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A Recombinant ω-Gliadin-like D-Type Glutenin and an α-Gliadin from Wheat (*Triticum aestivum*): Two Immunoglobulin E Binding Proteins, Useful for the Diagnosis of Wheat-Dependent Allergies

Hamza Mameri,[†,▽] Isabelle Bouchez,[†] Catherine Pecquet,[‡] Nadia Raison-Peyron,[§] Dominique Choudat,[∥] Habib Chabane,[⊥] Stephane Kerre,[#] Sandra Denery-Papini,[▽] Yann Gohon,[†] Pierre Briozzo,[†] Michel Laurière,[○] and Jacques Snégaroff*[,†]

[†]INRA, UMR1318, Institut Jean-Pierre Bourgin, F-78000 Versailles, France

[‡]AP-HP, Hôpital Tenon, Centre d'Allergologie, F-75020 Paris, France

[§]CHU Montpellier, Hôpital Saint-Eloi, Service de dermatologie, F-34295 Montpellier Cedex 05, France

[∥]AP-HP, Groupe hospitalier Cochin, Service de pathologie professionnelle, Université Paris Descartes, 75679 Paris Cedex 14, France

[⊥]Hôpital Delafontaine, F-93200 St-Denis, France

[#]Huidziekten, Gijmelsesteenweg 16, B-3200 Aarschot, Belgium

[▽]INRA, UR 1268 Biopolymères, Interactions, Assemblages, F-44316 Nantes, France

[○]INRA, UMR 206 Chimie-Biologique, F-78850 Thiverval-Grignon, France

Supporting Information

ABSTRACT: Among the wheat prolamins, D-type glutenins display a highly repetitive sequence similar to ω-gliadins, but they contain a cysteine, that allows them to be included in the gluten macropolymers. An ω-gliadin-like D-type glutenin, an α-gliadin, and an ω5-gliadin-like D-type glutenin were obtained as recombinant proteins and compared using synchrotron radiation circular dichroism. This technique evidenced the strong thermostability of the ω5-gliadin-like protein. The IgE reactivity of recombinant proteins was evaluated using 45 sera from wheat-allergic patients. The sera from patients diagnosed with cutaneous hypersensitivity to hydrolyzed wheat proteins often reacted with the ω-gliadin-like D-type glutenin and α-gliadin, whereas the IgE reaction was less frequent after dietary sensitization. So, these two proteins could be useful to diagnose these diseases. The sera from patients with exercise-induced anaphylaxis recognized the ω5-gliadin-like protein as a positive control and, less frequently, the other proteins tested. Only some sera from patients with baker's asthma reacted with the proteins tested.

KEYWORDS: *allergy, D-type glutenin, gliadins, IgE reactivity, synchrotron radiation circular dichroism, wheat (Triticum aestivum)*

INTRODUCTION

Bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) are major crops in Western countries where they constitute an important source of food. The grains contain starch as well as 8−15% proteins. Most of these proteins are gluten proteins, and they constitute a complex mixture of polymorphic components. They belong to the prolamin family, so-called because of their high content in proline and glutamine residues. Some of them are included into high size gluten polymers through intermolecular disulfide bonds.[1]

Individual wheat prolamins are divided into three groups according to their polymerization, their content in cysteine residues, their molecular weight, their N-terminal sequence, and their genetic relationships.[2] The first group contains the high molecular weight glutenin subunits (HMW-GSs), which are a part of the gluten polymers. The second group comprises the sulfur-rich (S-rich) prolamins, divided into the low molecular weight glutenin subunits (LMW-GSs), which are included in gluten polymers, and the essentially monomeric α- and γ-gliadins. The third group, the wheat S-poor prolamins, constitutes a very particular prolamin group: their sequences are almost entirely composed of a highly repetitive domain with short tandem peptides rich in proline and glutamine residues.[4] This group contains the ω-gliadins, which are monomeric and lacking in cysteine residues.

In addition, the LMW-GSs are divided according to their sequence into the B-type, the typical and most abundant LMW-GSs; the C-type, corresponding to modified α and γ-gliadins; and the D-type, the less abundant glutenins, corresponding to ω-gliadins but containing one cysteine,[3] available for binding into gluten polymers: such glutenins can constitute ends for the intermolecular disulfide-bridge chains.[5] So, the D-type glutenin subunits actually can be included in the S-poor prolamins group but found in gluten macropolymers. These glutenins are only known through their amino acid composition, their N-terminal sequence, the presence of a cysteine,[5] and expressed sequence tags (EST) analysis.[6]

In addition to their role in human nutrition, wheat grain proteins can unfortunately trigger several diseases including

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gluten intolerance, known as celiac disease, and various IgE-mediated allergies. These latter differ according to their sensitization route, with variations depending on the age of patients. Wheat ingestion can induce dietary allergy, involving classical symptoms like generalized urticaria, gastrointestinal symptoms, or anaphylaxis.[7,8] It can also be involved in atopic eczema dermatitis syndrome. Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a form of allergy resulting from both wheat ingestion and exercise.[9] Another kind of dietary allergy has been observed, not after ingestion of traditional flour products but after ingestion of food containing hydrolyzed wheat proteins (HWP) used as additives: this disease is called dietary immediate hypersensitivity to hydrolyzed wheat proteins (IHHWP).[10,11] In some cases, the reaction to HWP can be induced by exercise like in WDEIA.[12] Flour inhalation can trigger respiratory allergy including baker's asthma and rhinitis.[13] Skin contact with cosmetics containing HWP used as additives has been found to be responsible for cutaneous allergy, characterized by contact urticaria or edema (cutaneous IHHWP).[10] In rare cases, a cutaneous allergy has also been observed after skin contact with flour.[14]

Most of the wheat allergens identified belong to salt-soluble fractions and some of them to prolamins. The characterization in vitro of IgE-reacting prolamins using Western blot technique after one- or two-dimensional (1D or 2D) electrophoresis generally shows that only some proteins out of the numerous prolamins are involved and that they often differ according to the type of allergy, as shown, for example, in a previous work.[15] For wheat allergy diagnosis, the concentration of IgE specific to wheat in patient sera is usually considered as a poor marker. Indeed, it is well-known that prolamins are under-represented in the commercial wheat extracts used in diagnostic tests. To improve the available diagnostic tools, it is important to effectively identify the in vitro IgE binding proteins specifically involved in each type of wheat allergy: these proteins could be valuable as disease markers.

