Modifications of Wheat Flour Proteins during in Vitro Digestion of Bread Dough, Crumb, and Crust: An Electrophoretic and Immunological Study

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The proteins of wheat flour have several biological activities that can affect human health and physiology when wheat-based foods are consumed. The modifications of bread crumb and crust proteins during an in vitro peptic/pancreatic digestion process were studied by electrophoresis and immunoblotting with polyclonal antibodies specific for single proteins or groups of homologous proteins of the wheat flour, and the results were compared to those obtained for an unheated dough sample. The results show that baking affects the extent of proteolysis and the immunological and physicochemical features of the digestion products in relation to the level of the heat treatment. Therefore, the results concerning the digestion of the unheated wheat flour or dough are not representative of what happens when baked products enter the human digestive tract.

Keywords: *Wheat; dough; bread; baking; digestion; peptides*

INTRODUCTION

Wheat is a staple food for most of the world's population. The dietary importance of wheat flour is largely due to its unique property to give rise, after mixing with water, to a viscoelastic dough that can be processed into leavened products and pasta. The basic flour components that determine this possibility are the gluten proteins, the storage proteins of the seed. These proteins, also known as wheat prolamins, comprise the alcohol-soluble monomeric gliadins (comprising α/*β*-, *γ*-, and *ω*-gliadins) and the polymeric glutenin (partly soluble in diluted acetic acid or detergents). Upon reduction of disulfide (SS) bonds, glutenin polymers are broken down into polypeptides, the high and the low molecular weight glutenin subunits (HMW-GS and LMW-GS, respectively) (*1*). Another classification, based on biochemical criteria rather than on solubility properties, divides the storage proteins into high molecular weight (HMW) prolamins (corresponding to the HMW-GS), S (sulfur)-rich prolamins (corresponding to α/*β*- and *γ*-gliadins plus LMW-GS) and S-poor prolamins (the *ω*-gliadins) (*2*). The latter nomenclature will be followed in this paper. The water/salt-insoluble prolamins represent ∼80% of the total proteins of the wheat kernel. The remaining 20% is constituted by proteins soluble in water (albumins) and in salt solutions (e.g., 0.5 M NaCl) (globulins), which essentially have a physiological function in the seed (enzymes, enzyme inhibitors, ...).

Several proteins of the wheat flour possess different biological activities (*3*) that can affect, either adversely or beneficially, human health and physiology when wheat is consumed as a food. For example, certain amino acid sequences of the wheat storage proteins exhibit antihypertensive activity (*4*), and there are also indications that some mental disorders, such as schizophrenia and autism, could be related to a dietary overload of wheat proteins containing peptides with opioid-like activities (*5*−*7*). Other effects of wheat protein ingestion concern individuals suffering from specific pathologies. The most important one is celiac disease (CD), also known as gluten-sensitive enteropathy, which is caused by toxic peptides generated from the digestion of the wheat storage proteins (*8*, *9*) that lead, probably through an immunological mechanism, to a damaged intestinal mucosa and a malabsorption of nutrients. Moreover, some wheat flour proteins cause IgE-mediated hypersensitivity reactions after ingestion of wheat-containing products in sensitive individuals (*10*−*13*). Rather than from the intact wheat proteins, all of these effects should be considered to arise from the presence of wheat peptides capable interacting with the gut mucosa. These peptides originate from the digestive breakdown of the wheat proteins, which is initiated in the stomach by acid and pepsin and continued in the intestinal lumen by a variety of proteolytic enzymes, including chymotrypsin, trypsin, and carboxypeptidases. Amino acids and peptides are then absorbed within the intestinal mucosa and enter the circulatory system.

Because wheat is never used as a food without some type of heat processing, including baking, extrusion, cooking, etc., it is important to know how the different proteins (and their breakdown during digestion) are affected by these treatments. It is in fact well-known that the physicochemical and biological characteristics, including antigenicity, of proteins are changed after heating and that also the extent of their degradation during digestion can be either increased or lowered after

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heat processing (*14*). All of these modifications can affect the characteristics of the digestion products of wheat flour proteins and, consequently, their effects on human physiology. Some papers have been published on the variation of digestibility of wheat flour proteins as affected by heat processing (*15*, *16*). All of these studies were performed on model systems in which controlled moisture levels and temperatures were applied to the wheat flour. However, to the best of our knowledge, no data are available on the effects of the proteolytic digestion on the wheat flour proteins as they are actually consumed by humans, that is, after being transformed into foods. To clarify this point, in this paper we have studied, by electrophoresis and immunoblotting with specific antibodies, the fate of the different wheat flour proteins as they are present in the unheated wheat dough and in bread crumb and crust, after being digested in an in vitro system reproducing the process occurring in the human digestive tract.

