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

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The effect of baking and digestion on the allergenicity of wheat flour proteins has been studied. Pooled sera of patients suffering from food allergy to wheat products were tested for IgE binding to the proteins of the wheat dough and of the bread crumb and crust, before and after being in vitro digested. During in vitro digestion, the IgE binding protein components of the unheated dough tended to disappear, whereas a permanence of IgE recognition was evident for both the bread crumb and crust. This indicates that the baking process increases the resistance of the potential allergens of the wheat flour to proteolytic digestion, allowing them to reach the gastrointestinal tract, where they can elicit the immunological response. Therefore, the effects of baking must be carefully considered in studying food allergies to wheat products.

Keywords: *allergy, bread, baking, digestion, food processing, wheat proteins*

INTRODUCTION

It is estimated that more than 2% of the adult population of the developed countries suffers from IgE-mediated hypersensitivity reactions after ingestion of foods (food allergy), including wheat products, and the prevalence of this pathology seems to continuously increase.

Stability during digestion is considered an important feature in determining the allergenicity of food proteins (*1*). It is generally thought that food allergens are typically resistant to proteolysis (*2*), thus, reaching unaltered the intestine where they elicit the immune response. In contrast, digestible food proteins (as, for example, those of fruits and vegetables) can act as allergens only before entering the digestive tract and therefore are mainly involved in determining perioral symptoms, as, for example, the oral allergy syndrome (OAS) (*1*) and contact urticaria (*3*).

Protein digestibility can be strongly affected by thermal processing, which induces modifications of the physicochemical and immunological characteristics of the potential allergens of a given food (*4*). For example, it has been recently reported that the allergenic properties of peanuts are enhanced after heat treatment because of the formation of protease-resistant protein aggregates with novel IgE binding sites, arising from the Maillard reaction occurring during roasting (*5*).

Wheat is a typical example of a food that cannot be consumed without some type of thermal processing, including cooking, baking, extrusion, etc. Ingestion of wheat-based foods, such as bread or pasta, can cause gastrointestinal (GI) symptoms in allergic subjects (*6*), suggesting an involvement of wheat proteins in eliciting an intestinal IgE-mediated reaction. Indeed, the occurrence of IgE binding to both water/salt soluble (albumins and globulins) and insoluble (storage) proteins [HMW, S-poor and S-rich prolamins, (*7*)] has been detected, by immunoblotting, in the sera of patients suffering from allergic symptoms after wheat ingestion (*6*, *8*−*10*), indicating that different protein components of the wheat flour can contain IgE-reactive epitopes. However, before coming into contact with their target organ, the wheat flour allergens undergo extensive modification both before entering the mouth (i.e., during food preparation) and after being ingested (i.e., during proteolytic digestion in the GI tract) (*11*−*14*). Therefore, at least in the cases of food allergy with symptoms appearing after a time compatible with a more or less complete digestion process, the analysis of the raw (untreated) flour proteins for their binding to IgE should be considered of limited value for the elucidation of what actually happens when processed wheat products are ingested by individuals with food allergy to this cereal.

In a previous paper, we have shown that bread baking affects the digestibility of the wheat flour proteins by inducing the formation of protein aggregates stabilized by heat-induced interactions and we have hypothesized that this phenomenon would have an effect on the allergenic properties of wheat-based foods (*14*). To verify this assumption, we have studied the binding of the serum IgE of wheat allergic patients with GI symptoms to the proteins present in bread dough, crumb, and crust before and after being digested in an in vitro multienzymatic system reproducing the human digestive process.

MATERIALS AND METHODS

Chemicals. Pepsin (E. C. 3.4.23.1) from hog stomach (≈3000 U/mg) was from Fluka. Pancreatin from porcine

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pancreas (Sigma cat. P 1750) and anti-human IgE peroxidase-conjugate antibody were from Sigma. The GelCode Glycoprotein staining kit and the SuperSignal chemiluminescent substrate were from Pierce. Other chemicals were of analytical grade.

