Analysis of the Effects of Heat Treatment on Gliadin Immunochemical Quantification Using a Panel of Anti-prolamin Antibodies

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Antigen-labeled capture enzyme-linked immunosorbent assay with four different anti-gliadin monoclonal antibodies and an anti-gliadin serum and two different sample systems (purified gliadin fractions heat-treated in soluble phase and a model of dough simulating a baking process) were employed to study the effects of heat treatment on gliadin quantification. The analysis of purified gliadins showed that there is no particularly heat stable fraction. Remarkably, ω-gliadin did not present a differential heat stability. Reactivity varied depending on the time−temperature conditions of the treatment, the antibody employed, and the fraction analyzed. Heated dough samples showed an impairment of protein extraction depending on the intensity of the treatment. Capillary electrophoresis analysis of extracts showed that each gliadin group is affected to a different extent; ω-gliadin is less modified. Immunochemical analysis of the heat-treated samples using either of the five antibodies showed a decrease in the quantified gliadin, in concordance to the loss in the extracted proteins. Among the different sources of error in gliadin immunochemical quantification, the impairment in extraction efficiency in heat-treated samples appears as a major drawback to be overcome.

Keywords: *Gliadin; ELISA; heat treatment; coeliac disease*

INTRODUCTION

Coeliac disease is triggered in susceptible individuals by the ingestion of prolamins from wheat, barley, rye, and its hybrids, causing histological changes in the small intestine mucosa, leading to a malabsorption syndrome. All of the disturbances revert when a strict gluten-free diet is established, being the only treatment available so far (*17*, *16*). Coeliac patients present a broad range of sensitivity to gluten intake, with clinical manifestations to minimal amounts of gliadin (10 mg/day) reported in some cases (*8*). Consequently, to certify gluten-free products, the use of high detectability assays is mandatory. Among the different analytical techniques, immunochemical methods are the most used tools to control gluten-free products. These methods are based on the immunochemical recognition of toxic proteins by mono- or polyclonal antibodies (*10*, *5*).

Heat treatments, employed in food processing, do not affect prolamin toxicity but may affect quantification. However, the effect of treatment on antigen−antibody interaction has been only partially characterized due to the high complexity of this protein system. In some reports, a particular food matrix was chosen, spiked with a known amount of flour or gliadin, and treated under a particular time−temperature condition. The recovery of the spiked components was variable depending on the assay and the treatment condition (*7*, *9*, *28*). These approaches allowed the evaluation of the performance and the limitations of the assays but did not allow one to establish a general rule on the effects of heat treatment on the quantification. On the basis of its apparently unaffected solubility after heat treatment, ω-gliadins were accepted to be the most heat stable fraction (*9*, *26*, *27*).

Two simultaneous effects take place upon heating. First, new interactions among gliadins or between gliadins and other proteins or other components present in the food are established, which can impair gliadin extraction from the food matrix. On the other hand, heat has drastic effects on protein structure. If these treatments produce changes in the epitopes recognized by the antibodies used in the quantitative assay, determination can be affected.

Molecular studies of gliadin structure are very difficult to perform because of their unusual solubility properties and their high tendency to form macromolecular aggregates. Most of these studies were carried out in ethanol/water mixtures, with low pH or added organic solvents, such as dioxane, to avoid aggregation, but these conditions may themselves produce changes in the structure (*31*).

By means of circular dichroism, Tatham and Shewry (*30*) followed the changes introduced in secondary structures of purified gliadin fractions heated at different temperatures. They found a drop in the proportion of α-helix conformation after heating α-, β-, or γ-gliadins from 20 to 80 °C. On the contrary, ω-gliadins, which are almost entirely constituted by repetitive domains organized in β-turns, showed no other secondary structures when treated in the same conditions. They con-

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cluded that hydrogen bonds are the main interactions stabilizing α-, β-, or γ-gliadins, whereas hydrophobic interactions stabilize ω-gliadins. However, it is difficult to predict changes in immunochemical reactivity using data from these structural studies. In most cases, an intense protein−protein interaction could not be ruled out due to the high protein concentration (about mg/mL) needed to perform circular dichroism experiments. Most studies use aqueous ethanol 70% or 1 mM acetic acid as solvent, where the effect of heat treatment may be different than in conditions used in our work. Even circular dichroism detects changes in certain secondary structures, and immunoreactivity may depend on subtle local structures whose changes may not be detectable by other means.

It must also be considered that the different gliadin fractions may suffer differential changes when heated. Ellis et al. (*6*) analyzed the immunochemical stability of purified gliadins treated in soluble phase. They used a rabbit anti-gliadin polyclonal serum as the capture antibody and a monoclonal antibody (mAb) directed to a peptide overlapping with the N-terminal sequence of A-gliadin in a sandwich enzyme-linked immunosorbent assay (ELISA). After the serum was treated at 100 °C for 30 min, purified α-, β-, γ-, and ω-gliadin showed differential behavior. α- and β-gliadins retained the 54% of the original reactivity, whereas γ-gliadin and ω-gliadin retained 51 and 93%, respectively. However, these results may reflect the particular thermal stability of the epitopes recognized by the antibodies employed.

