The Usefulness of Rabbit Anti-QQQPP Peptide Antibodies to Wheat Flour Antigenicity Studies

Joanna LESZCZYŃSKA[1], Agata ŁĄCKA[1], Malgorzata BRYSZEWSKA[1] and Ewa BRZEZIŃSKA-BŁASZCZYK[2]

[1]Institute of General Food Chemistry, Faculty of Biotechnology and Food Sciences, Technical University of Łódź, Łódź, Poland; [2]Department of Experimental Immunology, Medical University of Łódź, Łódź, Poland

Abstract

Leszczyńska J., Łącka A., Bryszewska M., Brzezińska-Błaszczyk E. (2008): **The usefulness of rabbit anti-QQQPP peptide antibodies to wheat flour antigenicity studies**. Czech J. Food Sci., **26**: 24–30.

The aim of our work was to estimate the usefulness of rabbit polyclonal anti-QQQPP peptide antibodies to determination of gluten proteins and the usage of these antibodies to estimate the changes of allergenicity of wheat flour after enzymatic modification. For enzymatic modification of wheat flour trypsin, subtilisin and transglutaminase were used. By the use of rabbit antibodies against QQQPP peptide we have stated that the products of subtilisin and transglutaminase degradation exert strongly decreased immunoreactivity.

Keywords: food allergy; wheat flour; glutenins; antigenicity; enzymatic modification

Adverse immunologic reactions to wheat encompass a broad spectrum of disorders with different pathomechanisms and clinical manifestations. It is well known that wheat gluten may cause coeliac disease, T-cell mediated intestinal inflammation, or dermatitis herpetiformis, blistering skin eruption. It is also well established that wheat flour may induce IgE-mediated reactions, such as bakers' asthma, wheat-dependent exercise-induced anaphylaxis (WDEIA), or food allergy. Because of the extensive use of wheat and related cereals in our daily diet, cereal-associated hypersensitivity reactions are particularly considered as a serious problem (Palosuo 2003).

Wheat proteins can be classified as water/salt-soluble albumins and globulins and water/salt-insoluble prolamines i.e. gliadins and glutenins. Gliadins account for about 50% of wheat grain proteins, and glutenins account for about 35% of total proteins. Both water/salt-soluble and -insoluble proteins have been found to be involved in the development of cereal hypersensitivity. Recent studies provide evidence that gliadins and glutenins are particularly responsible for development of IgE-dependent reactions. Sandiford et al. (1997) have documented that out of all prolamines the most allergenic are α- and ω-gliadins. Maruyama et al. (1998) have established that low molecular weight (LMW) glutenin and α- and γ-gliadins are the major wheat allergens. Battais et al. (2003) have documented that sera from patients with food allergy to wheat had specific IgE antibodies against α-, β-, γ- and ω-gliadins and against LMW glutenin subunits.

Pentapeptide QQQPP was chosen for rabbit immunisations because it is known as the most active peptide allergen among the low molecular

weight fractions of wheat flour proteins (Tanabe *et al.* 1996b). Similar peptide (QQPFP) has been recently used to establish antibodies for gluten analysis (Valdés *et al.* 2003), however although this peptide is characteristic for all groups of gliadins it is not present in the sequence of glutenins or appears very seldom, usually inside LMW glutenins (Protein Information Resource of the National Biomedical Research Foundation, http://www-nbrf. georgetown.edu/). Rabbit antibodies were selected for the study because the human sera are not used for the detection of anti-glutenin antibodies.

The most common and the easiest method of treating the symptoms of food allergy is the entire elimination of edible products that cause the allergy from the everyday diet. The obligation to be on a rigorous diet without gluten for the person with intolerance of gluten proteins highly reduces the assortment of products allowed for consumption. It also causes restrictions in the content of protein, vitamins and essential microelements in everyday diet, of which corn is the basic source. That is why a number of steps are taken in order to obtain flour with a decreased antigenicity. The allergenicity of food could be altered by several processing procedures. A product with a decreased allergenicity can be obtained by elimination of the epitop for example by using the extraction, its destruction by the physicochemical or enzymatic process, masking or genetic modification. In the case of wheat flour traditional or microwave heating, extraction, ionic irradiation, enzymatic hydrolysis, chemical or enzymatic cross-linking have been used till now (Besler *et al.* 2001; Leszczyńska *et al.* 2002, 2003a, b). From all of these methods, the most promising seems to be enzymatic modification.

