showed generalized urticaria and, for some of them, anaphylaxis when exposed to wheat protein hydrolyzates but not to natural wheat proteins (*14, 15*). This study was approved by the committee for protection of human subjects in biomedical research of Cochin Hospital (Paris, France), and each patient gave informed consent before blood taking.

PCR Cloning of LMW-GSs. Genomic DNA was extracted from wheat seedlings (*Triticum aestivum*, cultivar Neepawa) using a Qiagen (Germany) Dneasy plant kit. The DNA part, coding for two mature LMW-GSs called here "P73" (*16*) and "B16", was obtained with PCR using genomic DNA as template and Accuprime pfx (Invitrogen, Carlsbad, CA). See **Table 1** for PCR details.

Two parts of the B16 gene, the N-end domain of the protein, containing mainly repetitive domains (we called it "NB16"), and the C-end moiety, containing mainly less-repetitive domains (called "B16C"), were also cloned after PCR amplification (**Table 1**). The plasmid pET101D/Topo (Invitrogen, Carlsbad, CA) was used for cloning B16 and NB16; the plasmids pET28b+ and pET20b+ (Novagen, Merck Biosciences, Darmstadt, Germany) were used for B16C and P73, respectively.

Chimeric Glutenins. Using direct primer specific for plasmid and reverse primer specific for a sequence exactly identical to the two glutenin genes (**Tables 1** and **2**), the N-end part of each gene was amplified with PCR. Using this fragment as direct and reverse primers, the plasmid containing the other gene was amplified, giving a chimeric plasmid containing recombined inserts, with nicks at the ends of strands. The parental plasmid was digested using DpnI enzyme, specific for methylated DNA, and the modified plasmid inserted directly by transformation in a *E. coli* strain, able to repair the plasmid.

Table 2. Sequences of Recombinant LMW-GS Compared to Known Sequences from the EMBL Database, and Sequences of Cloned Peptides from the B16 Protein[a]

```
                                  |—Nter—|———————————— Repetitive ————————————
                                  ▼
CAA316685                1   METRCIPGLERPWQQQPLPPQQTFP---------QQP-LFSQQQQQQLFPQQPSFSQQQ-PPPWQQQ-PP 58
P73 (embl TAE19835)      1   -ETRCIPGLERPWQQQPLPPQQTFP---------QQP-LFSQQQQ--LFPQQPSFSQQQ-PPPWQQQ-PP 55
TAE17845                 1   ---SHIPGLERPSQQQPLPPQQTLSHHHQQQPIQQQPQQFPQQQPCSQQQQQPPLSQQQQPPFSQQQQPP 67
B16 (embl AJ937920)      1   ---SHIPGLERPSQQQPLRPQQTLSHHHHQQPIQQQPQQFPQQQPCSQQQQQPPLSQQQQPPFSQQQQPP 67
                                                                                ▲
NB16                     1   ---SHIPGLERPSQQQPLRPQQTLSHHHHQQPIQQQPQQFPQQQPCSQQQQQPPLSQQQQPPFSQQQQPP 67
B16C

                                  ———————————————————— Repetitive ————————————————————
CAA316685               59   FSQQQ-PILPQQPPFSQQQQ--LVLPQQPPFSQQQQPVLPPQQSPFPQQQQQ--HQQIV----------- 112
P73 (embl TAE19835)     56   FSQQQ-PILPQQPPFSQQQQ--LVLPQQPPSSQQQQPVLPPQQSPFPQQQQ---HQQIV----------- 108
TAE17845                68   FSQQQQPVLPQQPSFSQQQLPPFSQQQQPPFSQQQQPVLP-QQPSFSQQQLPPFSQQLPPFSQQQQPVLP 136
B16 (embl AJ937920)     68   FSQQQQPVLPQQPSFSQQQLPPFSQQQQPPFSQQQQPVLP-QQPSFSQQQLPPFSQQLPPFSQQQQPVL- 135
NB16                    68   FSQQQQPVLPQQPSFSQQQLPPFSQQQQPPFSQQQQPVLP-QQPSFSQQQLPPFSQQLPPFSQQQQPVL- 135
B16C

                                  ———————————————— Repetitive ————————————————|—Cter I
CAA316685              112   ------------------------------------------------------------QQQIPVVQPSIL 124
P73 (embl TAE19835)    108   ------------------------------------------------------------QQQIPVVQPSIL 120
