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Hypoallergenic wheat bread: response to an emerging issue

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The increasing prevalence of gluten intolerance raises the need to broaden the range of products that are suitable for coeliac. Usually, this means creating gluten-free (GF) products. However, limited amounts of wheat can be consumed safely by some people with moderate gluten intolerance. In this study, we attempted to produce high-quality bread with reduced immunoreactivity of wheat flour proteins by the partial replacement of wheat flour with wheat starch and by use of lactic acid fermentation. The resulting bread immunoreactivity was determined using the enzyme-linked immunosorbent assay (ELISA) method and compared to that of regular wheat bread. The combination of flour mixture modification and fermentation lowered the immune response in digested bread samples by more than 90% in the case of anti-QQPP peptide antibodies and by over 40% for human anti-gliadin antibodies.

Keywords: gluten intolerance; dietetic bread; wheat; gliadins; immunoreactivity; lactic acid fermentation

Introduction

The consumption of bread, in genetically susceptible individuals, can provoke various undesirable effects. Typical reactions include gastrointestinal disorders such as chronic diarrhoea, malnutrition, weight loss, fatty stools, abdominal pains and bloating. These symptoms are diagnosed as celiac disease (CD), which is a chronic immunological disease induced by ingesting food containing gluten (Lohi et al., 2007; Shan et al., 2002). It is estimated that CD is one of the most widespread genetically induced diseases and that approximately 1% of the world's population suffers from gluten intolerance (Green & Jabri, 2003; Niewinski, 2008). The prevalence of CD has increased sharply in recent years, although improved detection rates are the most frequently cited cause (Lohi et al., 2007; Niewinski, 2008). In the face of the constantly growing number of people who suffer from gluten intolerance, attempts should be made to broaden the range of products that can be consumed.

Yet following a gluten-free (GF) diet can significantly affect the well-being of CD patients (Catassi & Fasano, 2008) since wheat flour is a fundamental component of the diet and wheat bread a staple food of the majority of the world population. Bread products made from GF raw materials have been proposed as a solution. However, the replacement of wheat flour with GF counterparts represents a difficult task since wheat gluten is responsible for the unique viscoelastic behaviour of the dough and its

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exceptional bread-making potential (Dobraszczyk & Morgenstern, 2003; Shewry, 2009). Therefore, the use of alternative flours results in breads of inferior quality (Gallagher, Gormley, & Arendt, 2004; Hager et al., 2012).

Fortunately, the treatment of CD does not always require total removal of gluten from the patient's diet. The degree of gluten intolerance varies from individual to individual. Some patients do need to live on a strict GF diet, with food from natural GF sources only, while there are others who can tolerate a low gluten diet (Catassi et al., 1993; Montgomery, Goka, Kumar, Farthing, & Clark, 1988). Thus, limited amounts of wheat with lowered immunoreactivity can be consumed safely by those who are gluten intolerant, but whose sensitivity is moderate.

Many studies have shown that wheat flour becomes hypoallergenic through the hydrolysis of peptide bonds. Various bio-processing methods have proven to be effective and to have different levels of impact on the rheological, biochemical and immunological characteristics of the flour (Susanna & Prabhasankar, 2011). Recently, sourdough fermentation has received increased interest as a tool for decreasing wheat flour immunoreactivity (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2007; Moroni, Dal Bello, & Arendt, 2009). Sourdough is a mixture of flour and water fermented by lactic acid bacteria (LAB) and yeasts, which not only determine the acidity, aroma and leavening of flours but also improve their baking and nutritional qualities (Gänzle, Loponen, & Gobbetti, 2008; Poutanen, Flander, & Katina, 2009; Thiele, Grassl, & Gänzle, 2004). It has been proven that sourdough-resident microorganisms reveal complex metabolic activity resulting in the hydrolysis of gliadins responsible for inflammations in CD (De Angelis et al., 2006; Di Cagno et al., 2002; Leszczyńska et al., 2009; Loponen, Mikola, Katina, Sontag-Strohm, & Salovaara, 2004; Rizzello, De Angelis, Coda, & Gobbetti, 2006; Rollan, De Angelis, Gobbetti, & de Valdez, 2005). Several laboratories have already begun research on the production of low immunoreactivity wheat bread for celiac (Di Cagno et al., 2004; Leszczyńska, Diowski, Lacka, Wolska, & Bartos, 2012; Moroni et al., 2009; Rizzello et al., 2006). However, due to the technical difficulties involved, there is still an urgent need for broader research on how to make breads that are suitable for people who are intolerant or allergic to gluten. The aim of this study was to produce high-quality bread with reduced immunoreactivity of wheat flour proteins, which could diversify meals consumed by those suffering from moderate gluten intolerance.