The content of certain fractions of protein in wheat flour can be determined by various physicochemical methods (spectroscopy, chromatography, electrophoresis), but for the estimation of its antigenicity immunometric methods based on antigen-antibody reaction *in vitro* must be adopted. Usually in that kind of research sera of patients suffering from wheat allergy are used (Palosuo *et al.* 2001; Simonato *et al.* 2001).

The aim of our work was to estimate the usefulness of rabbit polyclonal anti-QQQPP peptide antibodies to determination of gluten proteins and the application of these antibodies to determine the changes of allergenicity of wheat flour after the enzymatic modification.

MATERIAL AND METHODS

Materials. Commercial wheat flour was investigated. Lyophilised trypsin powder, transglutaminase and subtilisin A from *Bacillus* sp. with the activity of 7–15 units/mg from Sigma Chemical Co. (St Louis, USA) were used for the preparation of modified flour. Solutions of TMB (3,3',5,5'-tetramethylbenzidine, T 8665) and p-NPP (*p*-nitro phenol phosphate, N 7660) as well as Tween 20, 2-mercaptoethanol, dithioerythritol and carbonate-bicarbonate buffer pH 9.6, were obtained from Sigma. Phosphate buffer with Tween (PBS) pH 7.2, Tris-HCl buffer pH 7.5, 1M H_2SO_4 aqueous solution and 3M NaOH solution were prepared using redistilled water. All chemicals used were of analytical quality and were used without further purification. Anti-rabbit IgG conjugate with horseradish peroxidase (HRP) and anti-IgE conjugate with alkaline phosphatase were from Sigma.

Pentapeptide QQQPP was obtained in Institute of Organic Chemistry, Technical University of Łódź.

Immunisation procedure of rabbits. In order to obtain the conjugate of QQQPP peptide with the protein we gradually added, while mixing, the glutaraldehyde (final concentration 1%) to the ovalbumin and the peptide solution, in the proportions of 1:40 (10 mg QQQPP and 0.25 mg ovalbumin in 1 ml of PBS pH 7.2). The reaction was carried out 1 h at 37°C and was terminated using $NaBH_2$ (10 mg/ml). The product of the reaction was latest dialysed in PBS.

Ten 6-month-old rabbits were injected once on the back with QQQPP peptide conjugated with ovalbumin using incomplete Freund's adjuvant (Sigma). The total amount of conjugate used for immunisation was as follows: 1 µg for two rabbits (number 1 and 2), 10 µg for two rabbits (number 3 and 4), 50 µg for three rabbits (number 5, 6, and 7), and 100 µg for three rabbits (number 8, 9, and 10). Bleedings were obtained 8 weeks after immunisation.

The levels of anti-QQQPP peptide IgG and IgE antibodies were determined by ELISA method by the use of microtiter plates coated with conjugate QQQPP and ovalbumin.

Wheat flour modification and separation of wheat protein fractions. Samples of wheat flour (1 g) were mixed with 5 ml of water and after addition of trypsin, transglutaminase or subtilisin the enzymatic modification of wheat proteins was carried out under conditions given in Table 1. All

Table 1. Conditions of enzymatic modification

No.	Enzyme to flour ratio (g E/g S)	Time (h)	Temperature (°C)
1	1:10 000	2	37
2	1:10 000	18	20
3	1:10 000	18	37
4	1:100 000	18	37

wheat flour samples were centrifuged at 2500 rpm and the supernatant containing water soluble proteins (albumins) was collected. The extraction procedure from sediment was repeated using 5 ml of water and after next centrifugation both supernatants were collected. Other fractions of wheat proteins were extracted from the sediment according to the Osborne procedure employing their different solubility. Globulins were separated after two successive extractions with 5 ml of aqueous solution containing 0.4M NaCl and 0.067M NaKHPO$_4$, gliadins were obtained after two extractions with 5 ml of 60% aqueous ethanol solution and finally glutenins were separated by twofold extraction with 5 ml of 50% aqueous solution of 1-propanol containing 2M urea, 0.05M Tris HCl buffer pH 7.5 and 1% dithioerithritol or 10% mercaptoethanol at temperature 60°C.

