Evaluation of Immunoreactivity of Wheat Bread Made from Fermented Wheat Flour

Joanna LESZCZYŃSKA[1], Anna DIOWKSZ[2], Agata ŁĄCKA[1], Katarzyna WOLSKA[1] and Adrian BARTOS[1]

[1]Institute of General Food Chemistry and [2]Institute of Fermentation Technology and Microbiology, Technical University of Lodz, Lodz, Poland

Abstract

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Combined culture of lactic acid bacteria (*Lactobacillus brevis*, *L. plantarum* i *L. sanfranciscencis*) and baker's yeasts was used in order to reduce immunoreactivity of gluten from wheat. Flour and dough samples were analysed in terms of lactic acid fermentation and thermal processing. Their immunoreactivity was determined with ELISA method using both anti-gliadin antibodies from patients suffering from coeliac disease and rabbit anti-QQQPP peptide (main epitope of flour allergen) antibodies. Also, immunoreactivity was measured in the final products after simulated digestion. The obtained total effectiveness of the fermentation and digestion processes amounted to less than 30% relative to immunoreactivity of human anti-gliadin antibodies and less than 10% relative to immunoreactivity of anti-QQQPP peptide antibodies as compared to the baking made with non-fermented flour.

Keywords: gliadins; immunoreactivity; lactic acid bacteria; bread, wheat

The consumption of wheat products or those supplemented with wheat proteins can evoke various undesired reactions in the organism. The most common symptoms include: urticaria, atopic dermatitis (AD), and wheat-dependent exercise-induced anaphylaxia (WDEIA). However, AD mainly occurs in children whereas urticaria and WDEIA affect adults (Palosuo 2003; Battais *et al.* 2005). Apart from food allergy, there are disorders associated with digestive gluten intolerance, also known as coeliac disease (CD). In this case, gluten intake results in small intestinal disorders and in the end does harm to epithelium and triggers inflammatory response, with the strongest response being caused by peptides received from the digestion of wheat gliadins – one of the fractions of gluten proteins (Shan *et al.* 2002).

Fermented grocery products are significant for the sake of their dietary function and can display different immunoreactivity values compared to non-processed foods. Lactic acid fermentation conducted with the use of lactic acid bacteria is a safe, natural, and favourable process. Its objective is to create specific aromatic compounds, to stop undesirable fermentation by other microorganisms, to delay the expiration date of the obtained bread and improve its nutritional value, as compared to bread only made through the addition of baker's yeasts (Liljeberg & Björck 1996; Hammes & Gänzle 1998).

Numerous homo- and heterofermentative species of lactic acid bacteria were isolated from sourdough. The bacteria of *Lactobacillus* genus prevail in sourdough (De Vuyst & Neysens 2005; Rollan *et al.* 2005; Corsetti & Settani 2007).

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It was proved that these bacteria synthesise a set of proteolytic enzymes capable of hydrolysing gliadin peptides that cause inflammation in coeliac disease (Di Cagno *et al.* 2002; Rollan *et al.* 2005; Rizello *et al.* 2006).

In this work, we applied a mixed culture of selected lactic acid bacteria able to reduce the immunoreactivity of wheat flour proteins. Also, we assessed their potential for obtaining ready-made products from fermented wheat flour exposed to thermal processing (baking and boiling). The immunoreactivity of these products was estimated before and after the simulated digestion.

The immunoreactivity values of wheat flour, dough and its derivative products were determined with ELISA method using anti-gliadin antibodies from patients' sera which were made available owing to the co-operation with Polish Mother's Memorial Hospital Research Institute, Lodz. Also, rabbit sera containing anti-QQQPP peptide antibodies were utilised. The peptide is considered by some authors as the main epitope in wheat allergy (Tanabe *et al.* 1996).

MATERIAL AND METHODS

Type 500 wheat flour containing 18.8% gluten, 0.51% ash and 14.9% humidity was exposed to lactic acid fermentation. The fermentation assays with wheat flour were conducted with dough preparation at efficacy of 200 (1 kg flour + 1 kg water, which makes 2 kg dough).

