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High Molecular Weight Entities in Industrial Wheat Protein Hydrolysates Are Immunoreactive with IgE from Allergic Patients

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Hydrolyzed wheat proteins (HWP) can induce immediate hypersensitivity through skin contact and/or food ingestion. Such patients develop IgE against unmodified wheat proteins without allergy to wheat. Our objective was to study the IgE-reacting content of HWP. We compared the reactivity of HWP and unmodified wheat proteins with IgE from patients suffering from immediate hypersensitivity to HWP. We studied the cross-reactivity between one HWP preparation and wheat proteins using immunoblot inhibition experiments. This showed that the tested HWP carried mainly unmodified epitopes originating from wheat proteins. The size distribution of polypeptides from two HWP preparations was analyzed by size-exclusion−high performance liquid chromatography (SE-HPLC), and their reactivity with IgE was studied. This showed that they contained highly IgE-reacting high molecular weight entities, likely resulting in a rearrangement of peptides issued from gluten processes. These multiepitopic entities could explain the high immunogenicity of HWP for sensitized people.

KEYWORDS: Immediate hypersensitivity to hydrolyzed wheat proteins; hydrolyzed wheat proteins; wheat protein isolate; SE-HPLC; skin prick test

INTRODUCTION

Proteins are an important part of the human diet. They may also be used as functional ingredients, and incorporated in formulated foods or in cosmetics. Because of the expansion of the wheat starch industry, wheat gluten, a byproduct of this industry, is available in large amounts and at relatively low cost. Gluten is an insoluble protein complex at neutral pH and shows visco-elastic properties. It is used to enhance the strength of flours in bakeries and to texturize food. To enlarge its industrial applications in food and the cosmetic industry, gluten can be modified through partial hydrolysis using acids or proteolytic enzymes (such as Neutrase). The hydrolyzed wheat proteins (HWP) thus obtained are a mixture of polypeptides with various sizes. Their lengths depend on the type and extent of hydrolysis. They may also contain glucides, lipids, and mineral salts, but the content of polypeptides is usually between 80 and 90%. For this reason, they belong to wheat protein isolates. HWP are more soluble than gluten and develop emulsifying and foaming properties. They are widely used as functional ingredients in cosmetics and food products such as soft drinks, soups, sauces, snacks, or meat products.

Wheat proteins are divided in two main classes: the albumins and globulins soluble in water and saline solutions, respectively, and prolamins, which are insoluble in these solutions. The latter are the main components of gluten. Prolamins are further divided in two groups according to their degree of polymerization (*1*). The first group is composed of gliadins, which are monomeric proteins named α/β-gliadin, γ-gliadins, and ω-gliadins, soluble in alcoholic solutions without reducers. In the ω-gliadin family, ω1,2-gliadins and ω5-gliadins designate slow and fast moving ω-gliadins (*2*), respectively. The second group is composed of glutenins, which are polymeric proteins, soluble in alcoholic and dilute acids or alkali solutions containing a reducing agent. Glutenins are composed of two classes of subunits, namely, high (HMW-GS) and low molecular weight glutenin subunits (LMW-GS).

The different classes of wheat proteins are involved in various wheat allergies. In baker's asthma, allergens belong mainly to the albumin−globulin fraction (*3*), but gliadins and glutenin subunits have also been shown to be IgE-reacting (*4*). In food allergy, wheat proteins belonging to each class have been described as IgE-reacting, with some differences between children and adults (*3*, *5*, *6*, *7*). Among these allergies, wheat-dependent exercise-induced anaphylaxis (WDEIA) is well known (*8*). It involves ω-5 gliadins, HMW-GS (*9*), or LMW-GS (*7*, *10*, *11*). In immediate hypersensitivity to hydrolyzed wheat proteins (IHHWP) by either skin contact (c-IHHWP) or skin contact and food ingestion (c/f-IHHWP) (*7*−*9*), ω1,2- and γ-gliadins have been shown to be the main IgE-reacting proteins (*8*).

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Some effects of gluten processing on wheat allergenicity have been studied (*3*). Enzymatic treatments have especially been developed to produce hypoallergenic wheat, by focusing on the digestion of known allergenic epitopes carried by some glutenin subunits (*12*). Conversely, it has been suggested that heating could increase the allergenicity of wheat gluten proteins (*6*). The question of the allergenicity of the HWP has not been well studied. With the increasing use of these preparations in food productions, new wheat-related allergies have been described. Several cases of contact urticaria or contact dermatitis to HWP present in cosmetic cream without food allergy associated have been shown (*13*, *14*). The first case of c/f-IHHWP has been described by Pecquet et al. (*15*). Gluten-derived products have then been shown to be responsible for immediate hypersensitivity by both cutaneous and oral contact but the chronology of reactions was in favor of cutaneous sensitization. Some cases of food allergy to wheat protein isolates have also been described (*16*, *17*).

In a previous study, we reported six cases of c/f-IHHWP and three cases of c-IHHWP (*18*, *19*). We showed that patients specifically sensitive to HWP differed from those suffering from other food and contact wheat allergies in that they tolerated traditional wheat food products, even if they developed IgE against unmodified wheat proteins, as well as against HWP. We showed also that, contrary to what could be expected, HWP made high molecular weight aggregates not dissociated by SDS or a reducer.

The aim of the present article was to bring new data about the reactivity of commercial HWP with IgE from sensitized patients. We focused especially on their immunoreactive polypeptide content in order to answer the following questions: What is the origin of the neo-allergenicity of HWP compared to normal gluten, and do hydrolysis processes produce new-epitopes or new arrangements of the pre-existing epitopes already found?

Some patients suffering from IHHWP were selected, and the IgE reactivity of their sera toward HWP and unmodified wheat proteins was studied using skin prick tests (SPT) and western blots. The cross-reactivity between HWP and unmodified wheat proteins, i.e., normal gluten, was analyzed using immunoblot inhibition experiments to detect the presence of new epitopes. Special attention was taken about the size distribution of the IgE reactive polypeptides contained in industrial HWP preparations, estimated both with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and size-exclusion−high performance liquid chromatography (SE-HPLC), followed by the IgE reactivity testing of the separated components.