The aim of this work is to analyze the effects of heat treatment on the quantification of prolamin on foods. A panel of four anti-gliadin mAb's and an anti-gliadin serum of a different reactivity pattern was employed. To separately analyze the effects of heat treatment on gliadin solubility and the changes in gliadin immunochemical reactivity, two different approaches were used. To avoid alterations in extractability, the reactivity of purified gliadin fractions was studied after treatment in soluble phase under different time−temperature conditions. Moreover, a model system of dough with different gliadin content exposed to different heat treatments was also analyzed.

MATERIALS AND METHODS

Samples and Treatments. *Heat Treatment in Soluble Phase.* Purified gliadin fractions were obtained by preparative electrophoresis at acid pH following a procedure recently described (*24*). Fractions consisting of α-, β-, γ-, and ω-gliadins were divided in aliquots and stored at −80 °C until they were used.

Immediately before they were heat-treated, fractions were diluted in phosphate buffer (PBS: 140 mM NaCl; 2.7 mM KCl; 1.5 mM KPO_4H_2; 8.1 mM $NaPO_4H_2$; pH 7.4) at concentrations ranging from 1 μg/mL to 200 ng/mL and then heated in a water bath at 70, 80, 90, or 100 °C for different times (effective time: 10 min for 70 and 80 °C treatments and 5, 10, or 20 min for 90 and 100 °C ones). The effective time of heat treatment was calculated as previously described (*21*). Briefly, sample temperature was recorded with a constantan/copper thermocuple. The time−temperature curve was plotted, and the area under the curve was measured. The effective time employed in the results expression was obtained as the ratio between the measured area and the bath temperature.

Heat Treatment on a Model System. Samples were prepared with wheat flour, *Triticum aestivum* L. Oasis, and dough (1 g wheat flour + 0.6 mL of water). Samples were heated in a drying scale Mettler LP16. Dough samples (0.5 g) were extended in thin layers to improve heat transference and to minimize temperature differences among different regions of the sample. Samples were treated for 20 min at a temperature of 100, 130, or 160 °C. Subsequently, they were ground and extracted with 70% (v/v) aqueous ethanol (1 mL/100 mg) for 3 min at half of the maximal power in a Sorvall omnimixer. Extracts were immediately separated by centrifugation (12 000*g*, 2 min, 15 °C). As controls, untreated wheat flour and dough samples were processed simultaneously in every determination.

Two matrixes, controlled as gluten-free by ELISA, of corn flour and corn starch were added with 1% wheat flour and, after the addition of water (0.6 mL/g), were heat-treated and extracted in the same conditions as described above.

Protein Quantification. Protein content of purified gliadin solutions and ethanol extracts from samples was determined by the method described by Lowry et al. (*15*). As a standard, a gliadin (Sigma Chemicals, St. Louis, MO)/ethanol solution quantified by Kjeldahl's method was used.

Electrophoretic Analysis. Purified fractions and ethanol extracts of heat-treated samples were analyzed by electrophoresis at acid pH (A-PAGE, pH 3.1; acrylamide 7%) as described by Lafiandra and Kasarda (*12*). Samples were analyzed immediately after they were extracted. Correspondingly, all samples were also analyzed by capillary electrophoresis (CE) using a capillary column of 25 μm ID (Polymicro Technologies, Phoenix, AZ) with a phosphate buffer (0.1 M, pH 2.5) as previously described (*23*). A CE equipment MDQ from Beckman (Fullerton, CA) was employed. Samples were injected at 2 psi for 3 s.

Immunochemical Quantification of Gliadin. Samples were quantified following the procedure described by Chirdo et al. (*3*) by means of a capture antigen-labeled sequential ELISA using biotinilated gliadin. Briefly, polystyrene microplates (Maxisorp, Nunc) were incubated with the capture antibody dissolved in PBS, 100 μL/well, for 16 h at 4 °C. Then, wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T), and nonspecific binding sites were blocked by incubating with 200 μL/well of 3% (w/v) dry skimmed milk powder in PBS for 2 h at 37 °C. After each incubation step, wells were washed with PBS-T solution. Gliadin standard solution or samples were dissolved in PBS-T containing 1% (w/v) of dry skimmed milk (diluent solution) and 1 μg/mL of biotinilated gliadin. When heat-treated purified prolamin fractions were analyzed, the quantification curve used was made using different dilutions of the purified fraction as reference. A solution of commercial gliadin (Sigma) quantified by Kjeldahl's method was used as a standard when heat-treated dough samples were analyzed. Samples under analysis were divided in aliquots, each one treated at different time−temperature conditions. An aliquot of unheated sample was always quantified in the same assay as the heat-treated samples. Of each sample, 100 μL/well was incubated at 37 °C for 1 h. Then, wells were washed with PBS-T and streptavidin-alkaline phosphatase conjugate (Sigma Immunochemicals), diluted 1:1500 in diluent solution, and incubated (100 μL/well) for 1 h at 37 °C. The color reaction was developed by adding a solution containing *p*-nitrophenil-phosphate (1 mg/mL, Sigma) in 10% (v/v) diethanolamine, 0.01% (w/v) $MgCl_2$, pH 9.8. The enzymatic reaction was stopped after 30 min with 40 μL/well of 0.1 M ethylenediaminetetraacetic acid. The optical density was determined at 405 nm using a Rainbow reader (SLT, Wien, Austria). As the capture antibody, four different mAb's and a purified anti-gliadin rabbit serum were alternatively used. The characterization of the anti-gliadin serum and the mAb's employed, 1B4E9, 1C4D9, 2A1C4, and 3B4H1, was described in previous reports (*2*, *3*, *25*). In each experiment, three replicates of heat-treated samples and six replicates of control unheated sample were included. Each experiment was performed at least five times.