TAE17845               137   QQPPFSQQQLPPFSQQLPPFSQQQQPVLPQQPPFSQQQQQPILPQQPPFSQQQQPVLLQQQIPFVHPSIL 206
B16 (embl AJ937920)    135   ------------------------------------------------------------LQQQIPFVHPSIL 148
NB16                   135   ------------------------------------------------------------LQQQIPFVHPSIL 148
B16C

                                  ———————————————— Cter I ————————————————————|——
                                  ▼     ▼                ▼     ▼▼
CAA316685              125   QQLNPCKVFLQQQCSPVAMPQRLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIILQEQQ 194
P73 (embl TAE19835)    121   QQLNPCKLFLQQQCSLVAMPQRLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIIYSIILQEQQ 190
TAE17845               207   QQLNPCKVFLQQQCSPVAMPQSLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIVYSIILQEQQ 276
B16 (embl AJ937920)    149   QQLNPCKVFLQQQCSPVAMPQSLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIVYSIILQEQQ 218
                                  ▲     ▲                ▲    ▲▲
NB16                   149   Qkgelnsklegkpipnpllgldstrtghhhhhh                                       181
B16C                     1   --vNPCKVFLQQQCSPVAMPQSLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIVYSIILQEQQ 68

                                  ———————————— Cter II ————————————|——— Cter III
                                          ▼
CAA316685              195   QVQGSIQSQQQQPQQLGQCVSQPQQQSQQQLGQ--------------QPQQQQLAQGTFLQPHQIAQLEV 250
P73 (embl TAE19835)    191   QVQGSIQSQQQQPQQLGQCVSQPQQQSQQQLGQ--------------QPQQQQLAQGTFLQPHQIAQLEV 246
TAE17845               277   QVQGSIQTQQQQPQQLGQCVSQPQQQSQQQLGQ--------------QPQQQQLAQGTFLQPHQIAQLEL 332
B16 (embl AJ937920)    219   QVQGSIQTQQQQPQQLGQCVSQPQQQSQQQLGQCSFQQPQQLQQLGQQPQQQQIPQGIFLQPHQISQLEV 288
                                                  ▲                ▲
NB16
B16C                    69   QVQGSIQTQQQQPQQLGQCVSQPQQQSQQQLGQCSFQQPQQLQQLGQQPQQQQIPQGIFLQPHQISQLEV 138

                                  ——————— Cter III ———————|
                                          ▼
CAA316685              251   MTSIALRILPTMCSVNVPLYRTTTSVPFGVGTGVGAY 287
P73 (embl TAE19835)    247   MTSIALRILPTMCSVNVPLYRTTTSVPFGVGTGVGAY 283
TAE17845               333   MTSIALRTLPTMCNVNVPLYRTTTRVPFGVGTGVGAY 369
B16 (embl AJ937920)    289   MTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGGY 325
                                          ▲
NB16
B16C                   139   MTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGGYlehhhhhh 183
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	Number of amino acids	Deduced molecular weight	pI	Aliphatic index
P73	284	32,465 Da	10.34	73.06
B16	325	37,017 Da	10.00	65.43
NB16	181	20,835 Da	9.2	49.3
B16C	183	20,642 Da	7.64	78.58

[a] Domains were those described by D'Ovidio and Masci (*21*). Parameters were computed from sequences including the amino acids brought by the vector, and the aliphatic index was computed using only glutenin amino acids. The amino acids brought by the vector are presented in lower case. The cysteine residues of proteins are marked with ▼ or ▲. The sequence of the reverse primer used for the N-end half-amplification to obtain the chimera is underlined.

Expression and Partial Purification of Recombinant Proteins and Peptides. The plasmids were introduced in the *E. coli* Rosetta (DE3) strain (Novagen, Madison, WI). This strain is adapted for expression through the T7 promoter and contains a plasmid pRare providing genes for tRNA specific for rare *E. coli* codons.