Materials and methods

Bread making

The composition of flour mixtures used in baking trials was based on wheat flour (type 500 'Szymanowska' wheat flour), wheat starch (Meritena 200) and fermented wheat flour (wheat sourdough – see *Sourdough fermentation* below) used in different proportions (Table 1). In some recipes, guar gum (Brenntag, Poland) was used as a gluten substitute.

All the ingredients (Table 1) were mixed at speed 1 for 3 minutes, and the dough was kneaded at speed 2 for 7 minutes using a Bosch ProfiMixx 46. The dough was then divided into 400 g portions and placed in the moulds. Leavening was performed at 30°C for 45 minutes. The bread was baked at 230°C for 30 minutes with low pressure steam introduced into the oven (Polin, Verona, Italy) at the beginning of the baking process.

A trained sensory panel evaluated the quality of the breads according to the Polish Standards scoring method (Polish Standards, 1996). Their inspections included sensory

Table 1. Composition of dough used in baking trials (g).

No.	Wheat flour	Wheat starch	Fermented wheat flour	Guar gum	Salt	Baker's yeast	Tap water
1	100	–	–	–	2	3	60
2	50	50	–	–	2	3	60
3	–	50	50	–	2	3	60
4	50	50	–	2	2	3	60
5	–	50	50	2	2	3	60

analysis and instrumental assessment of the bread's volume, crumb moisture and acidity. Serious bread defects result in the loss of points in the assessment. The breads were placed in categories according to their total score. First class corresponded to 40–36 points, second to 35–31, third to 30–26 and fourth to 25–8 points. Breads awarded fewer than 8 points were disqualified.

Sourdough fermentation

Wheat sourdough (Table 2) was prepared according to the one-stage fermentation process. The starter culture was composed of *Lactobacillus* LAB (*Lb.plantarum* LOCK-0858, *Lb. sanfranciscensis* DSMZ-20663 and *Lb.brevis* DSMZ-1267), cultivated at 30°C for 24 hours in lactobacilli MRS broth (Difco, USA), and the yeast *Saccharomyces cerevisiae* LOCK-K₁ cultivated beforehand at 30°C for 24 hours in YPG medium (BTL, Poland). The strains originated from the Pure Cultures Collection of the Institute of Fermentation Technology and Microbiology – LOCK 105 (LOCK) – and from the German collection Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

Fermentation was performed at a temperature of 30°C for 24 hours. Sourdough after fermentation served as a source of fermented flour (Table 1) used in baking trials.

Immunoreactivity determination

We applied the modified method of gliadin extraction proposed by Garcia et al. (2005). The samples were treated with ethanol solution containing guanidine and mercaptoethanol. Hundred-fold diluted supernatants were used to determine immunoreactivity. The indirect non-competitive enzyme-linked immunosorbent assay (ELISA) method was used to determine immunoreactivity of extracted and diluted gliadins, with human primary antibodies or rabbit anti-QQQPP peptide antibodies (POLGEN Ltd., Lodz, Poland) and secondary antibodies – anti-human IgG coupled with alkaline phosphatase or goat anti-rabbit immunoglobulin coupled with peroxidase. The human anti-gliadin antibodies (gliadin IgG antibodies in a concentration of 40 U/ml) originated from the serum of a

Table 2. Sourdough composition.