RESULTS AND DISCUSSION

Food manufacturing involving heat treatment can affect the results of immunochemical quantification of

gliadin. The tertiary structure of the protein may suffer important changes after it is heated, affecting the recognition by antibodies. On the other hand, gliadin extraction can be impaired by heat treatment, reducing the amount of proteins available for the immunochemical analysis. In the last two decades, the development in gliadin quantification has had to face the problems derived from heat treatment. However, the contribution of the two effects mentioned before to the errors on gliadin quantification was not fully elucidated.

To analyze changes in immunochemical reactivity independently from solubility changes, an useful approach is to heat proteins in soluble phase, where it is very simple to control heating conditions. As mentioned above, Ellis et al. (*6*) determined changes in reactivity of heat-treated purified gliadin fractions using a sandwich ELISA with an anti-gliadin peptide mAb. Because their study was made using only one anti-gliadin mAb, we selected a panel of four mAb's and a polyclonal anti-gliadin antibody presenting different reactivity patterns (*2*, *3*, *25*) to obtain a broader perspective of the changes in the immunochemical reactivity induced by heat treatment. Briefly, mAb's 3B4H1 and 1B4E9 presented a broad recognition pattern, which were able to react against α/*β*, *γ*, and *ω*-gliadins. 1B4E9 is able to recognize also prolamins from rye and barley, whereas 3B4H1 recognizes only oat prolamins. mAb's 1C4D9 and 2A1C4 have a more selective reactivity, binding only to *ω*-gliadins and to *γ*-gliadin. 2A1C4 reacts against rye and barley prolamins, whereas 1C4D9 recognizes only rye prolamins. The anti-gliadin serum employed presented high reactivity to α/*β*, *γ*, and *ω*-gliadins as well as to rye and barley prolamins.

In the present work, a capture-competitive ELISA format using biotinilated gliadin was employed since it allows a true competition between the biotinilated marker and the sample in soluble phase for the antibody binding sites. In this way, it is superior to the simple competitive format using antigen-coated wells, since the absorption to the well induces conformational changes in the antigen (*1*) and may produce misleading results when small changes in the conformation of the sample are being evaluated. Each treated purified gliadin was referred to a calibration curve made using the same fraction. Errors due to differences in the distribution of recognized epitopes among standard and sample were eliminated, since the standard and sample used in each case were the same. Because each antibody has a characteristic pattern of reactivity, each pair of analyzed and purified gliadin/antibody presents a different calibration curve. As an example, Figure 1 shows the calibration curves obtained for the 3B4H1 mAb that reacts strongly against the four gliadin groups. It can be seen that a particular calibration curve is obtained for each purified gliadin. 1B4E9 mAb and the polyclonal serum, which present broader reactivity, produced very close calibration curves. Conversely, 2A1C4 and 1C4D9 mAb's that showed differential reactivity against *γ*- and *ω*-gliadin produced curves shifted to lower antigen concentration for these fractions (not shown).

Effects of Heat Treatment on Purified Gliadin Fractions. Purified gliadin fractions were treated at different time−temperature conditions and quantified by ELISA using the panel of four mAb's and the anti-gliadin serum. Thermal treatments were performed on fractions diluted in phosphate buffer. Samples were diluted up to a concentration roughly corresponding to the middle part of the calibration curve in each case. Working at low concentrations, protein−protein interactions are discouraged. Fractions were heated in 0.5 mL plastic tubes into a thermostatized water bath. There was a delay until the bulk of the sample reached the temperature of treatment due to heat transfer. This delay could be relevant, especially for short time treatments. To minimize errors in the time of treatment, for each temperature, time−temperature plots were registered, and the effective length of treatment was calculated as described and used to express the duration of treatment (*21*).