MATERIALS AND METHODS

Chemicals. Pepsin (EC 3.4.23.1) from hog stomach (≈3000 units/mg) was from Fluka. Pancreatin from porcine pancreas (Sigma catalog no. P 1750), anti-rabbit IgG alkaline phosphatase conjugate, and BCIP/NTB alkaline phosphatase substrate tablets were from Sigma. Other chemicals were of analytical grade.

Antibodies. The polyclonal rabbit antibodies (PABs) against wheat flour proteins were a PAB specific for the HMW prolamins (anti-7), a PAB specific for the prolamin group (HMW and S-rich prolamins, i.e., LMW-GS and α/*β*- and *γ*-gliadins) (anti-12) (*17*), a PAB specific for *β*-amylase (*18*), and a PAB produced against the 0.19 albumin component (anti-A) but recognizing several members of the low M_r (14−16 kDa) albumins (*19*).

Bread Preparation. Fifty gram round bread loaves were prepared in a bakery according to the traditional Italian bread-making procedure with common wheat flour, water, salt, and yeast. Oven temperature was 220 °C, and baking time was 30 min.

Sample Preparation. Bread crumb was excised from the interior of a bread piece, whereas bread crust was scraped with a scalpel from the bread surface. A piece of dough, taken immediately before baking, was also used. All of the samples were freeze-dried, reduced to a fine powder by grinding in a mortar, and sieved.

Nitrogen Quantification. Samples were mineralized according to the method of Hach et at. (*20*), and nitrogen was quantified by using the AOAC method 33.056 (*21*).

Protein Digestion. Freeze-dried dough and bread crumb or crust (60 mg) were suspended in 4 mL of 0.2 N HCl (pH 2.0), containing 0.05 mg/mL pepsin. The final protein concentration was 1.5 mg/mL with an enzyme/protein ratio of 1:30 (w/w). Control (undigested) samples were treated in the same manner, but without pepsin. Proteolysis was carried out for 30 min. Afterward, the following was added to the samples: 1.15 mL of 1 M boric acid and 0.5 N NaOH, adjusted to pH 6.8 with 5 N HCl and containing 0.25 mg/mL of pancreatin. The resulting pH was 7.6, and the pancreatin to protein ratio was 1:21 (w/w) (*22*). 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 the addition of 1 volume of 20% (w/v) trichloroacetic acid (TCA). After standing for 1 h, the samples were centrifuged (8000*g*, 10 min) and the pellet was analyzed for nitrogen content.

Electrophoresis. Undigested samples (in 0.2 N HCl) and digested samples taken at the different times were made 0.2 M Tris-HCl, pH 7.4, 10% (w/v) glycerol, 2.0% (w/v) SDS, and 2% 2-ME (v/v) and immediately heated at 100 °C for 5 min. After centrifugation, the clear supernatants were analyzed by tricine−SDS-PAGE (T−SDS-PAGE) according to the method of Schägger and von Jagow (*23*) in a 16.5% total polyacrylamide gel.

Gels were stained with Coomassie or used for blotting. Molecular weight standard proteins (Bio-Rad) were phosphorylase 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).

Immunoblotting. Immunoblotting experiments were carried out as previously described (*17*), but anti-rabbit IgG−alkaline phosphatase conjugate goat antiserum was used as the secondary antibody. Immunoenzymatic activity on blots was revealed with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium.