In this work, we applied lactic acid bacterial monocultures of *Lactobacillus* genus. The research engaged the following homo- and heterofermentative strains:

Lactobacillus plantarum – LOCK 0860, AD 98, LOCK 0858 (Pure cultures collection of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz – LOCK 105) and 2648 from the collection of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brauschweig, Germany).

Lactobacillus brevis – MS-99 from the collection of LOCK 105 and 1267 from the collection of DSMZ.

Lactobacillus sanfranciscensis – MW-94 from the collection of LOCK 105 and 20663 from the collection of DSMZ.

Saccharomyces cerevisiae K1 yeasts were also used, originating from the collection of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz. Fermentation was run at 30°C for 24 hours.

After the mixture of wheat flour, water, and microorganisms was ready, the dough was mixed for 19 min in Aliphamix blender at the speed level 7, then incubated for 25 min in a grow chamber until fully grown. The dough samples were preserved thermally in three variants: (1) boiling in water at 100°C for 10 min; (2) steam-cooking for 25 min; (3) baking at 220°C for 15 minutes. Gluten was extracted from these samples by a modified method of ethanol extraction with the addition of guanidine and mercaptoethanol (Garcia *et al.* 2005).

Immunoreactivity determination. IgG antibodies from the sera of allergic patients with coeliac disease were used for wheat flour antigenicity determination. The determined concentration of anti-gliadin antibodies is presented in Table 1.

Pentapeptide QQQPP was received from the Institute of Organic Chemistry, Technical University of Lodz. In order to conjugate QQQPP peptide with a protein, glutaraldehyde was added into a solution containing ovoalbumine and the peptide in the ratio of 1:40 (10 mg QQQPP and 0.25 mg ovoalbumine in 1 ml phosphate buffered saline – PBS pH 7.4 buffer) until the final concentration reached 1%. The reaction was carried on for 1 h at 37°C and was stopped with the addition of $NaBH_4$ (10 mg/ml). The reaction product was subjected to dialysis against PBS. The antibodies against the complex of QQQPP peptide and ovoalbumine were received from the immunised rabbits by POLGEN Ltd. (Łódź, Poland).

The supernatants were diluted 100-fold for immunoreactivity determination. The coating step involved the use of 0.05M carbonate buffer pH 9.6 (Sigma, town, state) prepared by dissolution of 1 reagent capsule in 100 ml distilled water. To block the uncoated surfaces, we used 3% powdered skimmed milk solution in PBS. Indirect non-

Table 1. Human sera applied in research

Serum number	Antibodies concentration (u/ml)	
	IgG	IgA
219	130.3	1.1
459	25.7	1.5
875	40	0
892	47.1	1
903	96.8	1.5

competitive ELISA method was used with primary antibodies being patients' antibodies or anti-QQQPP peptide antibodies, produced by POLGEN Ltd. (Lodz, Poland), and secondary antibodies – anti-human IgG coupled with alkaline phosphatase or goat anti-rabbit immunoglobulin coupled with peroxidase. In the case of peroxidase conjugation, 3,3', 5,5'-tetramethylbenzidine (TMB) solution was used as the substrate *p*-nitrophenyl phosphate (*p*-NPP) served as the substrate for alkaline phosphatase. Enzymatic reaction was stopped with the use of 1M H_2SO_4 or 3M NaOH, respectively.

Phosphate buffer PBS (pH 7.2), with 0.1% Tween 20 was used to wash plates and for dilutions.