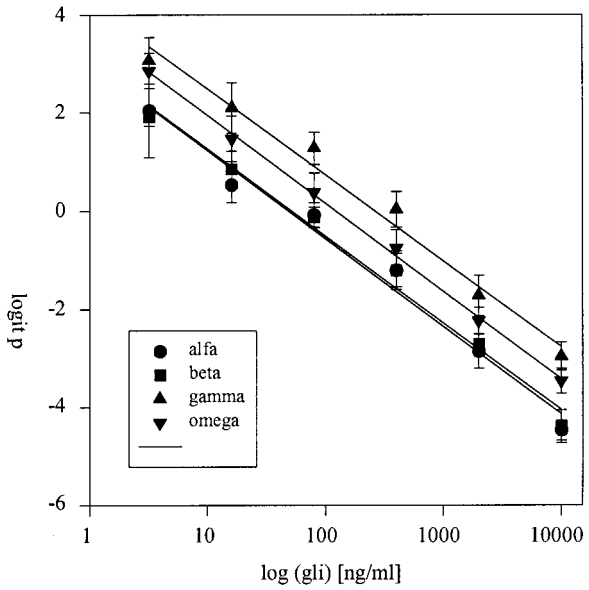

Figure 1. Regression lines of capture antigen-labeled ELISA using purified gliadin fractions and the 3B4H1 mAb. The mean value of five independent experiments and standard deviation are depicted. Symbols correspond to the gliadin fraction used for the calibration curve: (●) α-gliadin, (■) *β*-gliadin, (▲) *γ*-gliadin, and (▼) *ω*-gliadin.

Figure 2 shows the results of quantification of purified fractions after different time−temperature treatments. Results obtained using the 3B4H1 mAb on this system are shown as an example. In each experiment, an aliquot of the unheated fraction was used as a reference of 100% of reactivity to simplify expression results. As it can be observed, no important differences in the behavior of the different gliadin fractions tested were detected. According to time−temperature conditions, different responses can be obtained. Mild heat treatments such as 70 °C for 10 min or 80 °C for 10 min do not produce important changes in the reactivity of the fractions tested. Treatments at higher temperatures do not introduce changes in reactivity when short times are considered, such as 90 °C for 5 min or 100 °C for 5 min, whereas stronger treatments such as 100 °C for 20 min produce a drop in reactivity of approximately 30% on average.

The same study was performed using the other three mAb's and the anti-gliadin serum (Figure 3). In comparison, only two time−temperature conditions are depicted, and the results are grouped according to the gliadin fraction tested. It can be observed that the effects of heat treatment on reactivity can vary according to the antibody employed. α-gliadin reactivity is increased more than 2-fold after treatment at 80 °C for 10 min measured with 1B4E9 mAb. Reactivity decreases to

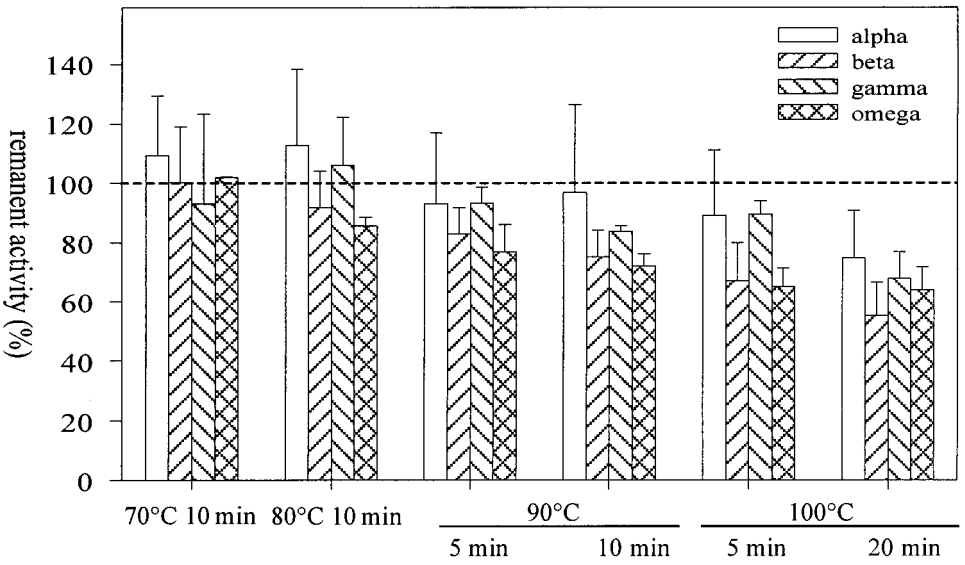

Figure 2. Immunochemical reactivity of purified gliadin fractions heat-treated in soluble phase. Reactivity of treated samples was measured using a calibration curve prepared with the homologous fraction by antigen-labeled capture ELISA employing 3B4H1 mAb. Time−temperature conditions and the fraction analyzed are indicated. Results are expressed as percentage (remanent reactivity) referred to the unheated control (100% of reactivity). The mean value of five independent experiments and standard deviation are depicted.

