JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Salt-Soluble Proteins from Wheat-Derived Foodstuffs Show Lower Allergenic Potency than Those from Raw Flour

MARTA DE GREGORIO,[†] ALICIA ARMENTIA,[‡] ARACELI DÍAZ-PERALES,[†] ARANTXA PALACÍN,[†] ANTONIO DUEÑAS-LAITA,[§] BLANCA MARTÍN,[§] GABRIEL SALCEDO,[†] AND ROSA SÁNCHEZ-MONGE*[,†]

Unidad de Bioquímica, Departamento de Biotecnología, ETS Ingenieros Agronomos, Madrid, Spain, Sección de Alergia, Hospital Universitario Rio Hortega, Valladolid, Spain, and Servicio de Farmacología Clínica-Unidad Regional de Toxicología Clínica, Hospital Universitario Rio Hortega, Valladolid, Spain

Salt-soluble proteins from wheat flour have been described as main allergens associated with both baker's asthma and food allergy. However, most studies have used raw flour as starting material, thus not considering potential changes in allergenic properties induced by the heat treatment and other industrial processing to produce wheat-derived foodstuffs. Salt extracts from different commercial wheat-derived products were obtained and their allergenic properties investigated by IgE-immunodetection, ELISA assays, and skin prick test. The IgE-binding capacity of salt-soluble proteins from commercial breads and cooked pastas was reduced around 50% compared with that of raw flour, the reduction being less dramatic in noncooked pastas and biscuits. Several wheat-derived foodstuffs showed major IgE-binding components of 20 and 35 kDa, identified as avenin-like and globulin proteins, respectively. These proteins, as well as most flour and bread salt-soluble proteins, were hydrolyzed when subjected to simulated gastrointestinal digestion. However, the digested products still exhibited a residual IgE-binding capacity. Therefore, processing of wheat flour to obtain derived foodstuffs decreases the IgE binding-capacity of the major salt-soluble wheat proteins. Moreover, simulated gastric fluid digestion further inactivates some heat-resistant IgE-binding proteins.

KEYWORDS: Allergens; immunoglobulin E; food allergy; wheat proteins; wheat foodstuffs

INTRODUCTION

The prevalence of wheat food allergy is increasing, ranging from 0 to 3.6% depending on diagnostic methods and geographic areas (*1*). Wheat is also one of the most frequent allergenic foods associated with food-dependent exercise-induced anaphylaxis (*2*). Another type of wheat IgE-mediated hypersensitivity is baker's asthma, an important occupational disease, caused by inhalation of wheat and other cereal flours (*3*).

On the basis of differential solubility, wheat grain proteins have been classified as salt-soluble albumins and globulins and gluten fraction or prolamins, which includes gliadins and glutenins (*4*). Both salt-soluble and insoluble proteins have been implicated in the development of wheat hypersensitivity (*5*). Among salt soluble proteins, members of the α-amylase inhibitor family seem to be the most important allergens responsible for baker's asthma (*6, 7*). They have also been described as wheat food allergens (*8, 9*). Some other salt-soluble proteins, such as a peroxidase and a non-specific lipid transfer protein, have been implicated in allergy to wheat both by inhalation and ingestion (*9*−*11*). Gliadins are involved mainly in IgE-mediated reactions to ingested wheat (*9, 12*−*16*) and recently in baker's asthma as well (*17*). High and low molecular weight subunits of glutenins have also been identified as potential wheat food allergens (*9, 18*−*21*).

It is remarkable that most patients with baker's asthma do not present wheat food allergy (*3, 22*). A difference in sensibilization (inhalation versus ingestion) and allergenic source (wheat flour versus wheat processed foodstuffs) could explain this fact, in spite of the fact that some wheat allergens, such as α-amylase inhibitors or lipid transfer proteins, are implicated in both types of allergy.

Heat treatment can modify the allergenicity of foods (*23*). Although raw wheat flour is rarely consumed, most of the in vitro and in vivo studies on wheat food allergy have been performed with flour and thus do not take into account the effect of heat treatment, during processing or cooking, on the IgE-binding capacity of potential wheat allergens. Only in one study,

* To whom correspondence should be addressed. Unidad de Bioquímica, Departamento de Biotecnología, ETS Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid, Spain. E-mail: mariarosa.sanchezmonge@upm.es

[†] ETS Ingenieros Agronomos.

[‡] Sección de Alergia, Hospital Universitario Rio Hortega.

[§] Servicio de Farmacología Clínica-Unidad Regional de Toxicología Clínica, Hospital Universitario Rio Hortega.

10.1021/jf803475v CCC: $40.75
Published on Web 03/10/2009

Table 1. Demographic and Clinical Data of Selected Patients

patient no.	age/sex	reported symptoms[a]	specific IgE: wheat (kU/L)	specific IgE: other foods[b] (sIgE > 0.35kU/L)	SPT wheat (mm^2)
1	30/F	As	7.7	ba, ry	20
2	45/F	As	>100	ba, ry, ri	78
3	20/F	As	27.9	ba, ri, co, pe	20
4	61/F	As, An	4.9	ba, ry, or	28
5	28/M	As, An	11.4	ba, ry, le, so	38
6	46/F	As	3.1	ba, ry	20
7	25/M	As,U	2.3	ba, ry	19
8	22/F	As	2.3	ba, ry	20
9	73/F	As, U	4.4	ba, ry	20
10	25/F	As, U	3.3	ba, co	20
11	27/M	As, An	4.2	-	38
12	32/M	As	5.3	ry	28
13	25/F	As	4.8	eg	28
14	2/M	As, U	3.2	le	28

[a] As, asthma; An, anaphylaxis; U, urticaria. [b] ba, barley; ry, rye; ri, rice; co, corn; pe, peach; or, orange; le, lentil; so, soybean; eg, egg.

cooked wheat flour was used in double-blind placebo-controlled challenges (*24*). In another study, some wheat food allergens lose the IgE-binding capacity in some patients, after a short treatment at 100 °C (*9*). However, this treatment does not mimic the complex process of baking or other processes to obtain wheat-derived foods.