Analytical procedure. Microtiter plates EB 92029330 (LabSystems, Helsinki, Finland) were coated overnight at 4°C with 100 µl of coating solution (conjugate QQQPP peptide with ovalbumin or diluted extract of glutenins obtained from samples of different flours) after mixing with 0.1M carbonate-bicarbonate buffer pH 9.6 in the ratio 1:1 (the amount of antigen was 3 µg for each well). Next the plates were washed four times with PBS buffer and free binding sites were blocked by incubation of the plates for 2 h at room temperature with 3% solution of low fat milk in phosphate buffer (pH 7.2), containing 0.1% Tween-20. This was followed by the removal of milk buffer solution, rinsing of the plates four times with PBS and further incubation with 100 µl of diluted rabbit serum from immunised rabbit containing anti-QQQPP peptide antibodies for 1 h at room temperature. The plates were washed again and 100 µl of antibodies against IgG conjugated with peroxidase or antibodies against IgE conjugated with alkaline phosphatase were added. After incubation of the plates for 1 h and rinsing with phosphate buffer the substrate solution (TMB for HRP or p-NPP for AP conjugates,

respectively) to the wells of platelets was introduced and incubated again for 1 h. Then the reaction was terminated by addition of 100 µl 1M solution of H$_2$SO$_4$ for TMB or 100 µl 3M solution of NaOH for p-NPP and the resulting absorbance was measured with Multiscan RC reader at 405 nm for p-NPP or 450 nm for TMB, respectively.

The immune response of all protein fractions was tested twice in three independent determinations. Although some gliadins are referred as containing QQQPP fragments in their sequences (Protein Information Resource of the National Biomedical Research Foundation), gliadin extracts were not recognised by rabbit anti-QQQPP antibodies (data not shown).

RESULTS AND DISCUSSION

In the first phase of the research we have estimated the levels of IgE and IgG antibodies specific for QQQPP peptide in rabbits' sera according to the procedure given above (Figure 1). We have stated that levels of IgE antibodies were accompanied by the increase in levels of IgG antibodies. The highest concentration of specific IgE antibodies was obtained in the case of rabbits 8, 9, 10, which were injected with the highest doses of antigen. Thus, in all further experiments, we have used sera 8 and 9. Additional experiments have shown that the optimal dilution of the sera for the reaction between peptide conjugate and antibodies, was 1:50 (Figure 2). To the next parts of the experiment the second antibodies anti-IgE were chosen because of correlation between data obtained using both anti-IgG and anti-IgE antibodies and higher sensitivity of enzyme conjugated with anti-IgE antibodies.

The obtained rabbit polyclonal antibodies were very selective. They reacted with glutenins, in a lower degree with gliadins, but did not react with other proteins: albumins and wheat globulins, ovalbumin, casein, and gelatine (data not shown). Standard curve of gluten determination using the
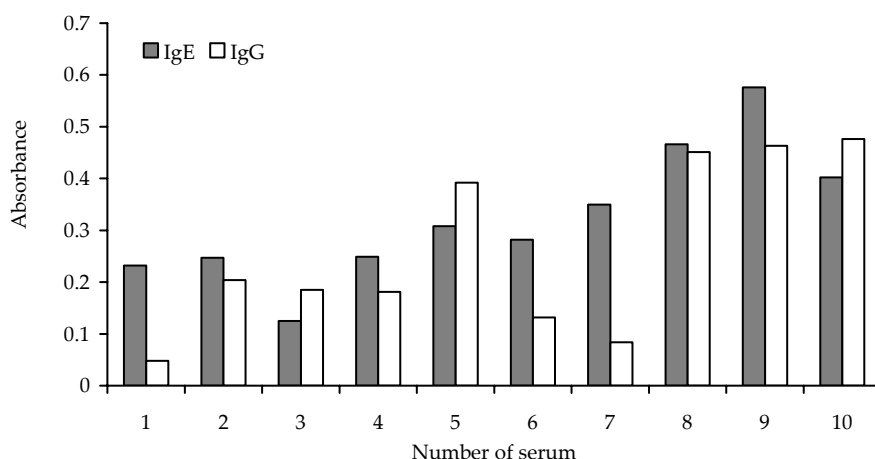
Figure 1. The levels of anti-QQQPP peptide IgE and IgG antibodies in rabbit sera (the plates were coated with the conjugate solution)

antibodies against QQQPP (serum 8) is shown in Figure 3.