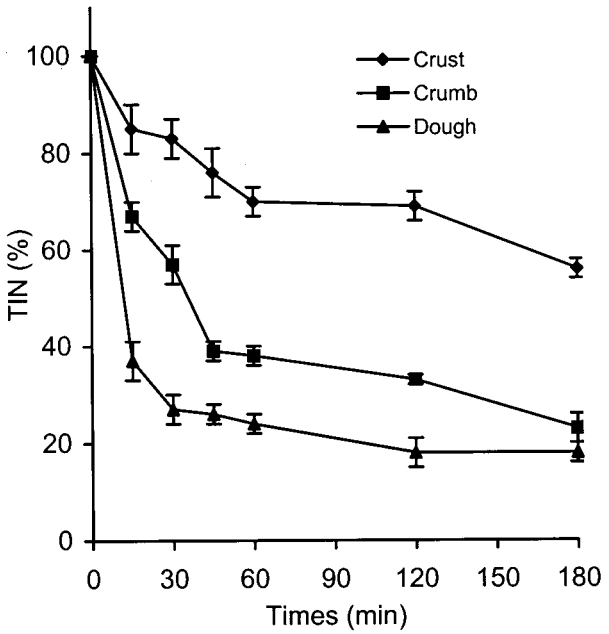

Figure 1. TCA-insoluble nitrogen (TIN) during enzymatic digestion of unheated wheat dough and bread crumb and crust.

RESULTS

In Vitro Protein Digestion. In vitro digestion experiments were performed on the unheated wheat dough and on both bread crumb and crust. The samples were treated sequentially with pepsin and pancreatin for different times, and the 10% TCA-insoluble nitrogen (TIN) was quantified. Therefore, with this system we measured not only the insoluble proteins remaining after digestion (*16*) but all of the protein material with a molecular weight >∼400 Da (*24*), including the albumin/globulin fraction and its proteolytic fragments and peptides generated by thermal protein breakdown, as occurs at >150 °C (*25*).

After 30 min of pepsin attack, the TIN of the dough sample was reduced to ∼27% of the initial value, whereas TINs of both the crumb and the crust decreased to only about 57 and 83%, respectively (Figure 1). These results indicated that the heat treatment affects the degree of hydrolysis of the samples by pepsin, confirming the results of Hansen and Johnston (*15*).

In the case of pancreatin attack following pepsin digestion, a different rate of hydrolysis could be noted for the proteins of the crust and those of the crumb and the dough. In fact, after the entire treatment, the TIN of the crust was found to be ∼56%, whereas TINs of the crumb and the dough both tended to approach the same value of 25% of the initial value (Figure 1).

Electrophoretic Analysis. The samples at the different times of digestion were analyzed by T−SDS-

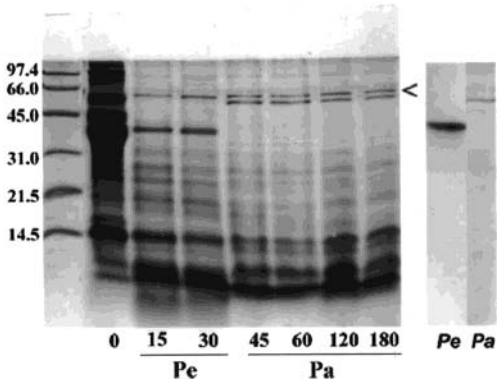

Figure 2. Tricine−SDS-PAGE analysis of unheated wheat dough before and during enzymatic digestion. Numbers indicate digestion times in minutes. Pe and Pa indicate pepsin and pancreatin, respectively. M_r standard proteins are on the left side.

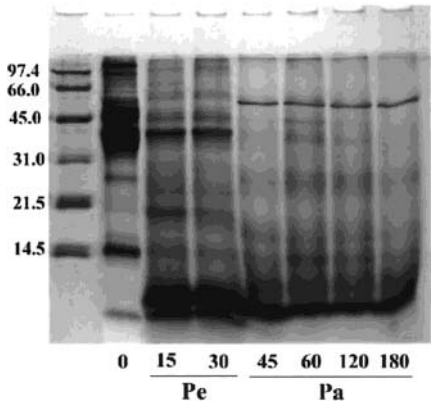

Figure 3. Tricine−SDS-PAGE analysis of bread crumb before and during enzymatic digestion. Lettering is as in Figure 1.

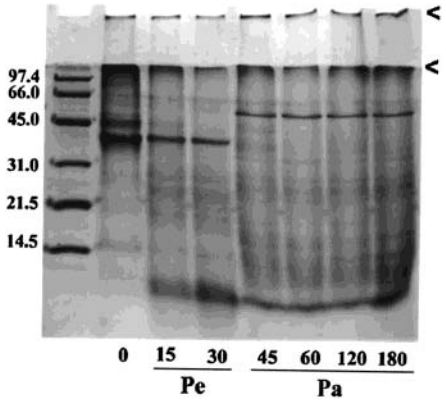

Figure 4. Tricine−SDS-PAGE analysis of bread crust before and during enzymatic digestion. Lettering is as in Figure 1.