The assays were done with indirect ELISA method. Microtitre plates EB 92029330 (Labsystem, Helsinki, Finland) were coated overnight at 48°C with 100 ml of the antigen solution (100 times diluted gliadins extracts obtained from the samples) in 0.1M carbonate buffer (pH 9.6), containing about 1.5 mg of protein. The plates were washed and the free binding sites were blocked by incubation of the plates with a 3% solution of low fat milk in phosphate buffer (pH 7.2) containing 0.1% Tween-20, for 2 hours. This was followed by the removal of the buffer solution, rinsing the plates five times and further incubation with human sera containing antigliadin antibodies or rabbit antibodies, diluted with phosphate buffer, at room temperature for 1 hour. The plates were washed again and 100 ml of 1000-fold diluted solution of anti-IgG antibodies conjugated with alkaline phosphatase or anti-rabbit immunoglobulin coupled with peroxidase was added. After the incubation of the plates for 1 h and rinsing with phosphate buffer, the bound phosphatase activity was determined by the reaction with *p*-NPP (phosphatase) or TMB (peroxidase) with Multiscan MC reader at 405 nm or 450 nm, respectively.

The remaining immunoreactivity was expressed in % relative to non-modified flour, referred to on a dry matter basis.

Simulated digestion. The experimental conditions: pepsin – incubation for 1 h, 37°C, pH 2.0; trypsin – 1 h, 37°C, pH 8.3; enzyme:substrate ratio 1:30 (digestion conditions by Berti *et al.* 2003)

RESULTS AND DISCUSSION

In the previous paper (Leszczyńska *et al.* 2009) a set of lactic acid bacterial strains was examined in respect of their capacity to produce L-lactic acid, which decreases pH, and the ability to destruct gliadin epitopes recognised by antibodies from the patients' sera. Dough acidity is significant as the prevailing proteolysis of wheat proteins is catalysed by endogenous aspartate proteases present in flour, requiring low pH to become activated (Spicher & Nierle 1988; Loponen *et al.* 2004). The strains of lactic acid bacteria also show an indirect property of destroying epitopes as in mixed cultures, where specific species cohabit in synergy, the resultant enzymatic activity turns out to be much higher than just the total activity from the individual species (De Angelis *et al.* 2006).

Earlier research made us propose four compositions of lactic acid bacterial starting cultures,

Table 2. Composition of starter cultures and acidity of dough obtained as a result of fermentation (expressed as a figure in milliliters of 0.1M NaOH per 10 g wheat dough)

Number of composition	Strains of lactic acid bacteria	Acidity of dough after fermentation	
		no addition of yeasts	addition of *S. cerevisiae* K_1
1	*L. plantarum* DSMZ 2648 *L. sanfranciscensis* DSMZ 20663 *L. brevis* DSMZ 1267	9.7 ± 0.1	11.2 ± 0.2
2	*L. plantarum* ŁOCK 0860 *L. sanfranciscensis* DSMZ 20663 *L. brevis* DSMZ 1267	10.6 ± 0.1	11.1 ± 0.1
3	*L. plantarum* AD 98 *L. sanfranciscensis* MW 94 *L. brevis* MS 99	10.5 ± 0.2	11.0 ± 0.1
4	*L. plantarum* ŁOCK 0858 *L. sanfranciscensis* DSMZ 20663 *L. brevis* DSMZ 1267	10.0 ± 0.1	11.7 ± 0.2

Table 3. Immunoreactivity of fermented dough in relation to the makeup of lactic acid bacterial starter culture as well as to applied human serum (expressed in relation to immunoreactivity of non-fermented dough)

Number of composition	Remaining immunoreactivity (%)			
	no addition of yeasts		addition of *S. cerevisiae* K_1	
	S 459	S 892	S 459	S 892
1	66.9 ± 3.1	30.3 ± 2.1	46.5 ± 2.2	52.7 ± 3.8
2	49.8 ± 2.2	19.1 ± 2.3	49.2 ± 3.2	42.0 ± 3.6
3	63.1 ± 3.0	10.1 ± 1.9	42.0 ± 3.9	46.0 ± 2.6
4	24.3 ± 3.9	19.5 ± 2.2	30.6 ± 2.4	39.3 ± 2.3

including homo- as well as heterofermentative bacteria. Especially the notable strain *L. sanfranciscensis* stimulates the growth of other lactic acid bacterial strains in sourdough due to its enzymatic properties. Phosphorolysis of maltose, which accounts for 60% of free sugars in dough, is an ability that favours the creation of populations associated with other microorganisms that require glucose and other monosaccharides for the growth and metabolism (Gobbetti & Corsetti 1996).