Among the described enzymes we can find: bromelain, chymotrypsin, trypsin, as well as cellulase used together with actinase (Tanabe *et al.* 1996a; Watanabe *et al.* 2000). Because epitops of wheat glutenins have a Gln-Gln-Gln-Pro-Pro (QQQPP) motifs (Tanabe *et al.* 1996b), they can be sensitive to hydrolysis caused by bromelain which catalyses breaking of bonds next to prolin. The research has suggested that hypoallergenic flour can be obtained that way. Similar results were obtained with the presence of collagenase, which suggests that the Gln-X-Y-Pro-Pro epitopes, where X and Y are any amino acid, can be sensitive to proteolysis, but the obtained product can disrupt the structure of dough, influencing the quality of baking. Some of the researchers report about the reduction of allergenicity with the use of cross-linking enzymes like transglutaminase (Watanabe *et al.* 1994; Zhu *et al.* 1995). This enzyme causes the origination

of large cross-linking aggregates, which have an influence on the change of properties of dough, similar as in the case of proteases.

According to own initial investigation (Leszczyńska *et al.* 2002) for the modification of wheat flour, enzymatic modification was used by the reaction of proteolysis catalysed by trypsin, subtilisine and transglutaminase. The enzymes used in the research were added in relation to the mass, but not their activity. The immunoreactivity of glutenins from the enzymatically modified wheat flours in the different conditions is presented in Figure 4.

Hydrolysis catalysed by trypsin in given conditions of modification has led to an small decrease of glutenin immunoreactivity, in the conditions of modification number 3 (Table 1) to about 80% of initial value of the immunoreactivity. Other conditions gave even an increased immunoreactivity compared to glutenins in the native form.

Trypsin hydrolyses peptide bonds from the side of carboxylic group of arginin and lysine. In the
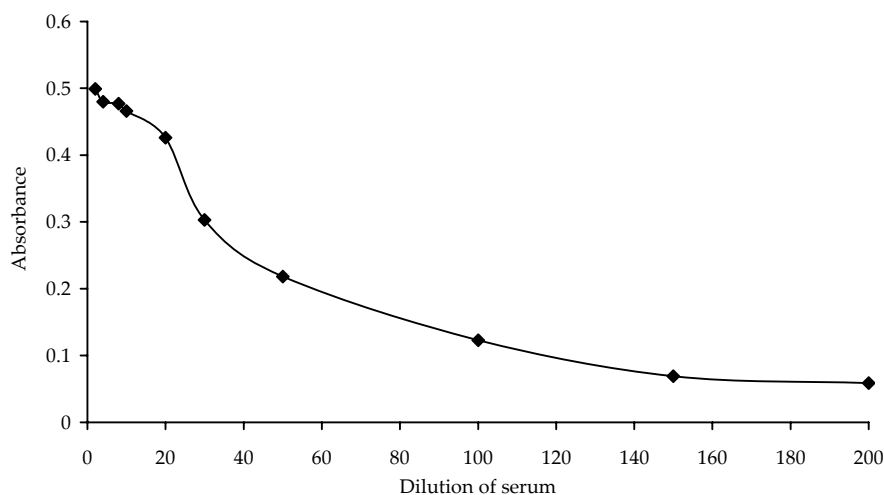

Figure 2. Curve of titration of antibodies in serum 8 (the plates were coated with the conjugate solution, anti-IgE second antibodies)
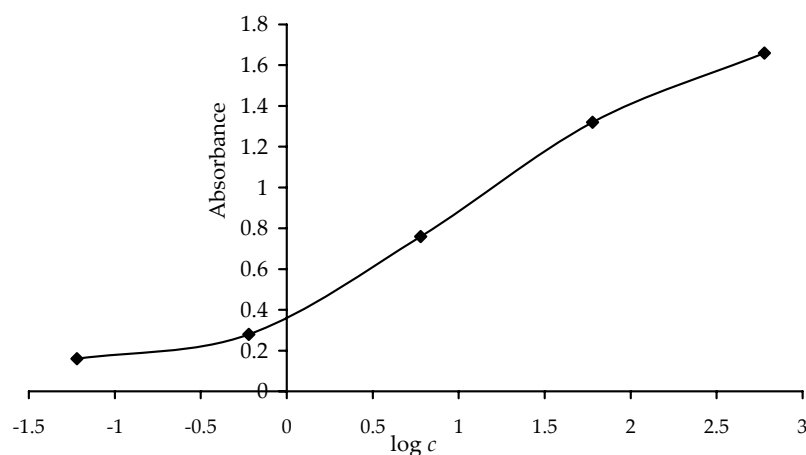
Figure 3. Standard curve for wheat gluten determination with the polyclonal rabbit anti-QQQPP peptide antibodies (c – concentration of gluten in mg/ml) (anti-IgE second antibodies)