Additionally, wheat food proteins suffer a gastrointestinal digestion, which could hydrolyze potential flour allergens. Simulated gastrointestinal digestion of wheat flour protein extracts shows that most of the salt-soluble and glutenin allergens were rapidly digested and not detected in immunodetection with a serum pool from wheat food allergic patients (*25*). Gliadins were stable to gastric enzymes but digested by simulated duodenal fluid. However, heat treatment (for instance during baking) can modify the digestibility of wheat proteins due to aggregation or Maillard reactions with sugars (*26*). In this context, a simulated gastrointestinal digestion assay has shown a persistent IgE-binding capacity of bread crumb and crust proteins, compared with those of bread dough, which were readily digested (*27*). In a similar study using boiled pasta (*28*), most of the wheat proteins were hydrolyzed, but the resulting peptides still bound IgE from wheat food allergic patients.

The in vitro IgE-binding capacity and the in vivo allergenic potency of salt-soluble proteins from raw flour and wheat-derived foodstuffs with different heat processing, as well as the effects of simulated gastrointestinal digestion, are reported here. Furthermore, two main potential allergens from wholemeal tin loaf bread have been also identified.

MATERIALS AND METHODS

Patient Recruitment and Collection of Serum Samples. A serum pool from 14 patients who have suffered from allergic symptoms (asthma, urticaria, and/or anaphylaxis) related to ingestion of cereal processed foods was used in the study. These patients were recruited from the database of cereal allergic patients (Hospital Rio Hortega, Valladolid, Spain) diagnosed by skin prick test (SPT), specific IgE, and challenge tests to wheat. Specific IgE antibodies to wheat, other cereals, and a battery of foods were determined using the ImmunoCAP 100 System (Phadia AB, Uppsala, Sweden). The clinical data of the included patients are summarized in **Table 1**. Informed consent was obtained from each patient and ethical approval from the Ethical Committee of the Hospital Rio Hortega.

Skin Prick Test. Skin prick tests were performed with a commercial panel of food allergenic extracts (Bial-Aristegui Laboratories, Bilbao, Spain). Additionally, salt-soluble protein preparations (2 mg of protein/mL) from raw flour, wholemeal tin loaf bread, wholemeal toast bread, and boiled pasta were tested in 10 patients.

Wheals were measured after 15 min and outlined by tracing on adhesive paper. The diluent of the prick solution (physiologic saline solution) was used as negative and histamine (10 mg/mL) as positive controls. A wheal area greater than 7 mm^2 was considered positive.

Salt-Soluble Protein Extracts. The following wheat-derived foods were purchased at a supermarket: French bread, wholemeal bread, white tin loaf bread, wholemeal tin loaf bread, toasted bread, wholemeal toast bread, white pasta, wholemeal pasta, fresh pasta, biscuit, pizza, baby cereal food, and breakfast cereals. The pastas were boiled during 10 min for dry and 2 min for fresh ones. Wheat flour and bran (*Triticum aestivum*, cultivar Astral) were also used.

All these wheat-derivatives were freeze-dried and defatted with cold acetone (2 × 1:10 [wt/vol] for 1 h at 4 °C) and, after drying, extracted (1:5 [wt/vol] for 1 h at 4 °C with 2 pulses of sonication) with 0.1 M Tris-HCl, pH 7.5, and 10 mM EDTA. After centrifugation (9000*g* for 30 min at 4 °C), the supernatants were dialyzed against H_2O (cutoff point, 3.5 kDa) and freeze-dried. Protein concentration was determined by the method of Bradford (*29*).

Specific IgE Determination and ELISA-Inhibition Assays. Direct ELISA to determine specific IgE was carried out as previously described (*30*), using 30 μg of protein/mL in solid phase and a serum pool (1:10 dilution) from 14 wheat-food-allergic patients. Bovine serum albumin (3 μg/mL) was used as negative control. All tests were performed in triplicate.

ELISA-inhibition assays were performed by the same method, using 30 μg of protein/mL of salt-soluble proteins from raw flour as solid phase and the serum pool preincubated with 30 μg of protein/mL of salt-soluble proteins from wheat foodstuffs, during 3 h at room temperature. All tests were performed in triplicate.

SDS−PAGE and IgE Immunodetection. Samples (30 μg of protein of salt-soluble protein extracts) were separated by SDS−PAGE on 10−20% precast gradient polyacrylamide gels (Invitrogen, Carlsbad, USA), using 0.1 M Tris-Tricine running buffer under reduction conditions, and stained by Coomassie Blue. Alternatively, protein bands were electrotransferred onto polyvinylidene difluoride (PVDF) membranes as previously reported (*31*). After blocking and washing, membranes were incubated with the serum pool from wheat-food-allergic patients (1:10 dilution) and then with peroxidase-conjugated antihuman IgE (Biosource, Camarillo, USA; 1:3000 dilution). Detection of IgE binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Thermo scientific, Rocford, USA). An immunodetection, in the same conditions, with a serum pool from eight patients allergic to mites but not to plant foods or pollens was performed as negative control.