Among the gliadins involved in allergy, specific attention has been turned to ω5-gliadin, which has been described as a major allergen in WDEIA[16−18] and in food allergy in children.[19] This allergen is now included in commercial specific IgE quantization tests, and so, it is helpful in diagnosis.[20,21] Other IgE binding gliadins have been detected in dietary IHHWP:[15] they belong to ω- and γ-gliadins. Few studies have revealed the IgE reactivity of the other groups of gliadins. For instance, the IgE binding to purified gliadins was analyzed and showed a non-negligible reactivity to all gliadin fractions, mainly to ω-gliadins but to α- and γ-gliadins as well;[22] in another study, phage display was used to identify gliadin fragments binding IgE in baker's asthma.[23] The α- and γ-gliadins are well-known to interact with the immune system mainly in celiac disease, in which α- and also some γ-gliadins bind HLA receptors and so induce this intolerance reaction (for example, see Kim et al.[24]).

The present work aimed at cloning the genes coding for some gliadins to produce recombinant proteins for IgE binding analysis. The authors obtained an α-gliadin and two recombinant D-type glutenins, one of them being ω5-gliadin-like, the well-known allergen. Since gliadins are known to be insoluble in aqueous solutions, there are no available data showing a high-resolution structure obtained by crystallography. Information on their secondary structures was therefore obtained using a synchrotron radiation circular dichroism experiment. The IgE reactivity of these recombinant proteins was analyzed using sera from patients allergic to wheat. The authors selected a cohort of patients who were suffering from IHHWP, which comprises a type of allergy known to involve ω-gliadin (dietary IHHWP), as mentioned above, and which is difficult to diagnose, and other types of allergy involving different specific sensitization routes (cutaneous IHHWP) and respiratory allergy to wheat. To obtain a comparison, the authors also selected some patients diagnosed with WDEIA and used the ω5-gliadin-like protein as a positive control for IgE binding.

MATERIALS AND METHODS

cDNA Synthesis. Grains from wheat (*T. aestivum*), cultivars Prinqual and Neepawa, were provided by G. Branlard, INRA, UMR ASP-UBP, Clermont-Ferrand, France, and grown at the Grignon INRA research center. Immature grains were harvested 14 days after anthesis and stored at −80 °C. The total RNA was extracted using a midi spin columns kit (Macherey-Nagel GmbH, Düren, Germany). cDNA was then synthesized with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using the adapter primer (Invitrogen, 3′RACE kit) containing an oligo-dT moiety.

Primer Design and PCR Cloning. A search using translated BLAST (Tblastn from US National Library of Medicine) in the database of Expressed Sequence Tags (dbEST) on the NCBI Web site made it possible to join the following EST into a consensus sequence of a D-type glutenin: *CA734224*, *BQ804665*, and *BQ804424*. This consensus was in agreement with other ESTs, *BJ235153*, *CJ635325*, and *BJ232292*. After identification of the translated signal peptide MKTFLIFVLLAMAMNIATA and of the consensus N-end of some mature ω-gliadins ARQL, specific primers were designed (Table S.1 in the Supporting Information).

The D-type glutenin (referred to as DG14) corresponding to this consensus and an α-gliadin were cloned using primers and PCR conditions shown in Table S.1 in the Supporting Information. The DNA polymerase used was Accuprime Pfx (Invitrogen). Another D-type glutenin close to ω5-gliadin and called A12 was obtained using cDNA from wheat Prinqual cultivar as previously described.[25]

Expression and Partial Purification of Recombinant Proteins. The T7express *E. coli* strain (New England Biolabs, Beverly, MA) was transformed with recombinant plasmids. After overnight preculture, the pelleted bacteria were resuspended in 200 mL of Turbo broth medium (AthenaES, Baltimore, MD), grown for 1 h, and further incubated for 5 h with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were harvested, washed with 0.9% (w/v) NaCl, pelleted, and frozen until used. Bacteria, resuspended in 50 mM Tris-HCl, pH 8.5, buffer containing protease inhibitors (Roche-Diagnostics, Mannheim, Germany), were disrupted using cell disrupter (Constant Systems, Daventry, United Kingdom). After 1 h of incubation at 37 °C with 0.1 mg of Dnase1 and another one with 1 M NaCl, inclusion bodies were pelleted and then solubilized in 50 mM Tris-HCl, pH 8.5, buffer containing 2% (w/v) sodium dodecyl sulfate (SDS) or 8 M urea. All proteins from these pellets were reduced using a 30 min incubation with 0.1 M DTT. After the addition of 2 volumes of ethanol, 1 h of incubation, and centrifugation, the reduced proteins were alkylated using a 30 min incubation in the dark with 0.5 M iodoacetamide. They were further recovered from supernatant using acetone precipitation (3 volumes and overnight incubation at 4 °C) and resolubilization in 50 mM Tris-HCl buffer, pH 7.5, containing 2% (w/v) SDS or 8 M urea. For dot-blot experiments, the proteins were extracted without SDS but were solubilized in urea. The N-terminal half of α-gliadin was cloned and expressed alone, and since it was soluble and not in inclusion bodies, it was directly extracted from crude lysates by adding 2% (w/v) SDS and then 0.75 volumes of ethanol and recovered from supernatant using acetone precipitation as described above.

Extraction of Natural Glutenins and Gliadins. Flours from wheat, cultivars Prinqual and Neepawa, were obtained by grinding grains for 1 min in a steel ball mill. Flours were submitted to extraction

Table 1. Summary of Clinical Features of the 45 Wheat-Allergic Patients Tested for IgE Binding to Recombinant Proteins

type of allergy	no. of patient sera	sensitization agent	symptoms	diagnosis elements
cutaneous IHHWP sera no. 1−9	9 adults	nos. 1−7, 9: HWP (cosmetics additive)	contact urticaria or edema	SPT[a] to cosmetics and HWP: positive
		no. 8: flour	no reaction after wheat-product ingestion	SPT to wheat and wheat flour: negative
				IgE specific to gluten, wheat flour: negative
dietary IHHWP sera no. 10−21	11 adults + a 10 year old girl (no. 20)	no. 10−21: HWP (food additive)	dietary allergy symptoms[b]	SPT to HWP: positive
		nos. 10−12, 14, 17: HWP (food and cosmetics additive)	no reaction after wheat-product ingestion	SPT to wheat: negative or weakly positive
		nos. 19−21: HWP + exercise		IgE specific to gluten, wheat flour: weakly positive
WDEIA sera no. 22−37	16 adults	wheat product ingestion + exercise no. 36: aspirin intake[c]	anaphylaxis	SPT to wheat and wheat flour: either positive or negative
				IgE specific to gluten, wheat flour and ω5-gliadin: positive
wheat respiratory allergy sera no. 38−45	8 adults	wheat flour inhalation	asthma or rhinitis	
			no reaction after wheat-product ingestion	