Wheat flour (g)	Starter culture (ml)		Tap water (g)
	Lactic acid bacteria	Yeast	
100	1	1	98

celiac patient treated at the Polish Mother's Memorial Hospital Research Institute. The pentapeptide QQQP was procured from the Institute of Organic Chemistry, Lodz University of Technology. In order to conjugate QQQP peptide with a protein, glutaraldehyde was added to a solution containing ovoalbumine and the peptide in the ratio of 1:40 (10 mg QQQP and 0.25 mg ovoalbumine in 1 ml phosphate buffered saline (PBS), pH 7.4) until a final concentration of 1% was reached. The reaction was allowed to continue for 1 hour at 37°C and was halted with the addition of NaBH₄ (10 mg/ml). The reaction product was subjected to dialysis against PBS.

Microtitre plates EB 92029330 (Labsystem, Helsinki, Finland) were coated overnight at 48°C with 100 µl of antigen solution (100 times diluted extracts of gliadins obtained from the samples) in 0.1 M carbonate buffer (pH 9.6), containing about 1.5 mg of protein. The plates were washed using PBS buffer (pH 7.2) containing 0.1% Tween 20. Free binding sites were blocked by incubating the plates for 2 hours with 3% skimmed milk powder solution in the PBS buffer. After removing the buffer solution, the plates were rinsed five times. Further incubation with either human serum, containing antigliadin antibodies, or rabbit antibodies, each diluted with phosphate buffer, was carried out for 1 hour at room temperature. The plates were then washed again before the addition of either 100 µl of 1000-fold diluted solution of anti-IgG antibodies conjugated with an alkaline phosphatase or anti-rabbit immunoglobulin coupled with peroxidase. After another 1 hour of incubation and subsequent rinsing of the plates with phosphate buffer, the bound phosphatase activity was determined. *p*-nitrophenyl phosphate (*p*-NPP) served as a substrate for alkaline phosphatase. In the case of peroxidase conjugation, 3,3',5,5'-tetramethylbenzidine (TMB) solution was used as a substrate. The enzymatic reaction was halted with the use of 3 M NaOH in the case of *p*-NPP substrate or 1 M H₂SO₄ for TBM, with measurements taken using a Multiscan MC reader at 405 nm or 450 nm, respectively. The remaining immunoreactivity was expressed as a percentage relative to non-modified flour, referred to on a dry matter basis.

Simulated digestion

In vitro digestion was performed according to the experimental conditions proposed by Berti, Trovato, Bardella, and Forlani (2003): 1 hour incubation with pepsin at 37°C, pH 2.0, followed by 1 hour incubation with trypsin at 37°C, pH 8.3, with an enzyme to substrate ratio of 1:30.

Statistical analysis

The results were calculated as the means and standard deviations of triplicate determinations. The statistical significance of the data was determined by a Student *t*-test. A *p* value below 0.05 was considered statistically significant.

Results and discussion

The main aim of our research was to obtain low-gluten product with sensory characteristics similar to those of regular wheat bread. Two alternative routes were explored: one using wheat starch to reduce the initial gluten content in the flour mixture and the other relying on LAB proteolytic activity during sourdough fermentation to decrease the immunoreactivity of the final bread. In the preliminary studies, it was shown that wheat flour can be replaced with wheat starch in quantities as high as

50% (dough recipe No. 2) with acceptable results (Figure 1). However, the overall quality of the bread was lowered by three quality classes in comparison to the control wheat bread (dough recipe No. 1). This was mainly due to the poor sensory quality, especially to the worse bread structure.