Identification of Major IgE-Binding Proteins. Fractions enriched in the two main IgE-binding proteins from wholemeal tin loaf bread were isolated by two sequential chromatographic methods. The salt-soluble proteins were first fractionated on a Sep-Pak Vac RC cartridge (500 mg; Acell Plus CM, Waters, Mildford, USA), using 20 mM formic acid (pH 4) as equilibration buffer and eluting the retained material in three steps with 0.2, 0.5, and 1 M NaCl in the same buffer. Fractions containing the main IgE-binding bands were subjected to reverse-phase HPLC on a Vydac 300 C4 column (22 × 250 mm; particle size 10 μm; The Separations group, Hesperia, USA), eluting with a two-step linear gradient of acetonitrile in 0.1% trifluoracetic acid (10% to 60% in 120 min and 60% to 85% in 30 min; 1 mL/min). Two HPLC chromatographic fractions, enriched in the IgE-binding proteins, were further fractionated by SDS−PAGE and transferred to PVDF membranes as described above. Bands corresponding to the IgE-binding proteins excised from the gel were characterized by fingerprinting after tryptic digestion using a Mascot search. Alternatively, membrane excised bands were N-terminal amino acid sequenced by using standard methods on an Applied Biosystem 447 gas-phase sequencer (Foster City, USA).

Simulated Gastrointestinal Digestion. Salt-soluble protein extracts from raw flour and wholemeal tin loaf bread were submitted to simulated gastrointestinal digestion following the method described by Moreno et al. (*32*). Digested samples were fractionated by SDS−PAGE and used for ELISA-inhibition assays as described above.

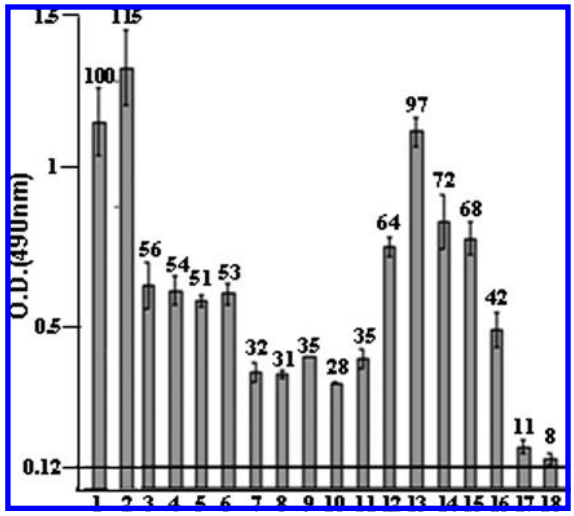

Figure 1. Specific IgE of a serum pool from patients with wheat food allergy to salt-soluble proteins from wheat flour (1) and bran (2) and the following wheat-derived products: French bread (3), wholemeal bread (4), white tin loaf bread (5), wholemeal tin loaf bread (6), toasted bread (7), wholemeal toasted bread (8), boiled white pasta (9), boiled wholemeal pasta (10), boiled fresh pasta (11), white pasta (12), wholemeal pasta (13), fresh pasta (14), biscuit (15), pizza (16), baby cereal food (17), and breakfast cereals (18). Means ($n = 3$) and SD (bars) are represented. Numbers above bars represent the percentage of IgE levels to each sample with respect to that of raw flour (1). Values were considered positive when greater than 0.12 OD units (mean + 3 × SD of BSA as solid phase).

Statistical Analysis. SPT responses (wheal areas) to commercial and salt-soluble protein extracts from raw flour and wheat-derived foodstuffs were compared by using a two sided T Student's test. A level of significance <5% ($p = 0,05$) was considered to be significant. The Kolmogorov−Smirnov test was previously used to ascertain the normal distribution of data.

RESULTS

Clinical Characteristics of Patients with Food Allergy. Thirteen adult and one pediatric patient (mean ± SD age, 32.9 ± 18; range, 2−73 years) were included in the study (**Table 1**). Clinical manifestations reported by patients after ingestion of wheat-derived products consisted of asthma (7/14), asthma and urticaria (4/14), and asthma and anaphylaxis (3/14). All included patients showed specific IgE to wheat (mean, 16.02 kU/L; range, 2.3 to >100 kU/L)), and positive SPT responses to a wheat commercial extract (mean ± SD wheal areas: 29 ± 15.5 mm^2). Most patients were also sensitized to other plant foods, particularly barley (10/14) and rye (9/14).

IgE-Binding of Salt-Soluble Proteins from Wheat-Derived Foodstuffs. *1. Specific IgE Determination.* The specific IgE to salt-soluble proteins from wheat raw flour and bran, and 16 wheat-derived foodstuffs was determined by ELISA assays, using a serum pool from 14 selected patients (**Figure 1**). Samples from biscuit and nonboiled pastas (numbers 12−15) retained a high proportion (97−64%) of the IgE-binding capacity of raw flour, whereas those from boiled pastas (numbers. 9−11) displayed a substantial decrease (35−28%). Similar results, but with lower values, were found for breads (numbers 3−6; 56−51%) and toasted breads (numbers 7 and 8; 32−31%). Extracts from baby cereal food and breakfast cereals showed the lowest IgE-binding capacity (numbers 17, 18; 11−8%).