MATERIALS AND METHODS

Reagents and Materials. Analytical grade reagents were from Sigma-Aldrich Chimie (St. Louis, MO, USA). Precast 12% Bis-Tris polyacrylamide gels and 3-(*N*-morpholino)propane sulfonic acid buffer were manufactured by Novex (San Diego, CA). Polyvinylidene difluoride (PVDF) blotting membranes were from Millipore (St-Quentin-en-Yvelines, France). SuperSignal West Dura Extended duration substrate was from Pierce Biotechnology (Rockford, IL, USA). Rabbit antihuman IgE conjugated to horseradish peroxidase (HRP) was supplied by Dako S.A. (Trappes, France). Black Indian drawing ink was supplied by Pelikan AG (Hanover, Germany). Flours from wheat (*Triticum aestivum*) cv. Soissons, Cracklin, Recital, and Tamaro were from Dr. G. Branlard (UMR1095 Amélioration et santé des plantes, INRA, Université Clermont II, Clermont-Ferrand, France).

Commercial HWP Preparations. The four commercial HWP preparations (called HWP 1 to 4) used in the biochemical study were provided by four different industries. HWP 1 was an extensively hydrolyzed gluten. HWP 2 was an enzymatically hydrolyzed gluten (through Neutrase action). HWP 3 was an acid-hydrolyzed gluten with a low degree of deamidation. HWP 4 was an enzymatically hydrolyzed gluten treated to limit the size of the peptides. This treatment could not be specified in this article because of industrial secrets. No other details could be obtained on the hydrolysis procedures. The applications of these preparations were various. All four were included as functional ingredients either in cosmetics (for HWP 1) or in food products such as dietary supplements, functional foods, beverages, soups, sauces, and prepared meals (for HWP 2, 3, and 4). The amount of wheat polypeptides of each HWP preparations, estimated from nitrogen quantification by the producers, ranges from 82 to 87%.

Patients. Sera came from the patients described in **Table 1** (patients 1 to 5). One (patient 3) out of four IHHWP patients was atopic. The atopic condition was established by skin prick tests and clinical history. Patients 1 to 4 developed urticaria after exposure to products containing HWP, through skin contact or ingestion. These reactions were established by considering the clinical history. All could eat traditional wheat products without any problems. Patient 5, the control patient, was suffering from WDEIA. Patients were evaluated by SPT with the following products: wheat flour extracts; gluten; wheat protein isolate (= HWP); an industrial preparation of HWP (HWP 5), specifically provided by the producing industry for SPT and which was an ingredient of an industrial precooked food; a skin tensing cosmetic (name of the cosmetic triggering the allergy of the patient 1), which contained HWP 1. All products were solubilized and diluted in glycerol−saline solution. Histamine and the glycerol−saline solution were used as positive and negative controls, respectively. A positive SPT result was defined as a wheal greater than or equal to one-half the diameter of the histamine control and at least 3 mm larger than the diameter of the glycerol−saline solution. Two other control sera, a normal human serum and the serum from an atopic patient allergic to peanuts, were purchased from PlasmaLab Int. (Everett, WA, USA). 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 giving blood.

Wheat Protein Fractions. Wheat proteins were extracted sequentially from 40 mg of flour from *T. aestivum* cv. Soissons or from 40 mg of a blend of flours from *T. aestivum* cv. Craklin, Soissons, Recital, and Tamaro. Gliadins were extracted, using a nonclassical but very effective method, by gentle mixing for 1 h with 1 M Tris at pH 7.1 buffer. After centrifugation, the supernatant (containing gliadins and albumins/globulins) was recovered, and gliadins were precipitated overnight by adding three volumes of 7.5% (w/v) NaCl to it. After centrifugation, the supernatant (containing albumins/globulins) was recovered, and albumins/globulins were precipitated overnight by adding three volumes of acetone to it. Glutenin subunits were extracted from the first pellet by gentle mixing for 1 h with 35% (w/w) *n*-propanol and 0.1 M acetic acid containing 5% (v/v) 2-mercaptoethanol (*18*). After centrifugation, the supernatant (containing glutenin subunits) was recovered, and the glutenin subunits were precipitated overnight by adding three volumes of 7.5% (w/v) NaCl to it. All centrifugations lasted 5 min each at 11,000*g*. All pellets resulting from the precipitation of proteins were resolubilized with Laemmli's sample buffer (*20*), 63 mM Tris-HCl at pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, and 5% (v/v) of 2-mercaptoethanol and boiled for 5 min. Proteins from each fraction were quantified using a turbidimetric method adapted to microplates (*21*).

Total Wheat Protein and HWP Extracts. Total wheat proteins were extracted from 40 mg of a blend of flours from four varieties of wheat (*T. aestivum* cv. Craklin, Soissons, Recital, and Tamaro) by gently mixing with 1 mL of Laemmli's sample buffer containing 5% (v/v) 2-mercaptoethanol, for 1 h, then boiled for 5 min. After centrifugation for 5 min at 11,000*g*, the supernatant was recovered. For SDS−PAGE, the extract was used as well. For inhibition experiments, proteins from the supernatant were precipitated by adding three volumes of acetone to it. After centrifugation for 5 min at 11,000*g*, proteins from the pellet were solubilized with 0.02% (w/v) SDS and 0.1% (w/v) Tween 20. Proteins from the total wheat protein extract were quantified using a turbidimetric method (*21*).

HWP for SDS−PAGE were extracted from 10 mg of commercial HWP powder by gently mixing with 1 mL of Laemmli's sample buffer containing 5% (v/v) 2-mercaptoethanol, as described above. For inhibition experiments, commercial HWP powder was directly added to the solution containing the serum.

SDS−PAGE and Western Blotting. SDS−PAGE was performed on precast 12% Bis-Tris polyacrylamide gels using 3-(*N*-morpholino)-propane sulfonic acid SDS running buffer under reducing conditions

Table 1. Description of the Patients Included in the Study

patient number		1	2	3	4	5
age (years)		24	25	18	20	32
manifestations		contact urticaria	urticaria	contact urticaria	urticaria	anaphylactic shock
allergy		contact allergy to HWP-containing cosmetic	food allergy to HWP-containing processed food	contact allergy to HWP-containing cosmetic	exercise-induced food allergy to HWP-containing processed food	wheat-dependent, exercise-induced anaphylaxis
products triggering the manifestations		skin tensing cosmetic	cooked industrial meal	facial cream	sausage, ham, industrial sauce	traditional wheat food product
skin prick test (SPT)	wheat flour extract	−[a]	−[a]	nd	−[b]	+[a]
	gluten	−[b]	−[b]	−[b]	+[b]	+[b]
	wheat protein isolate (HWP)	nd	+[b]	+[b]	+[b]	−[b]
	HWP 5[c]	nd	nd	+	nd	nd
	facial cream	nd	nd	+	nd	nd
	skin tensing cosmetic[d] containing HWP 1	+	nd	nd	nd	nd

[a] Originated from Stallergenes S.A. (Antony, France). [b] Originated from ALK (Varennes en Argonne, France). [c] Ingredient of precooked food provided by the production industry. [d] Name of the cosmetic responsible for the contact urticaria of patient 1. nd = not determined.

according to the manufacturer's instructions. Gels were blotted onto PVDF membranes, under semidry conditions using the protocol of Laurière (*22*). Twenty micrograms of proteins (total wheat proteins and fractionated proteins) and 200 μg of HWP were loaded onto the gel. These amounts were chosen to obtain a correct pattern on SDS−PAGE.