Gan, Rafael, Cato, and Small (2001) have claimed that, to achieve acceptable bread quality and without the addition of a gluten substitute, a maximum of 30% of wheat flour can be replaced with rice flour. The rheology of wheat doughs and the resulting loaf volume are mainly determined by gluten proteins (Ganzle et al., 2008). The properties of gluten become apparent when flour is hydrated, giving extensible dough, with good gas-holding properties and a good crumb structure when baked (Gallagher et al., 2004). It was, therefore, to be expected that reducing the gluten content by half would have an adverse impact on bread quality and appearance.

The results were even poorer when, as well as partially replacing the wheat flour, wheat sourdough was used instead of non-fermented flour (dough recipe No.3). Thiele et al. (2004) have indicated that proteolysis during sourdough fermentation and the rheological consequences of gluten degradation are related mainly to the pH-mediated activation of cereal enzymes. Acidification and the reduction of disulfide gluten bonds by LAB increase the activity of cereal proteases (Ganzle et al., 2008). Primary proteolysis is caused by wheat endogenous enzymes, which are activated by low pH. Further hydrolysis of peptides is then caused by intracellular peptidases of LAB in a strain-specific manner (Di Cagno et al., 2002). The degradation of gluten protein structure in sourdoughs is a crucial factor in the loss of viscoelastic properties in the final dough. Gluten is crucial for the water-holding capacity of dough and for gas retention during fermentation (Arendt, Morrissey, Moore, & Dal Bello, 2008). Although in our experiments the bread volume seemed to increase, a cross section of the loaves revealed abnormally large gas cells and crust projecting over the crumb, indicating that the increase in loaf volume was only apparent. Such severe defects resulted in the bread being disqualified in the quality assessment. Thus, as some authors have suggested (Di Cagno et al., 2004; Rizzello et al., 2006), sourdough with extended fermentation time is not suitable for bread production and may only be incorporated as a baking compound into GF recipes.

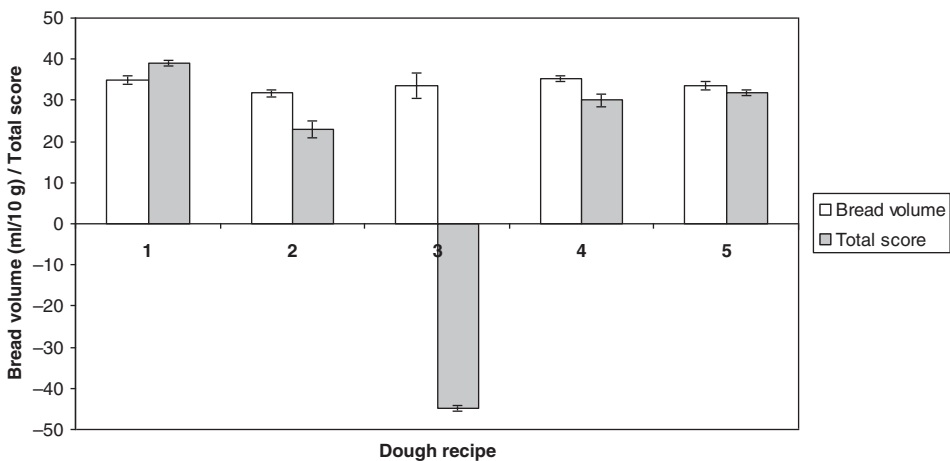


Figure 1. Volume and overall quality of breads obtained from the doughs prepared according to different recipes.

Since the rheological characteristics of the dough were strongly affected by both the partial replacement of wheat flour with starch and by the fermentation process, the addition of structure-building additives was necessary to make the dough suitable for bread production. The use of hydrocolloids represents the most widespread approach used to mimic gluten in manufacture of GF bread (BeMiller, 2008). To produce the desired texture and appearance in the processed bread, guar gum was added (dough recipes No. 4 and No. 5). The use of guar resulted in a highly significant increase in loaf volume and improvement of crumb structure with a more even cell size distribution. The impact was especially marked in the case of bread prepared from a mixture of wheat starch and fermented flour (dough recipe No. 5). The specific bread volume was as high as in the control sample and the texture and flavour were comparable to those of traditional wheat breads. The inclusion of guar as an alternative to gluten was found to significantly enhance crumb grain quality and the overall bread score.