To explore whether or not the IgE-binding components from raw flour and wheat-derived products shared common epitopes,

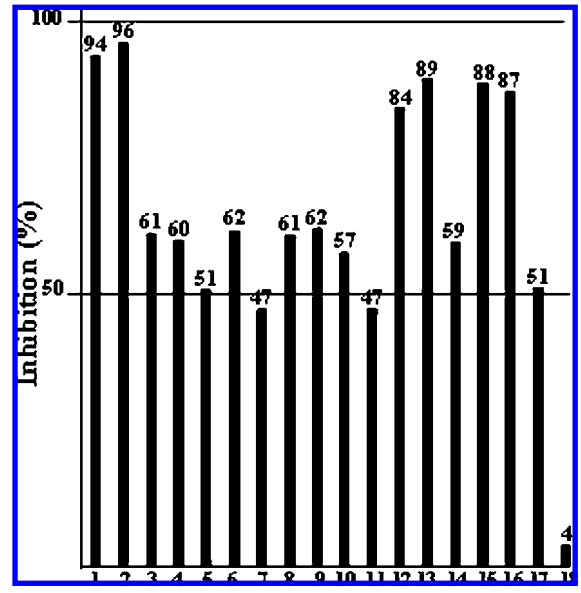

Figure 2. Inhibition (%) of specific IgE-binding capacity to salt-soluble proteins from wheat raw flour by the equivalent extracts from wheat raw flour (1) and bran (2), French bread (3), wholemeal bread (4), white tin loaf bread (5), wholemeal tin loaf bread (6), toasted bread (7), wholemeal toasted bread (8), boiled white pasta (9), boiled wholemeal pasta (10), boiled fresh pasta (11), white pasta (12), wholemeal pasta (13), fresh pasta (14), biscuit (15), pizza (16), baby cereal food (17), and breakfast cereals (18). Assays were carried out using the same samples and serum pool as described in **Figure 1**.

ELISA-inhibition assays were preformed using the extract from raw flour as solid phase and those from foodstuffs as inhibitors (**Figure 2**). High inhibition values (89−47%) were observed in most extracts from wheat-derived products, including baby cereal food (number 17), toasted breads (numbers 7 and 8), and boiled pastas (numbers 9−11). Only the breakfast cereal sample (number 18) exhibited no inhibition.

2. IgE Immunodetection of Salt-Soluble Proteins from Wheat Foodstuffs and Identification of IgE Binding Proteins. The salt-soluble proteins from the different wheat-derived products were separated by SDS−PAGE and stained by Coomassie Blue (**Figure 3A**). A complex pattern of protein bands with apparent molecular mass from 5 to 120 kDa was found in all samples analyzed, except in breakfast cereals, which did not present appreciable bands. In contrast, the immunodetection of replica gels with the serum pool from wheat food allergic patients rendered differential IgE recognition patterns for most extracts (**Figure 3B**). Raw flour and bran (numbers 1 and 2), biscuit (number 9), and nonboiled pasta (numbers 13−15) samples exhibited from 5 to 10 prominent IgE-binding components, covering approximately a 5−100 kDa range of apparent molecular mass. Extracts from boiled pastas (numbers. 10−12) showed a few number of reactive bands (from 5 to 2), mainly in the 20−50 kDa range. The qualitative and quantitative decrease of IgE recognition was even greater in the case of pizza, baby cereal food, and breakfast cereal extracts (numbers 16−18). A clear-cut pattern was uncovered in breads (numbers 3−6), with two main IgE-binding bands of 20 and 32 kDa, whereas no band was detected in the toasted breads (numbers 7 and 8) preparations.

The identification of the two major reactive proteins from bread samples, which have retained the IgE-binding capacity after the baking process, seems to be warranted. Therefore, chromatographic fractions enriched in the 20 and 32 kDa bands were obtained from the wholemeal tin loaf bread extract and