PAGE in reducing conditions. This electrophoretic system allows a good visualization of the polypeptides with very low M_r (*23*). In the case of both the dough and the crumb samples the proteins were completely solubilized in the PAGE sample buffer, as assessed by measuring the nitrogen content of the undissolved material, consisting mainly of starch (not shown). On the contrary, it was not possible to completely solubilize the nitrogen of the crust samples. At the end of the digestion process, this unsolubilizable material was ∼27% of the total nitrogen of the sample.

Dough and Its Digestion Products. The electrophoretic pattern of the undigested dough (Figure 2) showed the classical main protein components of bread wheat flour, that is, HMW-GS and LMW-GS (M_r values of around 100 and 31−45 kDa, respectively), ω-gliadins (M_r around 60 kDa), and α/β- and γ-gliadins (M_r around 30−40 kDa). Moreover, some bands belonging to the albumin/globulin fraction were particularly evident, corresponding to β-amylase(s) (M_r of 60 kDa) (*18*, *26*) and to the protein family of the α-amylase/trypsin inhibitors (M_r values around 14−16 kDa) (*27*). Rapid disappearance of the bands corresponding to the storage protein components (HMW and S-rich prolamins) was evident even with short (15 min) pepsin digestion, confirming that gluten proteins are sensitive to proteolytic enzymes (*28*). On the contrary, the protein band with a M_r around 15 kDa seemed to be more resistant to proteolysis, although some polypeptides derived from the proteolytic digestion of gliadins were shown to accumulate at this M_r (*28*). Also, a band with an M_r of ∼60 kDa (arrowhead in Figure 2) was not affected until the last time of digestion. The M_r of this band was similar to those of both wheat β-amylases (*26*) and chromosome 1D-encoded ω-gliadins (*29*), which comigrate in SDS-PAGE. Some bands with M_r <14 kDa were detected in the undigested dough sample. In the same gel region, however, protein bands with similar M_r values were reinforced during digestion, indicating an accumulation of proteolytic products. A similar pattern was reported by Popineau et al. (*28*) as an example of pepsin degradation of a γ-type gliadin, suggesting that the low M_r bands originated from gliadin breakdown. In addition, several other minor bands with M_r values ranging from 14 to ∼40 kDa were detectable until the last step of protein digestion.

Bread and Its Digestion Products. The T−SDS-PAGE pattern of the undigested crumb (Figure 3) was rather similar to that of the undigested dough but with a lower band definition, which was particularly evident for the digested samples. This had to be related to the effect of baking on the crumb proteins, which resulted in heat-induced protein modification and breakdown (*25*, *30*). However, as observed with the dough samples, the storage proteins rapidly disappeared during digestion. A main qualitative difference concerned the 60 kDa band, which, in the case of the crumb, was undetectable not only in the digested samples but also in the undigested one, indicating a major effect of heating.

As mentioned above, the samples deriving from the bread crust digestion could not be totally solubilized for denaturing T−SDS-PAGE. However, the electrophoretic pattern of the proteins that could be solubilized from the undigested bread crust (Figure 4) was different from those of the corresponding samples of the dough and the crumb, showing a heavy stain at the top of both the stacking and resolving gels (arrowheads). This material was likely to correspond to protein aggregates with an M_r so high that they could not enter the gel pores. It was interesting to note that these high M_r aggregates were also detectable after the proteolytic treatment, indicating that they were, at least in part, not degraded by the digestive enzymes and confirming that the reason for the high values of TIN after digestion of the crust should be related to irreversible heat-induced protein aggregation. Furthermore, a lower quantity and a poorer resolution of the other bands were evident when the crust was analyzed.