Proteins were stained by incubating the gel overnight with 2 volumes of 0.4% (w/v) Coomassie Brilliant blue G-250 in ethanol and 8 volumes of 2% (w/v) orthophosphoric acid and 10% (w/v) ammonium sulfate according to Neuhoff (*23*).

Immunoblot and Immunoblot Inhibition Experiments. *Immunoblot Experiments.* According to Snégaroff et al (*24*), PVDF membranes were first saturated for 2 h in 50 mM (pH 7.4) phosphate buffer, 150 mM NaCl, 0.1% (w/v) Tween 20 (PBST), and 2% (w/v) polyvinylpyrrolidone-40 (PBST-PVP-40). They were then incubated overnight at 4 °C with patient serum diluted 1:10 in PBST-PVP-40. After three washing steps in PBST-PVP-40, they were incubated for 2 h with the rabbit antihuman IgE-HRP conjugate diluted 1:25,000 in PBST containing 3% (w/v) dried cow's milk. After five washing steps in PBST-PVP-40 and two washing steps in PBS, the membranes were incubated for 5 min with the SuperSignal West Dura Extended duration substrate. All washing steps lasted 10 min each. All steps were performed under gentle rocking, at room temperature, except for the incubation with the patient serum that was at 4 °C.

Immunoblot Inhibition Experiments. According to Snégaroff et al (*24*), experiments were performed as described above except that inhibitors were added to the solution containing the serum from patient 1, diluted 10 times, gently mixed for 2 h, then incubated with the membrane overnight. We chose to use the higher concentrations of inhibitors as far as possible in the limit of their solubility in the buffer used. For inhibition using HWP 1, HWP 1 was directly solubilized at 10 mg/mL in the solution containing the patient serum. For inhibition using unmodified wheat proteins, total wheat proteins were added at 250 μg/mL in the solution containing the patient serum. The assay without inhibition was performed in the same way, with the patient serum but without inhibitor. Total proteins were stained after immunoblotting with Indian ink at 10 μL/mL in PBS containing 0.3% (v/v) Tween 20 according to Eynard (*25*) and Hancock (*26*). For all immunoblot experiments, a control membrane without patient serum was processed in the same way as the assays.

Chemiluminescence Detection and Quantification. Chemiluminescence was recorded using the luminescent image analyzer LAS-3000 supplied by Fujifilm (USA). Quantification was done using Bio 1D software (Vilbert-Lourmat, Torcy, France) by calculating the volume of the chemiluminescent responses, in arbitrary units. Light emission is directly proportional to the amount of peroxidase conjugates fixed on the blot. The use of a specific luminescence recorder with a high dynamic linear range detection signal, specially designed for Western blot analysis, allowed a quantitative evaluation of the IgE fixed on their antigens. The volume was the sum of the intensity of each pixel contained in the delimited area. For the immunoblot inhibition experiment, the calculation of the inhibition was 100 − (volume assay with inhibition × 100/volume assay without inhibition).

SE-HPLC. SE-HPLC was performed on a HPLC GOLD system from Beckman Coulter Inc. (California, USA) fitted with the Diode Array Detector module 168 and the Pump system 126. The column was the Superose 6 10/300 GL Tricorn (10 mm ID X 300 mm) from GE Healthcare (Buckinghamshire, GB) with an optimal separation range for globular proteins from 5×10^3 Da to 5×10^6 Da and an exclusion limit of 4×10^7 Da. The mobile phase was PBS with 0.1% (w/v) SDS. The calibration of the column was achieved by an injection of 100 μL of a solution containing 1 mg of each standard: bovine serum albumin (66.3 kDa), carbonic anhydrase (29 kDa), cytochrome *C* (12.4 kDa), and aprotinin (6.5 kDa). The retention time was well correlated with each molecular weight. For polypeptides with molecular weights higher than 66.3 kDa, an extrapolation of the retention time was made, in the optimal separation range of the column. We obtained estimated relative molecular weights. Five hundred microliters of HWP 2 or HWP 3 (20 mg/mL in PBS containing 4% (w/v) SDS) were injected, at a 2 mL/min flow rate. Detection was done at 214 and 280 nm. For each injection, eluted polypeptides were collected every minute between 14 and 70 min. The content of these subfractions was pooled into eight fractions (I to VIII) according to the chromatographic profile obtained at 214 and 280 nm. Polypeptides from these fractions were

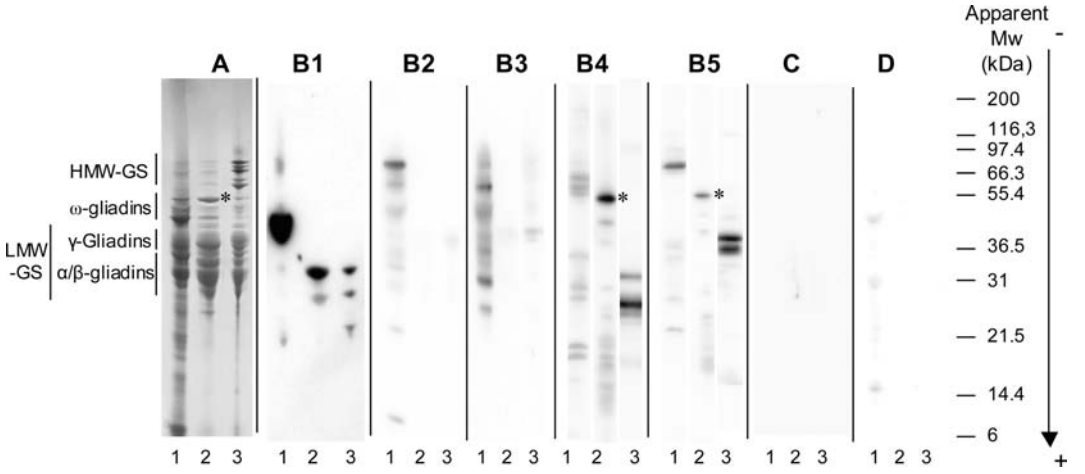

Figure 1. Sensitization pattern of patients toward wheat proteins. Albumins/globulins (1), gliadins (2), and glutenins (3) from *T. aestivum* cv. Soissons were analyzed using SDS−PAGE and either stained with Coomassie Brilliant Blue (**panel A**) or blotted onto a PVDF membrane and immunoprobed with the serum from patients 1 to 5 (**panels B1** to **B5**, respectively), with normal human serum (**panel C**) and with a serum from an atopic patient allergic to peanuts (**panel D**). The detection of IgE binding was done using chemiluminescence. Twenty micrograms of protein was loaded onto the gel. The asterisk denotes ω5-gliadin.

quantified using the ninhydrin method, then analyzed using dot-blot and immunoblot experiments.