A success in terms of the achieved quality, however, was not enough to complete the task. Confirmation of the effectiveness in terms of immunoreactivity reduction was required. First of all, the sourdough fermentation process was tested. The immunoreactivity of the dough before (non-fermented dough) and after sourdough fermentation (fermented dough) were compared (Table 3).

The results obtained with both types of antibody were highly consistent. The fermentation of wheat flour started by a bacteria-yeast starter culture resulted in ca. 45% reduction of the immune response. Previous studies (De Angelis et al., 2006; Di Cagno et al., 2002; Leszczynska et al., 2009; Loponen et al., 2004; Rizzello et al., 2006; Rollan et al., 2005) also confirm the usefulness of LAB as an efficient tool for reducing wheat allergens, although not all strains reveal the same capacity for gliadin fraction hydrolysis. The starter culture applied in the fermentation process was not composed at random. The selected strains had been examined previously for their ability to destruct gliadin epitopes recognised by antibodies from the sera of CD patients (Leszczynska et al., 2009). De Angelis et al. (2006) noted that mixed cultures reduced immunoreactivity more efficiently than single strains due to the complex action of proteolytic enzymes with strain-dependent specificity. Such programmed proteolysis in combination with partial flour

Table 3. Comparison of the immunoreactivity of non-fermented and fermented dough and bread prepared from wheat flour and starch mixture (means $\pm$ SD; $n = 3$).

Sample	Rabbit anti-QQPP antibodies		Human serum antibodies	
	Immunoreactivity (OD/B)	Fermentation effectiveness (%)	Immunoreactivity (OD/B)	Fermentation effectiveness (%)
Non-fermented dough	194.0 ^a $\pm$ 4.2	–	48.8 ^d $\pm$ 3.1	–
Fermented dough	104.0 ^b $\pm$ 4.6	46.4	22.2 ^e $\pm$ 2.2	44.5
Non-fermented bread (dough recipe No. 4)	168.0 ^c $\pm$ 4.4	–	38.1 ^f $\pm$ 3.2	–
Fermented bread (dough recipe No. 5)	94.0 ^b $\pm$ 4.7	44.0	20.6 ^e $\pm$ 2.1	45.9

Note: Means signed with different letters are statistically significantly different ($p < 0.05$).

replacement by wheat starch may reduce gluten levels to such an extent that the resultant products are tolerated by celiac patients.

However, it should be taken into account that cereal allergens undergo further extensive modifications depending on the type of food processing they undergo. Thermal processing, in particular, to which baked goods are subjected during manufacture may influence the antigenicity of food proteins. Baking is thought to increase wheat flour immunoreactivity by introducing new antigens as a result of the Maillard reaction (Davis, Smales, & James, 2001; Simonato et al., 2001). These observations were confirmed by our study. Given that the amount of wheat flour in the bread recipes was reduced by half, it could be expected that the resultant bread immunoreactivity would also be 50% lower due to the ‘dilution’ of gluten by the addition of starch. The analysis of the bread samples (Table 3) demonstrated some decrease in their immunoreactivity, but the results were not as good as anticipated. Nevertheless, the immunoreactivity of the bread made with fermented flour corresponded to the difference obtained at the fermentation stage (ca. 45%).

The changes that occur in a patient’s digestive tract are just as important with regard to gliadin toxicity as thermal conversion during baking. Only the samples subjected to simulated digestion correspond with how the polypeptides they contain interact within an organism. During digestion, prolamin-derived polypeptides rich in proline and glutamine are liberated and elicit T-cell-mediated immune responses in celiac patients (Ganzle et al., 2008). The efficiency of the proposed method of wheat flour processing in lowering gliadins immunoreactivity in the bread was fully confirmed in the samples exposed to digestion (Table 4).

