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IMMUNOREACTIVITY REDUCTION OF WHEAT FLOUR PROTEINS MODIFIED BY THE TREATMENT WITH SUBTILISIN

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The immune response of wheat flour modified by the treatment with subtilisin under different conditions of temperature, incubation periods and the ratio of enzyme/wheat flour was investigated. Respective protein fractions, namely, albumins, globulins, gliadins and glutenins were obtained from the modified wheat flour on the basis of their diverse solubility. The particular wheat protein fractions were examined for the immune reaction by the use of an indirect non-competitive ELISA method. Commercially available antibodies, namely, monoclonal anti-human IgG and monoclonal anti-human IgE conjugates with alkaline phosphatase and human sera with elevated IgG as well as rabbit sera against QQQPP peptide were tested. The highest decrease in gliadins immunoreactivity was observed for wheat flour modified under following conditions: temperature 37°C, 18 h of incubation time and the enzyme/wheat flour ratio of 1:10 000.

INTRODUCTION

Allergy is a continuously increasing medical problem of recent years. As it was estimated, up to 10% of the population depending on the age is affected by food allergies [Estaban, 1992]. Food allergy is a symptom of incorrect response of the immunological system of a human organism to the consumption of particular proteins naturally occurring in food products. Wheat flour containing gluten is classified as one of the most recognized food allergens [Dohi *et al*., 1991; Hughes & Mills, 2001]. Among others wheat gluten consists of gliadin and glutenin fractions. The allergenic properties of gliadins are well documented, whereas those of glutenins are uncertain [Skerritt *et al*., 1990; Denery-Pappini *et al*., 1999].

Food allergic reactions resulting from the hypersensitivity of human organism to the offending compounds, are accompanied by the presence of immunoglobulin class E (IgE) antibodies in the sera of individuals with a genetic predisposition to allergy [Weiss *et al*., 1993; Santos *et al*., 1999]. Celiac disease which affects about 0.5% of the population belongs to the few diseases which are not connected with the production of IgE class antibodies [Catassi *et al*., 1994]. Recent studies have shown that celiac disease is rather a complex genetic illness induced by prolamines, *i.e.* the protein fraction soluble in aqueous ethanol. Prolamines derived from wheat are named gliadins, whereas secalins originate from rye and hordeins are derived from barley [Wieser, 2001; Bevan *et al*., 1999]. Gliadins can elicit allergic reactions if they are present even at very low concentration in a diet. Once ingested, they develop disappearance of small bowel tufts and troubles with intestinal digestion and absorption of food and food components. The application of an eliminative diet to patients with wheat proteins allergy does not help because wheat flour is broadly used as a component of various food products. The better solution seems to be the modification of wheat flour with the aim of obtaining products with hypoallergenic properties.

The earlier studies have indicated that food allergy can be reduced by technological processes such severe heating which induces physical, chemical and conformational alterations to the structure of proteins [Besler *et al*., 2001]. Also proteolysis of wheat proteins, as an effect of the proteolytic activity of enzymes, led to the reduction of immunoreactivity of gliadins. As it was found earlier, the reduction of immunoreactivity of pure gliadins treated with enzyme subtilisin reached a value of 70 percents [Leszczyńska *et al*., 2002].

The aim of our present study was to investigate the influence of proteolysis by subtilisin on the immune reaction of different fractions of wheat proteins, separated from wheat flour. The immunoreactivity of each protein fraction was examined by an indirect non-competitive enzyme-linked immunosorbent assay (ELISA) with antibodies from sera of celiac patients or rabbits antibodies against pentapeptide QQQPP.

MATERIALS AND METHODS

Material and chemicals

Commercial wheat flour ("Mąka Szymanowska 500") and lyophilized powder of subtilisin A from *Bacillus* sp. with the activity of 7-15 units/mg from Sigma Chemical Co. (St Louis, MO, USA) were used for the preparation of modified flour. Solutions of TMB (3,3',5,5'-tetramethylbenzidine,

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T 8665) and p-NPP (p-nitrophenol phosphate, N-7660) as well as Tween-20, 2-mercaptoethanol, dithioerythritol 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 H_2SO_4 aqueous solution and 3 mol/L NaOH solution were prepared using redistilled water. All chemicals used were of analytical grade and were used without further purification.

Antibodies and sera

Four commercial antibodies were employed, namely, monoclonal anti-human IgG conjugate with alkaline phosphatase (AP) from mouse clone GG-5 (Sigma, A 2064), monoclonal anti-human IgE conjugate with alkaline phosphatase (Sigma, A 3076), monoclonal, polyvalent anti-human IgG conjugate with alkaline phosphatase (Sigma, A 3313), and monoclonal anti-rabbit IgG conjugate with horseradish peroxidase (HRP) from Sigma (A 1052).