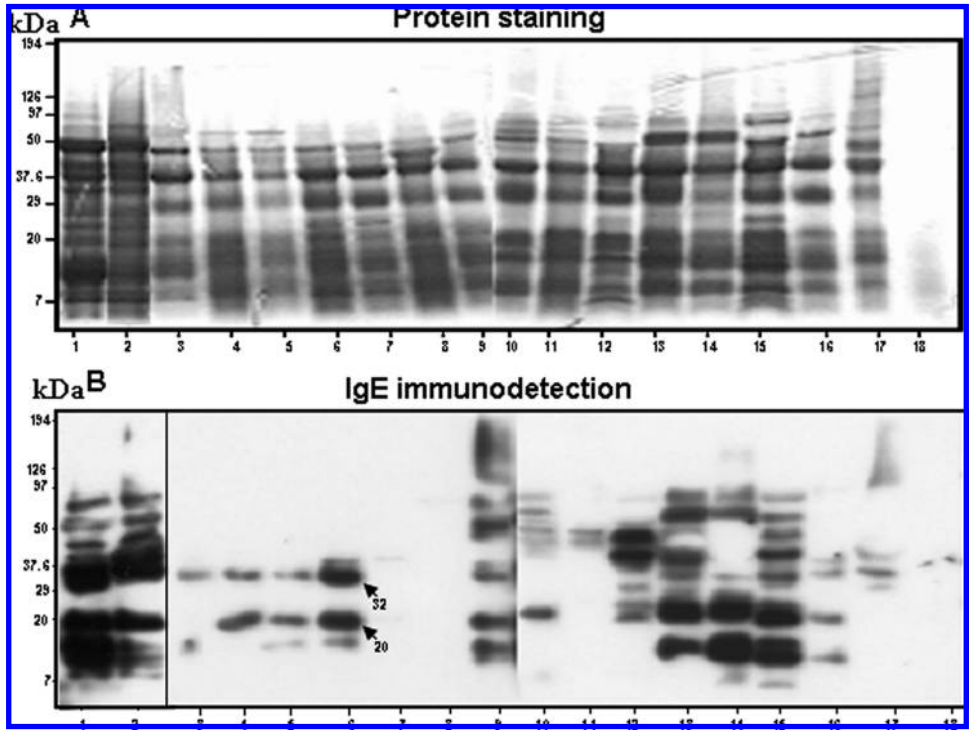

Figure 3. Protein staining (**A**) and immunonodetection with IgE of a serum pool from wheat food allergic patients (**B**) of the following SDS−PAGE separated samples: salt soluble protein extract from wheat raw flour (1) and bran (2), French bread (3), wholemeal bread (4), white tin loaf bread (5), wholemeal tin loaf bread (6), toasted bread (7), wholemeal toasted bread (8), biscuit (9), boiled white pasta (10), boiled wholemeal pasta (11), boiled fresh pasta (12), white pasta (13), wholemeal pasta (14), fresh pasta (15), pizza (16), baby cereal food (17), and breakfast cereals (18). The wheat raw flour and bran are represented by a 3-fold less exposition. Arrows point to the 20 and 32 kDa bands further characterized.

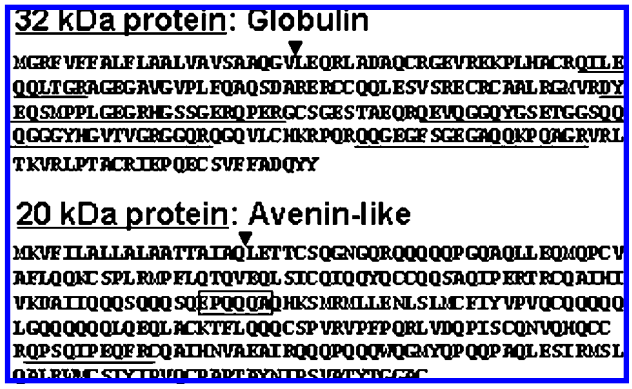

Figure 4. Identification of the major 32 and 20 kDa IgE-binding proteins from wholemeal tin loaf bread. The complete amino acid sequences deduced from nucleotide sequences encoding a globulin from *Triticum turgidum* (accession gi:37575362) and an avenin-like protein from *Aegilops sharonensis* (accession gi:156630214) are represented. Internal sequences obtained by fingerprinting analysis are underlined, and the N-terminal sequence of the 20 kDa protein is marked by a square. Arrows indicate the end of the predicted signal peptide.

the protein bands from PVDF membranes or SDS−PAGE gels subjected to N-terminal sequencing and fingerprinting analysis. The results are summarized in **Figure 4**. No clear N-terminal sequence of the 32 kDa protein was obtained, but 5 of its tryptic peptides corresponded (38% of sequence coverage) with internal amino acid regions of a putative *Triticum turgidum* globulin with a calculated molecular size of 24.5 kDa. Interestingly, a highly similar homologue to this globulin has been described in bread wheat (*T. aestivum*) as well (*9*). A tryptic peptide from the 20 kDa protein allowed us to identify an avenin-like protein from *Aegilops sharonensis*, which had a calculated molecular size of 32.4 kDa. However, the N-terminal sequence obtained for the 20 kDa protein (EPQQEA) is nearly identical to an internal sequence (EPQQQA) of the putative avenin-like protein. Furthermore, the calculated molecular size for a potentially processed fragment of the latter protein, with a N-terminus similar to that of the 20 kDa protein, was 19.4 kDa.

Table 2. Results of SPTs to Salt-Soluble Protein Extracts from Raw Flour and Wheat-Derived Foodstuffs

	SPT (mm^2 of wheal area) to				
patient no.[a]	commercial extract	raw flour	wholemeal tin loaf bread	wholemeal toasted bread	boiled pasta
1	20	78	78	18	20
2	78	78	79	23	20
3	20	79	50	7	38
4	28	21	28	(3)[b]	13
5	38	39	51	13	20
6	20	19	27	13	13
9	20	20	22	(5)	(5)
10	20	38	64	12	20
11	38	39	38	13	20
13	28	78	64	12	19

[a] Numbers as in **Table 1**. [b] Negative values are indicated in parentheses.

SPT Responses to Salt-Soluble Proteins from Wheat-Derived Foodstuffs. Extracts from raw flour, two bread samples, and boiled pasta were tested in 10 of the selected patients by SPTs (**Table 2**). The salt-soluble protein extract from wheat raw flour provoked higher responses than the commercial extract (mean, 48.9 versus 31 mm^2; $p = 0.09357$), but rather similar to those of the wholemeal tin loaf bread (mean, 50.1 mm^2; $r = 0.8296$; $p = 0.90162$). In contrast, the toasted bread (mean, 11.9 mm^2; $p = 0.00036$) and the boiled white pasta (mean, 18.8 mm^2; $p = 0.00283$) showed a strong decrease of allergenic potency compared to that of the raw flour extract.