Samples Preparation. Bread and samples preparations and in vitro protein digestion were performed as previously described (*14*). Briefly, unheated bread dough, bread crumb, and crust (60 mg) were suspended in 4 mL of 0.2 N HCl (pH 2.0) containing 0.05 mg/mL of pepsin. After 30 min at 37 °C, 1.15 mL of 1 M boric acid, 0.5 N NaOH, adjusted to pH 6.8 with 5 N HCl and containing 0.25 mg/mL of pancreatin was added. The resulting pH was 7.6. The reaction was allowed to proceed at 37 °C in a shaking water bath and stopped at different times (0, 15, and 30 min of pepsin attack and 15, 30, 90, and 150 min of pancreatic digestion) by addition of 0.5 volumes of 0.6 M Tris-HCl, pH 7.4, containing 30% (w/v) glycerol, 6.0% (w/v) SDS and 6% (v/v) 2-mercaptoethanol. Samples were immediately heated at 100 °C for 5 min and centrifuged.

Electrophoresis. Undigested and digested samples were analyzed by tricine−SDS−PAGE (T−SDS−PAGE) as previously described (*14*) in a 16.5% total polyacrylamide gel. Molecular weight standard proteins (Bio Rad) were phosphorilase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soy trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Gels were stained with Coomassie or used for immunoblotting.

IgE Immunoblotting. Detection of IgE binding to bread dough, crumb, and crust proteins, before and after digestion, was performed as previously described (*15*). A pool of sera from seven allergic patients, previously characterized as suffering from GI symptoms after ingestion of wheat-based foods (*6*) was used.

Carbohydrate Detection. Carbohydrates were stained on electrophoretic gels by the periodate method, using the GelCode Glycoprotein staining kit.

RESULTS

In Vitro Protein Digestion of Bread Dough, Crumb, and Crust. Bread dough, crumb, and crust were digested by an in vitro multienzymatic system simulating the digestion process occurring in the human GI tract, as recently described (*14*). The total proteins of the digested samples were analyzed by T−SDS−PAGE. Starting from the initial pepsin attack, digestion of the unheated bread dough apparently resulted in a rapid breakdown of the wheat flour proteins and gave rise to the accumulation of bands with Mrs lower than about 15 kDa (Figure 1A). The bread crumb seemed to be similarly affected by the proteolytic enzymes (Figure 2A), although some variations in the fate of the various protein components during digestion were detected in comparison with the unheated dough. Such variations depend on the heat treatment (*14*) that, during baking, approaches 100 °C (*15*). As previously reported (*14*), the crust sample, before and after digestion, could not be completely solubilized in SDS−PAGE sample buffer as a result of the strong modifying effect (mainly involving protein aggregation) of the very high temperature (i.e., 200−220 °C) reached by the exterior of the bread loaf during baking (*11*, *13*, *14*). However, the proteins extractable by the SDS−PAGE sample buffer in reducing conditions from the undigested and digested crust samples were analyzed by electrophoresis (Figure 3A). In addition to a loss of bands in the undigested sample and to a general decrease in band definition, the main difference noted with respect to the crumb was the presence of high molecular weight protein aggregates, blocked at the top of the gel, which apparently resisted