Ninhydrin Quantification of Polypeptides. The quantification of the polypeptidic content of each SE-HPLC fraction was performed using the ninhydrin method, according to Landry et al (*27*). Briefly, it consisted of an alkaline hydrolysis of polypeptides at 130 °C for 45 min followed by the reaction of the released amino acids with ninhydrine, which produced a colored chromophore (Ruhemann's purple, λ_{max} = 570 nm). Gluten was used as the standard.

Dot-Blot Experiments. A Millipore dot-blot device was used. Different amounts of polypeptides (0.8, 2, or 4 μg) from each SE-HPLC fraction (from I to VIII) were deposed in cups filled with 500 μL of a solution containing 25% (v/v) ethanol, 10% (v/v) acetic acid, and 0.5% (w/v) SDS. This solution was chosen to maintain proteins in a soluble state. After aspiration of the solution through the membrane, proteins were uniformly adsorbed onto the surface of the membrane. The latter was then dried to improve the fixation of polypeptides onto it. Immunoblot experiments and the quantification of the chemiluminescent signals were performed as described above.

RESULTS

SPT Results. As shown in **Table 1**, patient 1 developed positive SPT to a skin tensing cosmetic containing HWP and negative SPT to wheat flour extract and gluten. Patients 2 to 4 developed positive SPT to modified wheat proteins (wheat protein isolate for all and HWP 5 for patient 3). They developed negative SPT to wheat flour extracts (except for patient 3, who was not tested) and negative SPT to commercial gluten (except for patient 4, who developed a positive response). This positive response could be explained by the fact that the commercial preparations of gluten may contain slightly modified wheat proteins, in addition to the unmodified ones. This had been demonstrated in a previous study (*18*). Perhaps, this minor part of the gluten was IgE-reacting in the case of patient 4. Thus, all four patients were identified as suffering from IHHWP, either through skin contact with HWP-containing cosmetics (patients 1 and 3) or through ingestion of HWP-containing food products, associated with exercise (patient 4) or not (patient 2). The control patient (WDEIA) developed both positive SPT to wheat flour extracts and gluten, and negative SPT to wheat isolate, which was characteristic of WDEIA (*8*). Specific IgE to wheat flour (f4) and gluten (f9) (UniCap Pharmacia Diagnostics AB, Uppsala, Sweden) were determined for all IHHWP patients, except patient 4. They showed no significant level of specific IgE to wheat flour (f4) and gluten (f9).

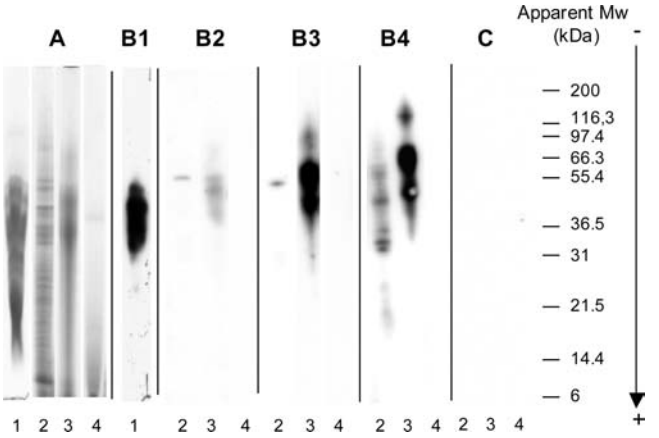

Figure 2. IgE reactivity of the sera from the four patients toward HWP. HWP 1 (1), HWP 2 (2), HWP 3 (3), and HWP 4 (4) were analyzed using SDS−PAGE and either stained with Coomassie Brilliant Blue (**panel A**) or blotted onto a PVDF membrane and immunoprobed with the serum from patients 1 to 4 (**panels B1** to **B4**, respectively) and with normal human serum (**panel C**). The detection of IgE binding was done using chemiluminescence. HWP 1 was analyzed only with the serum from patient 1. Two hundred micrograms of HWP was loaded onto the gel.

Sensitization Pattern of Patients toward Wheat Proteins. The sensitization pattern of all patients included in this study was analyzed in vitro using wheat protein fractions. Albumin/globulin, gliadin and glutenin fractions from *T. aestivum* cv. Soissons were analyzed using SDS−PAGE (**Figure 1**) and stained with Coomassie Brilliant Blue (**Figure 1**, **panel A**). Wheat proteins analyzed on the gel displayed a characteristic pattern, with bands in the range 6 to 116 kDa. In the gliadin fraction, ω5-gliadins (indicated by an asterisk) gave one band around 60 kDa, and α/β and γ-gliadins displayed several bands in the range from 25 to 45 kDa. In the glutenin fraction, HMW-GS and LMW-GS displayed bands in the range from 66 to 97 kDa and from 25 to 45 kDa, respectively.

Immunoprobing on PVDF membranes with patient sera (**Figure 1**, **panels B1** to **B4** for patients 1 to 4, respectively) was also performed. The pattern of IgE-binding to wheat proteins and the response intensities differed, according to the patient and the analyzed fraction. This reflected some heterogeneity between the