The following human sera from sensitive patients diagnosed with elevated IgG-factor were obtained from the Polish Mother's Memorial Hospital: No. S461 (IgG 320 u/mL, IgA 26 u/mL), No. S462 (IgG 136 u/mL, IgA 0 u/mL), No. S467 (IgG 50 u/mL, IgA 16 u/mL), No. S468 (IgG 60 u/mL, IgA 20 u/mL), No. S474 (IgG 40 u/mL, IgA 0 u/mL), No. S480 (IgG 40 u/mL, IgA 0 u/mL), No. S482 (IgG 95 u/mL, IgA 28 u/mL), No. S625 (IgG 48.5 u/mL, IgA 0 u/mL), No. S721 (IgG 115.3 u/mL, IgA 70.9 u/mL), No. S729 (IgG 101.3 u/mL, IgA 0 u/mL), No. S748 (IgG 41.5 u/mL, IgA 24.4 u/mL), and No. S750 (IgG 29.4 u/mL, IgA 85.8 u/mL).

Rabbit sera (RS) containing antibodies against peptide QQQPP (gln-gln-gln-pro-pro) [Leszczyńska *et al*., 2008] were used as well.

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 subtilisin the enzymatic modification of wheat proteins was carried out under conditions given in Table 1 (without corrections of pH). Then, all wheat flour samples were centrifuged at 2500×*g* 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 combined and used for the determination of the immune response of albumins. Other fractions of wheat proteins were extracted from the sediment according to the Osborne procedure [Osborne, 1907] employing their diverse solubility. Globulins were separated after two successive extractions with 5 mL of aqueous solution containing 0.4 mol/L NaCl and 0.067 mol/L $NaKHPO_4$, 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 2 mol/L urea, 0.05 mol/L Tris HCl buffer pH 7.5 and 10% mercaptoethanol at a temperature of 60°C.

Total protein content in all separated fractions of wheat proteins was determined with the Pierce method using a ready-to-use test of Biomedicals Co. (Rockfort, USA).

Sandwich ELISA for protein extracts

Microtiter plates EB 92029330 (Labsystems, Helsinki, Finland) were overnight coated at 4°C with 100 μL of 1:10 or 1:100 diluted extracts of proteins after mixing at the ratio of 1:1 with 0.1 mol/L carbonate-bicarbonate buffer pH 9.6 (the cells contained about 1.5 mg of proteins). Next the plates were washed four times with PBS buffer and free binding sites were blocked by incubating the plates with 3% solution of low fat milk in phosphate buffer (pH 7.2), containing 0.1% of Tween-20 for 2 h at room temperature. This was followed by the removal of milk buffer solution, rinsing the plates four times with PBS and further incubation with 100 μL of 100-fold diluted human serum or 10-fold diluted rabbit serum for 1 h at room temperature. The human sera were used for gliadin fractions, the rabbit sera – for glutenins, both groups of sera for albumins and globulins. The plates were washed again and 100 μL of monoclonal antibodies conjugated with peroxidase (HRP) or alkaline phosphatase (AP) were added (1000 x diluted). The monoclonal anti-human IgG with AP or the monoclonal polyvalent anti-human Ig with AP were added to the samples with human sera, the monoclonal IgE with AP or the monoclonal anti-rabbit IgG with HRP were added to the cells with the rabbit sera added. As no commercial anti-rabbit IgE antibodies are available, the monoclonal anti-human IgE were used (reactions of those antibodies with both human and rabbit IgE gave the same results – data not shown). 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) was introduced and incubated again for 1 h. Then the reaction was stopped by adding 100 μL of 1 mol/L solution of H_2SO_4 for TMB or 100 μL of 3 mol/L solution of NaOH for p-NPP and the resulting absorbance was measured using a Multiscan RC reader at 405 nm for p-NPP or 450 nm for TMB, respectively. The immunoreactivity was normalized in the relation to the content of protein. Immune responses of all protein fractions were tested twice in three independent determinations.

A-PAGE of protein extracts

Gliadin peptides extracted from the obtained samples were exposed to A-PAGE electrophoretic separation. Electrophoresis was performed in polyacrylamide gel (aluminium lactate buffer, 2.5 g/L, lactic acid up to pH 3.10). A volume of 50 μL lactate buffer, containing 0.6 mg of methyl green, and 0.5 mL of glycerol were mixed with 50 μL of gliadin extract, then transferred onto the gel. Electrophoresis was run in a minigel setup of Desaga apparatus (Desaga, Heidelberg, Germany) at 300 V for 30 min.