almost the same level of unheated control for stronger treatments (in Figure 3, 100 °C for 10 min is depicted). A similar behavior was observed for this fraction using 1C4D9 mAb. Conversely, almost no changes in reactivity were observed using the anti-gliadin serum, whereas a slight decrease was detected using either 3B4H1 or 2A1C4 mAb's. Treatment of β-gliadin at 80 °C for 10 min produced a rise to almost twice the reactivity of the control using 1C4D9 and 1B4E9 mAb's. Similar to the case of α-gliadin, treatments at higher temperatures moderated the changes observed for this fraction with both 1B4E9 and 1C4D9 mAb's. Reactivity of β-gliadin measured using the other antibodies showed minor changes. Treatment at 80 °C for 10 min introduced almost no changes in reactivity, whereas at 100 °C for 10 min a reduction up to almost 80% of reactivity of the unheated control was observed. Both γ- and ω-gliadins remained almost unaffected after an 80 °C for 10 min treatment when analyzed using the set of antibodies. Only small changes were observed after more intense time−temperature treatments.

To summarize, stronger treatments, such as 100 °C for 10 min, produce a decrease in reactivity but in any case lowering below 70% of the reactivity of the untreated sample. Each purified gliadin/antibody pair shows a particular pattern of changes. Mild treatments do not produce major changes, and in some cases, an increase in reactivity can be observed. Similar findings were described for mAb's or polyclonal antibodies in other systems, such as soy proteins (*19, 20*), β-lactoglobulin (*11*), or ovalbumin (*21*). Even if each purified gliadin showed some particular changes in reactivity after treatments at different time−temperature conditions, one of the most striking observations is that no important differences among α-, β-, γ-, and ω-gliadin fractions could be detected.

Effects of Heat Treatment on a Food Model System. To study the effects of heat treatment on a food model system, dough samples were heated under conditions simulating a baking process. Samples were extended in thin layers, to minimize differences in temperature among parts of the sample, and heated in an open system. Heat-treated samples were extracted with 70% aqueous ethanol as usually employed for immunochemical determination of gliadin. Protein concentration of extracts of unheated flour or dough was similar (Figure 4). A decrease in protein content of extracts of heat-treated samples is evident; the content is more intense as the temperature of treatment rises. Dough samples heated at 160 °C for 20 min presented the lowest amounts of extractable protein.

The effects of thermal treatment depend on the time−temperature conditions applied and the characteristics of the food matrix that contains the wheat proteins. Moisture content has a critical role on the extent of changes introduced by heating (*32*). Intense heat treatments, such as 160 °C for 20 min, have a limited impact on solubility when applied to flour (low moisture content), whereas on dough the decrease in solubility is much more evident (*22*).

Changes in solubility are mainly due to covalent cross-linking of gliadin fractions promoted by heat treatment. Schofield et al. (*26*) showed the role of disulfide bonds in gliadin thermal alteration. They observed that heat treatment increases the disulfide−sulfydryl exchange, promoting the formation of intermolecular bridges in sulfur-rich gliadins. Much experimental evidence was accumulated showing the central role of this covalent reaction in the decrease in solubility of wheat proteins after heat treatment in different food systems (*4*, *13*, *18*). Hill and Skerritt (*9*) analyzed the effect of heating on gliadins, finding that ω-gliadin was the less effected fraction by heat treatment. The lack of cysteine in its primary structure prevents the covalent cross-linking involving sulfydryl groups. Most of these studies were performed by A-PAGE and immunoblotting, which did not allow a quantitative estimation of the changes. It must also be considered that these results do not show a particular structural stability of ω-gliadins when submitted to thermal treatment.

To characterize the composition of ethanol extracts from heated dough samples, CE was employed (Figure 5). Mobility regions were assigned using purified α-, β-, γ-, and ω-gliadin as previously described (*24*). Samples

