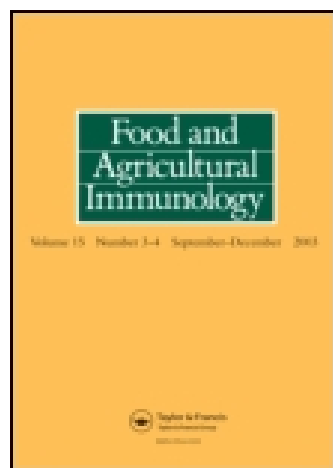




Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/cfai20>

The effect of enzymatic modification and genetic background on wheat gliadin immunological properties

Joanna Leszczyńska^a, Jacek Waga^b, Agata Łącka^a, Katarzyna Wolska^a, Iwona Majak^a & Adrian Bartos^a

^a Institute of General Food Chemistry, Faculty of Biotechnology and Food Sciences, Technical University of Lodz, 4/10 Stefanowskiego Street, 90-924, Lodz, Poland

^b Plant Breeding and Acclimatization Institute, Cereal Department, 4 Zawila Street, 30-423, Krakow, Poland

Published online: 11 May 2012.

To cite this article: Joanna Leszczyńska, Jacek Waga, Agata Łącka, Katarzyna Wolska, Iwona Majak & Adrian Bartos (2012) The effect of enzymatic modification and genetic background on wheat gliadin immunological properties, Food and Agricultural Immunology, 24:2, 217-230, DOI: [10.1080/09540105.2012.682564](https://doi.org/10.1080/09540105.2012.682564)

To link to this article: <http://dx.doi.org/10.1080/09540105.2012.682564>

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms &

The effect of enzymatic modification and genetic background on wheat gliadin immunological properties

Joanna Leszczyńska^a, Jacek Waga^b, Agata Łacka^{a*}, Katarzyna Wolska^a, Iwona Majak^a and Adrian Bartos^a

^a*Institute of General Food Chemistry, Faculty of Biotechnology and Food Sciences, Technical University of Lodz, 4/10 Stefanowskiego Street, 90-924 Lodz, Poland;* ^b*Plant Breeding and Acclimatization Institute, Cereal Department, 4 Zawila Street, 30-423 Krakow, Poland*

(Received 2 November 2011; final version received 2 April 2012)

The immune response to gliadins extracted from 35 wheat cultivars, strains and species was investigated. With the use of a non-competitive ELISA method, we sought to examine if immune reaction occurs between wheat proteins and sera from patients sensitive to gluten, as well as between wheat proteins and rabbit antibodies against pentapeptide QQQP. Results showed considerable differences among cultivars. Breeding strains STH 183, STH 650, STH 2804, *Triticum aethiopicum* and *Triticum durum* showed the lowest immunoreactivity of the native samples. Seventeen of selected wheat flour samples were subjected to enzymatic modifications by subtilisin and transglutaminase. Samples modified by subtilisin exhibited the most extensive change in the level of immunoreactivity of gliadin proteins. The lowest immunoreactivity was reported for the breeding strains STH 1596, STH 2804 and cultivars Tonacja and Zyta. One wheat cultivar (Tonacja), known for its high native gliadin immunoreactivity, was shown to be very susceptible to enzymatic digestion, which resulted in a reduction of its initial immunoreactivity level to low values. Immunoreactivity analysis was also conducted on extracted gliadin fractions from winter wheat strain Ostka strzelecka. The highest level of immunoreactivity to both types of antibodies we used was indicated for ω 5-gliadins. It was, however, abrogated in the aftermath of enzymatic modifications.

Keywords: wheat; gliadins; immunoreactivity; subtilisin; transglutaminase

1. Introduction

Foodstuffs made from wheat flour are the main components of daily diet, hence toxic and allergenic properties of gluten proteins are a serious issue for food science and medicine. Undesired properties of gluten are associated mainly with the presence of proteins called gliadins, which account for approximately 50% of all proteins in wheat grain (Shewry, Tatham, & Kasarda, 1992). It is a group of a few dozen proteins sharing similar amino acid sequences and it has a significant impact on rheological and organoleptic properties of wheat grocery products.

Research on decreasing unfavourable properties of gluten proteins is oriented to induce changes in protein structures, reduce affinity of epitopes to specific antibodies and prevent mutual binding. One example of a protein modification studied extensively in recent years is the enzymatic digestion. There is appreciable evidence

*Corresponding author. Email: agata.lacka@p.lodz.pl

that wheat flour treated with different proteolytic enzymes may change its immunoreactive properties in many ways. Research on gliadin modification using various proteolytic enzymes of different biological activities showed that most of them slightly decreased the level of immunoreactivity, while some, elastase for instance, increased it (Leszczyńska et al., 2002). Most of the patients with wheat allergy showed no response after ingestion of cakes made from such modified flour. So far, no successful technology was invented to produce wheat bakery products that would be tolerable for the afflicted with coeliac disease (CD).

Little attention has been drawn to the analysis of relation between gliadin polymorphism and immunoreactivity of modified proteins. Physico-chemical properties of gliadins are seen to vary among wheat genotypes: species, cultivars and breeding lines. These differences are determined genetically. Diversity within the composition may be an important cause for different immunoreactive properties and susceptibility to enzymatic treatment.

In the present study, two enzymes, subtilisin and transglutaminase, were used for digestion of 17 gliadin protein samples. These were extracted from winter wheat genotypes of different genetic backgrounds. The enzymes used in this experiment do not occur in the human alimentary tract. Subtilisin produced by *Bacillus licheniformis* exhibits low proteolytic specificity (Leszczyńska, Bryszewska, Łacka, Wolska, & Żegota, 2008a). Transglutaminase is often used in bread production to improve proteins functionality in flour by stimulating additional cross-linking via isopeptide bonds between lysine and glutamine residues (Bonet, Caballero, Gomez, & Rosell, 2005; Caballero, Bonet, Rosell, & Gomez, 2005; Larre et al., 2000).