RESULTS AND DISCUSSION

The modification of gliadins and glutenins of wheat gluten by heating in order to obtain the hypoallergenic wheat flour is difficult to perform because those proteins are characterised by high thermal resistance [Rumbo *et al*., 1996]. They occur as linear epitopes with high similarity of amino acids sequences. Therefore, the enzymatic hydrolysis of these wheat protein fractions by the treatment with proteolytic enzymes seems to be more promising. Subtilisin originating from

Bacillus licheniformis is classified as serine proteinase with the optimum of enzymatic activity in an alkaline medium at pH 9-11. This enzyme demonstrates a broad range of specificity, although the preferred site of its attack is the peptide bond at carboxylic ends of hydrophobic amino acids such tyrosine, phenylalanine and leucine [Rao *et al*., 1998]. This protease is characterised by a broad activity against various protein fractions and the commercial enzyme preparation is rather inexpensive. It has been reported that subtilisin could be applied for the production of protein hydrolysates with increased functional properties [Vioque *et al*., 2000] or with reduced immunoreactivity [Clemente *et al*., 1999]. Any alterations of proteins increase the risk of formation of peptides with unknown biological activity, for example, similar to opiate [Restani *et al*., 1996], or with antigenic activity. They may also deteriorate the organoleptic quality of the product.

The proteolytic hydrolysis of wheat flour proteins with subtilisin was carried out in relatively mild conditions (Table 1) in order to save its sensory and backing properties. Moreover, very small amounts of subtilisin were used to avoid an introduction of potentially-allergenic components of the enzyme preparation.

Immunoreactivity of each fraction of modified wheat flour proteins was determined by the reaction with human anti-gliadins antibodies taken from patients suffering from celiac disease or with rabbit antibodies against QQQPP peptide, which is recognized as the most allergenic peptide of glutenins [Tanabe *et al*., 1996]. The necessity of applying rabbit antibodies resulted from the fact that in medical examinations of human sera the detection of anti-glutenin antibodies is not carried out. The disadvantage of the chosen method of immunoreactivity determination is connected with a high differentiation in the specificity of human anti-gliadin antibodies present in sera of sensitive patients. The activity of serum immunoglobulins can be directed against various epitopes of a given antigen, therefore, the immunoreactivity changes for various products containing proteins may differ for particular patients as it was shown earlier [Van Eckert *et al*., 1997]. For this reason, our results – being tentative – may only help to define the approximate level of the immune reaction of modified wheat proteins.

In the first step of our study the conditions of modification procedure of wheat flour with subtilisin were optimized by the investigation of temperature effect, reaction time and the ratio of quantity of enzyme to the amount of wheat flour.

TABLE 1. Parameters of temperature, reaction time and enzyme:wheat flour ratio utilized for the enzymatic modification of wheat flour by the treatment with subtilisin. Each of series contains modified and control samples.

Sample	Subtilisin:wheat flour ratio (g of enzyme powder:g of flour)	Time of treatment (h)	Temperature (°C)
A	1 : 10 000	2	37
B	1 : 10 000	18	37
C	1 : 10 000	18	20
D	1 : 100 000	18	37

The results presented in Figure 1 show the level of residual immunoreactivity of gliadins, which were obtained from wheat flour modified under different conditions (see Table 1). The results were obtained in the reaction of gliadins against human sera antibodies or rabbit antibodies against peptide QQQPP. Gliadins from wheat flour submitted to the treatment under conditions A (reaction time 2 h, temperature 37°C, the subtilisin:wheat flourratio of 1:10,000, w/w) have exhibited even 20% increase in the immunoreactivity against antibodies present in serum No. S461 in comparison with gliadins obtained from untreated wheat flour. The highest decrease in gliadins immunoreactivity was obtained for wheat flour modified under conditions B (37°C, 18 h of incubation and the enzyme:wheat flour ratio of 1:10,000). Therefore the protein fractions separated from wheat flour modified upon conditions B were used for further investigations.