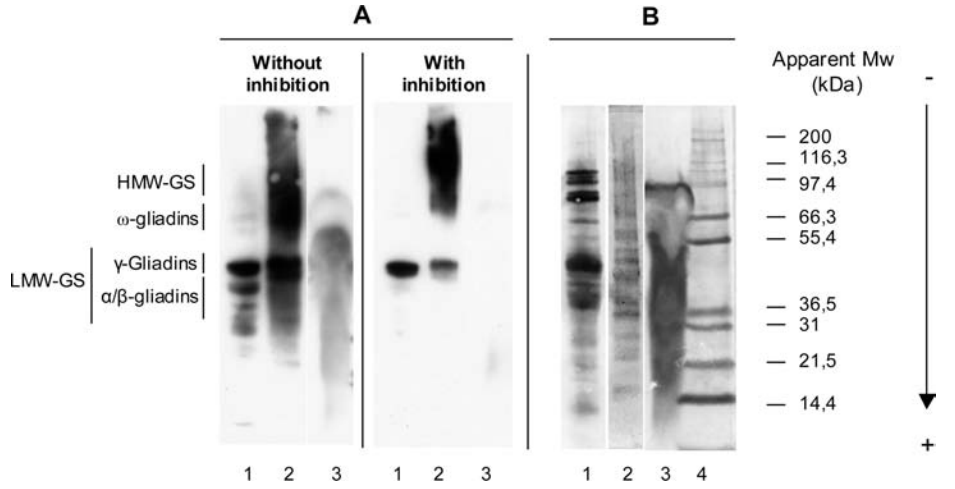

Figure 3. Cross-reactivity between HWP 1 and unmodified wheat proteins revealed by immunoblot inhibition experiments using HWP 1 as an inhibitor. Total wheat proteins (1) and albumins/globulins (2) from a blend of flours from *T.aestivum* cv. Soissons, Cracklin, Recital, Tamaro, HWP 1 (3), and the molecular weight standard Mark 12 from Novex (4) were analyzed using SDS−PAGE, blotted onto a PVDF membrane, then immunoprobed with the serum from patient 1 (**panel A**), incubated alone (assay without inhibition) or with HWP 1 (assay with inhibition). The detection of IgE binding was done using chemiluminescence. Indian ink staining was performed on the membrane from **panel A** after the immunoblot inhibition experiment (**panel B**). Twenty micrograms of proteins and 200 μg of HWP 1 were loaded onto the gel.

studied patients. The albumin/globulin fraction was roughly more IgE-reacting than the gliadin and glutenin fractions. HMW-GS were not IgE-reacting. ω5-Gliadin reacted only with the IgE from patient 4, who was suffering from exercise-induced food allergy due to HWP. Each experiment was run along with a control membrane immunoprobed without the patient serum, which showed no signal (results not shown). Two other controls were carried out (**Figure 1**, **panels C** and **D**). Under the used conditions, no reaction was observed with wheat proteins, except for a very weak signal observed with albumins/globulins for the patient allergic to peanuts. In addition, the serum from a patient allergic to wheat and suffering from WDEIA was used for comparison with IHHWP sera (**Figure 1**, panel **B5**). It displayed a pattern of IgE-reactivity specific to WDEIA patients, especially with the recognition of ω5-gliadins (indicated by an asterisk) (*8*) and a cluster of LMW-GS around 36 kDa. All these controls showed the specificity of the IgE responses.

IgE Reactivity of Patient Sera toward HWP. HWP preparations 1 to 4 were analyzed using SDS−PAGE under reducing conditions (**Figure 2**) and stained with Coomassie Brilliant Blue (**Figure 2**, **panel A**). Compared to unmodified wheat proteins (**Figure 1**), the SDS−PAGE protein pattern of HWP was strongly modified, to show smears with no well-defined bands or multiple bands as in HWP 2. That showed the efficacy of hydrolysis in industrial HWP and that almost all the original proteins underwent one or more hydrolytic events. Except for HWP 4, which was chosen for the high degree of hydrolysis of its components and showed a smear around 40 kDa to less than 6 kDa, HWP 1 to 3 displayed the remains of large amounts of components with relatively high molecular weights, similar to that of the original gluten proteins.

Immunoprobing of these HWP preparations on PVDF membranes with patient sera is shown **Figure 2**, **panels B1** to **B4** for patients 1 to 4, respectively. A normal human serum was used as the negative control (**Figure 2**, **panel C**). HWP 1 (lane 1) was only immunoprobed with serum from patient 1. HWP 2 to 4 (lanes 2 to 4, respectively) were immunoprobed with sera from patients 2 to 4 (**panels B2** to **B4**, respectively). The pattern of IgE-binding and the response intensities differed for each patient and each analyzed HWP. HWP 1 displayed a highly IgE-reacting smear in the molecular weight range 30 to 55 kDa, with patient serum 1. HWP 2, which exhibited multiple individualized bands upon Coomassie staining, displayed weak signals with patient sera 2 and 3, and a smear in a separation range from 20 to 97 kDa with additional fuzzy IgE-reacting bands with patient serum 4. HWP 3 displayed strong signals, with smears highly IgE-reacting observed in a separation range from 30 to 116 kDa for patient 3 and from 35 to 200 kDa for patient 4. HWP 4 did not show any IgE-reactions with patient sera 2 to 4. Interestingly, all HWP preparations showed no IgE reactions of their polypeptides below 30 kDa. No nonspecific reactions with nonspecific IgE toward HWP were observed (**Figure 2**, **panel C**).

Table 2. Study of the Cross-Reactivity between Unmodified Wheat Proteins and HWP 1

	type of proteins/HWP blotted on membrane	% of inhibition[a]
using HWP 1 as an inhibitor	total wheat proteins	46
	albumins/globulins	44
	HWP 1	73
using total wheat protein extract as an inhibitor	albumins/globulins	97
	gliadins	65
	glutenin subunits	0
	HWP 1	86

[a] Calculation of the inhibition is 100 − (volume assay with inhibition × 100/volume assay without inhibition); volumes were calculated using the Bio 1D software from the intensity and the area of the chemiluminescent responses, in arbitrary units.

Cross-Reactivity between One HWP Preparation and Unmodified Wheat Proteins. Cross-reactivity between HWP 1 and unmodified wheat proteins were studied through immunoblot inhibition experiments to search for the presence of new epitopes in HWP. Experiments were performed successively using HWP 1 or a total wheat protein extract as inhibitors. HWP 1 was chosen because it showed strong and clear reactions with the IgE from patient 1, making this couple of reagents suitable for inhibition experiments. The chemiluminescent signals given by each group of proteins or by the HWP were recorded.