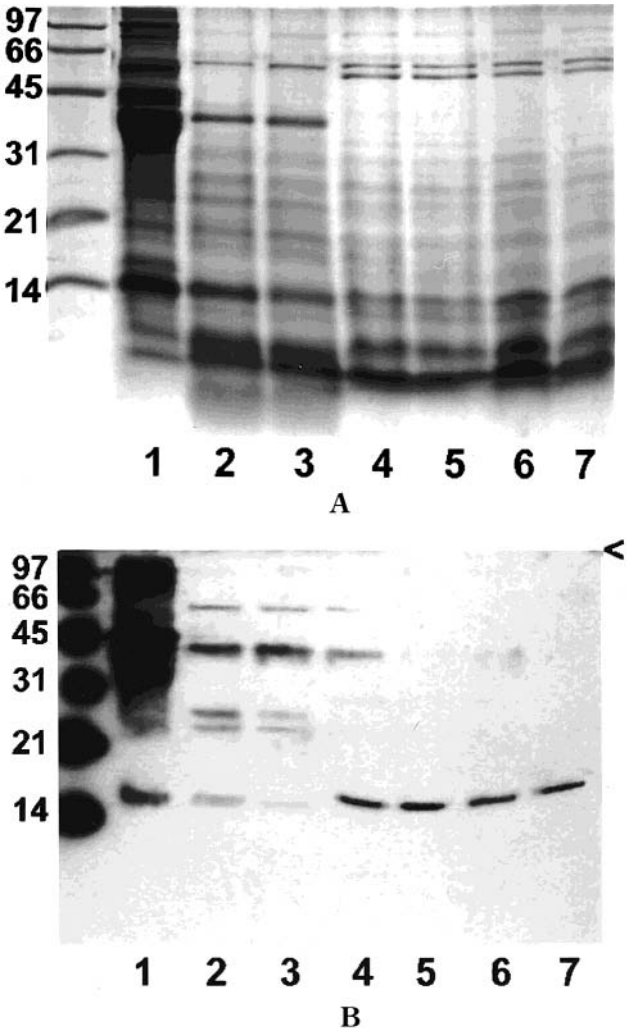

Figure 1. Tricine−SDS−PAGE analysis of bread dough proteins before and during in vitro enzymatic digestion. Lane 1: undigested sample. Lanes 2 and 3: samples taken after 15 and 30 min of pepsin digestion, respectively. Lanes 4−7: samples taken after 30 min of pepsin digestion followed by 15, 30, 90, and 150 min of pancreatin digestion, respectively. A: gel stained with Coomassie. B: IgE immunoblot with pooled sera of patients with food allergy to wheat products. The arrowhead in B indicates the top of the resolving gel. Mr standard proteins [marked with the method of Eberini and Puglisi (*27*) in B] are on the left side.

unaffected during the entire digestion process (*14*) (Figure 3A, arrowheads).

Serum IgE Binding to the Proteins of Bread Dough and to Its Digestion Products. Proteins from the bread dough and its in vitro digestion products were analyzed by immunoblotting in order to detect the IgE binding of pooled sera from seven atopic patients previously characterized as suffering from GI symptoms after wheat ingestion (*6*). These patients were chosen on the basis of their clinical history, RAST and SPT positivity to wheat, and because they typically showed GI manifestations of food allergy, including abdominal pain and diarrhea, some time after eating wheat-based food products (like bread and pasta) (*6*).

When the pooled sera were tested by immunoblotting with the enzymatic preparations (pepsin and pancreatin) used in the in vitro digestion experiments, no IgE binding was detectable (not shown).