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

Bacteria were resuspended in 25 mM Tris-HCl pH 8 buffer and incubated with 0.3 μg/μL lysozyme for 30 min at 37 °C. After addition of 2% w/v SDS and 100 mM 2-mercaptoethanol (final concentration), the lysate was incubated at 65 °C for 1.5 h with shaking. After chilling, three volumes of ethanol were added (75% v/v final concentration) and incubation lasted for 1 h, before centrifugation (14000*g*, at room temperature). The ethanol extract was stored in a freezer until use. Proteins were precipitated overnight by adding 2 volumes of 7.5% w/v NaCl. The pellet was resuspended with Laemmli sample buffer (*17*)

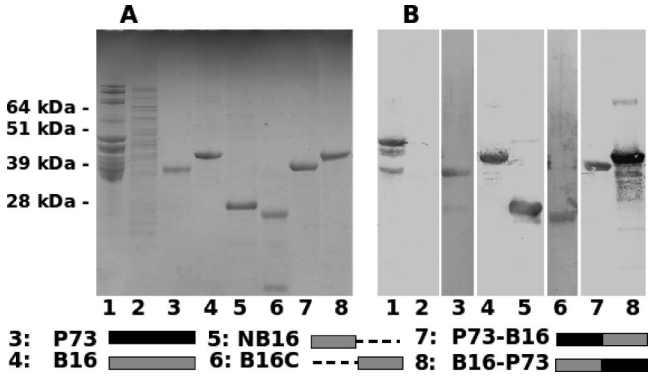


Figure 1. Expression of recombinant LMW-GSs and peptides, and their immunoreactivity with rabbit anti-LMW-GS serum. (**A**) Coomassie staining of SDS-PAGE. (**B**) Western blot and alkaline phosphatase detection using rabbit anti-LMW-GS serum. Lanes: 1, glutenin fraction from Neepawa wheat cultivar; 2, extract from *E. coli* grown with empty plasmid and after the same protocol purification as that for recombinant proteins; 3, recombinant LMW-GS P73; 4, recombinant LMW-GS B16; 5, recombinant peptide NB16; 6, recombinant peptide B16C; 7, chimeric glutenin P73-B16; 8, chimeric glutenin B16-P73.

and used in SDS-PAGE or in dot blot. Alkylation could be obtained through 30-min incubation of an alcoholic extract at 60 °C with 2% v/v 2-mercaptoethanol and another 30-min incubation at room temperature in the dark with 1 M iodoacetamide.

B16C and chimeric proteins were extracted using the same protocol. NB16 was extracted in the same way but did not precipitate with addition of salt solution. Then, the ethanol extract was evaporated under an air stream, and the sample was resuspended as above.

The protein (or peptide) content was quantified according to Vera's protocol (*18*), using TCA precipitation, adapted to the microplate format.

SDS-PAGE analysis was done using Novex NuPAGE precast gels from Invitrogen, Carlsbad, CA. For the natural glutenin fraction, large-size Tris-glycine classical gels were used. In some experiments, mass spectrometry was done using either a Maldi-Tof apparatus or a LC-MS instrument using an electrospray device.

Blotting Proteins on Membranes. Proteins were separated using SDS-PAGE under reducing conditions and electroblotted on PVDF membranes, using semidry conditions (*19*). For dot blot analysis, partially purified recombinant proteins were spotted directly on a PVDF membrane, using a Millipore dot blot device: about 10 μg of protein in solution, purified using 5% v/v 2-mercaptoethanol with or without reducer, was loaded into a solution containing 25% v/v ethanol, 10% v/v acetic acid, and 0.5% w/v SDS.

Rabbit IgG Binding Detection. A classical Western blot analysis was done using serum from a rabbit immunized with a natural LMW-GS fraction. Rabbit IgG proteins were detected using a secondary antibody (a goat antirabbit IgG alkaline phosphatase conjugate, purchased by Bio-Rad Laboratory. Inc.) and NBT/BCIP staining. Proteins were stained on a membrane with Indian ink (*20*) after immunodetection.

Human IgE Binding Detection. The membranes were incubated for 2 h in the blocking solution (2% w/v PVP 40, 0.1% w/v Tween 20, PBS) and overnight with serum from a patient allergic to wheat (dilution 1/12). After three washings in blocking solution, the membranes were incubated in 3% w/v dry milk, 0.1% w/v Tween-20 PBS with rabbit antihuman IgE, and HRP-conjugated antibody (from Dako S.A., Trappes, France), 1/25000 diluted. After five washings in the blocking solution and two washings in PBS, chemiluminescence was detected using a Supersignal West Dura extended duration substrate (Pierce Biotechnology) on a X-OMAT AR-5 film from Sigma-Aldrich, St Louis, MO) (*20*). All washing steps lasted 10 min each. In one experiment, the chemiluminescence signal was recorded using a Fuji Las3000 camera, and quantitative analysis was done using Bio1D software from Vilber-Lourmat, Torcy, France.

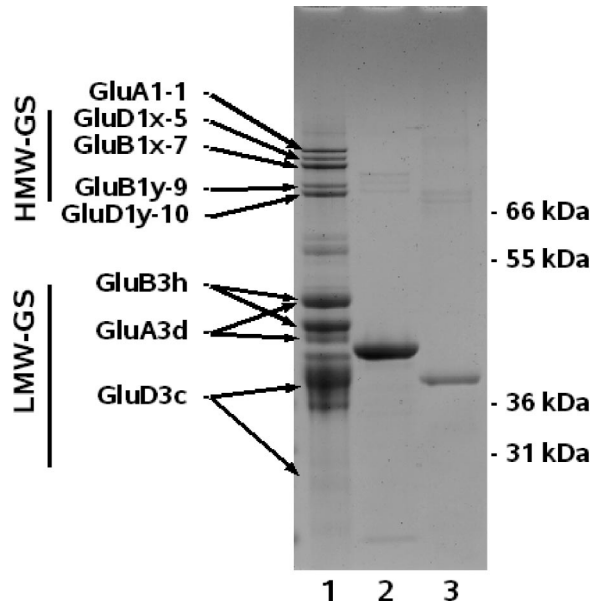


Figure 2. Comparison of recombinant glutenins with natural glutenins. Coomassie staining of SDS-PAGE. Lanes: 1, natural fraction of glutenins (HMW-GSs and LMW-GSs) from wheat (*Triticum aestivum*, cultivar Neepawa); 2, recombinant LMW-GS B16; 3, recombinant LMW-GS P73.