In Table 2, we show the contents of the applied compositions of the mixed lactic acid bacterial cultures as well as the acidity of dough that was fermented by them. Sourdough acidity varies only slightly and equals 9.7 to 11.7, with the yeast addition contributing to its increase. The discrepancy is associated with symbiotic cohabitation of lactic acid bacteria and yeasts (Gobbetti 1998).

The application of mixed cultures in fermentation (Table 3) reduces immunoreactivity much more significantly than when only one strain is used for wheat flour fermentation (Leszczyńska *et al.* 2009). Good results were obtained for starter composition No. 4, where we observed a decrease in immunoreactivity by approximately 20%. Therefore, in further research this particular lactic acid bacterial composition was utilised. The yeasts addition contributed to the drop in immunoreactivity being more substantial. Unfortunately, some sera indicated cross-reactivity between gluten and *Saccharomyces cerevisiae* yeast proteins. Similar conclusions were reported by other researchers (Watanabe *et al.* 1995). Indigenous yeasts presence in flour supports the necessity of making yeasts addition despite the prospective slight increase in immunoreactivity (Stolz 1999). If there is no competition from baker's yeasts, they can grow and thus negatively affect the dough quality.

To confirm the viability of fermented wheat flour in the production of foods with reduced immunoreactivity, flour samples were exposed to thermal processing and subsequent analysis of immuno-

Table 4. Remaining immunoreactivity of the products made from fermented flour (starter composition 4) (effectiveness of fermentation – remaining immunoreactivity compared to analogous sample that was not subject to fermentation)

Sample	Immunoreactivity		Effectiveness of fermentation (%)
	OD/B	(%)	
Human anti-gliadin antibodies, serum S 875			
Non-fermented dough	36.1 ± 3.4	100.0 ± 4.0	
Bread made from non-fermented flour	27.1 ± 2.2	76.5 ± 3.0	
Dough made from fermented flour	16.7 ± 2.0	46.2 ± 2.3	46.2 ± 4.1
Bread made from fermented flour	15.2 ± 2.0	42.1 ± 2.7	55.0 ± 3.9
Rabbit anti-QQQPP antibodies			
Non-fermented dough	102.0 ± 4.1	100.0 ± 4.2	
Bread made from non-fermented flour	80.0 ± 3.3	78.4 ± 3.4	
Dough made from fermented flour	52.0 ± 3.6	50.9 ± 3.6	50.9 ± 4.0
Bread made from fermented flour	33.8 ± 2.9	33.1 ± 3.1	42.2 ± 4.1

Table 5. Remaining immunoreactivity of the products made from fermented dough and exposed to thermal processing

Type of thermal processing		Remaining immunoreactivity (%)		
		human antibodies		rabbit anti-QQQPP antibodies
		S 903	S 219	
Non-fermented dough (reference)		100.0 ± 2.3	100.0 ± 2.6	100.0 ± 3.1
Fermented dough	boiled in water	35.7 ± 3.2	69.5 ± 3.4	63.5 ± 3.1
	cooked by steam	5.5 ± 3.4	66.9 ± 3.7	15.2 ± 2.6
	batch (bread)	37.9 ± 2.9	48.7 ± 3.6	41.8 ± 2.9

reactivity was conducted of bread made from the fermented dough. Apart from human sera, rabbit anti-QQQPP pentapeptide antibodies were applied. These antibodies had been successfully applied before to determine the immunoreactivity of gluten proteins subjected to modifications (Leszczyńska *et al.* 2008). As can be seen in Table 4, fermentation allowed to halve immunoreactivity of the examined proteins relative to the initial values, no matter if rabbit or human serum was used.