Immunoblotting Analysis of Dough and Its Digestion Products. Although the possibility of epitope destruction during proteolysis and/or baking must be

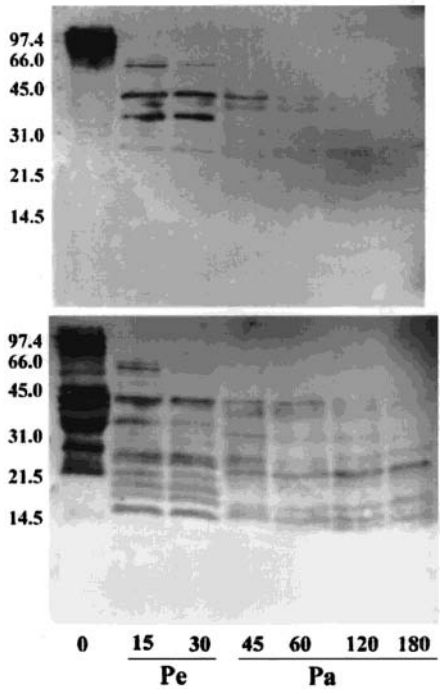

Figure 5. Immunoblottings showing the binding of the anti-7 (top panel) and anti-12 (bottom panel) antibodies to the proteins of the bread dough before and during enzymatic digestion. Lettering is as in Figure 1.

taken into account, the use of PABs specific for particular wheat flour single protein components or groups of homologous proteins gave some qualitative information on the effects of baking on flour proteins and on their susceptibility to be degraded by the enzymes of the digestive system.

The immunoblotting results obtained with the two anti-prolamin PABs with the proteins extracted from the dough at all of the examined digestion stages are shown in Figure 5. Due to the different specificities of the two PABs, comparison between the two blots allowed the evolution of the HMW prolamins to be distinguished from that of the S-rich prolamins. During the pepsin attack the former were rapidly converted into a small number of bands with M_r values between 66 and 31 kDa, which tended to disappear after the addition of pancreatin (Figure 5, top panel). On the contrary, protein fragments deriving from components of the group of the S-rich prolamins could be detected until the last stages of the digestion process (Figure 5, bottom panel), some of them maintaining a relatively high M_r, which corresponded to that of the polypeptides detectable by Coomassie staining in the gel region between 14 and 40 kDa (see Figure 2).

Similar experiments were performed with the PAB specific for wheat β-amylase (Figure 6, top panel). This protein is one of the major components of the salt-soluble wheat protein fraction but can be detected also in a bound form as part of the glutenin polymers (*18*). Its immunological detection was possible only for the undigested sample, being completely absent after the first step of pepsin digestion. This result indicated that the 60 kDa band that could be clearly detected by Coomassie staining of the dough samples during the entire digestion process (see Figure 2) was likely to belong to the slow-moving ω-gliadins, although a small proteolytic modification of the β-amylase epitope(s) recognized by the PAB could not be excluded.

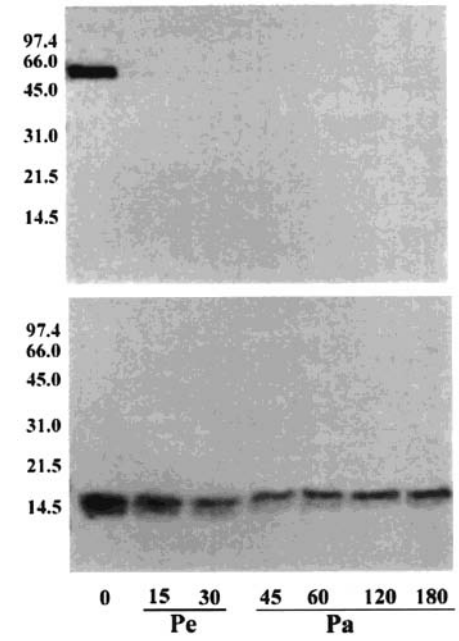

Figure 6. Immunoblottings showing the binding of anti-β-amylase (top panel) and anti-A antibodies to the proteins of the bread dough before and during enzymatic digestion. Lettering is as in Figure 1.

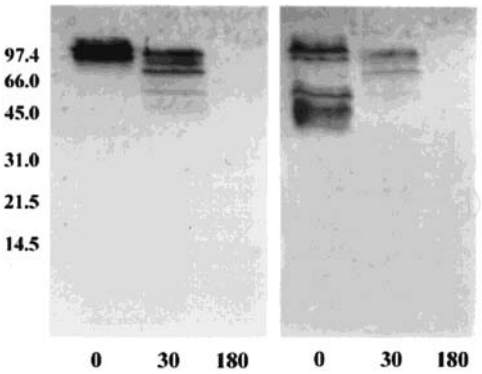

Figure 7. Immunoblottings showing the binding of the anti-7 (left panel) and anti-12 (right panel) antibodies to the proteins of the bread crumb before and during enzymatic digestion. Lettering is as in Figure 1.