RESULTS AND DISCUSSION

Cloning of LMW-GSs, Fragments, and Chimeras. Two successive PCR runs, using the primers described in **Table 1**, led to cloning of the coding parts for two mature LMW-GSs, P73 and B16, respectively. Their sequences, shown in **Table 2**, were recorded in the EMBL nucleotide sequence database under the numbers *TAE519835* and *AJ937920*.

These sequences showed typical features of the LMW-GSs and high homologies. They displayed 70% identity when calculated on the whole amino acid sequence. They differed mainly in their repetitive parts and their number of cysteine residues. The repetitive domains showed only 57% identity, while their C-terminal parts showed 81% identity. They showed the typical repetitive motif PFSQ characteristic of B-type LMW-GSs (*21*). Moreover, with the N-end of B16 being SHIPGL. . . and that of P73 being METRCIP. . ., they belong to LMW-GS-s and -m, respectively. Concerning the cysteine residues, they where present at the canonical positions for LMW-GSs. According to ref *21*, the P73 sequence belongs to the first group of LMW-GSs with the first Cys in the N-terminus and B16 belongs to the second group with the first Cys in the repetitive domain. However, B16 is, to our knowledge, original, due to the presence of Cys residues at both positions, 237 and 252. Classically, LMW-GSs show a Cys residue at either one or the other position (*21*). Thus, B16 had nine Cys residues instead of eight, as usual.

Two fragments of the B16 gene were expressed, the N-end part (NB16), which contains mainly the repetitive domain, and the C-end part (B16C), which is less repetitive with interspersed short repetitive stretches (**Table 2**). The two sequences B16 and P73 also displayed the well conserved sequence apart from the fifth and sixth Cys's of the first domain of the C-terminal sequence. This sequence could be obtained using PCR chimeric constructs combining the N-end part of one protein and the C-end part of the other.

Expression, Purification, and Characterization of Recombinant Proteins and Peptides. Expression of B16, P73, and their derived constructs in a standard *E. coli* strain led to poor

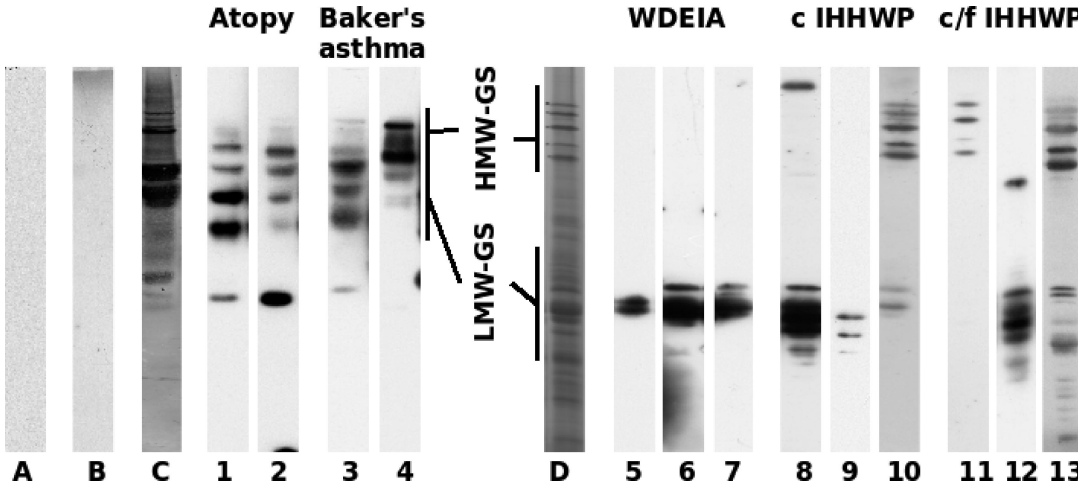


Figure 3. Examples of IgE binding patterns with natural LMW-GS fractions using 13 sera from patients suffering from different wheat-dependent allergies. Immunoblot analysis using natural glutenin fractions from wheat, cultivar Soissons, and chemiluminescence IgE detection. Two SDS-PAGE systems were used (lanes A–C and 1–4 using precast NuPAGE gels from Invitrogen; lanes D and 5–13 using large-size Tris-glycine gels). A, control without serum; B, control using a serum from a nonallergic person; C and D, Indian ink staining of proteins on the membranes. Numbers 1–13 correspond to individual sera.