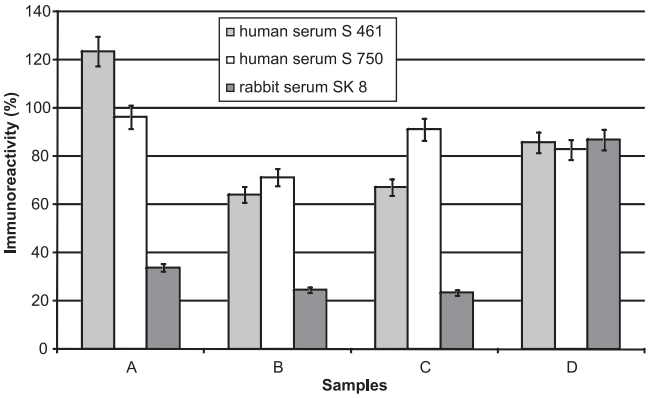

FIGURE 1. Changes of immunoreactivity of gliadins from wheat flour modified by subtilisin (for the modification parameters see Table 1) assayed in the reaction against human anti-gliadin antibodies (sera No S461 and S750) and rabbit antibodies against peptide QQQPP (SK8) (changes shown in respect of control samples).

Albumins and globulins

Albumins and globulins are protein fractions soluble in water or in an aqueous salts solution [Van Eckert *et al*., 1997]. There is little data on the allergenic properties of albumins and globulins. It was only reported that the allergy occurred after intake of these proteins by inhalation [Weiss *et al*., 1997]. The methods of immunoreactivity determination used in our experiments allowed only the observation of immune responses of gliadins and glutenins, components of wheat gluten known from their allergenicity [Walsh & Howden, 1989; Weiss *et al*., 1993]. Therefore, the direct assay of the immune activity of albumins and globulins was not possible. Nevertheless, it was reasonable to assume that cross reactions between albumins, globulins, gliadins and glutenins may occur resulting in the formation of new epitopes, but any spurs of such reactions in wheat flour modified with subtilisin have been found (data not shown).

Gliadins

Food allergy is connected with the production of IgE class antibodies. Immunization with a high dose of antigen generates the immune response characterised by the production

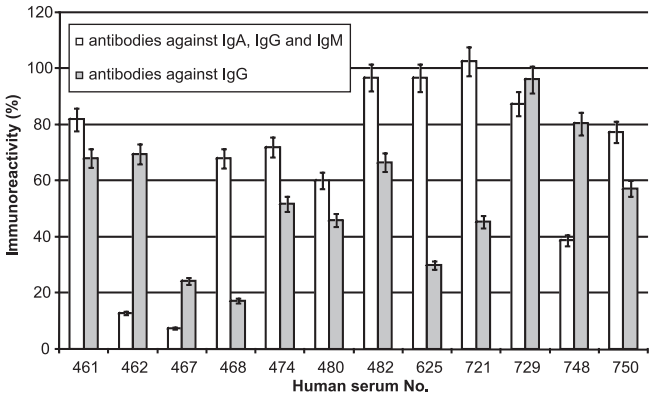

FIGURE 2. Immunoreactivity changes of gliadins from wheat flour modified by the treatment with subtilisin (temperature 37°C, 18 h of incubation, the enzyme:wheat flour ratio of 1:10,000) determined in the reaction with antibodies present in human sera of twelve immunosensitive patients. The results for monoclonal antibodies against IgG and polyvalent antibodies against IgA, IgG and IgM immunoglobulins are included (changes shown in respect of control samples).

of IgG antibodies without the presence of IgE [Chapman *et al*., 2000]. Gliadins are cited in the literature as an example of a food allergen eliciting a very low level of IgE immunoglobulins [Aalberse, 1997]. Furthermore, in the organism of individuals suffering from coeliac disease of genetic origin with immunological response to ingestion of prolamines, besides of IgG, immunoglobulins of other classes, mainly IgA, are produced. Therefore, the immunoreactivity of gliadins was examined by the reaction of human sera antibodies and the antibodies against monoclonal IgG and monoclonal, polyvalent antibodies against IgG, IgA and IgM immunoglobulins.

Changes in the immune activity of gliadins from modified wheat flour were assayed using sera of many sensitive patients and the results for twelve of these sera are presented in Figure 2.

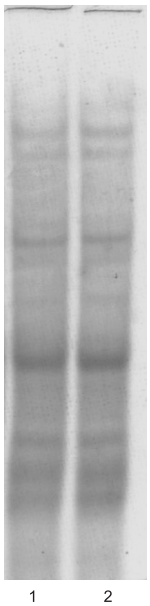

FIGURE 3. A-PAGE of gliadins (1 – reference, 2 – hydrolysed gliadins).