The present study was aimed to identify strains and lineages of wheat with the lowest level of immunoreactivity. It also attempts to define the relation between certain enzymatic modifications and wheat genotypes.

2. Experimental

2.1. Plant material

Thirty winter wheat cultivars and strains from the collection of Plant Breeding Co. (Strzelce, Poland) were chosen for this study (Table 1). The selected genotypes varied in regard of agronomical properties, baking quality and gliadin protein composition. Research also included other wheat species: diploid einkorn (*Triticum monococcum*), tetraploid emmer (*T. diccicum*), durum wheat (*T. durum*), *T. aethiopicum* and hexaploid spelt cultivar (Oberkummeler Rotkorn, *T. aestivum* ssp. *spelta*).

Kernel samples (about 1.5 kg) were harvested in 2006 and milled in a Quadrumat Senior (Brabender GmbH, Germany) laboratory mill.

2.2. Antibodies

Four samples of human sera from celiac patients were obtained from the Polish Mother's Memorial Hospital in Lodz (Poland). They were:

- (1) No S721 (IgG 115.3 u/ml, IgA 70.9 u/ml)
- (2) No S748 (IgG 41.5 u/ml, IgA 24.4 u/ml)
- (3) No S892 (IgG 47.1 u/ml, IgA 1 u/ml)
- (4) No S929 (IgG 96.8 u/ml, IgA 1.5 u/ml)

Table 1. Immunoreactivity of gliadins from different wheat cultivars, strains and species.

No.	Sample	Immunoreactivity (EI)				Rabbit serum	Mean relative immuno-reactivity
		S 721	S 748	S 892	S 929		
1	60A			18.0±0.9	20.4±1.1	46.2±3.3	1.29
2	Elena	29.0±1.5	5.09±0.25	15.6±0.5	17.7±0.9	50.1±3.6	1.18
3	Fraza			13.6±0.5	12.9±0.6	37.0±2.9	0.92
4	Jawa	25.2±1.4	3.98±0.15	21.1±0.8	19.2±1.2	36.2±3.4	1.11
5	Kobra	28.3±1.4	3.88±0.12				0.99
6	Korweta			19.5±0.9	14.1±0.6	40.7±2.1	1.10
7	Kris			13.0±0.7	11.1±0.5	44.4±2.7	0.93
8	Nepal			20.5±0.7		40.8±2.9	1.12
9	Nutka	27.5±1.6	4.01±0.18				0.99
10	Ostka strzelecka	22.9±1.6	6.53±0.27	14.8±0.4	13.2±0.4	32.3±3.2	1.03
11	Pavon				5.7±0.2	62.9±3.3	1.06
12	Rywalka	23.8±1.5	4.55±0.21	14.7±0.4	10.3±0.3	44.4±2.7	0.96
13	Soraja	26.7±1.7	5.38±0.22	16.4±0.5	9.5±0.4	33.3±2.8	0.97
14	STH 146			18.9±1.1	15.4±0.4	36.7±2.9	1.09
15	STH 1596	25.7±1.1	3.57±0.17			27.4±1.4	0.84
16	STH 183			12.7±0.8	10.9±0.2	22.1±1.2	0.73
17	STH 2703	28.5±1.7	3.62±0.14			29.2±1.9	0.89
18	STH 2804	20.5±1.1	2.98±0.12			22.4±1.3	0.69
19	STH 3104	24.1±1.2	3.49±0.13			26.2±1.2	0.81
20	STH 490			18.4±1.2	17.2±0.8	35.2±1.4	1.12
21	STH 5295			17.6±0.9	11.8±0.4	31.9±1.5	0.93
22	STH 5338			16.7±0.7	9.8±0.3	32.9±1.6	0.87
23	STH 626			29.5±1.3	18.6±0.6	49.9±2.4	1.49
24	STH 650			10.9±0.5	7.2±0.3	30.5±1.8	0.67
25	STH 764			15.4±0.5	16.2±0.8	35.5±1.4	1.03
26	Sukces	31.9±1.8	4.57±0.16	19.4±1.0	20.5±1.3	43.7±1.3	1.23
27	Symfonia	25.9±1.6	5.07±0.19				1.08
28	Tonacja	31.8±1.7	6.15±0.24	20.4±1.1	7.9±0.3	26.4±1.3	1.02
29	TS 24076			21.3±0.9		55.9±2.3	1.35
30	Zyta	26.7±1.7	4.09±0.20				0.98
31	<i>Triticum monococcum</i>			17.5±0.7	10.0±0.3	35.3±1.2	0.91
32	<i>Triticum dicoccum</i>			23.9±0.9	10.2±0.3	64.4±2.2	1.29
33	<i>Triticum aethiopicum</i>			18.9±0.8	8.1±0.2	26.6±1.6	0.81
34	<i>Triticum durum</i>	24.5±1.5	3.41±0.19	8.11±0.5	7.9±0.2	27.7±1.7	0.71
35	Spelt	21.3±1.4	3.50±0.12	19.6±1.0	8.4±0.2	45.1±1.9	0.92
	Average	26.1±3.2	4.4±1.0	17.6±4.3	12.6±4.4	37.9±11.0	1.00

Rabbit serum containing IgG and IgE antibodies against QQQPP peptide was obtained according to the protocol described in previous work (Leszczyńska, Łacka, Bryszewska, & Brzezińska-Błaszczyk, 2008b).