On the contrary, the anti-A PAB was able to bind a band with an M_r around 15 kDa present in the undigested dough sample, and this binding persisted throughout the in vitro digestion process (Figure 6, bottom panel), indicating that at least one component of the α-amylase/trypsin inhibitors protein family (*27*) was not modified.

Immunoblotting Analysis of Bread and Its Digestion Products. *Anti-prolamin PABs.* The binding of the PABs was tested on samples of both bread crumb and crust taken before digestion, after 30 min of pepsin attack, and at the end of the digestion treatment. In the crumb sample, the results obtained with the anti-HMW prolamins PAB (anti-7) indicated that the thermal treatment did not affect the HMW prolamins binding pattern in the undigested sample but had an effect on the degradability of these subunits by pepsin (Figure 7, left panel; compare with Figure 5, top panel). In fact, the M_r values of the crumb bands recognized by the antibody after 30 min of digestion remained closer to those of the HMW prolamins of the undigested sample in comparison to what was observed for the

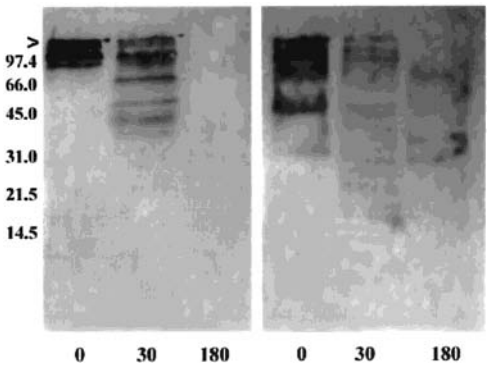

Figure 8. Immunoblottings showing the binding of the anti-7 (left panel) and anti-12 (right panel) antibodies to the proteins of the bread crust before and during enzymatic digestion. Lettering is as in Figure 1.

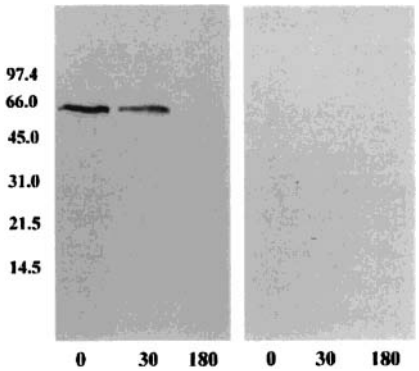

Figure 9. Immunoblottings showing the binding of the anti-β-amylase to the proteins of the bread crumb (left panel) and crust (right panel) before and during enzymatic digestion. Lettering is as in Figure 1.

dough, indicating an increased degree of resistance of HMW prolamins to pepsin hydrolysis after baking. However, after pancreatin attack, no bands recognized by the anti-7 PAB could be detected in the crumb sample, resembling the result obtained for the dough.

Immunoblotting experiments with the anti-12 PAB indicated that, different from the dough, at least some of the crumb gliadins (M_r <31 kDa) were not detected in the undigested sample, whereas at least some of the LMW-GS components were (Figure 7, right panel). This result confirmed the tendency of gliadins to be heat-denatured (*31*), thus losing their immunological reactivity (*32*). On the contrary, the LMW-GS seemed to be immunologically more heat-resistant, probably because of the stabilizing effect of their intermolecular SS bonds within the glutenin polymers. Moreover, during digestion, several of the bands detected in the digested dough sample with the anti-12 PAB were absent in the crumb, suggesting that those bands were derived from gliadins (compare with Figure 5, bottom panel).

By using the anti-7 PAB a variation in the SDS-PAGE mobility of the HMW prolamins could be detected in the undigested crust sample with respect to both the dough and the crumb (Figure 8, left panel). In particular, some of the material bound by the PAB was blocked at the boundary between the stacking and the resolving gels (arrowhead), indicating that the HMW prolamins were involved in those protein aggregates formed by the drastic thermal treatment producing the bread crust. Moreover, these aggregates seemed to be resistant, at least in part, to pepsin hydrolysis, being detected also after 30 min of digestion, but, although present in the Coomassie-stained gel (Figure 4), they were immunologically undetectable at the end of the digestion process.