[a]SPT, skin prick test. [b]Dietary allergy symptoms: generalized urticaria, gastrointestinal symptoms, or anaphylaxis. [c]Similar reactions also have been described.[36]

according to a protocol derived from Singh et al.,[26] using 50% (v/v) 1-propanol in water. The supernatant contained the gliadin fraction. Reduction and alkylation were obtained using two consecutive 30 min incubations of the gliadin fraction, first at 65 °C with 0.2 M β-mercaptoethanol, and second, in dark, at room temperature with 1 M iodoacetamide. The pellet resulting from gliadin extraction was incubated with 35% w/w 1-propanol in 0.1 M acetic acid containing 0.2 M β-mercaptoethanol. After centrifugation, the supernatant was alkylated as above, and it contained the glutenin fraction. To run SDS−polyacrylamide gel electrophoresis (PAGE), gliadin and glutenin fractions were recovered from 1-propanol extracts after a speed-vac evaporation and resuspension in 50 mM Tris-HCl, pH 8, buffer containing 2% (w/v) SDS.

Protein Quantization and SDS-PAGE. Protein quantization was performed using Vera's protocol adapted to microplate format,[27] and SDS-PAGE was run with precast gels from Invitrogen (Carlsbad, CA), following the manufacturer's recommendations. Gels were stained using Neuhoff's method.[28]

Synchrotron Radiation Circular Dichroism. The recombinant D-type glutenins and N-terminal half of α-gliadin, obtained after precipitation from alcoholic extract, were resuspended in 50 mM sodium phosphate buffer, pH 6.0, containing 2% (w/v) SDS and 10 mM sodium sulfate. After a centrifugation for 30 min at 20800*g* at room temperature, the supernatant was used in synchrotron radiation circular dichroism (SRCD) measurements, carried out on the DISCO beamline at the Soleil synchrotron (Gif-sur-Yvette, France).[29] Scans using wavelengths from 170 to 280 nm with 1 nm steps were measured four times for each sample. Buffer spectra 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 (CSA)[30] using the CDtool software.[31] Spectra were normalized with protein concentrations. Ratios of secondary structures were determined using the ContinLL program[32] in Dichroweb,[33] with SP175 and set 5 as references. The SP175 database contains the largest set of spectra (72 soluble proteins) to date.[34] The set 5 contains 17 proteins, and it allows calculation of the poly(Pro)II helix (PPII) content.[35] Normalized root-mean-square deviation (NRMSD) provided insight into the fitting accuracy for each spectrum.

Human Sera from Allergic Patients. The IgE reactivity toward recombinant D-type glutenins and α-gliadin was tested in dot-blot experiments using 45 human sera: the patients belonged to four groups of wheat-allergic patients that differed by wheat allergy type. The patients' clinical features are summarized in Table 1.

Nine patients were diagnosed with cutaneous IHHWP, 12 patients were diagnosed with dietary IHHWP, 16 patients were diagnosed with WDEIA, and eight patients were diagnosed with wheat respiratory allergy (asthma or rhinitis). In the cutaneous IHHWP group, all patients reacted to HWP, except for one (patient no. 8) who had reacted to wheat flour contact. The dietary IHHWP group was more heterogeneous, since some patients displayed both dietary and cutaneous reactions, and for some of them, the reaction was induced by exercise. For these two groups, the patients did not react after wheat ingestion.[10,11] In the WDEIA group, one patient reacted after aspirin intake as already described.[36] This study was approved by the Committee for the Protection of Human Subjects in Biomedical Research of Cochin Hospital (Paris, France), and each patient gave informed consent before blood sampling.

Protein Blotting. A Western blot experiment was carried out after SDS-PAGE and transfer to a membrane according to Laurière's protocol.[37] For dot-blot experiments, recombinant proteins were solubilized in a 50 mM Tris-HCl, pH 8.5, buffer containing 8 M urea. The proteins were dot blotted on a PVDF membrane using the Millipore dot-blot device, the proteins being added to a carrier solution (25% v/v ethanol, 10% v/v acetic acid, and 1 M urea). The amounts of A12 ω5-gliadin-like D-type glutenin were 16 μg, and the amounts of the other proteins were proportional to their molecular weight to obtain equimolar deposits.

IgE Binding Detection. Membranes were incubated for 1 h in the blocking solution: TBS (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl) containing 2% (w/v) polyvinylpyrrolidone 40 and 0.1% (w/v) Tween 20 and further incubated overnight with patient sera (final dilution 1/20). After 10 min washes in blocking solution, membranes were incubated for 2 h with rabbit HRP-conjugated antibody against human IgE (DakoCytomation, Denmark) at a final dilution of 1/15000 in TBS containing 3% (w/v) dry milk and 0.1% (w/v) Tween 20. Membranes were washed in blocking solution and further incubated with the Super Signal West Dura Extended Duration substrate (Pierce-Thermofisher, Rockford, IL). IgE chemiluminescence was recorded using a Fuji Las3000 camera (Fujifilm, Tokyo, Japan), and semiquantitative analysis was done with the Multi-Gauge software (Fujifilm). As a negative control and to assess the specificity of the chemiluminescence signal, a membrane was always incubated without serum and processed in the same way.