2.3. Reagents

Transglutaminase and lyophilised powder of subtilisin A from *Bacillus subtilis* with the activity of 7–15 units/mg from Sigma Chemical Co. (St Louis, MO, USA) were used for the preparation of modified gliadins. Solutions of TMB (3,3',5,5'-tetramethylbenzidine, T 8665) and p-NPP (p-nitrophenol phosphate, N-7660) as well as Tween-20 and carbonate-bicarbonate buffer pH 9.6 were obtained from Sigma. Phosphate buffer (PBS) pH 7.2, Tris-HCl buffer pH 7.5, 1 mol/L H₂SO₄ aqueous solution and 3 mol/L NaOH solution were prepared using redistilled water. Commercial antibodies were employed, namely, monoclonal anti-human IgG conjugate with alkaline phosphatase (AP) from mouse clone GG-5 (Sigma, A 2064) and antibodies against rabbit immunoglobulin. All reagents were of analytical grade and were used without further purification.

2.4. Preparative electrophoresis

Isolation and purification of gliadin proteins were carried out by a method of preparative polyacrylamide gel electrophoresis in lactate-aluminium buffer (pH 3.1), using Model 491 PrepCell by BioRad. Methodology of Bushuk and Zillman (1978) was utilised.

The preparative column (37 mm in diameter and 10 cm high) was filled with polyacrylamide gel with a total monomers concentration 8% (w/v) and crosslinker concentration 0.29% (w/v). The highly exothermic, polymerisation reaction required intensive cooling of chamber during all stages of gel preparation. A mixture (6 ml) containing gliadins extract and saturated saccharose solution in lactate-aluminium buffer (1:1) was put on the prepared column. Separation was carried out at constant voltage (500 V) and electric current (50 mA). Fraction collecting was initiated 5 hours after electrophoresis had been started. Seven millilitre volume fractions, containing eluent (lactate-aluminium buffer, pH 3.1) and gliadins that left the column were aliquoted into 60 vials. Preparation was finished 12 hours after the collection was initiated. Each fraction was labelled so that the first number refers to the cycle of gliadins purification and the second one is the fraction number obtained in that cycle.

2.5. Protein extraction for ELISA tests

Gliadins of wheat were extracted from flour with 70% ethanol (1:10 w/v). Samples were gently shaken during 1 hour at room temperature, and then centrifuged for 10 minutes, 12,000 rpm.

Total protein content in all gliadin extracts was determined with kit for protein assay using bicinchoninic acid (Biomedicals Co., Rockford, IL, USA).

2.6. Enzymatic modification of gliadin proteins

Samples of wheat flour (1 g) were mixed with 3 ml of water. Following the addition of 10 μ l solution of the chosen enzyme at concentration 1 mg/ml (the ratio of enzyme to substrate was 1:10⁵), the modification of wheat proteins was carried out for 18 h in the water bath at 37°C. Then 7 ml ethanol was added and all wheat flour samples were centrifuged at 2500 g. In the end, supernatant containing proteins was collected.

2.7. ELISA

Gliadin extracts diluted 1:100 were mixed at a 1:1 ratio with 0.1 mol/L carbonate-bicarbonate buffer pH 9.6. 100 μ l solution aliquots were used to coat Microtiter plates EB 92029330 (Labsystems, Helsinki, Finland) overnight at 4°C. Next, the plates were washed four times with PBS buffer and free binding sites were blocked by incubation with 3% solution of low fat milk in PBS (pH 7.2), containing 0.1% of Tween-20, for 2 h at room temperature. This was followed by removal of milk buffer solution, rinsing the plates four times with PBS and further incubation with 100 μ l of 100-fold diluted human serum (or 20-fold diluted rabbit serum) for 1 h at room temperature. The plates were washed again and subjected to addition of 100 μ l antibodies against human IgG conjugated with AP (2000)-fold diluted) or antibodies against rabbit IgG coupled with peroxidase. After incubation of the plates for 1 h and rinsing with PBS, the substrate solution (p-NPP for phosphatase or TMB for peroxidase) was introduced and plates were incubated again for 1 h. Then, the reaction was stopped by adding 100 μ l 3 mol/L solution of NaOH (p-NPP) or 100 μ l 1 mol/L solution of H₂SO₄ (TMB) and the output absorbance was measured using Multiscan RC reader at 405 nm (p-NPP) or 450 nm (TMB). The immunoreactivity was normalised relative to the content of protein. The immune response of gliadins was tested twice in three independent assays.

2.8. Evaluation of the obtained data

Immunoreactivity level of the native protein samples was calculated as a ratio of absorbance (OD_{native}) to protein content (B_{native}). The obtained values were used as indicators of the effective immunoreactivity (EI) of native samples.

Immunoreactivity level of enzyme-treated proteins was estimated in the form of two indicators: effective residual immunoreactivity (ERI) and relative residual immunoreactivity (RRI). ERI was calculated as a ratio of absorbance obtained for hydrolysed samples (OD_{modified}) to the protein content in modified samples (B_{modified}). This indicator reflects a residual affinity of proteins after enzymatic modification. Lower values of ERI reflect stronger effect of enzyme treatment on immunoreactivity. RRI was calculated as the ratio of ERI to EI and expressed as a per cent. It reflects the susceptibility to enzymatic digestion of gliadin proteins from individual wheat genotypes. This indicator measures the influence of genetic background on the efficacy of enzymatic modification. Lower values of RRI confirm an effect sustained from enzymatic modification, while higher RRI values indicate resistance of gliadins to hydrolysis.

3. Results

Gliadin proteins of winter wheat genotypes presented considerable differences in regard of their immunoreactive properties, as shown by ELISA (Table 1). Due to the very individual character of response from specific antibodies, we determined a mean relative immunoreactivity in order to compare the samples. Mean relative immunoreactivity is an average taken of relative immunoreactivity values of the samples relative to the average value, calculated separately for each serum. Cultivars Elena, Sukces, 60A, STH 626, TS 24076 and *T. dicoccum* showed the highest mean relative immunoreactivity level, while breeding strains STH 183, STH 650, STH 2804, *T. aethiopicum* and *T. durum* showed the lowest level of immunoreactivity in the native samples.