Table 3. Ratio of Sera from Various Wheat-Allergic Patients Where IgE Bound Recombinant Glutenins and Peptides[a]

recombinant protein	disease				
	atopy	Baker's asthma	WDEIA	contact IHHWP	contact/food IHHWP
P73	0/2	5/7	5/5	3/4	4/6
B16	2/2	6/7	4/5	2/4	2/6
NB16	2/2	3/4	1/3	0/2	1/4
B16C	2/2	1/4	2/3	0/2	0/4
chimeric P73-B16		0/2	3/3	0/1	0/2
chimeric B16-P73		0/2	3/3	0/1	0/2

[a] Number of positive reactions compared to number of tested sera. Results from all dot blot or Western blot experiments were fused without consideration of signal intensity.

whole protein yields, with shorter fragments (data not shown). This was due mainly to differences in codon usage between wheat endosperm and *E. coli.* The use of the *E. coli* Rosetta (DE3) strain, which supplied tRNAs for rare codons in *E. coli*, increased the yield of full length proteins. The B16 and P73 sequences contain respectively 17 and 10 of these codons.

The proteins and peptides expressed in *E. coli* were isolated from the bacteria by taking advantage of their specific solubility properties: LMW-GSs were solubilized from lysed bacteria using SDS and 2-mercaptoethanol and were prepurified using ethanol extraction, which induced precipitation of most bacterial compounds while keeping glutenins in solution. The addition of salt induced the precipitation and the recovery of glutenins from the ethanol extract. The protocol was applied successfully to P73, B16, and B16C, but not to NB16, which did not precipitate from the salt solution. This showed that the properties of solubility are not distributed homogeneously on the molecules. The B16C peptide, which displayed a more diversified amino acid composition but with a high aliphatic index (**Table 2**), was salt insoluble, as was the whole protein. Surprisingly, the NB16 peptide, which corresponds to the main repetitive domain but with a lower aliphatic index (**Table 2**), was clearly more soluble. A similar observation was reported with HMW-GS. A peptide containing a nonrepetitive domain of a HMW-GS shared the solubility characteristics of the whole protein (*22*). On the contrary, the repetitive domain from the same HMW-GS was found to be water-soluble (*23*).

The proteins and peptides specifically extracted from bacteria displayed a satisfying purity. Every preparation showed one main band using SDS-PAGE and Coomassie blue staining or after immunoblotting using an anti-LMW-GS rabbit anti serum (**Figure 1**), except for the chimeric construct, which displayed minor additive products. The specific reaction with these antiglutenin antibodies confirmed that all the products produced belonged to the glutenin family. No reaction occurred with bacterial proteins after the same extraction protocol (**Figure 1**, panel **B**). The molecular weight analyses of B16 and P73 products using mass spectrometry confirmed that the proteins were expressed in full length and correspond to the sequence measured using mass spectrometry. They gave 32458 Da for P73 via LC-MS and 37763 Da for B16 using MALDI-TOF analysis. This last value slightly exceeds the expected mass for B16, suggesting the presence of some adducts still attached to the proteins.

Attempts to Identify the B16 and P73 Genes. The sequence of genes coding for B16 and P73 was compared to already published sequences. P73 exhibited a 98% identity with the gene described by Colot et al. (*24*) (EMBL accession number: *CAA316685*), which is located on chromosome 1 from the D genome. In addition, the P73 protein was shown to comigrate with the major LMW subunit of the Glu-D3c allele (**Figure 2**). This multiallele displays two main characteristic bands with specific mobilities using SDS PAGE, which allow its identification (*25*). These features strongly suggest that P73 is an expressed sequence from the Glu-D3c locus.