The immune reaction of gliadins from modified wheat flour, determined using monoclonal antibodies against IgG, decreased more markedly, on average to 50% of the initial level, in comparison with 60% of residual activity, when determined in the reaction with monoclonal, polyvalent antibodies against IgG, IgA and IgM. The results presented in Figure 2 show very high discrepancy in immune reactions developed by particular sera of individuals suffering from celiac disease. For example, the immunoreactivity against IgG determined for the serum No. 729 was reduced slightly, by only about 5%, whereas the immunoreactivity reduction measured for the serum No. 468 reached nearly 75%. The differences in immunoreactivity of gliadins were even higher when determined using monoclonal, polyvalent antibodies. The sera No. 482, 625 and 721 developed immune reactions comparable with those measured for untreated gliadins, whereas, for sera No. 462 and 467 the reduction of immunoreactivity reached 90% in comparison with that measured for control gliadins. The small changes of the immunoreactivity were an effect of a limited hydrolysis of the gliadins, as shown in Figure 3. Electrophoregrams of gliadins in acidic conditions did not indicate any changes of the protein pattern. From the results obtained it can be concluded that the modification of wheat flour by the treatment with subtilisin is not very promising for the patients suffering from celiac disease.

The previously carried out experiments [Leszczyńska *et al*., 2002] concerning the modification of pure gliadins by the treatment with subtilisin under similar conditions (temperature 37°C, 18 h of incubation, the enzyme:protein ratio of 1:100 or 1:10,000) showed a significant decrease in their immunoreactivity, reaching 40% of the initial level, determined in the reaction with rabbit antigliadin antibodies (fundamental test defining the content of gliadins in low gluten foods), and more than 20% when measured for sera of patients suffering from celiac disease. The difference in results obtained now for gliadins extracted from modified wheat flour in comparison with the results described earlier for modified pure gliadins could be explained by different susceptibility to the enzymatic modification of native proteins present in wheat flour and gliadins in pure state.

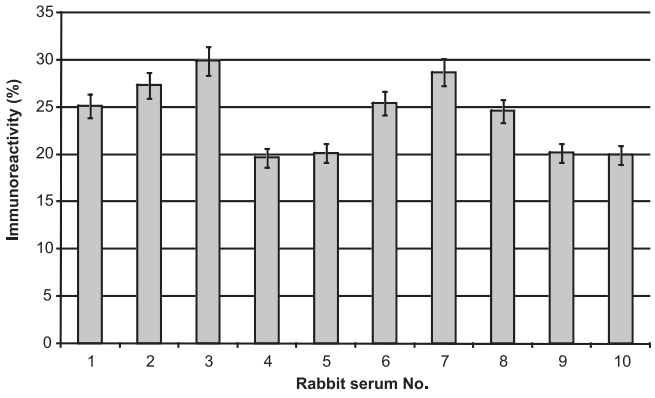

FIGURE 4. Level of residual immunoreactivity of glutenins from wheat flour modified by the treatment with subtilisin (temperature 37°C, 18 h of incubation, the enzyme:wheat flour ratio of 1:10,000) determined in the reaction with the rabbit sera immunized with QQQPP peptide/ovalbumin conjugate.

Glutenins

The immune reaction of glutenins from the modified wheat flour was investigated using IgE class rabbit antibodies, *i.e.* a typical immunoglobulin in food allergy. Rabbit sera containing antibodies against peptide QQQPP were used. QQQPP is known as the most active peptide allergen among the low molecular weight fraction of wheat flour proteins [Tanabe *et al*., 1996]. The rabbits were sensitized by an inoculation with QQQPP peptide/ovalbumin conjugate before taking their sera [Leszczyńska *et al*., 2008]. The results presented in Figure 4 show the residual immunoreactivity of glutenins from wheat flour modified by subtilisin in relation to the immune response of untreated control glutenins taken as 100%. The immune response of the rabbit sera measured with anti-human IgE antibodies and the monoclonal anti-rabbit IgG antibodies gave the same results. For all sera under investigation the residual immune reactions were found to be below 30% of the control value measured for the untreated glutenins.

CONCLUSIONS

The results obtained support the conclusion that the application of subtilisin for the enzymatic alteration of wheat flour proteins may be profitable from the technological point of view.

The results obtained for such modified wheat flour indicate that it should not be used as a component of products designated for children suffering from celiac disease because of relatively high residual immune responses of gliadin fraction of wheat proteins.

The decreasing of immunoreactivity is connected only with the reduction of the QQQPP fragments in the glutenin sequences. The low level of residual allergenicity determined for glutenins supports the conclusion that wheat flour modified with subtilisin may be added to food products destined for people with a classic food allergy developed by that protein fraction. However, reduction of the most toxic fragments of proteins does not promote the decrease of immunoreactivity of all proteins of this fraction.

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