RESULTS AND DISCUSSION

Recombinant D-Type Glutenins and α-Gliadin. The gene of a protein called DG14 was cloned and recorded in the EMBL sequence database under EMBL ID *FM212916*. This recombinant protein contains one Cys residue, the ω-gliadin N-terminal consensus ARQL resulting from the signal peptide

Table 2. Comparison between DG14 Sequence and Translated ESTs from a Database of Expressed Sequence Tags (dbEST) on the NCBI Website[a]

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                10        20        30        40        50        60        70
CA734224  hegqasssnhksnmktflifvllamamniataARQLNPSNKELQSPQQSFSHQQQPFPQQPYPQQPYPSQ
BQ804665  ----------------------------------------------------QQPFPQQPYPQQPYPSQ
BQ804424  ------------------------------------------------------------------
Dg-14     --------------------------------ARQLNPSNKELQSPQQSFSHQQQPFPQQPYPQQPYPSQ

                80        90       100       110       120       130       140
CA734224  QPYPSQQPFPTPQPQFPQQSQQPFpQPQQPiPLQPQQPFPQQPQQPQQPFPQPQQPFPWQPQQPFPQTQQ
BQ804665  QPYPSQQPFPTPQPQFPQQSQQPFTQPQQPTPLQPQQPFPQQPQQPQQPFPQPQQPFPWQPQQPFPQTQQ
BQ804424  ------------------------------------------------------------------
Dg-14     QPYPSQQPFPTPQPQFPQQSQQPFTQPQQPTPLQPQQPFPQQPQQPQQPFPQPQQPFPWQPQQPFPQTQQ

               150       160       170       180       190       200       210
CA734224  SFPLQPQQPFPQQPQQPFsQPQLQFPQQPEQIIPQQPQQPFLLESQQPFPQQPQQPFsQPQQLIPMQxxQ
BQ804665  SFPLQPQQPFPQQPQQPFPQPQLQFPQQPEQIIPQQPQQPFLLESQQPFPQQPQQPFPQPQQLIPMQPQQ
BQ804424  ----------------------------------------------------------fPRlnnPnnqfp
Dg-14     SFPLQPQQPFPQQPQQPFPQPQLQFPQQPEQIIPQQPQQPFLLESQQPFPQQPQQPFPQPQQLIPMQPQQ

               220       230       240       250       260       270       280
CA734224  ------------------------------------------------------------------
BQ804665  PFPQQSQQSQQPFPGPQQLFPELQQPIPQQPQQPFPLQPQQPFPQQSQQPFPQQPQQPCPLQPQQPFPQQ
BQ804424  qqPQQpfpqtQqpqqPfpqlqQpQQPIPQQPQQPFPLQPQQPFPQQSQQPFPQQPQQPCPLQPQQPFPQQ
Dg-14     PFPQQSQQSQQPFPGPQQLFPELQQPIPQQPQQPFPLQPQQPFPQQSQQPFPQQPQQPCPLQPQQPFPQQ

               290       300       310       320       330       340       350
CA734224  ------------------------------------------------------------------
BQ804665  PQQPFPQ--------------------------------------------------------------
BQ804424  PQQPFPQQPQQPFPLQPQQPFPLRPQQPFSQQPQQSQQSFPQPQPQQPQQPSILQPQQPFLQPQQQLSQQ
Dg-14     PQQPFPQQPQQPFPLQPQQPFPLRPQQPFSQQPQQSQQSFPQPQPQQPQQPSILQPQQPFLQPQQQLSQQ

               360       370       380       390
CA734224  ----------------------------------------------
BQ804665  ----------------------------------------------
BQ804424  LEQTISQQPQQPFPQQPHQPQQPYPQQQPYGSSLTSIDGQ*-------
Dg-14     LEQTISQQPQQPFPQQPHQPQQPYPQQQPYGSSLTSIDGQlehhhhhh
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[a]In lowercase letters, amino acids differing from consensus. *: stop.

removal,[38] and the C-terminal sequence PYGSSLTSIDGQ, similar to those of ω-gliadin. To evaluate the existence of this protein in wheat, translated Expressed Sequence Tags (ESTs) from the EST database on the NCBI Web site were manually assembled, because the alignment software cannot correctly take highly repetitive sequences into account, and moreover, ESTs can display some uncertainty at the end of sequences. Although the ESTs in the database correspond to different alleles and wheat cultivars, the recombinant DG14 protein displayed a sequence that could correspond to an EST consensus (Table 2): this indicated that such a protein actually existed in wheat. This result was also in agreement with the results of EST analysis done by Anderson et al.[6] So, it was clear that the DG14 protein was a recombinant D-type glutenin. If we refer to the N-terminal consensus ARQL, the DG14 D-type glutenin is probably encoded by chromosome 1 from the A or D genome.[6] The motif of tandem repeats is the octapeptide PFPQQPQQ with some variations (sequence shown in Table S.2 in the Supporting Information).

Different electrophoresis techniques have been tried to visualize the natural protein corresponding to the recombinant DG14 D-type glutenin. The gliadin fraction and the reduced-alkylated glutenin fraction were compared with the DG14 D-type glutenin using 1D SDS-PAGE, acid PAGE, and even a 2D electrophoresis acid PAGE—SDS-PAGE (data not shown): the natural protein equivalent to D-type glutenin was not clearly visible, because it was expressed at a low level, and moreover, it could be divided up between both glutenin and gliadin fractions, because the binding to gluten polymers through a disulfide bridge could be incomplete. Without any D-type glutenin-specific staining, it was not possible to accurately identify it among other prolamins. We also cannot discard the hypothesis that an artifact occurred in the reverse transcription or PCR used in the cloning process, leading to a recombinant protein that displayed a size slightly different from that of the natural one. This could be due to some deletion difficult to identify as well as the EST consensus itself that was obtained from highly repetitive sequences.

We also obtained a recombinant D-type glutenin similar to an ω5-gliadin and called A12 (EMBL ID *FM212917*), whose cloning was previously described[25] (sequences are shown in Table S.2 in the Supporting Information). This recombinant A12 protein was almost identical to the ω5-gliadin (Genebank ID AB181300) cloned by Matsuo et al.[18] except for a deletion (sequences shown in Tables S.2 and S.3 in the Supporting Information). Moreover, almost all of the peptides from this deletion are repeats found elsewhere in the A12 sequence (peptide identities are shown in Figure S.1 in the Supporting Information using dottup software from the EMBOSS package,[39] a software useful with highly repetitive sequences). This deletion could be due to either natural polymorphism among ω5-gliadins or, more likely, an artifact due to reverse

transcription or PCR in relation to repeats in DNA, given that there was no evidence of a natural ω5-gliadin of this size (data not shown, but this will be confirmed later in this study). Another clone of ω5-gliadin-like protein was similarly obtained (EMBL ID *FM212918*), displaying another deletion compared to the ω5-gliadin cloned by Matsuo et al.,[18] but this protein was not chosen for IgE binding studies, because some linear epitopes found in ω5-gliadin[40] were missing. A12 displayed a C-terminal end containing a Cys residue, resulting in its classification in the D-type glutenin group. It was probably a simple mutation of Ser into Cys, which is also found in some translated ESTs like *CD896770* and *CD919298*, that display the sequence: ...SPYQQYPQQQPSGSDVISICGL*. Since the natural protein corresponding to the recombinant A12 ω5-gliadin-like D-type glutenin displayed the N-terminal consensus SRLL sequence (the Ser residue was modified into Gly by cloning), it was probably encoded at the *Gli-B1* locus (chromosome 1 from the B genome)[38,6]

Even if some repeats in the recombinant A12 ω5-gliadin-like D-type glutenin were missing and if this corresponded to the loss of some epitopes in our experiments, this protein containing the linear epitopes[40] from ω5-gliadin could nevertheless be useful for IgE binding characterization, as a positive control in WDEIA.