The B16 sequence exhibited a 76% identity with the gene described by Masci et al. (*26*) (EMBL accession number: *TAE17845*), which is located at the *Glu-B3* locus. This last gene is remarkable due to the regularity of the repeats in the repetitive domain. Stretches of glutamines (Q_{2-5}) are part of the motives of five to eight amino acids starting with proline and ending with two to five glutamines. There are 24 repeats, and 20 of them form five successive clusters of 29–30 amino acids starting with PPF/LS and ending with PVLP/PQQ. The two last clusters are lacking in B16. Without taking into account these two clusters and the B16 additional peptide with the ninth cysteine, a 95% identity is observed between the two genes. Using SDS-PAGE, B16 exhibited a similar mobility to that of an unidentified band with a lower M_r than those of the two bands identified

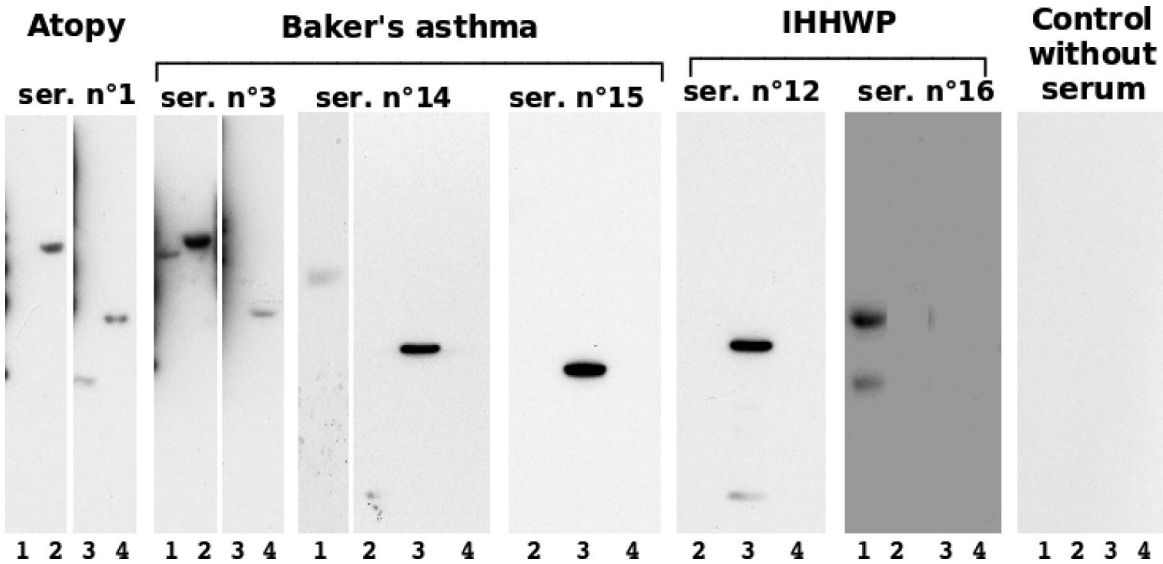


Figure 4. Examples of IgE binding patterns to recombinant LMW-GSs and peptides. Western blot analysis using chemiluminescence detection of IgE. The serum numbers correspond to those in **Figure 3**, with numbers above 13 corresponding to sera not used in **Figure 3**. Lanes: 1, recombinant LMW-GS P73; 2, recombinant LMW-GS B16; 3, recombinant peptide NB16; 4, recombinant peptide B16C. SDS-PAGE analyses were run in the Nupage Invitrogen system, using 12% gels and MES buffer for serum nos. 1 and 13, and 4–12% gels and MES buffer for the others.

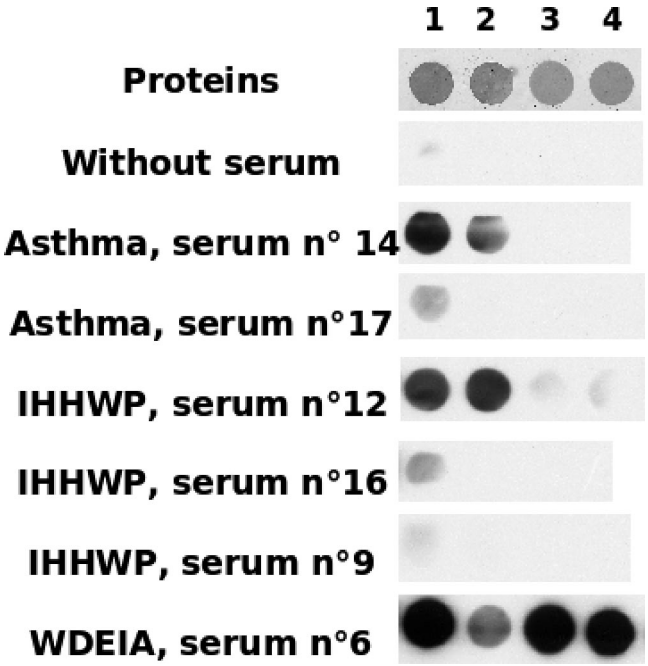


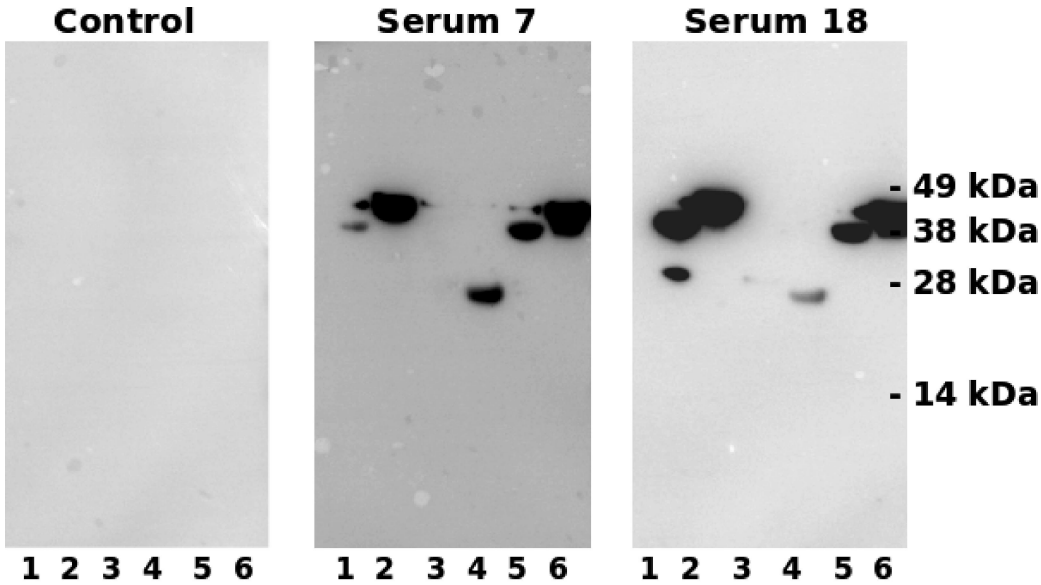
Figure 5. Dot blot analysis of IgE binding to recombinant proteins and chimeric constructs. Proteins were stained using amido black. IgE were detected using chemiluminescence. Serum numbers correspond to the numbers used in **Figures 3** and **4**. Other numbers are sera not shown in **Figures 3** and **4**. Lanes: 1, recombinant LMW-GS B16; 2, recombinant LMW-GS P73; 3, chimeric construct P73-B16; 4, chimeric construct B16-P73.