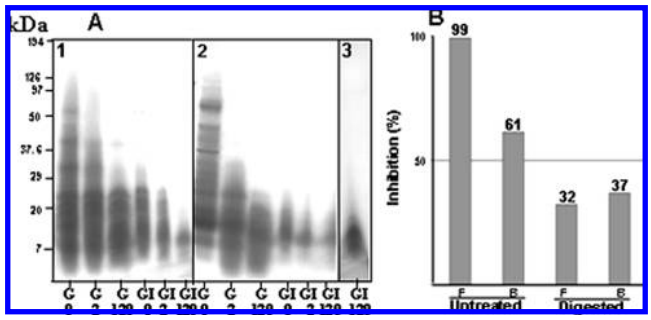

Figure 5. (**A**) Protein staining of SDS−PAGE separated samples of salt-soluble proteins from raw flour (A1) and wholemeal tin loaf bred (A2) subjected to simulated gastric (G; 0, 2, and 120 min) followed by intestinal (GI; 0, 2, and 120 min) digestion. Simulated gastrointestinal fluid (120 min) is included as control (A3). (**B**) ELISA-inhibition assays with salt-soluble proteins from wheat raw flour as solid phase, and untreated and digested (120 min of simulated GI) salt-soluble proteins from raw flour (F) and wholemeal tin loaf bread (B) as inhibitors.

Simulated Gastrointestinal Digestion of Flour and Bread Salt-Soluble Proteins. Extracts from raw flour and wholemeal tin loaf bread were selected as models to evaluate the effect of simulated gastrointestinal digestion on the IgE-binding capacity of wheat-derived products. Most salt-soluble proteins from both samples were readily hydrolyzed after 120 min of gastrointestinal digestion (**Figure 5A**). The lack of resolution by SDS−PAGE of the digestion products suggested that IgE-immunodetection should be discarded as a suitable method to evaluate the effects of digestion on IgE-binding capacity. ELISA-inhibition assays were chosen as an alternative approach (**Figure 5B**). The simulated digestion drastically decreased, but did not fully abolish, the IgE-binding potency of both raw flour (3-fold) and bread (1.6-fold) salt-soluble proteins.

DISCUSSION

Most studies on wheat food allergens have used raw flour as the reference wheat product, but almost all wheat-derived foodstuffs that are extensively consumed suffer heat treatment (and protein hydrolysis in some cases) during industrial processing and home cooking. These processes can modify the allergenic potency of wheat allergens as well as their solubility properties. The IgE-binding capacity of salt-soluble proteins from up to 16 different wheat-derived foodstuffs has been evaluated here and compared to that of raw flour, by means of several in vitro and in vivo assays.

A decrease in IgE-binding capacity, compared with that of raw flour has been detected in all wheat-derived products analyzed, both in native (ELISA) and denaturalizing (immunodetection after SDS−PAGE) conditions. The loss of allergenic potency seems to be related to the intensity of the heat treatment used to make the product. Thus, toasted breads showed the highest decrease (around 70%), whereas other types of breads, with less drastic heat treatment, retained approximately half of the IgE-binding level of raw flour. Furthermore, no reactive band was detected in toasted bread samples when immunodetected with sera from wheat food allergic patients. An even more emphasized difference has been found in pasta products. Boiled samples behaved as around half allergenic than raw pastas, probably due to heat denaturation of allergenic proteins and/or their flow to the cooking water. The loss of 12−15 kDa bands in boiled pastas is noticeable, which probably corresponds to allergens of the α-amylase inhibitor family. The inactivation (by putative denaturation) of these allergens by heat treatment has been previously described (*27, 33*).

Interestingly, the presence of common IgE-epitopes in raw flour and wheat-derived foodstuffs has been ascertained by ELISA-inhibition assays. Salt-soluble proteins from the processed products inhibited from 89% to 47% the IgE-binding to the raw flour extract, except that from breakfast cereals. Even cereal baby food exerted a remarkable inhibition value (51%), although its proteins are hydrolyzed and not detected by IgE-immunodetection. The enzymatic hydrolysis of wheat proteins renders peptides greater than 1 kDa, which are potentially allergenic, as has been previously reported (*34*).

The in vitro results discussed above were confirmed by in vivo tests. Similar SPT responses were reached by the raw flour and wholemeal tin loaf bread extracts, whereas a strong reduction (over 2.5-fold, statistically significant) was observed for those of boiled pastas and toasted breads, as expected.

Besides being subjected to heat (and proteolytic) treatment during industrial processing, the potential allergens of wheat-derived foodstuffs undergo gastrointestinal digestion after consumption. Therefore, the effect of simulated gastric followed by intestinal juices on the salt-soluble proteins from raw flour and wholemeal tin loaf bread samples was also tested. Most, if not all, proteins from both extracts were hydrolyzed. However, the digested products (peptides) retain a residual IgE-binding capacity (2−3- fold decrease with respect to that of the untreated samples).

Finally, the identification of the two major IgE-binding proteins detected in different bread, but not toasted, samples should be emphasized, thus indicating their partial heat-resistance. A 32 kDa putative allergen has been identified as a globulin, whose coding DNA sequence is linked to the Glu-1 locus (*35*), which encoded HMW-glutenin subunits, also described as potential wheat allergens (*20*), and pivotal components determining flour quality (*36*). This globulin family has been previously located as wheat food allergens by Pastorello et al. (*9*) in raw flour extracts. The second putative allergen identified corresponds to an avenin-like protein, a group of seed storage proteins, which seems to have a high expression in wheat endosperm (*37*). Therefore, the avenin-like proteins of wheat flour could represent a novel group of heat-resistant allergens with a potential role in wheat-derived foodstuffs. Interestingly, this protein group shows around 40% of amino acid sequence identity to wheat prolamin allergens previously reported (*16, 21*).