A recombinant α-gliadin was also obtained (Table S.2 in the Supporting Information): it shared 90% and 87% identity, respectively, with the sequences recorded under Genbank ID ABS72144 and EMBL ID *M11075* (Table S.4 in the Supporting Information). This protein contained two moieties: a N-terminal half containing tandem repeats rich in glutamine and proline residues, without a cysteine residue, and a C-terminal half with a more varied amino acid composition and containing six cysteine residues. Moreover, each half of α-gliadin contained a large cluster of 15 and 12 glutamine consecutive residues, respectively. Since the N-terminal α-gliadin half displays an amino acids composition close to ω-gliadins and contains repeats rich in proline and glutamine residues, it was interesting to compare its secondary structure content to that of D-type glutenins. So, structural SRCD analysis was carried out with this N-terminal α-gliadin half, cloned and expressed alone (see the sequences in Table S.2 in the Supporting Information): this moiety contained the first cysteine of α-gliadin. The structural analysis of whole α-gliadin seemed outside the scope of this study because the C-terminal half differed considerably from ω-gliadin-like proteins. The whole α-gliadin was only used in IgE binding experiments, because both α-gliadin halves were significantly less reactive with all sera (data not shown).

Some physicochemical properties of the recombinant proteins used in this work, as well as their content in linear epitopes described in the literature, are summarized in Table S.5 in the Supporting Information. The two recombinant D-type glutenins are characterized by a lower isoelectric point compared to that of α-gliadin and that of B-type LMW glutenin subunits, which is typically higher than 8.[4,3] All of these proteins contain about 40% of hydrophobic amino acids that are evenly distributed along the sequence and only few ionizable amino acids. The high content of glutamine residues can favor aggregation through hydrogen bounds. This explains the poor solubility of recombinant proteins in salt solution and their solubility in alcohol.

Sequence comparisons between these recombinant gliadins are shown in Figure S.2 in the Supporting Information, using dottup software from the EMBOSS package.[39] The length of peptides used as the parameter in dottup software was seven amino acids, the same as the length of the observed ω5-gliadin linear epitopes.[40] Only few peptides from the A12 ω5-gliadin-like D-type glutenin were shared by the recombinant DG14 D-type glutenin (Figure S.2A in the Supporting Information) and only one by α-gliadin (QQQILQQ). The recombinant DG14 D-type glutenin shares only three peptides with α-gliadin (Figure S.2B in the Supporting Information).

SRCD Analysis of Secondary Structure Contents. The recombinant D-type glutenins, α-gliadin and N-terminal half of α-gliadin, were analyzed using SRCD (Figure 1). In this experiment,the spectra displayed by the recombinant DG14 and A12 ω5-gliadin-like D-type glutenins were clearly different.

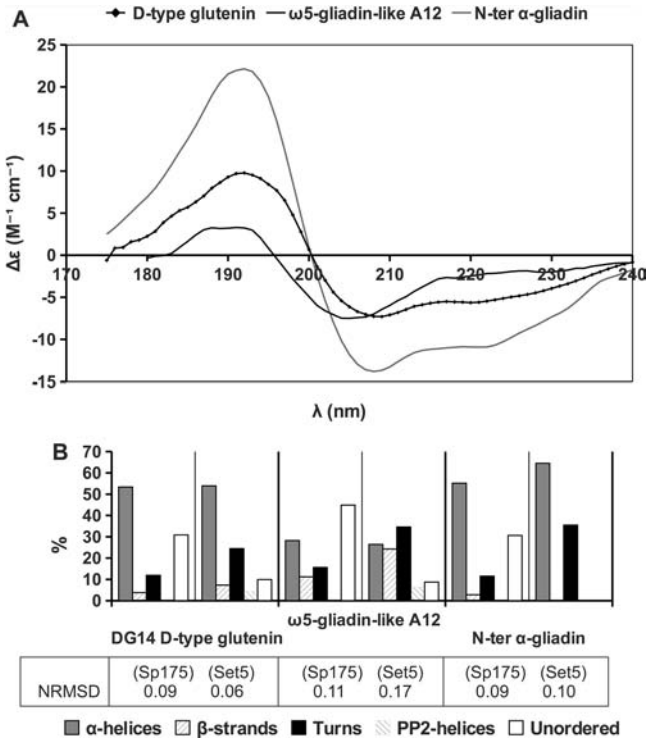

Figure 1. SRCD spectra and secondary structure contents for the recombinant DG14 and A12 ω5-gliadin-like D-type glutenins and N-terminal half of α-gliadin. (A) SRCD spectra. (B) Secondary structure contents calculated using the ContinLL program on the DichroWeb site, using Sp175 and set 5 as reference sets of structurally known proteins.

Their secondary structure contents were calculated using two spectra databases as a reference. The first one, Sp175, contains the structure of 72 soluble proteins,[34] but it does not identify PPII-helices, whereas the second one, set 5, contains 17 proteins[35] and allows the calculation of the PPII ratio. The two calculation methods led to an equivalent estimation of α-helix and β-strand contents, except for the A12 ω5-gliadin-like D-type glutenin, which showed differences in β-strand content. Depending on the calculation method, differences appeared in the ratios of turn and unordered structures. The second calculation method decreased the unordered structure ratio, increasing the turn ratio and identifying PPII helices. Under our experimental conditions, where SDS could favor the α-helix content as described elsewhere,[41] the recombinant DG14 D-type glutenin contained a high level of α-helices and a low ratio

of β-strands, whereas the A12 ω5-gliadin-like D-type glutenin displayed a significantly lower ratio of α-helices and a higher ratio of β-strands.

According to the second calculation method, the recombinant DG14 and A12 ω5-gliadin-like D-type glutenins contained high levels of turns, when PPII-helices were under 10%, despite their high proline residue content. The N-terminal half of α-gliadin almost always displayed a high level of α-helices and turns: this was probably due to the Gln cluster that formed an α-helix as predicted on the basis of several softwares (data not shown).