In Tables 2 and 3 we present immunoreactivity values from the analysis of enzymatically modified flour samples of selected lineages and strains of wheat. Independent modifications were made by two non-digestive enzymes: subtilisin (protease) (Table 2) and transglutaminase (cross-linking enzyme) (Table 3). Values were grouped into ERI and RRI that describes susceptibility of a certain strain of gliadin to proteolysis or cross-linking.

Enzymatic digestion of gliadins strongly altered their EI. The observed differences were dependent on the human sera, wheat genotype and enzyme used. The strongest effects of modification were observed when gliadin samples of breeding strains, Tonacja, Jawa, Sukces, STH 3104 and spelt, were modified by subtilisin. Immunoreactivity values of gliadin samples modified by transglutaminase and tested with human and rabbit sera were shown in Table 3. The strains that were most susceptible to modification by TG include: Tonacja, Zyta, Jawa, STH 2703 and spelt, while the least susceptible were Nutka, Elena, Symfonia and STH 2804.

Another stage of the experiment consisted in preparative separation of gliadins from a given winter wheat strain, as well as analysis of immunoreactivity of individual gliadin fractions – both wild-type and enzymatically modified. Strain Ostka strzelecka, with its average EI, ERI and RRI values, was chosen from the previously examined strains. Results of analysis on individual gliadin fractions from this strain are presented in Table 4 (wild-type proteins) and 5 (enzymatically modified proteins). Gliadin fractions $\omega 2$ and $\omega 5$ were the most immunogenic. Both these gliadin fractions were also most susceptible to enzymatic modifications, especially to hydrolysis catalysed by subtilisin.

4. Discussion

In the described research, immunogenic properties of gliadin proteins from various strains of winter wheat were analysed. Analysis was undertaken with the use of two types of antibodies: human and rabbit. Human antibodies (anti-gliadin, class IgG) were obtained from sera of patients diagnosed with CD. Differences between specific sera indicate fundamental problems: high individual discrepancies in antibody specificity as well as in immunological reaction intensity (examples: Tonacja and Ostka strzelecka, marked by either low or high immunoreactivity, depending on the type of serum). Additional analysis was performed with the use of rabbit anti-QQQPP pentapeptide antibodies, which is considered as one of the main epitopes in

Table 2. Immunoreactivity of flour made from different strains and lineages of wheat, subjected to proteolysis by subtilisin.

		Immunoreactivity (ERI and RRI)						
		S721		S748		Rabbit serum		
No.	Sample	ERI	RRI (%)	ERI	RRI (%)	ERI	RRI (%)	Average%
1	Elena	25.7 ± 1.3	88.7 ± 5.0	4.02 ± 0.21	78.9 ± 5.0	4.08 ± 0.14	71.3 ± 6.0	79.7 ± 8.7
2	Jawa	15.8 ± 0.9	62.5 ± 4.8	2.77 ± 0.12	69.6 ± 5.1	3.21 ± 0.17	67.1 ± 5.6	66.4 ± 3.6
3	Kobra	22.2 ± 1.2	78.4 ± 4.9	3.63 ± 0.14	93.6 ± 4.9	2.72 ± 0.12	57.0 ± 4.5	76.3 ± 18.4
4	Nutka	25.9 ± 1.2	94.4 ± 5.1	3.48 ± 0.22	86.8 ± 5.1	2.71 ± 0.12	30.4 ± 4.3	70.5 ± 34.9
5	Ostka strzelecka	20.0 ± 1.3	87.3 ± 5.1	4.66 ± 0.23	71.4 ± 4.9	4.93 ± 0.21	51.9 ± 4.7	70.2 ± 17.7
6	Rywalka	18.8 ± 0.9	79.2 ± 4.8	3.06 ± 0.22	67.3 ± 4.9	4.83 ± 0.22	70.0 ± 5.6	72.2 ± 6.3
7	Soraja	20.8 ± 0.9	77.8 ± 4.7	4.51 ± 0.31	83.8 ± 5.6	4.83 ± 0.32	59.1 ± 3.9	73.6 ± 12.9
8	STH 1596	23.7 ± 1.1	92.4 ± 4.5	3.16 ± 0.24	88.5 ± 4.1	3.11 ± 0.23	45.0 ± 5.2	75.3 ± 26.3
9	STH 2703	25.4 ± 1.2	89.1 ± 4.6	2.72 ± 0.14	75.1 ± 4.2	3.11 ± 0.25	45.0 ± 3.9	69.8 ± 22.5
10	STH 2804	19.6 ± 1.1	95.6 ± 4.2	2.74 ± 0.15	91.9 ± 5.2	2.71 ± 0.13	41.1 ± 3.8	76.2 ± 30.5
11	STH 3104	21.8 ± 1.2	90.5 ± 4.3	2.54 ± 0.13	72.8 ± 4.1	2.72 ± 0.13	32.5 ± 3.9	65.3 ± 29.7
12	Sukces	28.9 ± 1.4	90.4 ± 4.9	2.80 ± 0.14	61.3 ± 4.1	3.67 ± 0.23	47.6 ± 6.2	66.4 ± 21.9
13	Symfonia	20.2 ± 1.1	74.2 ± 3.8	3.05 ± 0.21	89.4 ± 4.7	4.22 ± 0.24	79.1 ± 5.6	80.9 ± 7.8
14	Tonacja	20.2 ± 0.9	63.6 ± 3.9	3.62 ± 0.25	58.9 ± 4.8	3.41 ± 0.21	30.3 ± 4.8	50.9 ± 18.0
15	Zyta	20.4 ± 0.8	76.6 ± 3.6	3.63 ± 0.22	88.7 ± 5.1	4.12 ± 0.23	63.3 ± 4.9	76.2 ± 12.7
16	<i>Triticum durum</i>	18.2 ± 0.8	77.8 ± 3.7	4.27 ± 0.33	84.2 ± 5.2	3.72 ± 0.15	63.7 ± 5.6	75.3 ± 10.5
17	Spelt	15.8 ± 0.7	74.3 ± 3.8	2.65 ± 0.12	75.7 ± 5.1	3.16 ± 0.16	32.9 ± 5.0	60.9 ± 24.3
	Average	21.4 ± 3.6	81.9 ± 10.1	3.4 ± 0.7	78.7 ± 10.8	3.6 ± 0.8	52.2 ± 15.7	70.9 ± 7.4

Table 3. Immunoreactivity of flour made from different strains and lineages of wheat, subjected to modifications by transglutaminase.