Bread immunoreactivity initially reduced by the addition of starch to the wheat flour used for baking was further decreased by the fermentation process. The level of remaining immunoreactivity depended on the type of antibodies used in the measurements. The final drop in immune response exceeded 90% in the case of anti-QQQPP peptide antibodies and 40% for human anti-gliadin antibodies. Gastrointestinal enzymes are normally said not to be able to degrade cereal allergens which reach the intestine unaltered, where they elicit an immune response (Astwood, Leach, & Fuchs, 1996). But it has also been reported that gliadins which remained unhydrolysed during fermentation are more susceptible to proteolysis in the digestive tract and that the products of this reaction indicate little or no celiac toxicity (De Angelis et al. 2006; Di Cagno et al., 2004).

Table 4. Remaining immunoreactivity of bread samples exposed to simulated digestion (means $\pm$ SD; $n = 3$).

Bread	Rabbit anti-QQQPP antibodies		Human serum antibodies	
	Immunoreactivity		Immunoreactivity	
	OD/B	%	OD/B	%
100% non-fermented wheat flour	328.0 ^a $\pm$ 2.2	–	122.0 ^c $\pm$ 1.4	–
50% non-fermented wheat flour 50% starch	111.0 ^b $\pm$ 7.6	33.8	95.7 ^b $\pm$ 6.1	78.4
50% fermented wheat flour 50% starch	30.0 ^c $\pm$ 1.2	9.1	72.5 ^d $\pm$ 8.2	59.4

Note: Means signed with different letters are statistically significantly different ($p < 0.05$).

Rizzello et al. (2006) confirm that wheat proteins extracted from fermented wheat bread after simulated digestion cease to bind IgE from patients with wheat allergy. Also Leszczyńska et al. (2012) show that the immunoreactivity of QQQPP epitopes in digested bread is strongly attenuated. Although both determinations in our study – using anti-gliadin antibodies from CD-affected patient serum and rabbit anti-QQQPP peptide antibodies – indicated a decrease in immunoreactivity, there were significant differences in the results. The reduction was much more substantial when rabbit anti-QQQPP antibodies were used, rather than antibodies from human sera. Rabbit anti-QQQPP pentapeptide antibodies had been applied successfully to determine the immunoreactivity of gluten proteins subjected to modifications (Leszczyńska, Lacka, Bryszewska, & Brzezinska-Błaszczyk, 2008). However, the data presented here clearly shows a need to confirm such results with human antibodies. What is more, the individual character of the immune response to gluten requires the application of a wide range of human sera (Leszczyńska et al., 2009, 2012), as the exact amount of toxic prolamins that celiac patients may consume without damaging the mucosa of their small intestines has still not been determined scientifically (Thompson, 2000). Therefore, long-term *in vivo* tests prior to the implementation of hypoallergenic bread into the diet are needed to confirm the safety of these products for patients intolerant to gluten.

Conclusions

The effectiveness of sourdough fermentation in the reduction of wheat immunoreactivity offers a promising prospect for the manufacture of baked goods that can be consumed by people moderately intolerant to gluten. The fermentation of wheat flour with selected LAB and its combination with starch added to the dough could represent a novel method of baking quality breads that are low in gluten. Such a wheat-based hypoallergenic recipe would represent a safe and effective alternative for gluten intolerant individuals. Controlled proteolysis in wheat dough may reduce the gluten level to such an extent that the resultant product is well tolerated. However, the estimated level of residual immunoreactivity in the final product depends on the type of antibodies used in the analysis. Whether the proposed method of hypoallergenic bread production could be used in future to manufacture safe products for gluten-sensitive people remains to be decided through clinical trials.

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