Some authors have thoroughly described[42,43] the conformation of C hordein, a barley protein homologous to ω-gliadins, and of its repeat motif: they observed that the peptide and the whole protein, solubilized in trifluoroethanol solutions, contained mainly β-turns at room temperature and PPII helices at very low temperature. Since our measurements were done in SDS solution, at 25 °C and in a wider wavelength range, our results displayed some discrepancies with those found in the quoted literature, mainly concerning the α-helix content. However, it is worth indicating that until now, no high-resolution structure of wheat prolamins has been available in the protein data bank.

A SRCD experiment was carried out with the A12 ω5-gliadin-like D-type glutenin and the N-terminal half of α-gliadin to evaluate their stability when heated. The samples were submitted to a temperature gradient that increased from 25 °C to 90 or 95 °C and then decreased back to 25 °C by 5 °C steps, with SRCD spectra recorded at each step (Figure 2). While the N-terminal half of α-gliadin displayed a progressive decrease of peaks, with complete and irreversible denaturation occurring at around 85 °C, the A12 ω5-gliadin-like D-type glutenin displayed a strong stability to temperature, with all of the spectra being highly superposable. So, in spite of similar amino acid compositions, these two proteins displayed very different physicochemical properties.

This observed high structural stability of the A12 ω5-gliadin-like D-type glutenin was a confirmation of some of the observations found in the literature:[44,45] ω5-gliadin was found to be stable when heated. This feature could explain that it is able to maintain its allergenicity during the technological processes applied to wheat products for food elaboration.

IgE Reactivity of the Recombinant D-Type Glutenins and α-Gliadin. The recombinant DG14 and A12 ω5-gliadin-like D-type glutenins and the corresponding natural gliadin or glutenin fractions from Prinqual cultivar were transferred to membranes after SDS-PAGE. These latter were incubated with three sera. The glutenin fraction was of course reduced and alkylated, whereas the gliadin fraction was not, to visualize the monomeric proteins extracted with 1-propanol solution. A membrane processed without serum was used to verify the IgE specificity of chemiluminescence signal (not shown): moreover, the differences observed in Figure 3 between the signal obtained from the three membranes indicated that the chemiluminescence signal was clearly related to sera reactivity.

The first serum was a supplementary serum described previously[15] and not used in the dot-blot experiment. It was taken from a patient diagnosed with dietary and cutaneous IHHWP (Figure 3A): the IgE clearly recognized the recombinant DG14 D-type glutenin (panel A, lane 1) but not the A12 ω5-gliadin-like D-type glutenin (panel A, lane 4). The lower bands weakly recognized in lane 1 could be protein fragments resulting from incomplete synthesis or cleavage

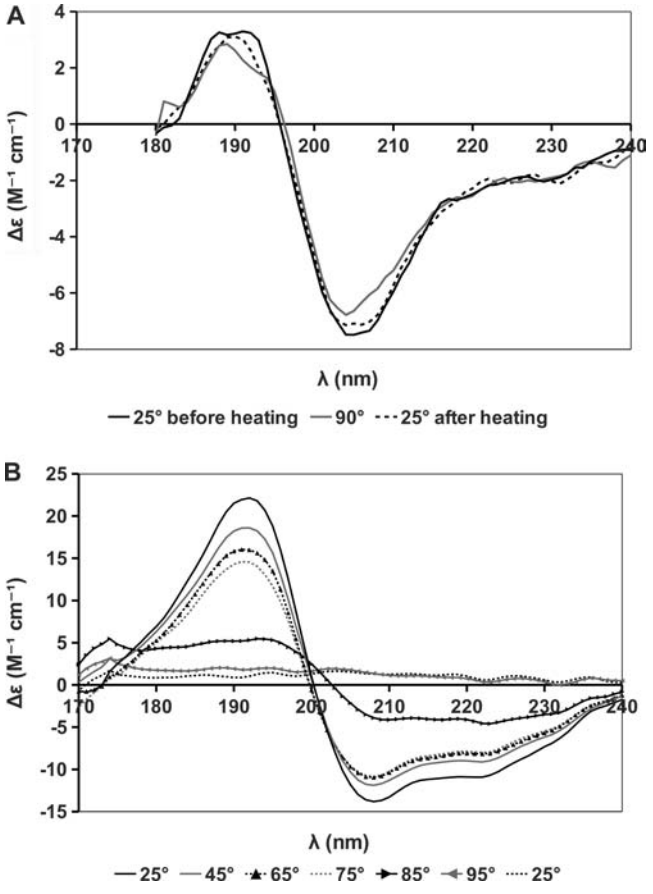

Figure 2. Thermostability of the A12 ω5-gliadin-like D-type glutenin (A) and N-terminal half of α-gliadin (B). SRCD spectra were recorded at each step of a temperature increasing gradient from 25 °C up to 90 or 95 °C and then decreasing back to 25 °C by 5 °C steps. To increase graphic legibility, all curves are not displayed.

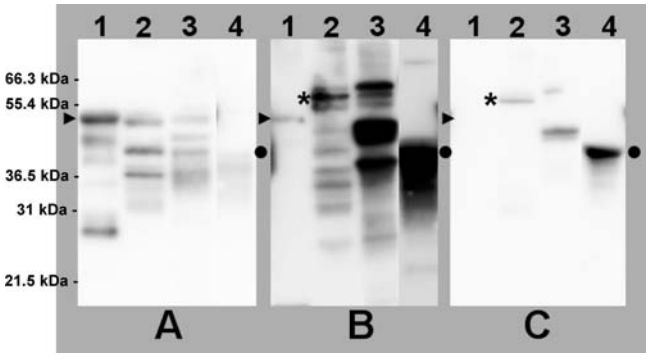

Figure 3. IgE reactivity to the recombinant DG14 and A12 ω5-gliadin-like D-type glutenins as compared to natural gliadin and glutenin fractions. Western blot IgE detection using chemiluminescence. (A) Serum (not used in dot-blot experiment and so not corresponding to any number) from a patient reacting to HWPs (dietary and cutaneous IHHWP). (B) Serum no. 19 from a patient reacting to HWPs after exercise. (C) Serum no. 28 from a patient diagnosed with WDEIA. Lane 1, the recombinant DG14 D-type glutenin (4.9 μg); lane 2, not reduced gliadin fraction from Prinqual cultivar (11 μg); lane 3, reduced-alkylated glutenin fraction from Prinqual cultivar (11 μg); and lane 4, the recombinant A12 ω5-gliadin-like D-type glutenin (4 μg). The recombinant DG14 D-type glutenin (▶), the recombinant A12 ω5-gliadin-like D-type glutenin (●), and native ω5-gliadin (*).