The presence of immunologically detectable protein aggregates in the crust was confirmed also by the anti-12 PAB (Figure 8, right panel), which bound only to smears in the upper region of the gel. In the case of the crust, however, not only the gliadins, as in the crumb, but also the LMW-GS were not detected by the anti-12 PAB. This would indicate that these subunits were modified during baking and possibly took part in the formation of at least those protein aggregates detected by the PAB. Thirty minutes of pepsin hydrolysis resulted in a fainter immunostaining, but in a wider gel region, whereas some material was bound by the anti-12 PAB also at the end of the digestion process.

Antisoluble Proteins PABs. β-Amylase was immunologically detectable in the undigested crumb (Figure 9, left panel), indicating that, although being heat denatured within a few minutes at 65 °C (*33*), its molecule remained immunologically active after baking. Moreover, contrary to what was shown for the dough, some immunological detection was possible in the crumb even after 30 min of pepsin attack. This could be an indication of a higher resistance to proteolysis after heat denaturation of the enzyme. However, after pancreatin treatment, β-amylase became immunologically undetectable.

Contrary to both the dough and the crumb, immunological detection of β-amylase was never possible for the crust sample (Figure 9, right panel), confirming the strong protein modifying effect of the high-temperature treatment.

The major difference between dough and bread, however, concerned the results obtained with the anti-A PAB. The protease-resistant 15 kDa protein band that was bound by this PAB in the dough until the last step of the digestion process (Figure 6, bottom panel) was, in fact, undetectable in all of the samples of both bread crumb and crust (not shown), indicating a major effect of the heat processing on its immunological reactivity.

DISCUSSION

It is well-known that heat processing can affect the protein digestibility of wheat flour. However, the studies published to date have been done with model systems, in which different temperatures were applied to flour at different moisture levels (*15*, *16*). On the contrary, literature data on the digestibility of bread are lacking. In this paper we have compared the effect of pepsin/pancreatin digestion on the bread dough to that on the bread crumb and crust, which can be considered as examples of two heat treatments of different intensities (<100 and >180 °C, respectively). Quantification of the TCA-insoluble nitrogen of the samples treated with pepsin indicated that the digestibility of both the bread crumb and crust was reduced in comparison to that of the unheated dough. However, following the action of the pancreatic proteases, the differences in protein digestibility between the crumb and the dough were reduced, leading to values approximately similar at the end of the digestion process, whereas the crust always maintained its relatively low protein digestibility. The different behaviors of the samples were obviously related to the heat-induced modifications of the wheat proteins during baking. Depending on the level of the heat treatment, these modifications have been shown to involve heat-induced protein breakdown, thermal

protein aggregation and cross-linking, and reactions with sugars (Maillard-type reactions) (*25*, *34*). For the crumb, in which, due to the presence of residual water during baking, the internal temperature does not exceed 100 °C (*35*), protein aggregation probably involved mainly SS bond formation and hydrophobic interactions (*25*, *36*, *37*). This was confirmed by SDS-PAGE analysis in reducing conditions of the undigested crumb sample, in which no protein aggregates were present (Figure 3). Therefore, the decreased pepsin degradability of the crumb proteins could be due, at least in part, to the reduced accessibility of the protease to its sites of action, deriving from chemically reversible heat-induced protein aggregation. On the other hand, when the pepsin-digested crumb sample was treated with pancreatin, its proteolytic degradation led to values more similar to those of the unheated dough. This might be due to the different mode of action of the two enzymatic preparations. Pepsin is in fact an endopeptidase that breaks the peptidic bonds inside the protein molecules, whereas pancreatin is a mixture of various proteases containing also enzymes with exopeptidase activity (carboxypeptidases A and B), which can result in an improved action on protein aggregates.