to be coded by the *Glu-B3 h* locus (**Figure 2**). These features did not lead to a clear allelic identification of B16 but showed a strong homology to the glutenin described by Masci et al. (*26*).

IgE Reactivity of Natural and Recombinant Glutenin Products. Glutenin fractions extracted from wheat grains and B16, NB16, B16C, P73, and the two chimeras were compared for their reactivity with IgE from the 24 selected human sera. They were analyzed using immunoblotting after SDS-PAGE or after dot blotting. Two different SDS-PAGE systems using large or small gels were used, depending on the amount of serum available. Dot immunoblotting was used only with recombinant proteins and peptides and after verification that IgE reacted specifically with only each recombinant product and not with bacterial proteins which could contaminate the preparations.

With almost all sera presented in **Figure 3**, IgE reacted with LMW-GSs extracted from grains. Some of them reacted with HMW-GSs. The pattern of glutenins binding IgE differed between patients, and diseases, in terms of the number and intensity of bands. This suggested differences in IgE reactivity between the subunit variants of LMW-GSs. This was confirmed when comparing the IgE reactivities of patients suffering from the same type of disease with the different recombinant products produced (**Table 3** and **Figures 4–6**). Clearly, despite the homologies of sequences, differences of reactivity were systematically observed between B16 and P73. The expressed peptides, NB16 and B16C, often showed strong differences in IgE binding. Interestingly, strong reactions with NB16 were observed in many cases where the whole B16 protein did not react (see sera 12, 14, and 15; **Figure 4**). This suggested that the epitopes involved were hidden in the whole molecule. The chimeric constructs reacted only with patients with WDEIA, among the patients tested (**Table 3** and **Figures 5** and **6**).