		Immunoreactivity (ERI and RRI)						
		S721		S748		Rabbit serum		
No.	Sample	ERI	RRI (%)	ERI	RRI (%)	ERI	RRI (%)	Average%
1	Elena	26.2 ± 1.9	90.3 ± 7.1	3.92 ± 0.15	77.0 ± 4.4	4.82 ± 0.12	84.2 ± 3.3	83.8 ± 6.6
2	Jawa	17.4 ± 1.2	69.2 ± 6.2	2.81 ± 0.12	70.6 ± 4.3	2.95 ± 0.13	61.7 ± 3.4	67.2 ± 4.8
3	Kobra	23.1 ± 1.9	81.5 ± 7.1	3.54 ± 0.12	91.2 ± 5.2	2.73 ± 0.11	57.3 ± 3.3	76.7 ± 17.5
4	Nutka	27.5 ± 1.8	100.0 ± 5.5	3.51 ± 0.11	87.5 ± 4.5	7.14 ± 0.14	80.1 ± 3.9	89.2 ± 10.1
5	Ostka strzelecka	17.1 ± 1.7	74.4 ± 4.5	4.50 ± 0.14	68.9 ± 4.5	5.94 ± 0.12	62.5 ± 3.7	68.6 ± 5.9
6	Rywalka	20.1 ± 1.7	84.4 ± 5.5	4.04 ± 0.13	88.8 ± 5.1	4.15 ± 0.12	60.1 ± 3.4	77.8 ± 15.4
7	Soraja	20.5 ± 1.8	76.8 ± 5.5	4.68 ± 0.13	86.9 ± 3.3	4.92 ± 0.13	60.2 ± 3.8	74.6 ± 13.5
8	STH 1596	21.4 ± 1.6	83.6 ± 5.6	2.69 ± 0.11	75.45 ± 2.9	3.20 ± 0.11	46.3 ± 3.0	68.4 ± 19.6
9	STH 2703	23.0 ± 1.7	80.7 ± 5.2	2.46 ± 0.11	67.9 ± 3.1	3.88 ± 0.11	56.2 ± 3.2	68.3 ± 12.3
10	STH 2804	19.4 ± 1.2	94.5 ± 5.3	2.78 ± 0.12	93.3 ± 3.4	4.24 ± 0.12	64.4 ± 2.8	84.1 ± 17.1
11	STH 3104	22.3 ± 1.3	92.4 ± 4.9	2.35 ± 0.11	67.3 ± 4.2	4.96 ± 0.14	59.2 ± 2.9	73.0 ± 17.3
12	Sukces	26.4 ± 1.3	82.8 ± 4.4	3.16 ± 0.13	69.2 ± 2.8	4.71 ± 0.14	61.1 ± 3.1	71.0 ± 10.9
13	Symfonia	20.4 ± 1.6	82.9 ± 4.9	2.80 ± 0.13	82.1 ± 3.7	4.34 ± 0.13	81.4 ± 3.9	82.1 ± 0.8
14	Tonacja	19.5 ± 1.5	61.2 ± 5.6	4.14 ± 0.14	67.3 ± 3.8	4.82 ± 0.14	42.8 ± 2.7	57.1 ± 12.8
15	Zyta	17.0 ± 1.3	63.9 ± 5.5	2.96 ± 0.12	72.4 ± 4.2	3.34 ± 0.12	51.3 ± 3.1	62.5 ± 10.6
16	<i>Triticum durum</i>	16.3 ± 1.2	76.6 ± 5.7	2.73 ± 0.12	78.0 ± 3.8	3.91 ± 0.12	66.9 ± 3.4	73.8 ± 6.0
17	Spelt	21.4 ± 1.8	82.6 ± 6.1	2.98 ± 0.13	58.8 ± 4.1	6.03 ± 0.14	62.9 ± 3.4	68.1 ± 12.7
	Average	21.1 ± 3.4	81.1 ± 10.3	3.3 ± 0.7	76.6 ± 10.1	4.5 ± 1.2	62.3 ± 11.3	73.3 ± 8.3

Table 4. Immunoreactivity of individual gliadin fractions of Ostka strzelecka strain expressed as an absorbance of immunoenzymatic reaction caused by 1g of protein.

No.	Fraction	Immunoreactivity (EI)	
		Rabbit serum	Human serum (S892)
1	α 11.4	40.3 ± 6.6	10.55 ± 5.1
2	α 11.8	43.8 ± 6.5	9.16 ± 2.9
3	β 8.1	38.4 ± 5.9	15.07 ± 3.4
4	β 8.6	11.2 ± 2.5	4.48 ± 2.1
5	γ 6.17	15.7 ± 2.6	17.95 ± 3.3
6	ω 5 10.47	1270 ± 9	645.7 ± 9
7	ω 1,2 8.53	135 ± 7	59.25 ± 3.2
8	ω 1,2 8.73	322 ± 9	84.1 ± 7.8

food allergy to wheat (Tanabe et al., 1996). Anti-QQQP antibodies were successfully applied in modified gliadin analyses (Leszczyńska et al., 2008b).