The results of the inhibition using HWP 1 as an inhibitor are shown in **Figure 3** and **Table 2**. Total wheat proteins and albumins/globulins from a blend of four wheat varieties and HWP 1 were analyzed using SDS−PAGE and immunoprobed on a PVDF membrane (**Figure 3**, **panel A**) using the serum from

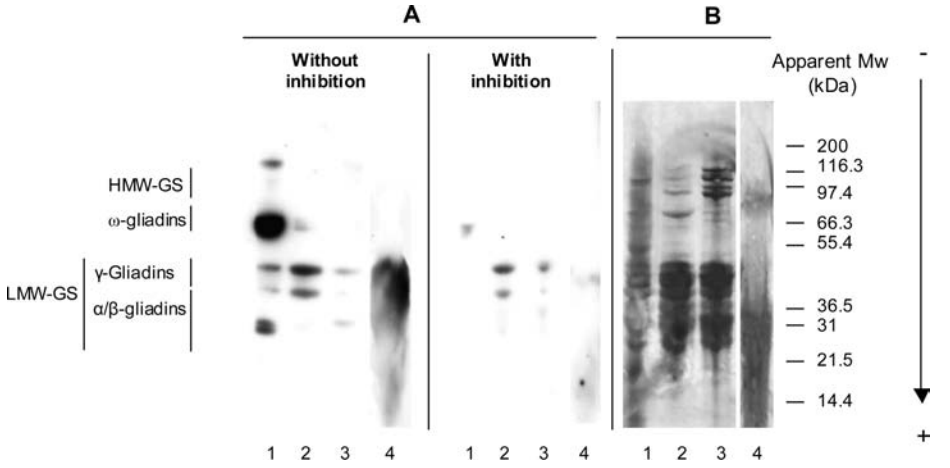

Figure 4. Cross-reactivity between HWP 1 and unmodified wheat proteins revealed by immunoblot inhibition experiments using a total wheat protein extract as an inhibitor. Albumins/globulins (1), gliadins (2), and glutenins (3) from *T. aestivum* cv. Soissons and HWP 1 (4) were analyzed using SDS−PAGE, blotted onto a PVDF membrane, then immunoprobed with the serum from patient 1 (**panel A**), incubated alone (assay without inhibition) or with the total wheat protein extract (assay with inhibition). The detection of IgE binding was done using chemiluminescence. Indian ink staining was performed on the membrane from **panel A** after the immunoblot inhibition experiment (**panel B**). Twenty micrograms of proteins and 200 μg of HWP 1 were loaded onto the gel.

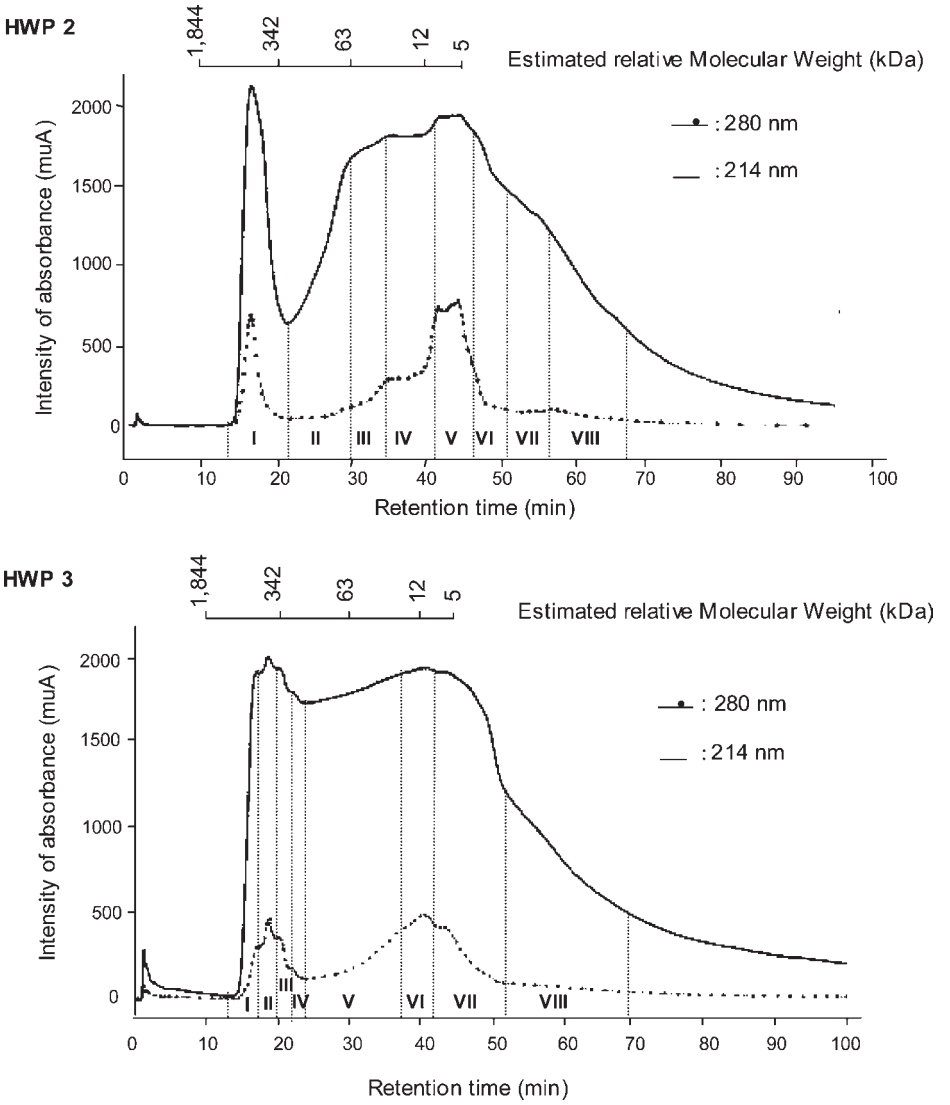

Figure 5. SE-HPLC elution profiles of HWP 2 and HWP 3. SE-HPLC elution profiles of HWP 2 and HWP 3 eluted each from a Superose 6 column into eight fractions (I to VIII). The detection of polypeptides was performed using two wavelengths (214 and 280 nm). Molecular weights were estimated from the calibration curve obtained with standard proteins. Ten milligrams of each HWP was injected onto the column.

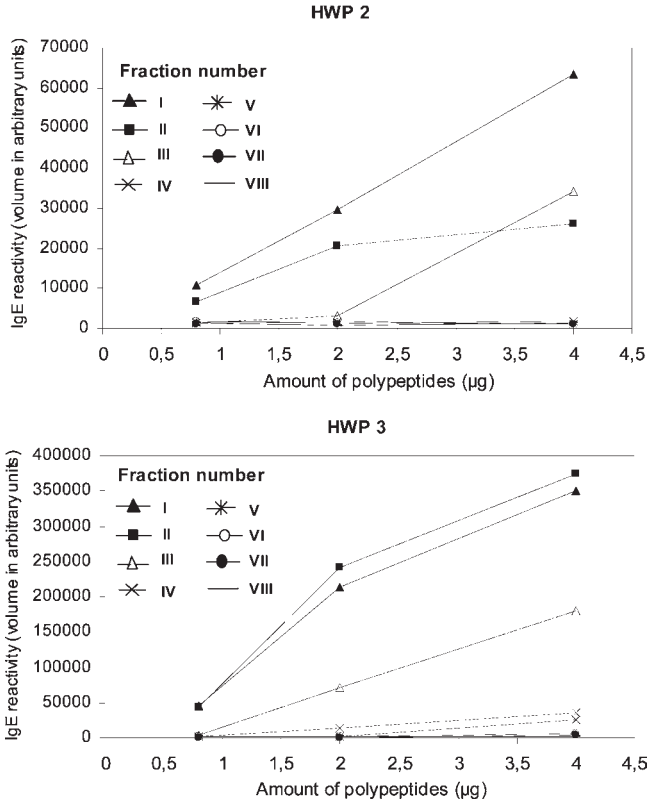

Figure 6. Reactivity of the IgE from the patient 4 toward the SE-HPLC fractions. Three different amounts (0.8, 2, and 4 μg) of polypeptides from the eight SE-HPLC fractions from HWP 2 and 3 were spotted onto PVDF membranes using dot-blot, then immunoprobed with patient 4 serum. Volume was calculated using the Bio 1D software and was the sum of the intensity of each pixel contained in a delimited area, in arbitrary units.