The different behaviors of the crust and the crumb proteins during proteolysis were clearly due to the different levels of heating of the two samples. In the case of the crust proteins, the heat treatment of baking affecting the bread surface (the temperature of which approaches that of the oven, i.e., 200−220 °C) led to the formation of high molecular weight protein aggregates stabilized by strong irreversible interactions, which had to be different from SS bonds and/or hydrophobic. In fact, it was not possible to completely solubilize the nitrogen of the samples deriving from the digestion of the crust even in reducing conditions and in the presence of a detergent. Moreover, also in the solubilized protein fraction, some Coomassie-stained material appeared to be blocked at the top of the SDS gels (arrowhead in Figure 4). The nature of the interactions involved in this phenomenon has not been precisely defined, although reactions with sugars (Maillard-type reactions) and/or formation of interpeptide cross-linking are likely to occur over certain temperatures (*38*). The accessibility of the proteases to their sites of action had to be reduced when they acted in a strongly aggregated structure, thus explaining the resistance of the crust proteins to digestion. Moreover, it was also shown that the action of carboxypeptidase B, an important pancreatic protease, was impaired during the digestion of heat-treated flour proteins, due to the formation of undegradable Maillard-like compounds (*34*).

The comparison of the undigested samples by SDS-PAGE and immunoblotting with PABs with different specificities confirmed that baking affected the physicochemical and immunological features of wheat flour proteins, giving some information on the fate of the most important protein components. This was possible to some extent also for the digested samples, although the possibility of epitope destruction during proteolysis had to be taken into account. In the unheated dough, it was possible to confirm that the storage proteins were susceptible to protease actions (*28*, *40*) but that the HMW prolamins (or, at least, their epitopes), were degraded more easily by the enzymes of the digestive system than the other prolamins. In fact, S-rich prolamins, due to their amino acid composition, seem to be a poor substrate for the pancreatic proteases (*34*). Moreover, also the native structure of the different prolamins possibly would affect their susceptibility to proteases. In gluten polymers, the HMW prolamins are supposed to be present as essentially "linear" subunits, whereas the LMW-GS should have a more compact molecular conformation, due to the presence of a relatively high number of intramolecular SS bonds. Moreover, also the monomeric gliadins have a molecular structure that seems to be quite compact due to the presence of intramolecular SS bridges (*41*). Therefore, it is possible that the different behaviors of HMW prolamins and S-rich prolamins (i.e., LMW-GS and α/β- and γ-gliadins) during proteolysis were related to the different densities of intramolecular SS bonds, which gives to the native molecules different degrees of exposition to the action of the digestive enzymes.

After baking, the SDS-PAGE patterns and the immunological reactivity of the prolamins indicated major changes in both the bread crumb and crust, and also their digestion products were bound by anti-prolamin PABs differently compared to the unheated dough. This indicated that the results obtained with the unheated wheat flour prolamins cannot, in general, be considered as completely representative of what actually happens when baked wheat products are ingested. Among other things, this could have a certain importance in the evaluation of the digestive production of peptides toxic for people suffering from celiac disease, because the degree of proteolysis and the immunological characteristics of the prolamin components are changed after heat processing. Moreover, the baking process modified also the main components of the water/salt-soluble protein fraction and their evolution during digestion, as demonstrated by specific PABs. The anti-A PAB recognized more than one component of the protein family with M_r values around 14−16 kDa (*19*), reflecting the similarities existing among the members of the α-amylase/trypsin inhibitors protein family of wheat flour (*27*). This family of proteins is particularly important because it comprises the major allergens causing IgE-mediated hypersensitivity reactions occurring in wheat-allergic individuals. These reactions occur after wheat flour inhalation (bakers' asthma) (*42*), but it was suggested that the α-amylase/trypsin inhibitors are important allergens also when the allergic symptoms occur after ingestion of wheat products (*11*). The results here presented indicate that at least one of these proteins seemed to be unaffected during digestion of the unheated dough, confirming the general characteristic of allergens to show some degree of resistance to proteolysis (*43*). However, when both the bread crumb and crust were analyzed with the same PAB, no bands were detected. This is an indication that a variation occurs after heating and that the results obtained with the raw flour should be taken with caution when food allergies are concerned.

In conclusion, it appears that the wheat proteins and their digestion products are heavily modified after heat treatments in both their physicochemical and immunological features. This means that some attention to these modifications must be paid when the possible effects of the digestion products of wheat proteins on human health and physiology are studied and that the results obtained with the unheated wheat flour or dough cannot be representative of what happens when baked products enter the human digestive tract.

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