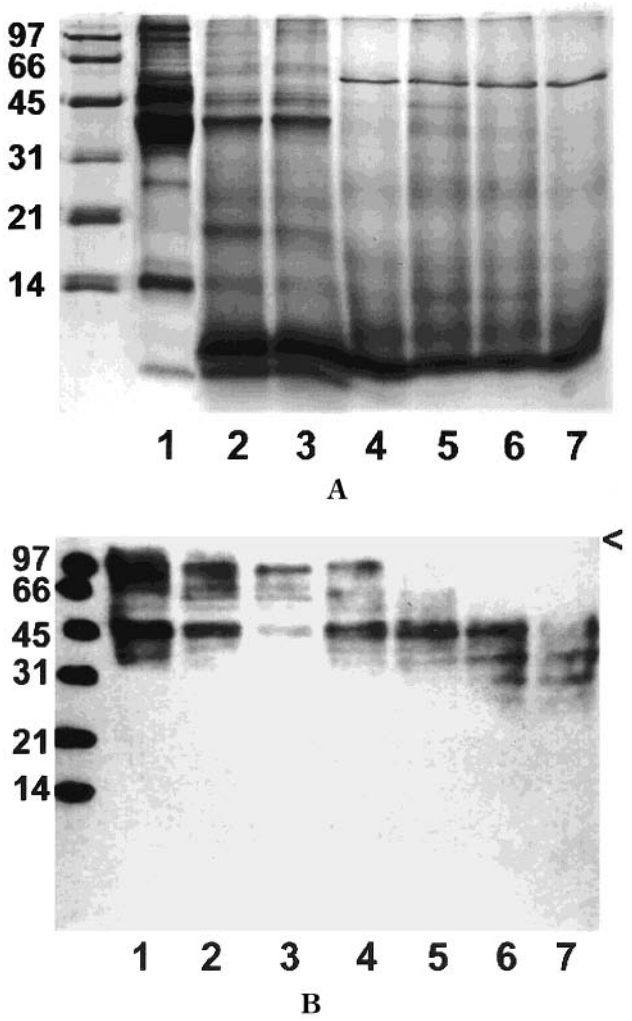

Figure 2. Tricine−SDS−PAGE analysis of bread crumb proteins before and during in vitro enzymatic digestion. The rest of the legend as in Figure 1.

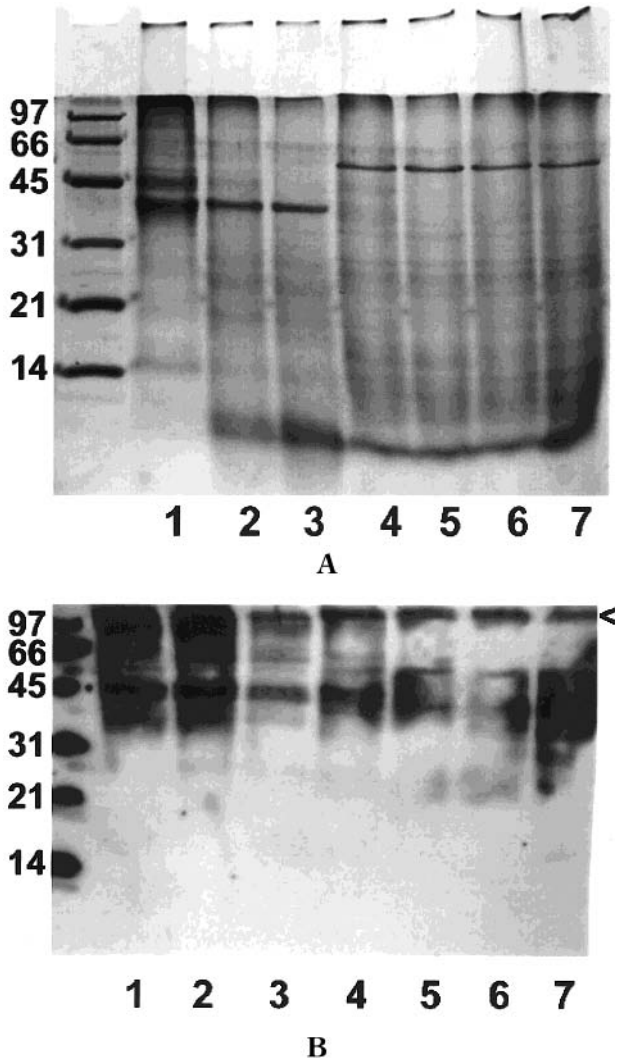

Figure 3. Tricine−SDS−PAGE analysis of bread crust proteins before and during in vitro enzymatic digestion. The rest of the legend as in Figure 1.