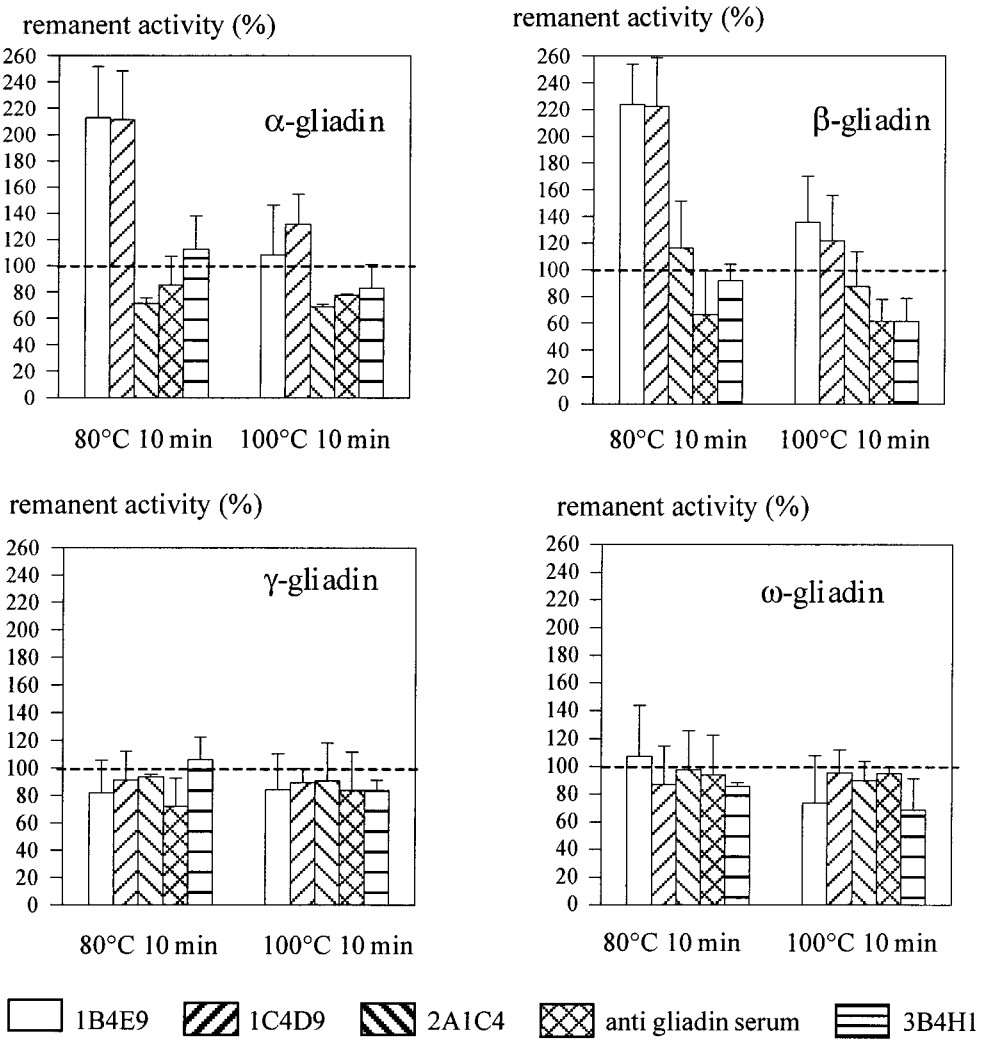

Figure 3. Immunochemical reactivity of heat-treated purified gliadin fractions measured using four different mAbs and the anti-gliadin serum as described in Figure 2. The time−temperature conditions, fraction analyzed, and antibody used are indicated. Results are expressed as percentage (remanent reactivity) referred to the unheated control (100% of reactivity). The mean value of five independent experiments and standard deviation are depicted.

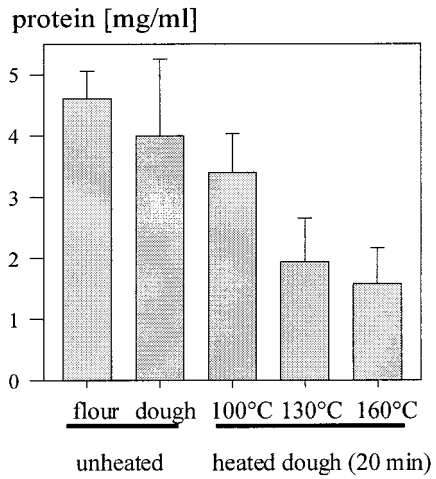

Figure 4. Protein content of extracts from control and heat-treated dough samples measured by Lowry's method. Time−temperature conditions of treatment are indicated. The mean value of five independent experiments and standard deviation are depicted.

were injected in the same conditions (2 psi for 3 s), allowing direct comparison of the protein concentration in the extracts. The electropherogram of gliadins from unheated dough or flour is almost identical. Gliadins from the four groups were present in the analyzed extracts. However, a slight impairment in resolution was observed for heat-treated samples. As injection conditions were the same in all cases, the decrease in the peak area indicates a decrease in concentration of the extract. Albumin and globulin fraction, detected between 2 and 3 min of run, are not present in extracts from heated dough samples.

Because peaks corresponding to ω-gliadin are clearly differentiated from the rest, it is simple to compare their proportion in the sample as the ratio between the area of ω-gliadin region and the total area under the electropherogram. The relative area of ω-gliadin increases from 14% in unheated dough or flour to 28% in the extracts from dough heated at 160 °C for 20 min. Even if the proportion of ω-gliadin increases with heat treatment, the amount of α/β- and γ-gliadin remains as the majority in all cases analyzed.

By reversed-phase high-performance liquid chromatography analysis, Wieser (*33*) described the presence of all gliadin types in aqueous ethanol extracts from bread crumb and crust. In both cases, all gliadin types were extracted, but samples from crust contained a higher proportion of ω-gliadin than the flour. In the present work, similar results were observed with dough samples that simulated bread crust (160 °C for 20 min).