sequences of glutenins the residues of both amino acids are present in a slight number among all residues in the protein (http://www-nbrf.georgetown.edu/). The increase of glutenins' immunoreactivity after the modification with trypsin may be caused by hydrolysis in a region located on the surface of the protein and uncovering of QQQPP epitopes from the internal fragments.

In literature the phenomenon of hydrophobic interaction between gluten proteins and other proteins was described (Osman *et al.* 1994; Calderon *et al.* 1996). Strong immunological response to extracts from flour in relation to native proteins may be connected to the formation of a new conformation of epitopes connecting the gliadins and glutenins with proteins soluble in salt solution, in analogy with formation of denaturised α-and β-lactoglobulin complex (Williams *et al.* 1998).

In previous research (Leszczyńska *et al.* 2002) the chemically pure gliadins were enzymatically modified, among others with trypsin. The obtained hydrolysates had less than 60% of the initial value of immunoreactivity in reaction with commercial

polyclonal antigliadin antibodies and less than 40% of the initial value in reactions with antibodies in human sera from subjects allergic to gliadins. The results obtained in this research show that glutenins were hydrolysed in nonantigenic fragments of proteins. Although flours prepared by hydrolysis with trypsin have lower immunoreactivity of gliadins, they should not be consumed both by coeliac and allergic persons.

Stronger hydrolysis may not cause a decrease of wheat flour allergenicity. Hydrolysates of milk, despite containing only polypeptides with mass molecule less than 3 kDa, have bitter taste and at the same time show certain immunoreactivity (Restani *et al.* 1996). Strong proteolysis of gluten proteins engenders a loss of unique organoleptic and physiochemical properties of flour. To sum up, the hydrolysis of proteins does not cause their destruction, but reveals new epitops that were previously hidden inside the protein.

The bigger decrease of the glutenins' immunoreactivity was obtained in the case of subtilisin and transglutaminase. Subtilisin, which belongs to
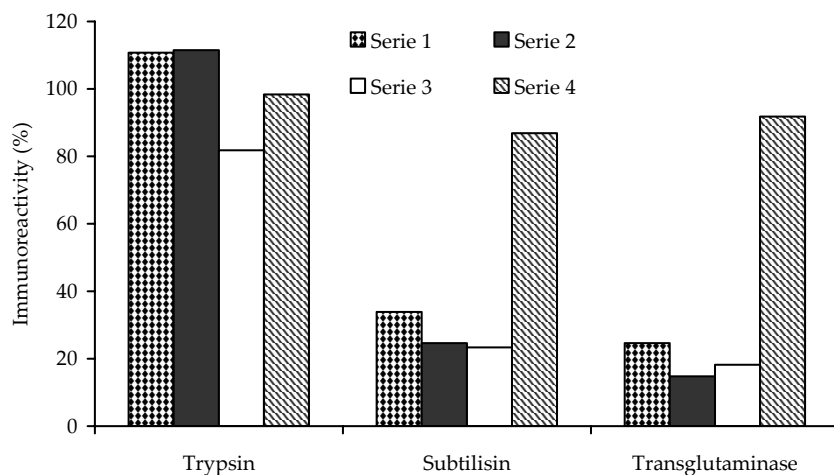

Figure 4. The change of immunoreactivity of wheat flour proteins under the influence of selected enzymes determined with anti-QQQPP peptide antibodies (plates were coated with of 100× diluted glutenin extract, second antibody – conjugat IgE with alkaline phosphatase; number of series refers to the number of conditions in Table 1)

serine proteases, decreases immunoreactivity of the proteins in a much higher degree then trypsin. Unfortunately, the use of subtilisin involves a risk of creation of peptides with opiate activities or many other biological activities, for example other allergens. It can also cause a decrease in organoleptic quality of the product. Such a risk does not exist in the case of using transglutaminase to decrease the immunoreactivity. Transglutaminase mainly catalyses cross-linking reaction, transferring acyl group from glutamine to lysine built in the structure of the same or other peptide chain, causing the creation of agglomerates, and thus that allergen epitopes are masked.