In the case of the unheated bread dough, IgE immunoblotting with pooled sera of the allergic patients showed a complex IgE binding pattern and confirmed that bands with Mrs corresponding to those of the storage proteins (namely, HMW and S-poor and S-rich prolamins) were recognized by the IgE of the pool (*6*) (Figure 1B). A protein with a Mr of about 16 kDa, belonging to the wheat α-amylase inhibitors protein family, was also recognized. This protein class was previously shown to comprise major allergens responsible for bakers' asthma (*17*) and to be involved also in food allergy to wheat (*9*). During pepsin digestion IgE binding to most of the protein bands seen in the undigested sample disappeared, and only a few bands maintained their IgE binding ability (Figure 1B). The Mrs of the IgE binding bands larger than 31 kDa corresponded to those of the proteolytic fragments deriving from the HMW prolamins, whereas the bands of lower Mr could be the products of the S-rich prolamins proteolysis, as previously shown by immunoblotting with specific antibodies (*14*). Moreover IgE binding to the 16 kDa wheat flour allergen was still detectable during the initial phase of pepsin proteolysis.

During the pancreatic attack, however, all of these bands tended to disappear, and a new band with Mr around 16 kDa was bound by the IgE of the pooled sera until the last step of the digestion process (Figure 1B). This 16 kDa band does not seem to belong to the α-amylase inhibitors, being generated only after the action of the pancreatic enzymes. This indicates that, despite the possibility to immunologically detect at least one of the components of the low Mr α-amylase inhibitors after in vitro digestion (*14*), the major wheat flour allergens involved in bakers' asthma lose their ability to bind the IgE of our wheat allergic patients.

The bands accumulating during proteolysis at Mrs lower than 14 kDa were not recognized by the IgE (Figure 1B), indicating a complete absence of allergenic activity for the low Mr wheat protein fragments generated by the in vitro digestion process.

Serum IgE Binding to the Proteins of Bread Crumb and Crust and to Their Digestion Products. The pooled sera were also tested for IgE binding to the bread crumb and crust proteins before and after in vitro digestion. In the undigested bread crumb, the IgE binding pattern was similar to that obtained with the unheated dough, although a lower number of S-rich prolamins was recognized and no binding to the α-amylase inhibitors was detectable (Figure 2B). This latter result was consistent with the loss of immunological detection obtained after bread baking with an antibody specific for components of the α-amylase inhibitors of the wheat flour (*14*).

In contrast, compared to the unheated dough, much different results were obtained when the digestion products of the crumb were examined, indicating a strong effect of the baking process on the digestion of the IgE binding protein components. In fact, with reference to the undigested crumb sample, despite the heavy modification of the protein pattern since the first 15 min of pepsin action (Figure 2A), the IgE binding pattern was apparently affected only slightly during 30 min of pepsin hydrolysis (Figure 2B), indicating a permanence of IgE reactive bands that could be only barely detected by Coomassie staining. Major bands involved in IgE recognition at this time showed a mobility corresponding to that of the HMW prolamins. Indeed, by the use of a specific antibody, only a slight modification of these glutenin subunits has been shown to occur in the bread crumb after 30 min of pepsin hydrolysis (*14*). Moreover, in the pepsin-digested crumb samples, other bands were bound by the IgE, showing a Mr corresponding to that of the group of the S-rich prolamins (Figure 2B).

Also the pancreatin attack seemed to be rather ineffective during the first 15 min of action and modified the IgE binding pattern only in the subsequent steps of digestion (Figure 2B). In this case, IgE binding to the HMW prolamins disappeared completely only after 90 min of pancreatin hydrolysis, but some binding to a protein fraction with a mobility similar to that of the S-rich prolamins was detected until the last step of digestion. In particular, a LMW glutenin subunit, that is similar to the band that has been shown to be strongly bound in the water/salt-insoluble protein fraction of the wheat flour by most of the wheat allergic patients suffering from GI symptoms (*6*), seemed to be particularly resistant to the action of the digestive enzymes (Figure 2B).

The same experiments were then performed on the bread crust (Figure 3B). Resembling the results recently shown for roasted peanuts (*5*), some IgE binding was detectable at the level of the protein complexes of the crust appearing as smears at the top of the resolving gel (*14*), suggesting that they contained some flour allergens maintaining their allergic properties also in an heat-induced aggregated form. Moreover, these protein aggregates were recognized during the entire digestion process, indicating that the allergens were stabilized against proteolytic degradation by the molecular interactions formed by the high temperature of baking. Other bands were recognized in the crust samples, suggesting that the thermal treatment did not destroy the IgE binding ability of some wheat flour proteins. Also in the crust samples, IgE binding to the 16 kDa wheat flour allergen was never detectable.