patient 1 incubated alone (assay without inhibition) or with an added amount of HWP 1 (assay with inhibition). In the conditions of the assay, free soluble HWP 1 in the medium was able to strongly inhibit (73%) IgE-binding to the same HWP 1 components fixed on the PVDF. This was considered as a correct positive control. At the same time, IgE binding to unmodified wheat proteins fixed on the PVDF was only partially inhibited by HWP 1 (46 and 44% for total wheat proteins and albumins/globulins, respectively).

The results of the inhibition using the total wheat protein extract from cv. Soissons as an inhibitor are shown in **Figure 4** and **Table 2**. Albumins/globulins from the cv. Soissons, gliadins, glutenins, and HWP 1 were analyzed under the same conditions as described above, except that a total wheat protein extract was solubilized in the medium with the patient serum. The IgE binding to glutenin subunits and to gliadins fixed on the PVDF was not or partly inhibited, respectively. This was likely due to the limited solubility of these groups of proteins in the medium. This was verified with albumins and globulins, which are more soluble. The IgE binding to fixed albumins/globulins was fully inhibited (97%) by the albumins and globulins present in the total protein extract used as an inhibitor. This was a correct positive control. In the same conditions, IgE-binding to HWP 1 was almost completely inhibited by the total wheat protein extract (86%). This strongly suggested that almost all of the epitopes detected on the HWP components fixed on blot were already present on unmodified wheat proteins used as inhibitors. A control using Indian ink staining of proteins on the membranes after immunoblot inhibition experiments (**Figures 3** and **4**, **panel B**) showed no noticeable leaking of proteins from the membrane during the experiment.

SE-HPLC Analysis. HWP 2 and 3 were analyzed using SE-HPLC under nonreducing and nondissociating conditions to evaluate the molecular size distribution of their polypeptide content. These HWP were chosen because each of them stood for a kind of hydrolyzing process, enzymatic and acid, respectively. **Figure 5** shows the typical chromatograms obtained. The relative molecular weight of polypeptides was estimated from the standard curve (not shown). The size profile of the two HWP preparations ranges from about 1,000 to less than 5 kDa. These results show that in conditions which preserved S−S bonds and noncovalent interactions, high molecular weight entities were still present in large amounts in HWP despites the hydrolysis processes. Eight fractions were collected from each HWP chromatographic profile, and their protein content was determined.

IgE-Binding Ability of the SE-HPLC Fractions. To assess the IgE-binding ability of polypeptides from each of the previous fractions collected from the SE-HPLC, their content was spotted on a PVDF membrane and immunoprobed with the serum from patient 4 (**Figure 6**). This serum was chosen for the ability of its IgE to react significantly with the two HWP tested (see **Figure 2**, **panel B4**) but also for the large amount available. The same amounts of polypeptides (0.8, 2, and 4 μg) were spotted for each fraction to allow the comparison of their IgE-binding ability. An Indian ink staining of the membrane after immunodetection was performed as a control to verify whether polypeptides were still present on the membranes after the experiments, especially low molecular weight ones. As for inhibition experiments, no noticeable leaking of proteins from the membrane during the experiment was observed (results not shown). As already observed (**Figure 2**, **panel B4**), the polypeptides from HWP 2, which was an enzymatically hydrolyzed gluten, were less IgE-reacting than those from HWP 3, which was an acid-hydrolyzed gluten. Despites these differences, similar results were observed with the two types of HWP. The highest IgE binding ability was observed with fraction I (about 1,000 to 300 kDa) from HWP 2 and with fractions I and II (about 1,000 to 400 kDa) from HWP 3. A weaker IgE binding ability was observed with fractions II and III (from about 300 to 30 kDa) from HWP 2 and with fraction III (from about 400 to 300 kDa) from HWP 3. Other fractions, either faintly reacted or did not react at all. These experiments showed that the highest molecular weight entities contained in the two HWP were the highest IgE-reacting entities, with the used serum.

DISCUSSION

All patients described in this study were suffering from IHHWP. These patients developed their symptoms after exposure to products containing HWP, through skin contact with HWP-containing cosmetic products (such as a facial cream), or through ingestion of HWP-containing food (such as cooked industrial meals). However, they could eat traditional wheat products without any problems. Clinical skin testing effectively confirmed their tolerance to unmodified wheat proteins and their intolerance to these proteins after hydrolysis. IgE from the sera of the four IHHWP patients bound HWP (**Figure 2**) but also unmodified wheat proteins (**Figure 1**). This observation contrasted with the tolerance of these patients to wheat flour and traditional baked products. This showed that at least some epitopes were originated from unmodified wheat proteins and were conserved in HWP, as suggested by Laurière et al. (*18*, *19*). These epitopes could be conserved because gluten polypeptides are never completely hydrolyzed by the industrial processes in order to maintain rheological functionalities. However, HWP and unmodified wheat proteins showed different allergenic status in specific allergies to HWP. Two main explanations can be proposed: either the pre-existing epitopes are newly organized in

HWP, or they are associated with neo-epitopes appearing following the hydrolysis processes. To choose between these two hypotheses, we carried out immunoblot inhibition experiments which allowed us to compare the similarity between epitopes present in HWP and unmodified wheat proteins. The inhibition of IgE binding would mean that the same IgEs recognize the same epitopes on both inhibitor and blotted proteins.