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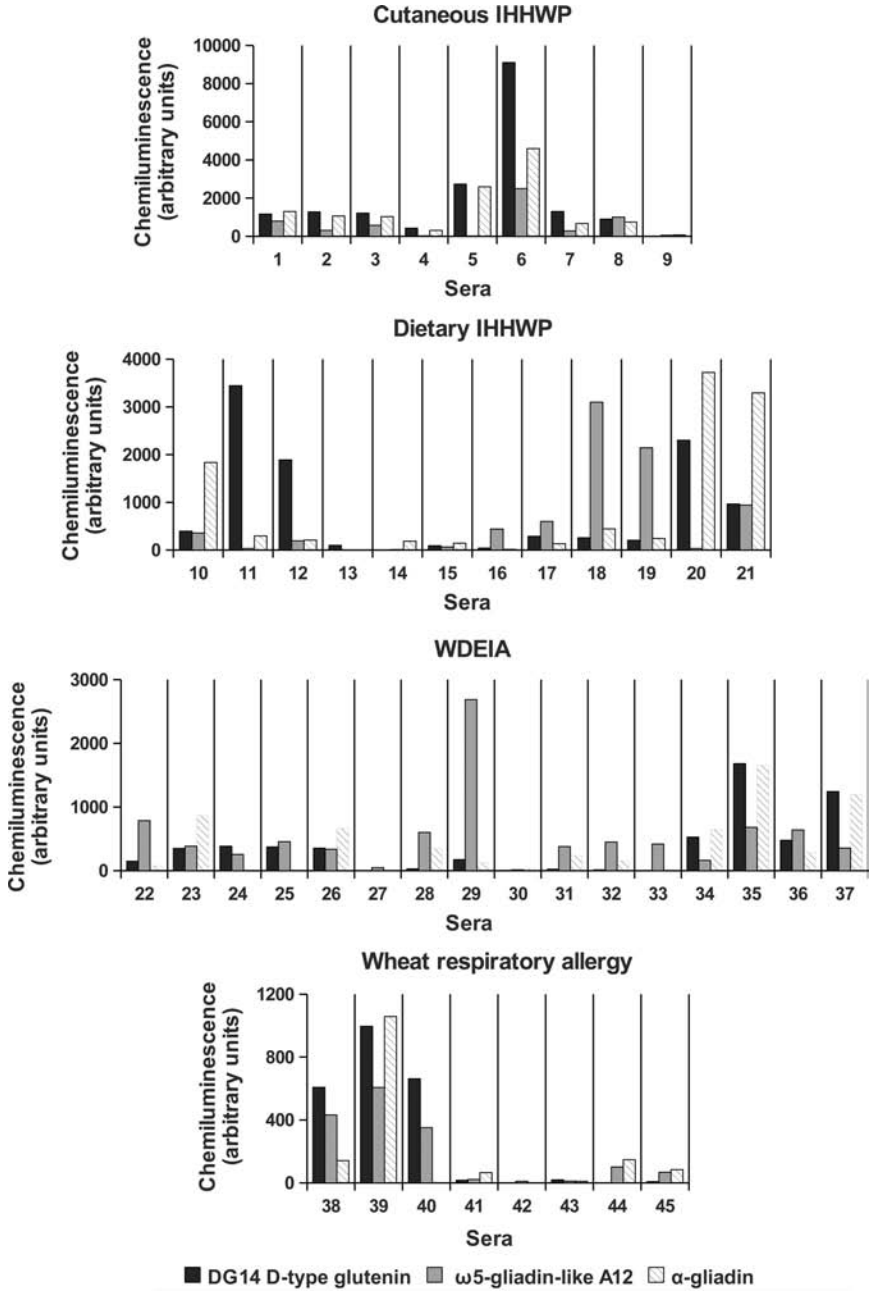

Figure 4. IgE reactivity of patient sera toward recombinant D-type glutenins and α-gliadin using dot-blot technique. Semiquantitative chemiluminescence intensity data are shown.

during extraction. The hypothesis of bacterial contaminants reacting with IgE could be eliminated because the equivalent bands were missing in lane 4 (panel A) when the extraction of the A12 ω5-gliadin-like D-type glutenin was done under the same conditions. Protein bands from the gliadin or glutenin fractions were recognized by IgE at a size similar to that of the recombinant DG14 D-type glutenin (Figure 3A, lanes 2 and 3): they could mark the protein equivalent to the recombinant DG14 among natural fractions. This experiment was in total agreement with the observations previously published[15] where sera from patients diagnosed with dietary IHHWP did not recognize ω5-gliadins but reacted with some other ω-gliadins. There was no indication that the IgE reactive protein in the gliadin fraction was a true ω-gliadin or its equivalent D-type glutenin, in the case of an incomplete binding to gluten polymers. On the other hand, the IgE binding protein from the glutenin fraction could be a D-type glutenin equivalent to the recombinant DG14. The small amount of the natural D-type glutenin equivalent to DG14 and its possible partition into each fraction probably accounted for the low chemiluminescence signal. This could make this natural protein difficult to identify in IgE binding analysis using the Western blot technique. So, the recombinant protein appears to be of greater use in IgE binding experiments.

The second serum (serum no. 19, Figure 3B) was obtained from a patient who was reacting to food HWP after exercise. The IgE reacted weakly with the recombinant DG14 D-type glutenin (lane 1) and strongly with the A12 ω5-gliadin-like D-type glutenin (lane 4), while the observed chemiluminescence was clear but lower with the native ω5-gliadin (lane 2, marked

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with an asterisk). The reactions observed in glutenin fractions (lane 3) were due to LMW-GSs as well as to proteins of the size of ω-gliadins, which could be ω-gliadins contaminating the glutenin fraction, or ω-gliadin-like D-type glutenins.

The third serum (serum no. 28) was obtained from a patient diagnosed with WDEIA (Figure 3C). There was no reaction with the recombinant DG14 D-type glutenin (lane 1), whereas the native ω5-gliadin reacted weakly (lane 2) and the A12 ω5-gliadin-like D-type glutenin more strongly (lane 4). The IgE reactivity of the latter two sera was also an indication that the recombinant A12 protein was not the result of the polymorphism among ω5-gliadins, but that it actually contained a deletion due to cloning as compared to the corresponding natural protein.