Overall, the results presented here indicate that wheat-derived foodstuffs have a lower allergenic potency than raw flour and that substantial differences in the decrease of their IgE-binding capacity are found among such commercial products. A further analysis of the allergenic properties of the most consumed wheat-derived foods as well as a detailed identification of their main allergens will help to clarify the actual status of wheat food allergy.

LITERATURE CITED

(1) Zuidmeer, L.; Goldhahn, K.; Rona, R. J.; Gislason, D.; Madsen, C.; Summers, C.; Sodergren, E.; Dahlstrom, J.; Lindner, T.; Sigurdardottir, S. T.; McBride, D.; Keil, T. The prevalence of plant food allergies: a systematic review. *J. Allergy Clin. Immunol.* **2008**, *121*, 1210–1218.

(2) Morita, E.; Kunie, K.; Matsuo, H. Food-dependent exercise-induced anaphylaxis. *J. Dermatol. Sci.* **2007**, *47*, 109–117.

(3) Brant, A. Baker's asthma. *Curr. Opin. Allergy Clin. Immunol.* **2007**, *7*, 152–155.

(4) Osborne, T. *The Proteins of Wheat Kernel*; Carnegie Inst.: Washington DC,1907; p 84.

(5) Palosuo, K. Update on wheat hypersensitivity. *Curr. Opin. Allergy Clin. Immunol.* **2003**, *3*, 205–209.