The case of WDEIA patients is interesting. The results presented here (**Figure 3**) clearly show that IgE of the tested patients reacted with natural and recombinant protein and peptides, but not with HMW-GSs. WDEIA patients were first described to react with a major allergen, ω-5 gliadins (*4*), and more recently also with HMW-GSs (*27*). Neither P73 nor B16 sequences showed the epitopes QQPGQ, QQPGQGQQ, and QQSGQGQ, found in the HMW-GS (*27*). The epitope sequences involved in ω-5 gliadin have been determined (*28–30*). They correspond to the consensus structure QQX1PX2QQ (X1 being L, F, S, or I with X2 being Q, E, or G) (*28, 29*). One of these epitopes ($Q_{35}QFPQQQ_{41}$) was found exactly in B16 and NB16, but not in the P73 sequence. This could explain the higher reactivity of B16 compared to that of P73 (**Figure 6**). Surprisingly, NB16 did not react in two out of three patients while B16C did (**Figure 6** and **Table 3**). This could suggest



IgE chemiluminescence quantitative analysis (arbitrary units)

Lane		Serum n° 7	Serum n° 18
1	P73	3.6	54.4
2	B16	68.1	102.7
3	NB16	0	0
4	B16C	9.6	1.1
5	P73-B16	13.5	12.8
6	B16-P73	63.7	80.8

Figure 6. IgE binding patterns to recombinant LMW-GSs and peptides, and chimeric glutenins, using two WDEIA patients (serum no. 7, from **Figure 3**, and serum no. 18, not shown in other figures). Western blot using chemiluminescence IgE detection recorded through a camera, allowing quantitative analysis. The data shown in the table represent IgE response intensity, in arbitrary units. The control membrane was incubated without serum. Lanes: 1, recombinant LMW-GS P73; 2, recombinant LMW-GS B16; 3, recombinant peptide NB16; 4, recombinant peptide B16C; 5, chimeric glutenin P73-B16; 6, chimeric glutenin B16-P73. Protein amounts were 2.5 μg per lane.

the involvement of other structures in WDEIA. Conversely, the presence of NB16 in the chimeras strongly enhanced the reactivity of the construct.

Evidence on the Role of Protein Conformation for IgE Binding. As mentioned above, the results observed with B16, NB16, B16C, P73, and the two chimeras on the IgE reactivity of WDEIA patients suggested the involvement of other structures in WDEIA, specific to LMW-GS. They also suggested a different role of NB16 and B16C in the reactivity of the whole molecules and the chimeras. Until now, no post-translational modifications were found on LMW-GSs. Thus, the hypothesized specific structures could be more likely peptidic and/or conformational epitopes. However, the differences of reactivity observed between B16, NB16, P73, and the two chimeras suggested conformational effects on the reactivity of IgE. Indeed, if we except the additional peptide in the B16 C-terminal, the differences between B16 and P73 lied mainly in the N-terminal halves of the molecules. So, P73 and the P73–B16 chimera had rather similar sequences and probably structures. This is in accordance with their low reactivity (**Figure 6**). On the other hand, with the same restriction, B16 and chimeric B16-P73 showed strong homologies, which are translated into higher reactivity with IgE. The fact that NB16 alone did not react with IgE of sera 7 and 18 and that its presence in B16 and B16-P73 increased the reactivity suggested a different role of the N-terminal halves in the IgE binding properties of LMW-GS and the implications of conformational changes.

Conclusion. Due to their similar properties and their relatively high polymorphism, gliadins and LMW-GSs are rather difficult to separate. Until now, the possible involvement of LMW-GSs in the allergic reactions in wheat allergies was based only on the observation of IgE binding in semipurified fractions. Well characterized recombinant proteins are possible tools to get unbiased results.

Indeed, as natural LMW-GSs do not present post-translational modifications and are necessarily studied after reduction of their disulfide bridges, heterologous expression in *E. coli* was well adapted. This was confirmed by the results presented here, which demonstrate that the LMW-GS protein and fragments heterologously expressed in *E. coli* are also IgE reactive as natural proteins. As a consequence, LMW-GSs are potential allergens, either intrinsically or through cross-reactivity with other prolamins. Common epitopes were found with gliadins, but evidence on the involvement of other specific antigenic structures was obtained.

Comparing two different recombinant whole molecules of LMW-GS, peptidic fragments, and chimeras showed clearly the role of the conformation of the molecules in their IgE reactivity.

Also, the IgE binding and the physicochemical properties, such as water insolubility, depended on the specific parts of the LMW-GS sequences. The repetitive domain in the N-terminal part of the molecule, which is richer in proline and glutamine, was more soluble than the C-terminal part. Also, the presence of the N-terminal half of B16 (NB16) in the structure of B16 and the chimera B16-P73 increased the reactivity with IgE. These observations evidenced the plasticity of the LMW-GS structures and show that they can be manipulated either through genetic selection or processing treatments to modify the IgE reactivity of the LMW-GS group and thus the allergenicity of grain proteins.

ABBREVIATIONS USED

LMW-GS, low-molecular-weight glutenin subunits; HMW-GS, high-molecular-weight glutenin subunits; WDEIA, wheat-dependent exercise-induced anaphylaxis; IHHWP, immediate hypersensitivity to hydrolyzed wheat proteins, after skin contact (c IHHWP) and after skin contact or food ingestion (c/f IHHWP).

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