Similar activity of transglutaminase against different flour proteins was observed by Gerrard *et al.* (2001). SDS-PAGE electrophoresis after fractional extraction of proteins underwent the action of that enzyme (5000 ppm) shows the strongest activity of the enzyme was observed among the peptides below 55 kDa (albumins and globulins) and in the range of 60–120 kDa (glutenins).

Simultaneously, contrary to proteolysis (from technological point of view) cross-linking process initiated by transglutaminase is very profitable, if used for modification of wheat flour. It improves the quality of pasta, especially obtained from flour of poorer quality and because of plasteinisation reaction enables the enriching of native proteins with necessary amino acids and reduction of bittering caused by hydrolysis with other enzymes. The use of this enzyme is more and more extended over other food groups. So, it seems that in the case of allergy, where glutenin fraction is an allergen, the use of transglutaminase appears to be a good solution.

However, in the conditions of insufficiency of lysine residues, which are needed for the formation of covalent bonds, transglutaminase catalyses the deamination of carboxyamide groups of glutamine residues, which can result in an increase in allergenicity of some fragments of the peptide (Arentz-Hansen *et al.* 2000), although rabbit antibodies would not recognise such fragments. Because of this reason the immunoreactivity of glutenins modified with transglutaminase measured by this antibodies is not connected directly with the allergenicity of wheat flour.

CONCLUSION

The antibodies against QQQPP peptide are very sensitive and selective tools for detection of gluten proteins in wheat, especially for glutenins. They can also be used for investigations of changes of antigenicity of glutenins.

Using the anti-QQQPP antibodies the influence of trypsin, subtilisin and transglutaminase on immunoreactivty of glutenin fraction of wheat flour was investigated. In the case of the last two enzymes, a product with strongly decreased immunreactivity was obtained. This decreasing is connected only with the reduction of QQQPP fragments in the glutenin sequences, however reduction of the most toxic fragments of proteins influences with decreasing of immunoreactivity of proteins. It is observed for wheat flour after modification, especially after the subtilisin hydrolysis.

Using transglutaminase for modification seems to be a good solution because the depletion of glutenin immunoreactivity is accompanied by improvement in the sensoric properties of product. But in this case usefulness of the antibodies against QQQPP peptide is limited because the modification can produce new epitopes outside QQQPP fragments.

References

Arentz-Hansen H., Körner R., Molberg O. (2000): The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. Journal of Experimental Medicine, **191**: 603–612.

Battais F., Pineau F., Popineau Y., Aparicio C., Kanny G., Guerin L., Moneret-Vautrin D.A., Denery-Papini S. (2003): Food allergy to wheat: identification of immunoglobulin E and immunoglobulin G-binding proteins with sequential extracts and purified proteins from wheat flour. Clinical and Experimental Allergy, **33**: 962–970.

Besler M., Steinhart H., Paschke A. (2001): Stability of food allergens and allergenicity of processed foods. Journal of Chromatography B: Biomedical Sciences and Applications, **756**: 207–228.

Calderon De La Barca A.M., Yepiz-Plascencia G.M., Bøg-Hansen T.C. (1996): Hydrophobic interactions between gliadin and proteins and celiac disease. Life Science, **59**: 1951–1960.

Gerrard J.A., Fayle S.A., Brown P.A., Sutton K.H., Simons L., Rasiah I. (2001): Effects of microbial transglutaminase on the wheat proteins of bread and croissant dough. Journal of Food Science, **66**: 782–786.

Leszczyńska J., Łącka A., Pytasz U., Lukamowicz J., Szemraj J., Lewiński A. (2002): Influence of proteolysis on gliadin immunoreactivity. Polish Journal of Food Nutrition Science, **11/52** (SI 2): 145–148.