Carbohydrate detection on the gel (Figure 4) indicated the presence of sugars at the level of both the loading point (double arrowhead) and the top of the resolving gel (single arrowhead). This latter staining was completely absent when the unheated dough sample was analyzed, suggesting that at least some of the protein complexes recognized by the IgE could be formed in the crust during baking as a result of protein interactions with the sugars present in dough.

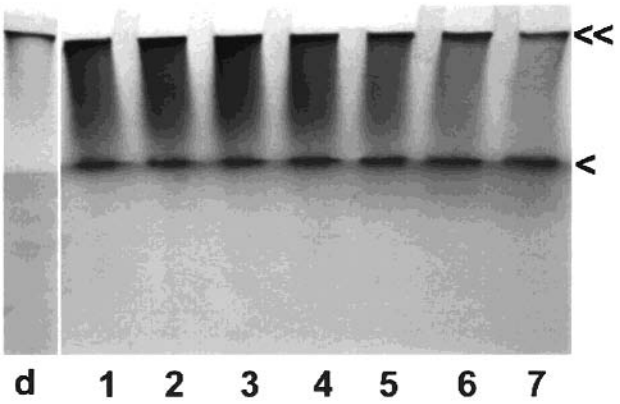

Figure 4. Carbohydrate detection after Tricine−SDS−PAGE of bread crust proteins before and during in vitro enzymatic digestion (as in Figure 3A). The double and the single arrowheads indicate the loading points and the top of the resolving gel, respectively. For comparison, the undigested bread dough is shown in lane d.

DISCUSSION

It is well-known that wheat flour proteins are modified after heat processing to an extent which depends on the intensity of the heat treatment. These modifications have been shown to involve not only protein breakdown, but also aggregation, cross-linking, and Maillard-type reactions (*11*, *13*), which result in a decreased protein digestibility (*12*, *18*). In a previous paper (*14*) we have shown that bread baking has a strong effect on the immunological and physicochemical characteristics of the wheat flour proteins and, as a consequence, on their fate during an in vitro proteolytic digestion process. This led us to hypothesize that also a variation of the allergic properties had to be expected as a result of baking. The same idea was also proposed by Hansen and Millington (*13*), who indicated the indigestible wheat protein species generated by the Maillard reaction as factors of antibody production in the intestinal cells. In this paper, we confirm this assumption by showing that the binding of IgE from sera of wheat allergic patients to the digestion products of wheat flour proteins can be strongly modified by the heat treatment. In fact, differently from the unheated bread dough, where a rapid breakdown of the IgE binding components could be detected, a persistent IgE binding to the flour allergens was evident for both the bread crumb and crust. This can be ascribed to the thermally induced wheat protein modifications occurring during baking that result in both the production of protein-sugar adducts that resist to the digestion process, mainly trough blockage of the action of carboxypeptidase-B (*13*) and the formation of macromolecular complexes in which the accessibility of the proteases to their sites of action has to be reduced (*14*). However, these effects seem to be different in the crumb and in the crust, which, during baking, undergo to temperatures lower than 100 °C and higher than 180−200 °C, respectively. In the crumb, the IgE binding protein components of the protease-treated samples show an electrophoretic mobility similar to that of the original proteins. This should indicate that protein aggregation can be in some way reversed by the treatment with SDS in reducing conditions (made before the electrophoretic separation), suggesting that the crumb protein aggregates are stabilized mainly by SS bonds and hydrophobic interactions (*11*, *14*, *19*, *20*). In contrast, the crust proteins could not be completely extracted by SDS and 2-mercaptoethanol, neither in the undigested sample nor in the digested ones (*14*). Moreover, the extractable fraction of all of the crust samples, when subjected to SDS−PAGE analysis, showed the presence of protein