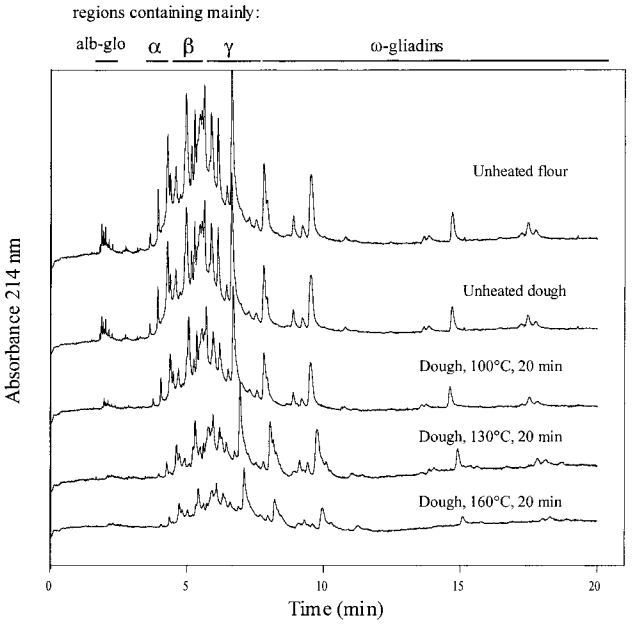

Figure 5. Capillary electrophoresis analysis of extracts from heated and unheated samples. α-, *β*-, *γ*- and *ω*-gliadin regions and time−temperature conditions are indicated. All samples were injected in the same conditions (2 psi, 3 s).

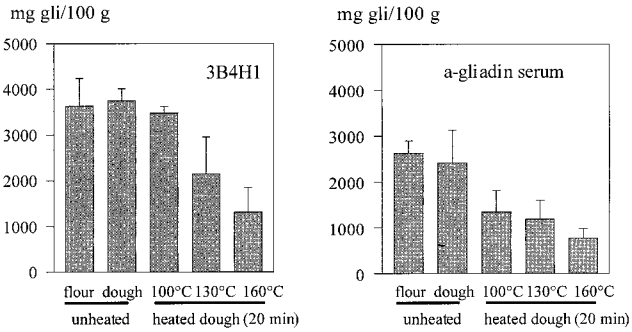

Figure 6. Immunodetected gliadin in heated and control samples determined by capture antigen-labeled ELISA using 3B4H1 mAb and the anti-gliadin serum. All samples were made using wheat flour and heat-treated at different conditions. Sample and time−temperature conditions are indicated. Results are expressed as milligrams of immunodetected gliadin in 100 g of sample (dry basis). The mean value of five independent experiments and standard deviation are depicted.

Extraction of *ω*-gliadin is less affected than other fractions. However, when heated and unheated samples are compared, the absolute amount of *ω*-gliadin is reduced.

The model system of heat-treated dough samples was studied using a panel of antibodies by antigen-labeled capture ELISA. Results obtained using 3B4H1 mAb and the anti-gliadin serum are shown in Figure 6. Even though the actual gliadin content in all samples was the same, differences in the quantified gliadin according to the heat treatment are evident. Similar results were obtained using either the 3B4H1 mAb or the anti-gliadin serum. Unheated flour and dough samples showed similar levels of immunoreactive gliadin, showing that the dough formation affects neither gliadin extraction nor its immunochemical reactivity. A decrease in immunodetected gliadin, more intense at higher temperatures, was observed. Treatment at 160 °C for 20 min caused a drop to approximately 30% of the levels of unheated dough. The other mAb's employed, 1C4D9, 1B4E9, and 2A1C4, presented similar

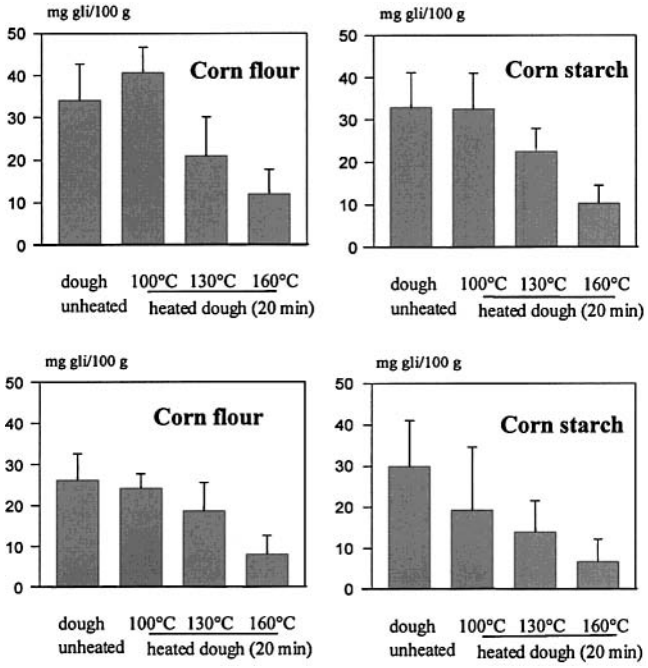

Figure 7. Immunodetected gliadin in heated and control samples made from gluten-free matrixes (corn flour or corn starch) spiked with 1% of wheat flour determined by capture antigen-labeled ELISA using 3B4H1 mAb and the anti-gliadin serum. The type of matrix, sample, time−temperature conditions, and antibody used are indicated. Results are expressed as milligrams of immunodetected gliadin in 100 g of sample (dry basis). The mean value of five independent experiments and standard deviation are depicted.