The significant inhibition of the IgE binding to unmodified wheat proteins by HWP 1 confirmed the presence of common epitopes carried by both HWP 1 and unmodified wheat proteins. However, it did not allow for excluding the presence of neo-epitopes in this HWP. To verify their possible presence in the HWP 1 preparation, a symmetrical inhibition experiment of the IgE reactivity on HWP1 components using unmodified total wheat protein extract as an inhibitor was performed. The IgE binding to HWP 1 was strongly inhibited by unmodified proteins, suggesting that most of the epitopes involved were already present on these unmodified proteins and that there were no IgE-reacting neo-epitopes in HWP 1 or that if they were present, they had a minor contribution. These results also demonstrate that deamidation of glutamine or asparagines residues of polypeptides, which can generate new allergenic epitopes and which were hypothesized by Leduc et al. (*16*) in the cases of mild acid treatment of wheat protein isolates, is not a general phenomenon responsible for HWP allergenicity. Furthermore, HWP obtained by enzymatic treatment, like HWP 1, do not contain normally significant amounts of deamidation products. Also, other authors (*28*) showed that deamidation of gliadins by using a cation-exchange resin reduced their reactivity toward the sera of patients with wheat allergy and the oral administration of deamidated gliadins to rats suppressed the production of gliadin-specific IgE (*28*). As experiments were carried out using only one HWP preparation and one patient serum, the results could not be extrapolated to other HWP preparations, nor to other patients. However, these results highlight that neo-epitopes were not necessarily involved in the allergenicity of HWP and that hydrolysis could maintain some pre-existing epitopes and not destroy all of them.

We analyzed the polypeptide composition and the IgE-binding ability of polypeptides from four HWP preparations using SDS−PAGE (**Figure 2**). For all four HWP, the original polypeptide pattern of unmodified gluten disappeared, and smears characteristic of random degradation were observed (**Figure 2**, **panel A**). Even if some fuzzy bands were still observed in HWP 2, these patterns were characteristic of efficient hydrolyses of gluten proteins. As expected after hydrolysis, the resulting components had molecular weights spanning over a wide range, up to very low values (less than 6 kDa). Interestingly, there was no IgE reactivity with polypeptides below 31 kDa (**Figure 2**, **panels B1** to **B4**). This contrasted with the IgE reactivity with normal gluten proteins (see **Figure 1**). All IgE-reacting components of HWP had molecular weights higher than 31 kDa, with some of them exceeding 116 kDa (**Figure 2**, **panels B3** and **B4**). All these results suggested either that the epitopes present on polypeptides smaller than 31 kDa were destroyed and those present on larger ones were maintained or that the polypeptides below 31 kDa were associated with other polypeptides to participate in the formation of the components making smears over 31 kDa. These results confirmed those previously obtained by Laurière et al. (*19*), with sera from patients suffering from allergies to HWP. They have shown that several commercial HWP preparations from different origins displayed high molecular weight IgE-reacting smears formed by denaturation/aggregation phenomena and resistant to SDS and reducers. HWP 4, which displayed no reactivity with IgE in the range of observation, provided an indirect proof of this hypothesis. It was treated by the producer to eliminate high molecular weight polypeptides, in order to contain essentially small and soluble peptides. As expected, HWP 4 did not react with any of the four sera tested (**Figure 2**).

To provide new data favoring the role of the association of polypeptides in HWP, HWP preparations were fractionated according to the molecular weights of their components using SE-HPLC performed under nonreducing and nondissociating conditions. In these conditions, the heterogeneous high molecular weight entities observed could correspond to the remaining large polymers of glutenins not fully hydrolyzed, but also to newly aggregated polypeptides resulting from the entanglements of their chains, S−S bond interchanges, or multiple noncovalent interactions formed during the heating or the drying of HWP. For these reasons, SE-HPLC fractions provide more realistic samples than SDS and reducer treated fractions to analyze the interactions of their components with IgE. Furthermore, the isolation and the testing of each SE-HPLC fraction in the same conditions allowed the determination of the relative reactivity of the separated components with IgE. Clearly, the fractions corresponding to the lowest molecular weight entities showed a null or weak IgE binding ability, while the fractions corresponding to the highest molecular weight entities showed a high one, for both tested HWP. Several hypotheses can be proposed to explain the high IgE reactivity of the highest molecular weight entities present in HWP. There is a low probability that the remaining polymers of glutenins could solely explain the IgE-reactivity of the fractions because patients were tolerant to unmodified proteins, which contain large amounts of glutenin polymers. Also, we could not exclude the demasking of buried epitopes due to partial hydrolysis of proteins, the more likely hypothesis, which was compatible with experimental results concerning the newly organized polypeptides more or less strongly linked together, as those resistant to dissociating and reducing conditions observed by SDS−PAGE. We suggest that these polypeptides could be composed of wheat polypeptides of different sizes, aggregated together by numerous linkages. They could be able to form multiepitopic entities of high molecular weight involving the pre-existing epitopes. Moreover, the creation of new terminal NH_3^+ and COO^- charged groups due to the hydrolysis of peptidic bonds could increase the solubility and the possibilities of interactions with the environment of gluten polypeptides (*18*). These structures could be responsible for the IgE-reactivity of HWP on the blot but also in vivo. Indeed, knowing that multivalency was necessary to trigger inflammation (*29*), this suggested that the high molecular weight entities contained in HWP would be more suitable for the bridging of IgE on mastocytes and basophiles than unmodified proteins. Palosuo et al. (*30*) have shown that the cross-linking of a peptidic fraction of omega5-gliadin by transglutaminase enhanced IgE-reactivity in WDEIA. The linkages involved in the polymers they obtained were not the same as those involved in the commercial HWP preparations we analyzed. However, they made the hypothesis that large polymers could have a better ability to bind IgE, as we observed.

Despite the limited number of sera and HWP used, the present study provides new data on the IgE-reacting content of HWP, which allow the proposal of a general mechanism explaining the neoallergenicity of HWP. It shows that, at least for the HWP and the serum used, the IgE-reacting epitopes mostly originated from the unmodified wheat proteins. Moreover, it highlights the importance of the size of the polypeptides contained in HWP on their IgE-binding properties. It shows that, contrary to what could be expected, hydrolysis does not destroy all pre-existing epitopes and can rather lead to the creation of multiepitopic entities of high molecular weight and high IgE-reaction. The

formation of these multiepitopic structures associated with a change in their presentation to the immune system was hypothesized to be the main basis of the allergenicity of HWP.

ABBREVIATIONS USED

HMW-GS, high-molecular weight glutenin subunits; HWP, hydrolyzed wheat proteins; IHHWP, immediate hypersensitivity to HWP; LMW-GS, low-molecular weight glutenin subunits; PBST, PBS with 0.1% (w/v) Tween 20; PVDF, polyvinylidene difluoride; PVP-40, polyvinylpyrrolidone-40; SE-HPLC, size-exclusion−high performance liquid chromatography; SPT, skin prick test.

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