aggregates, containing also sugars, smearing at the top of the gel and therefore having a size so large to prevent them from entering the electrophoretic gel pores. These results indicate that, in the case of the crust, protein aggregation involves also strong molecular linkages, different from hydrophobic interactions and/or disulfide bonds, including those deriving from Maillard-type reactions and, possibly, inter-peptide cross-linking (*21*). The products of the Maillard reaction have been recently reported to increase the allergenic properties as well as the resistance to digestion of roasted peanut proteins (*5*). Our results show that also in baked wheat products the thermally induced protein aggregation prevents a complete proteolytic degradation of the allergenic proteins, potentially allowing the passage of large IgE binding protein fragments through the GI tract, where they can elicit the allergic response. Therefore, for individuals suffering from wheat food allergy, the thermal treatment of the ingested products must be considered as an important factor affecting their allergenic action at the level of the intestine.

An other interesting result concerns the wheat flour allergens with Mr around 16 kDa, belonging to the cereal α-amylase inhibitors protein family. These proteins have been shown to represent major allergens in determining the occupational allergic disease known as bakers' asthma that results from the inhalation of flour particles (*17*). More recently, by IgE immunoblotting with a wheat flour extract, the same proteins have been indicated as being responsible for the allergic reactions occurring after wheat ingestion (*9*). The data here presented and others previously reported (*6*) confirm that the 16 kDa allergen is recognized by IgE of individuals with food allergy to wheat, but this occurs only when the unheated flour proteins are tested. In contrast, bread baking seems to result in a complete disappearance of IgE binding to the 16 kDa allergen, as can be seen in both the crumb and crust samples. This confirms the occurrence of relevant thermally induced changes of the immunological properties for some low molecular weight wheat flour albumins (*14*) and also the results of O'Connor and McGeeney (*22*), who reported that heating at 100 °C completely eliminated the activity of four purified members of the wheat α-amylase inhibitors family. Therefore it can be concluded that, although acting as allergens in the raw wheat flour, thus determining respiratory symptoms after flour particles inhalation, it is unlikely that members of the wheat α-amylase inhibitors protein family are involved in food allergy with symptoms arising after ingestion of heat-processed wheat products. This would explain the reason of the absence of allergic reactions after ingestion of wheat-based foods, as for example bread, in most of the individuals suffering from bakers' asthma (*23*).

When the allergens reach the intestine, they can either act locally or cross the intestinal mucosa, where their uptake is facilitated by specialized "M cells" overlying Peyer's patches (*24*). In the former case, the clinical manifestations of the hypersensitivity reactions can be acute, local symptoms at the level of the GI tract (i.e., GI anaphylaxis manifested by nausea, abdominal pain, bloating, and/or diarrhea). In contrast, if the allergens enter the circulatory system, they are likely to induce systemic anaphylaxis, as generalized urticaria, angioedema, hypotension, and shock (*25*). Because the present study has been carried out by using the sera of patients whose allergic reactions to wheat-based foods resulted in GI symptoms, it is likely that the indigestible bread allergens causing the onset of such reactions acted locally in the GI tract. In fact, at least for the IgE binding protein aggregates, it can be postulated that they are not able to cross the intestinal mucosa because of their high molecular size.

In conclusion, the results here presented indicate that some wheat allergens, as, for example the wheat 16 kDa protein, can be destroyed after baking, whereas others can be made more resistant to digestion by the same heating process, thus allowing their interaction with the gut mucosa in an immunologically active form. Therefore, the laboratory diagnostic tests commonly used for the diagnosis of food allergies, if prepared with the raw offending material, appear of limited use when the allergic symptoms arise after a time compatible with a more or less complete digestion of processed food products. Moreover, the assessment of the allergenic potential of a given protein through the evaluation of its stability to digestion (*26*) should be performed not only on the raw protein preparation, but also after processing the whole food containing the suspected allergen into the form in which it will be actually consumed by humans.

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