In Table 5, we show the immunoreactivity values of the fermented products exposed to thermal processing. Both the anti-gliadin antibodies from coeliac disease-afflicted patients' serum and rabbit anti-QQQPP peptide antibodies caused the immunoreactivity to decrease. The largest drop in immunoreactivity was reported for steam-cooked. Complete reduction is more substantial with human sera antibodies, rather than with rabbit anti-QQQPP antibodies.

The products made from wheat flour are consumed only if processed thermally, however, the bread immunoreactivity does not correspond with the way in which the polypeptides it contains interact with an organism. The changes occurring in the digestive tract are significant for the allergenic and immunogenic properties of peptides.

To confirm our results, we exposed the samples of baking made from both fermented and non-fermented dough to simulated digestion (Berti *et al.* 2003). The fermented baking samples made from flour exposed to digestion showed a much lower remaining immunoreactivity, approx. 1/4 relative to baking made from non-fermented flour in the assays with human serum and around 10% in those with rabbit serum (Table 6).

Our research proved that lactic acid bacteria are not capable of complete eradication of gliadins immunoreactivity. This is not always crucial. Gliadins that were not hydrolysed in fermentation are more susceptible to proteolysis in the digestive tract and the products of the reaction indicate little or no celiac toxicity (Di Cagno *et al.* 2004; De Angelis *et al.* 2006).

Baking is considered as conducive to allergenicity increase when new epitopes are created as a result

Table 6. Immunoreactivity of bread made from either fermented or non-fermented dough and exposed to simulated digestion

Sample	Immunoreactivity (OD/B)		Effectiveness of fermentation (%)
	non-fermented bread	fermented bread	
Human serum S 219			
Unexposed to digestion	295.6 ± 1.7	202.7 ± 1.3	68.6 ± 3.3
Exposed to digestion	122.0 ± 1.4	84.4 ± 0.8	69.2 ± 3.4
Effectiveness of digestion (%)	41.3 ± 2.2	41.6 ± 2.3	28.5 ± 2.4 (*)
Rabbit anti-QQQPP antibodies			
Unexposed to digestion	1323 ± 3.4	1046 ± 2.4	79.1 ± 3.3
Exposed to digestion	328.0 ± 2.2	127.8 ± 0.9	38.9 ± 3.4
Effectiveness of digestion (%)	24.8 ± 3.5	12.2 ± 2.8	9.6 ± 3.3 (*)

(*) – total effectiveness of fermentation and digestion processes

of Maillard reaction (Davis *et al.* 2001; Simonato *et al.* 2001). However, bread postdigestional immunoreactivity is subject to change. After simulated digestion, wheat proteins extracted from fermented wheat bread cease to bind IgE from patients with wheat allergy (Rizzello *et al.* 2006). Our results also indicate that immunoreactivity of QQQPP epitopes in digested bread is strongly attenuated.

Our research allows to affirm that the proposed way of reducing allergenicity and immunogenicity of wheat flour is viable, even more so as fermentation is a natural process, which improves the organoleptic and healthy properties of the product.

CONCLUSIONS

The addition of baker's yeast can entail an increase in gliadins immunoreactivity. This is probably due to cross-reactivity.

The effectiveness of immunoreactivity reduction for wheat proteins caused by lactic acid fermentation amounts to 20% up to 60% depending on the type of antibodies used.

The residual immunoreactivity of the final products exposed to fermentation during the preparation depends on the type of thermal processing used for dough.

For human antibodies, the effectiveness of the bread digestion does not depend on fermentation. For rabbit antibodies it is in comparison, two-fold higher.

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Corresponding author:

PhD. Joanna Leszczyńska, Technical University of Łodź, Faculty of Biotechnology and Food Sciences, Institute of General Food Chemistry, Stefanowskiego 4/10, 90-924 Łodź, Poland
tel. + 48 426 313 425, e-mail: joanna.leszczynska@ p.lodz.pl