Leszczyńska J., Łącka A., Szemraj J., Lukamowicz J., Zegota H. (2003a): The influence of gamma irradiation on the immunoreactivity of gliadin and wheat flour. European Food Research and Technology, **217**: 143–147.

Leszczyńska J., Łącka A., Szemraj J., Lukamowicz J., Zegota H. (2003b): The effect of microwave treatment on the immunoreactivity of gliadin and wheat flour. European Food Research and Technology, **217**: 387–391.

Maruyama N., Ichise K., Katsube T., Kishimoto T., Kawase S., Matsumura Y., Takeuchi Y., Sawada T., Utsumi S. (1998): Identification of major wheat allergens by means of the *Escherichia coli* expression system. European Journal of Biochemistry, **255**: 739–745.

Osman A.A., Braunersreuther I., Mothes T. (1994): Investigation of gliadin binding to different selected proteins using a biotin-streptavidin system. Zeitschrift für Lebensmittel-Untersuchung und -Forschung A, **198**: 249–252.

Palosuo K. (2003): Update on wheat hypersensitivity. Current Opinion in Allergy and Clinical Immunology, **3**: 205–209.

Palosuo K., Varjonen E., Kekki O.M., Klemola T., Kalkkinen N., Alenius H., Reunalda T. (2001): Wheat omega-5 gliadin is a major allergen in children with immediate allergy to ingested wheat. Journal of Allergy and Clinical Immunology, **108**: 634–638.

Protein Information Resource of the National Biomedical Research Foundation, Protein Sequence Database. http://www-nbrf.georgetown.edu/

Restani P., Plebani A., Velona T., Cavagni G., Ugazio A.G., Poiesi C., Muraro A., Galli C.L. (1996): Use of immunoblotting and monoclonal antibodies to evaluate the residual antigenic activity of milk protein hydrolysed formulae. Clinical and Experimental Allergy, **26**: 1182–1187.

Sandiford C.P., Tatham A.S., Fido R., Welch J.A., Jones M.G., Tee R.D., Shewry P.R., Newman Taylor A.J. (1997): Identification of the major water/salt insoluble wheat proteins involved in cereal hypersensitivity. Clinical and Experimental Allergy, **27**: 1120–1129.

Simonato B., Pasini G., Giannattasio M., Peruffo A.D., De Lazzari F., Curioni A. (2001): Food allergy to wheat products: the effect of bread baking and *in vitro* digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. Journal of Agricultural and Food Chemistry, **49**: 5668–5673.

Tanabe S., Arai S., Watanabe M. (1996a): Modification of wheat flour with bromelain and baking hypoallergenic bread with added ingredients. Bioscience, Biotechnology and Biochemistry, **60**: 1269–1272.

Tanabe S., Arai S., Yanagihara Y., Mita H., Takahashi K., Watanabe M. (1996b): 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.

Valdés I., García E., Llorente M., Méndez E. (2003): Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol. European Journal of Gastroenterology & Hepatology, **15**: 465–474.

Watanabe M., Suzuki T., Ikezawa Z., Arai S. (1994): Controlled enzymatic treatment of wheat proteins for production of hypoallergenic flour. Bioscience, Biotechnology and Biochemistry, **58**: 388–390.

Watanabe M., Watanabe J., Sonoyama K., Tanabe S. (2000): Novel method for producing hypoallergenic wheat flour by enzymatic fragmentation of the constituent allergens and its application to food processing. Bioscience, Biotechnology and Biochemistry, **64**: 2663–2667.

Williams S.C., Badley R.A., Davis P.J., Puijk W.C., Meloen R.H. (1998): Identification of epitopes within beta lactoglobulin recognised by polyclonal antibodies using phage display and PEPSCAN. Journal of Immunological Methods, 213: 1–17.

Zhu Y., Rinzema M., Tramper J., Bol J. (1995): Microbial transglutaminase – a review of its production and application in food processing. Applied Microbiology and Biotechnology, **44**: 277–282.

Corresponding author:

PhD. Joanna Leszczyńska, Technical University of Łódź, Faculty of Biotechnology and Food Sciences, Institute of General Food Chemistry, Stefanowskiego 4/10, 90-924 Łódź, Poland
tel.: + 48 426 313 425, fax. + 48 426 362 860, e-mail: slezanko@snack.p.lodz.pl