results (not shown). The decrease in immunoreactive gliadin may be originated by an impaired extraction in addition to structural changes caused by the heat treatment. In concordance with results shown here, Ellis et al. (*7*) found that only 17% of the gliadin content in flour was measurable in the outer crust of a loaf of bread baked at 230 °C for 10 min.

To establish if an impaired extraction may occur in foods containing very low levels of gliadin, matrixes of corn flour or corn starch were employed. These matrixes, proven to be gluten-free by means of the five different ELISA systems, were added with 1% (w/w) of wheat flour, heat-treated, and analyzed as previously described. Results obtained using 3B4H1 mAb and the anti-gliadin serum are shown in Figure 7. In both cases, results are similar. Heat treatment produced a reduction in the detected levels, which depend on the intensity of the treatment. A strict correlation with the results obtained using wheat dough can be observed. No differences were observed between the two matrixes studied. Similar results were observed with the other mAb's (not shown). Results could be explained considering that gliadins would keep the same molecular environment in these matrixes than in wheat flour, reflecting the organization in protein bodies, favoring mainly gliadin−gliadin interactions. More complex food products may also present interactions with other components that may affect the extraction efficiency.

To obtain an accurate gliadin immunochemical quantification, even in heat-treated samples, Skerritt and Hill (*28, 29*) proposed an anti-*ω*-gliadin mAb-based assay. To assess the efficiency of the test, homemade hamburgers spiked with 1% wheat flour and cooked in a microwave oven (750 W) for 5 min were analyzed. Because the results obtained were not affected by the

heat treatment, this method was considered appropriate for the quantification of heat-treated samples. It must be considered that time−temperature conditions analyzed in their work never exceeded 100 °C for 5 min. Dough samples treated at the same conditions and analyzed using the set of five different antibodies described here showed almost no differences with unheated samples. Problems introduced by heat treatments are mainly observed after the samples are baked, a process that exposes food to more intense time−temperature conditions.

Ellis et al. (*7*) used a reducing buffer containing 50% aqueous 1-propanol, 1% 2-mercaptoethanol, tris-HCl 0.08 mol/l (pH 7.5), and 2 M urea, finding an enhanced efficiency to extract gliadins from a heat-treated matrix. However, this buffer affected the stability of antibodies resulting in an attenuated calibration curve. Even when samples were diluted 100-fold, a 74% reduction from the actual gliadin content was observed. The presence of agents such as urea or 2-mercaptoethanol even at high dilution may alter the biological activity of antibodies. To analyze the effect of reducing agents on the assay performance, standard gliadin solution was added with 1% dithiotreitol (DTT) and analyzed in the different systems (not shown). When samples were analyzed at 20-fold dilution, measured gliadin content showed 90% reduction as compared with the controls with no DTT added. Even when samples were diluted 100-fold, a 50% underestimation of the gliadin content persisted. The effects of DTT disappeared when samples were diluted 1000-fold. However, this procedure is not suitable to analyze products with low gluten content since the dilutions that can be used are 50-fold higher than commonly used to certify gluten-free products using ethanol (70%) as extractant.

To improve extraction, buffer additives may be used. However, for immunochemical methods, it will be restricted to those that have minimum impact on antibody biological activity. To overcome this problem, two approaches could be followed. First, assays with high detection capability will allow the use of denaturing solvents for extraction, since by subsequent high dilutions the effects of the additives could be eliminated. On the other hand, antibodies could be genetically engineered to keep their activity even in the presence of buffer modifiers.

CONCLUSION

The analysis of purified gliadins heat-treated in soluble phase showed that there is no particular heat stable fraction. Changes in reactivity depend on the time−temperature conditions of the treatment, the antibody employed, and the fraction analyzed. No differences between the ω-gliadins and the other gliadins were found in this study, even though ω-gliadins have been generally accepted as the heat stable fraction. An impairment in the extraction efficiency of all gliadin types from heated dough samples, depending on time−temperature conditions, was observed.

The impairment of the gliadin extraction caused by heat treatments during food manufacturing is the main source of errors in the immunochemical quantification. Moreover, it is not possible to estimate its magnitude since it depends on the time−temperature conditions used, the food matrix, and the antibodies used in the immunochemical assay.

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