As shown in Figure 3, the classical Western blots using natural fractions lead to underestimation of the IgE binding toward weakly represented natural proteins in glutenin or gliadin fractions compared to recombinant proteins; moreover, the natural reacting proteins in Western blot did not permit conclusive identification.

The dot-blot technique using recombinant proteins was further used because the recombinant proteins were obtained in large amounts at a convenient degree of purity, making it possible to process many samples at the same time.Since the amounts of proteins loaded on membranes corresponded to the same molar ratio, the results allowed correct comparisons between proteins incubated with the same serum. To avoid spontaneous aggregation of the tested recombinant proteins, they were reduced and iodoacetamide-alkylated. This suppressed any dimer formation for the recombinant DG14 and A12 ω5-gliadin-like D-type glutenins but strongly modified the structure of the recombinant α-gliadin exposed to IgE binding. However, this latter recombinant protein spontaneously displayed varying degrees of polymerization (data not shown) so that the target exposed to IgE binding could differ between experiments. This reduction and alkylation, which induce structural modification, could actually modify the epitope conformation and their accessibility, but the linear epitopes and conformational epitopes not related to disulfide bridges could maintain their IgE reactivity. The specificity of the chemiluminescence signal was verified using a dot-blot membrane that was processed like the other membranes but without serum (data not shown).

Figure 4 presents the semiquantitative results of IgE binding using sera from four groups of patients who were suffering from cutaneous IHHWP, dietary IHHWP, WDEIA, and wheat respiratory allergy, respectively. With the sera from patients diagnosed with cutaneous IHHWP, the IgE reactivity pattern was relatively similar: the reaction with the A12 ω5-gliadin-like D-type glutenin was mainly weak or null, when almost all of the sera (eight out of nine tested) reacted positively with both recombinant DG14 D-type glutenin and α-gliadin. This reaction was very strong with one serum and lower but nevertheless clear with the other sera. These two proteins display only two identical peptides, one present two times in each sequence (PQQPYPQ) and another one present once in α-gliadin and three times in the recombinant D-type glutenin (QPFPQPQ) (Figure S.2 in the Supporting Information). These peptides or closely related ones might be responsible for IgE binding on both proteins; however, IgE could also bind these two proteins through completely different epitopes without any cross-reaction. With sera from patients suffering from dietary IHHWP, the IgE pattern was more heterogeneous. Four out of 12 sera gave a high chemiluminescence signal with the recombinant DG14 D-type glutenin, three out of 12 with α-gliadin, and three out of 12 with the A12 ω5-gliadin-like D-type glutenin. The other signals were weak or null. However, the DG14 D-type glutenin was recognized by IgE at a detectable level with eight sera out of 12, and α-gliadin with seven sera out of 12. This heterogeneity did not correspond to the heterogeneity of patients, either reacting only after HWP ingestion, or reacting after both ingestion and skin contact, or reacting after ingestion and exercise. The present results are also the proof that the DG14 D-type glutenin, in spite of its proximity to ω-gliadins, is different from the ω-gliadins found in a previous work to be systematically IgE-reactive with dietary IHHWP patients.[15] These ω-gliadins are not completely characterized and sequenced, but it is possible that they share some epitopes with the DG14 D-type glutenin as shown in Table S.5 in the Supporting Information. The differences in the reactions observed are an indication that the epitopes involved were probably not the same between the proteins.

As expected from the literature,[17,18,20] almost all sera from patients diagnosed with WDEIA showed a reaction with the A12 ω5-gliadin-like D-type glutenin, but it was not always the strongest reaction. The recombinant α-gliadin bound IgE less frequently than A12, whereas the recombinant DG14 D-type glutenin reacted at a lower frequency, or not at all. So, as expected, the A12 ω5-gliadin-like D-type glutenin constituted a correct positive control.

The sera from patients suffering from wheat respiratory allergy displayed a contrasted IgE pattern: most of the sera displayed no IgE reactivity or a very weak one with the tested proteins, whereas a few sera reacted clearly with both recombinant DG14, A12 ω5-gliadin-like D-type glutenins, and α-gliadin.

Some studies used peptide mapping (Pepscan technique)[40,46−48] to determine linear IgE epitopes from ω5-gliadin and some other gliadins that react with sera from patients diagnosed with WDEIA. Several epitopes of ω5-gliadin are only shared by the A12 ω5-gliadin-like D-type glutenin. Other epitopes found in ω-2 gliadin and α-gliadin are also present in the recombinant DG14 D-type glutenin and α-gliadin used here, respectively (Table S.5 in the Supporting Information). They could, at least partly, explain the IgE reactivities observed, particularly with sera from patients diagnosed with WDEIA. Since no linear epitopes described in these studies were common to the tested recombinant proteins, they seemed to be independent of each other as potential allergens and without cross-reactivity. This is also suggested through the variability of IgE-reactivity patterns observed with the sera used. There are no experimental data that indicate that the epitopes involved in IgE reactivity for the same protein in the different diseases were identical or different. A previous work suggested that a protein, the barley γ3-hordein, a protein homologous to wheat γ-gliadin reacted through different epitopes in WDEIA and in IHHWP.[49]

In wheat-dependent allergies, the patients are sensitized to a mixture of different allergens, sometimes highly modified like HWP.[11] These various allergens may differ depending on the type of allergy. So, the identification of the proteins recognized in vitro by IgE from patient sera IgE is an increasingly common method to improve diagnosis. As far as we know, this work is the first to be focused on recombinant D-type glutenins and α-gliadin: our results showed that both of these proteins were IgE reactive, and so, they could be used as markers in diagnosis, particularly in cutaneous IHHWP. These proteins will actually

be considered as allergens after in vivo tests like SPT or in vitro tests like mastocyte degranulation or basophil activation.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Tables S.1−S.5 and Figures S.1 and S.2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jacques.snegaroff@gmail.com.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

EST, expressed sequence tags; HWP, hydrolyzed wheat proteins; IHHWP, immediate hypersensitivity to hydrolyzed wheat proteins; PPII, poly(Pro)II helix; SPT, skin prick test; S-poor, sulfur-poor; S-rich, sulfur-rich; SRCD, synchrotron radiation circular dichroism; WDEIA, wheat-dependent, exercise-induced anaphylaxis.

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