In recent years attempts were made to search for wheat strains that show reduced allergenicity or limited content of peptides responsible for inflammation in CD (Spaenij-Dekking et al., 2005). From our work, we can identify cultivar lineages of common wheat, STH 183, STH 650, STH 2804, and other species of wheat, *T. aethiopicum* and *T. durum*.

In recent years researchers paid special attention to spelt wheat (*Triticum aestivum* ssp. *spelta*). Spelt is considered a crop of greater health benefits and more resistance to abiotic stresses as compared with common wheat. Because of lower agricultural requirements, it is suitable for ecological agriculture (Campbell, 1997). A special trait of spelt is its relatively high low-quality protein content. Consequently, proteins form weak gluten of lower elasticity (Wieser, 2000). The harmfulness of spelt to celiac patients is comparable to that of common wheat (Forsell & Wieser, 1995; Kasarda & D'Ovidio, 1999). The allergenic properties of spelt have not yet been described. In our study immunoreactivity level shown by spelt was on a par with common wheat.

Surprisingly high IR values for antibodies from celiac disease patients were also reported for species of diploid wheat (einkorn, *T. monococcum*) and tetraploid wheat (emmer, *T. dicoccum* and *T. aethiopicum*). The species lack genome D, responsible for the largest number of encoded gliadin epitopes recognised by antibodies in CD-afflicted patients (Molberg et al., 2005; Van Herpen et al., 2006). Pizzuti et al. (2006) also defined gliadins from einkorn as less toxic than gliadins from hexaploid wheat. The outcome of our analysis did not confirm low harmfulness of other wheat species (except *T. durum*) in CD. Immunoreactivity of gliadins from these species fits in IR range of common wheat gliadins. Rabbit antibodies directed against QQQP pentapeptide also bound gliadins at the level comparable with these found for gliadins from various common wheat strains, indicating similar allergenic properties of examined wheat species. Tetraploid species *T. durum* and *T. aethiopicum* were the only exceptions with significantly lower immunoreactivity towards anti-QQQP antibodies.

Durum wheat *T. durum* proteins bear resemblance to proteins from hexaploid wheat regarding both molecular characteristics and allergenic properties (Simonato et al., 2001). Foregoing research confirmed that immunoreactivity values of spelt and

Table 5. Immunoreactivity of gliadin fractions of Ostka strzelecka strain exposed to enzymatic modification expressed as an absorbance of immunoenzymatic reaction caused by 1 g of protein.

No.	Fraction	Subtilisin				Transglutaminase			
		Rabbit serum		Human serum S892		Rabbit serum		Human serum S892	
		ERI	RRI (%)	ERI	RRI (%)	ERI	RRI (%)	ERI	RRI (%)
1	α 11.4	38.1 ± 0.8	94.5 ± 5.5	0.9 ± 0.8	8.2 ± 5.4	41.7 ± 0.8	103.6 ± 6.6	5.4 ± 0.4	49.6 ± 5.7
2	α 11.8	24.8 ± 0.8	56.6 ± 5.6	3.6 ± 0.7	39.7 ± 7.6	29.1 ± 0.9	66.5 ± 7.1	5.6 ± 0.5	61.2 ± 6.1
3	β 8.1	35.8 ± 0.7	93.1 ± 7.1	3.3 ± 0.5	22.0 ± 6.9	6.67 ± 0.8	17.1 ± 5.6	13.8 ± 0.6	91.6 ± 7.1
4	β 8.6	12.4 ± 0.6	111.2 ± 7.2	2.7 ± 0.5	61.2 ± 7.4	1.1 ± 0.7	10.1 ± 5.4	4.8 ± 0.5	107.6 ± 7.2
5	γ 6.17	16.4 ± 0.8	104.5 ± 6.8	10.0 ± 0.5	55.9 ± 7.4	9.7 ± 0.6	61.7 ± 7.2	15.0 ± 0.7	83.8 ± 6.9
6	ω 5 10.47	139.9 ± 0.8	11.0 ± 4.9	0 ± 0.5	0 ± 5.5	4.7 ± 0.6	0.44 ± 4.1	96.2 ± 0.8	14.9 ± 5.7
7	ω 1,2 8.53	17.4 ± 0.7	10.31 ± 5.2	0 ± 0.6	0 ± 5.5	63.4 ± 0.7	46.9 ± 5.4	56.8 ± 0.8	95.9 ± 6.8
8	ω 1,2 8.73	41.4 ± 0.8	12.8 ± 4.4	0.8 ± 0.5	9.4 ± 5.5	14.0 ± 0.8	4.3 ± 3.9	66.5 ± 0.9	79.0 ± 5.7

durum wheat are comparable with those of bread wheat strains, yet flours made from them appeared to evoke the lowest allergenic response. The immunological reaction is unaffected by enzymatic modification endeavours. *Triticum aethiopicum* wheat is considered to be one of the durum wheat species (Mac Key, 1988). In our research both types of wheat showed different immunogenic properties, indicating significant variety in amino acid sequences of the proteins therein.

Studies on biochemical modification using enzymatic hydrolysis of gliadins have been described by many authors. The goal of modifications is to elucidate the theoretical basis for technological processing and production of hypoallergenic food. Enzymatic digestion is the most economical method of wheat gliadin modification. The low cost and simplicity of digestion methodology facilitate adaptation of technological processing in the bakery industry. Supposing a positive correlation between immunoreactivity estimated *in vitro* and real allergenicity, our results allow formulation of some basic principles for modification and evaluation of raw material for production of hypoallergenic bread. One of the most important findings is the occurrence of strong diversity among wheat genotypes in regard of their usefulness for biochemical modification and susceptibility to hydrolysis. An important criterion of genotype selection for the production of bread for allergic people is that it has to have a low level of gliadin immunoreactivity after enzymatic modification. To meet this requirement, a genotype must be selected that exhibits low immunoreactivity of native gliadins and high susceptibility of proteins to hydrolysis.