(6) Gómez, L.; Martín, E.; Hernández, D.; Sánchez-Monge, R.; Barber, D.; del Pozo, V.; de Andrés, B.; Armentia, A.; Lahoz, C.; Salcedo, G. Members of the alpha-amylase inhibitors family from wheat endosperm are major allergens associated with baker's asthma. *FEBS Lett*. **1990**, *261*, 85–88.
(7) Armentia, A.; Sánchez-Monge, R.; Gomez, L.; Barber, D.; Salcedo, G. In vivo allergenic activities of eleven purified members of a major allergen family from wheat and barley flour. *Clin. Exp. Allergy* **1993**, *23*, 410–415.
(8) James, J. M.; Sixbey, J. P.; Helm, R. M.; Bannon, G. A.; Burks, A. W. Wheat alpha-amylase inhibitor: a second route of allergic sensitization. *J. Allergy Clin. Immunol*. **1997**, *99*, 239–244.
(9) Pastorello, E. A.; Farioli, L.; Conti, A.; Pravettoni, V.; Bonomi, S.; Iametti, S.; Fortunato, D.; Scibilia, J.; Bindslev-Jensen, C.; Ballmer-Weber, B.; Robino, A. M.; Ortolani, C. Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int. Arch. Allergy Immunol*. **2007**, *144*, 10–22.
(10) Sánchez-Monge, R.; García-Casado, G.; López-Otín, C.; Armentia, A.; Salcedo, G. Wheat flour peroxidase is a prominent allergen associated with baker's asthma. *Clin. Exp. Allergy* **1997**, *27*, 1130–1137.
(11) Palacin, A.; Quirce, S.; Armentia, A.; Fernández-Nieto, M.; Pacios, L. F.; Asensio, T.; Sastre, J.; Diaz-Perales, A.; Salcedo, G. Wheat lipid transfer protein is a major allergen associated with baker's asthma. *J. Allergy Clin. Immunol*. **2007**, *120*, 1132–1138.
(12) Sandiford, C. P.; Tatham, A. S.; Fido, R; Welch, A.; Jones, M. G.; Tee, R. D.; Shewry, P. R.; Newman Taylor, A. J. Identification of the major water/salt insoluble wheat proteins involved in cereal hypersensitivity. *Clin. Exp. Allergy* **1997**, *27*, 1120–1129.
(13) Palosuo, K.; Alenius, H.; Varjonen, E.; Koivuluhta, M.; Mikkola, J.; Keskinen, H.; Kalkkinen, N.; Reunala, T. A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J. Allergy Clin. Immunol*. **1999**, *103*, 912–917.
(14) Varjonen, E.; Vainio, E.; Kalimo, K. Antigliadin IgE-indicator of wheat allergy in atopic dermatitis. *Allergy* **2000**, *55*, 386–391.
(15) Palosuo, K.; Varjonen, E.; Kekki, O. M.; Klemola, T.; Kalkkinen, N.; Alenius, H.; Reunala, T. Wheat omega-5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *J. Allergy Clin. Immunol*. **2001**, *108*, 634–638.
(16) Morita, E.; Kameyoshi, Y.; Mihar, S.; Hiragun, T.; Yamamot, S. Gamma-gliadin: a presumptive allergen causing wheat-dependent exercise-induced anaphylaxis. *Br. J. Dermatol*. **2001**, *145*, 182–184.
(17) Bittner, C.; Grassau, B.; Frenzel, K.; Baur, X. Identification of wheat gliadins as an allergen family related to baker's asthma. *J. Allergy Clin. Immunol*. **2008**, *121*, 744–749.
(18) Simonato, B.; De Lazzari, F.; Pasini, G.; Polato, F.; Giannattasio, M.; Gemignani, C.; Peruffo, A. D.; Santucci, B.; Plebani, M.; Curioni, A. IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion. *Clin. Exp. Allergy* **2001**, *31*, 1771–1778.
(19) Battais, F.; Pinea, F.; Popineau, Y.; Aparicio, C.; Kanny, G.; Guerin, L.; Moneret-Vautrin, D. A.; Denery-Papini, S. Food allergy to wheat: identification of immunoglobulin E and immunoglobulin G-binding proteins with sequential extracts and purified proteins from wheat flour. *Clin. Exp. Allergy* **2003**, *33*, 962–970.
(20) Matsuo, H.; Kohno, K.; Niihara, H.; Morita, E. Specific IgE determination to epitope peptides of omega-5 gliadin and high molecular weight glutenin subunit is a useful tool for diagnosis of wheat-dependent exercise-induced anaphylaxis. *J. Immunol*. **2005**, *175*, 8116–8122.
(21) Akagawa, M.; Handoy, T.; Ishii, T.; Kumazawa, S.; Morita, N.; Suyama, K. Proteomic analysis of wheat flour allergens. *J. Agric. Food Chem*. **2007**, *55*, 6863–6870.
(22) Baur, X.; Degens, P. O.; Sander, I. Baker's asthma: still among the most frequent occupational respiratory disorders. *J. Allergy Clin. Immunol*. **1998**, *102*, 984–997.
(23) Mills, E. N.; Mackie, A. R. The impact of processing on allergenicity of food. *Curr. Opin. Allergy Clin. Immunol*. **2008**, *8*, 249–253.
(24) Scibilia, J.; Pastorello, E. A.; Zisa, G.; Ottolenghi, A.; Bindslev-Jensen, C.; Pravettoni, V.; Scovena, E.; Robino, A.; Ortolani, C. Wheat allergy: a double-blind, placebo-controlled study in adults. *J. Allergy Clin. Immunol*. **2006**, *117*, 433–439.
(25) Mittag, D.; Niggemann, B.; Sander, I.; Reese, I.; Fiedler, E. M.; Worm, M.; Vieths, S.; Reese, G. Immunoglobulin E-reactivity of wheat-allergic subjects (baker's asthma, food allergy, wheat-dependent, exercise-induced anaphylaxis) to wheat protein fractions with different solubility and digestibility. *Mol. Nutr. Food Res*. **2004**, *48*, 380–389.
(26) Gerrad, J. A. Protein-protein cross linking in food: Methods, consequences, applications. *Trends Food Sci. Technol*. **2002**, *13*, 391–399.
(27) Simonato, B.; Pasini, G.; Giannattasio, M.; Peruffo, A. D.; De Lazzari, F.; Curioni, A. Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. *J. Agric. Food Chem*. **2001**, *49*, 5668–5673.
(28) De Zorzi, M.; Curioni, A.; Simonato, B.; Giannattasio, M.; Pasini, G. Effect of pasta drying temperature on gastrointestinal digestibility and allergenicity of durum wheat proteins. *Food Chem*. **2007**, *104*, 353–363.
(29) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248–254.
(30) Díaz-Perales, A.; Sanz, M. L.; García-Casado, G.; Sánchez-Monge, R.; García-Selles, F. J.; Lombardero, M.; Polo, F.; Gamboa, P. M.; Barber, D.; Salcedo, G. Recombinant Pru p 3 and natural Pru p 3, a major peach allergen, show equivalent immunologic reactivity: a new tool for the diagnosis of fruit allergy. *J. Allergy Clin. Immunol*. **2003**, *111*, 628–633.
(31) Diaz-Perales, A.; Collada, C.; Blanco, C.; Sanchez-Monge, R.; Carrillo, T.; Aragoncillo, C.; Salcedo, G. Cross-reactions in the latex-fruit syndrome: a relevant role of chitinases but not of complex asparagine-linked glycans. *J. Allergy Clin. Immunol*. **1999**, *104*, 681–687.
(32) Moreno, F. J.; Mellon, F. A.; Wickham, M. S.; Botrill, A. R.; Mills, E. N. C. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. *FEBS J*. **2005**, *272*, 341–352.
(33) O'Connor, C. M.; McGeeney, K. F. Isolation and characterization of four inhibitors from wheat flour which display differential inhibition specificities for human salivary and human pancreatic alpha-amylases. *Biochim. Biophys. Acta* **1981**, *658*, 387–396.
(34) Akiyama, H.; Sakata, K.; Yoshioka, Y.; Murata, Y.; Ishihara, Y.; Teshima, R.; Sawada, J.; Maitani, T. Profile analysis and immunoglobulin E reactivity of wheat protein hydrolysates. *Int. Arch. Allergy Immunol*. **2006**, *140*, 36–42.
(35) Kong, X.; Gu, Y. Q.; You, F. M.; Dubcovsky, J.; Anderson, O. D. Dynamics of the evolution of orthologous and paralogous portions of a complex locus region in two genomes of allopolyploid wheat. *Plant Mol. Biol*. **2004**, *54*, 55–69.
(36) Anjum, F. M.; Khan, M. R.; Din, A.; Saeed, M.; Pasha, I.; Arshad, M. U. Wheat gluten: high molecular weight glutenin subunits: structure, genetics, and relation to dough elasticity. *J. Food Sci*. **2007**, *72*, R56−63.
(37) Laudencia-Chingcuanco, D. L.; Stamova, B. S.; Lazo, G. R.; Cui, X.; Anderson, O. D. Analysis of the wheat endosperm transcriptome. *J. Appl. Genet*. **2006**, *47*, 287–302.

Received for review November 7, 2008. Revised manuscript received January 20, 2009. Accepted February 11, 2009. This study was supported by Ministerio de Educacion y Ciencia, DGI (project Bio 2006-07472) and by Instituto de Salud Carlos III, Retics 2007 (RD07/0064).

JF803475V