Based upon our previous findings (Leszczyńska et al., 2002, 2008a; Leszczyńska, Łacka, & Bryszewska, 2006) we chose two enzymes for gliadin modifications: subtilisin and transglutaminase. Neither exists in the alimentary canal and their activities are different. Subtilisin is a protease, and transglutaminase is regarded as a cross-linking enzyme. The latter is used as a supplement in the baking industry.

Transglutaminase (EC 2.3.2.13) (TG, protein-glutamine- γ -glutamyltransferase) is an acyltransferase capable of catalysing intra- and intermolecular cross-linking with formation of peptide bond between lysine and glutamine residues (Seguro, Kumazawa, Kuraishi, Sakamoto, & Motoki, 1996). Transglutaminase utilised in bread production leads to formation of large insoluble polymers (Larre et al., 2000) and it enhances physico-chemical properties of dough, especially this made from poor-quality flour or tainted as a result of proteolysis by insects (Bonet et al., 2005; Caballero et al., 2005).

TG-modified gliadins showed a clear increase in immunoreactivity towards human anti-gliadin IgA (Berti et al., 2007). This is probably because of the absence of lysine residues or other amines, therefore, in these conditions TG catalyses deamination of glutamine residues (Nielson, 1995). On the other hand, drop in gluten IR caused by the activity of microbiological transglutaminase was described by many authors (Leszczyńska et al., 2006; Watanabe, Ikezawa, & Arai, 1994). In spite of the fact that applied, microbiological transglutaminase differs from human transglutaminase, as far as recognisable glutamine residues in gliadins are concerned, both enzymes deamidate polypeptides with formation of structures that are recognisable for antibodies (Berti et al., 2007; Cabrera-Chavez, Rouzaud-Sanchez, Sotelo-Cruz, & Calderon de la Barca, 2008; Gerrard & Sutton, 2005). It could significantly affect results obtained with the use of antibodies from CD-afflicted patients. Nevertheless, the results do not indicate strong deamination of gliadins in our experiment.

We succeeded in identifying one wheat cultivar (Tonacja) of high native gliadin immunoreactivity but also with very high susceptibility to digestion by enzymes. On the other hand, some genotypes with low initial EI value of native proteins (but only of moderate susceptibility to hydrolysis) were also found. Further research should concentrate on finding the most useful genotypes with low initial value of native protein immunoreactivity, along with high susceptibility to digestion.

In order to determine how enzymatic modifications influence properties of individual gliadin fractions, the fractions were extracted from selected strains of wheat and modification was carried out as described in the case of flour samples. This experimental approach greatly affected the modification by transglutaminase, as there was no binding to other proteins that are richer in lysine residues as compared to gliadins. Ostka strzelecka was chosen as a representative winter wheat strain. This strain was characterised by medium immunogenicity level in all previous analyses, both with wild-type and modified proteins. Results obtained were far from expected. Despite the fact that literature to date indicates that α -gliadin group of proteins is the main source of immunotoxic epitopes for those afflicted with CD, it turns out that all gliadin fractions may evoke inflammatory response (Shewry et al., 1992). The very high IR of ω fraction, especially $\omega 5$ -gliadins, may hint at the presence of potential epitopes on ω -gliadins molecular surface, while analogical fragments in other fractions are found in the inner structure and get released only as an outcome of proteolysis by digestive enzymes. Another explanation is based on the cross-reactivity between human antibodies and surface fragments of ω -gliadins, which, instead of releasing inflammatory peptides get completely hydrolysed into component amino acid residues. By analogy, it is only cross-reactivity that can explain the strong reaction between ω -gliadins and anti-QQQPP antibodies, as in this group of proteins there are no sequential fragments identified that match the recognisable epitope.

Hydrolysis by subtilisin gave results that suggest extensive hydrolysis of epitopes recognisable by specific antibody groups. A drop in ω -gliadin immunoreactivity, both $\omega 5$ and $\omega 1,2$, confirms presence of inflammatory peptide fragments on the molecular surface, which happens to be a preferential site for proteolytic enzymes activity. As detected by anti-QQQPP antibodies, small extent of changes following proteolysis by subtilisin is associated with modest susceptibility of this fragment to hydrolysis by the enzyme given. The clear change in response to ω -gliadins confirms the lack of QQQPP on the molecular surface. Instead, cross-reactive peptides with similar structure and greater susceptibility to proteolysis may be present.

Transglutaminase-dependent changes in gliadin immunoreactivity were found to be smaller than those caused by proteolysis. The largest reduction was reported for $\omega 5$ -gliadins (up to 15% of value shown by native proteins), yet human anti-gliadin antibodies gave strong antigenic response to all fraction ω proteins. Immunoreactivity of α -gliadin fraction is decreased by half, while the remaining fractions bound to antibodies only slightly weaker than in native form. Modified by transglutaminase, epitopes QQQPP were recognised by rabbit antibodies to a much more limited extent than the wild type, especially for the $\omega 5$ -gliadins fraction. The smallest loss of immunoreactivity was detected in α -gliadins (up to about 65% initial value), which along with $\omega 1,2$ was the most strongest fraction recognised by antibodies.

In our opinion, adaptation of a proper methodology for production of hypoallergenic food in an industrial scale should be based on screening tests similar

to those described in this paper. A test should employ a wide spectrum of wheat species, cultivars and breeding strains, more enzymes and more sera of different classes (mainly IgG and IgE) obtained from patients showing diverse symptoms of disease.

References

- Berti, C., Roncoroni, L., Falini, M.L., Caramanico, R., Dolfini, E., Bardella, M.T., et al. (2007). Celiac-related properties of chemically and enzymatically modified gluten proteins. *Journal of Agricultural and Food Chemistry*, 55, 2482–2488.
- Bonet, A., Caballero, P.A., Gomez, M., & Rosell, C.M. (2005). Microbial transglutaminase as a tool to restore the functionality of gluten from insect-damaged wheat. *Cereal Chemistry*, 82, 425–430.
- Bushuk, W., & Zillman, R.R. (1978). Wheat cultivar identification by gliadin electrophoregrams. I. Apparatus, method and nomenclature. *Canadian Journal of Plant Science*, 58, 505–515.
- Caballero, P.A., Bonet, A., Rosell, C.M., & Gomez, M. (2005). Effect of microbial transglutaminase on the rheological and thermal properties of insect damaged wheat flour. *Journal of Cereal Science*, 42, 93–100.
- Cabrera-Chavez, F., Rouzaud-Sanchez, O., Sotelo-Cruz, N., & Calderon de la Barca, M. (2008). Transglutaminase treatment of wheat and maize prolamins of bread increases the serum IgA reactivity of celiac disease patients. *Journal of Agricultural and Food Chemistry*, 56, 1387–1391.
- Campbell, K.G. (1997). Spelt: agronomy, genetics, and breeding. *Plant Breeding Reviews*, 15, 187–213.
- Forsell, F., & Wieser, H. (1995). Dinkel und Zöliakie. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, 201, 35–39.
- Gerrard, J.A., & Sutton, K.H. (2005). Addition of transglutaminase to cereal products may generate the epitope responsible for coeliac disease. *Trends in Food Science & Technology*, 16, 510–512.
- Kasarda, D.D., & D'Ovidio, R. (1999). Deduced amino acid sequence of an α -gliadin gene from spelt wheat (spelta) includes sequences active in celiac disease. *Cereal Chemistry*, 76, 548–551.
- Larre, C., Denery, P.S., Popineau, Y., Deshayes, G., Desserme, C., & Lefevre, J. (2000). Biochemical analysis and rheological properties of gluten modified by transglutaminase. *Cereal Chemistry*, 77, 32–38.
- Leszczyńska, J., Bryszewska, M., Łacka, A., Wolska, K., & Żegota, H. (2008a). The immunoreactivity reduction of wheat flour modified by the treatment with subtilisin. *Polish Journal of Food and Nutrition Sciences*, 58, 335–340.
- Leszczyńska, J., Łacka, A., & Bryszewska, M. (2006). The use of transglutaminase in the reduction of immunoreactivity of wheat flour. *Food and Agricultural Immunology*, 17, 105–113.
- Leszczyńska, J., Łacka, A., Bryszewska, M., & Brzezińska-Błaszczuk, E. (2008b). The usefulness of rabbit anti-QQQPP peptide antibodies to wheat flour antigenicity studies. *Czech Journal of Food Science*, 26, 24–30.
- Leszczyńska, J., Łacka, A., Pytasz, U., Szemraj, J., Lukamowicz, J., & Lewiński, A. (2002). The effect of proteolysis on the gliadin immunogenicity. *Polish Journal of Food and Nutrition Sciences*, 52(SI 2), 145–148.
- Mac Key, J. (1988). A plant breeder's perspective on taxonomy of cultivated plants. *Biologisches Zentralblatt*, 107, 369–379.
- Molberg, O., Uhlen, A.K., Jensen, T., Flaete, N.S., Fleckenstein, B., Arentz-Hansen, H., et al. (2005). Mapping of gluten T cell epitopes in the bread wheat ancestors: Implications for celiac disease. *Gastroenterology*, 128, 393–401.
- Nielson, P.M. (1995). Reactions and potential industrial applications of transglutaminase – Review of the literature and patents. *Food Biotechnology*, 9, 119–156.

- Pizzuti, D., Buda, A., D'Odorico, A., D'Inca, R., Chiarelli, S., Curioni, A., et al. (2006). Lack of intestinal mucosal toxicity of *Triticum monococcum* in celiac disease patients. *Scandinavian Journal of Gastroenterology*, 41, 1305–1311.
- Seguro, K., Kumazawa, Y., Kuraishi, C., Sakamoto, H., & Motoki, M. (1996). The ϵ -(γ -glutamyl)lysine moiety in crosslinked casein is an available source for lysine for rats. *Journal of Nutrition*, 126, 2557–2562.
- Shewry, P.R., Tatham, A.S., & Kasarda, D.D. (1992). Cereal proteins and coeliac disease. In M.N. Marsh (Ed.), *Coeliac disease* (pp. 305–348). Oxford: Blackwell Scientific Publications.
- Simonato, B., Pasini, G., Giannattasio, M., Dal Belin Peruffo, A., De Lazzari, F., & Curioni, A. (2001). Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergic proteins. A study with bread dough, crumb and crust. *Journal of Agricultural and Food Chemistry*, 49, 5668–5673.
- Spaenij-Dekking, L., Kooy-Winkelaar, Y., Van Veelen, P., Drijfhout, J.W., Jonker, H., Van Soest, L., et al. (2005). Natural variation in toxicity of wheat: Potential for selection of nontoxic varieties for celiac disease patients. *Gastroenterology*, 129, 797–806.
- Tanabe, S., Arai, S., Yanagihara, Y., Mita, H., Takahashi, K., & Watanabe, M. (1996). A major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding epitope. *Biochemical and Biophysical Research Communications*, 219, 290–293.
- Van Herpen, T.W.J.M., Goryunova, S.V., van der Schoot, J., Mitreva, M., Salentijn, E., Vorst, O., et al. (2006). Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes. *BMC Genomics*, 7, 1.
- Watanabe, M., Ikezawa, Z., & Arai, S. (1994). Fabrication and quality evaluation of hypoallergenic wheat products. *Bioscience, Biotechnology, and Biochemistry*, 58, 388–390.
- Wieser, H. (2000). Comparative investigations of gluten proteins from different wheat species. I. Qualitative and quantitative composition of gluten protein types. *European Food Research and Technology*, 211, 262–268.