Reduction of immunoreactivity of wheat and rye prolamins by Flavourzyme proteolysis

BARBARA MICKOWSKA – KVETOSLAVA ROMANOVÁ – PETER SOCHA – DANA URMINSKÁ

Summary
The aim of this study was to check the ability of Flavourzyme (enzyme complex from *Aspergillus oryzae*; Novozymes, Copenhagen, Denmark) to hydrolyse wheat and rye prolamins. The proteolysis was performed at pH 5.0 and pH 7.0 and at a temperature rising from 37 °C to 60 °C at various enzyme/prolamins ratios. Prolamins and products of their proteolysis were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, their immunoreactivity by Western blot and competitive enzyme-linked immunosorbent assay. Wheat gliadins and rye secalins were succesfully hydrolysed, the immunoreactivity was significantly decreased – residual immunoreactivity after proteolysis was less than 5.5 % and 6.5 % in wheat and rye hydrolysates, respectively. Amino acid analysis was performed to quantify the amount of free amino acids released from prolamin molecules during proteolysis. A portion of 6.3–9.2 % of free amino acids was released from wheat gliadins and 1.4–4.2 % from rye secalins at enzyme/prolamins ratio of 1 : 250 and proteolysis duration of 2 h. Proline and glutamine were released at the lowest degree in spite of high contents of these amino acids in prolamins. Obtained results proved that Flavourzyme is efficient in cleavage of gliadins and secalins and is able to significantly reduce their celiac toxicity.

Keywords
prolamins; Flavourzyme; proteolysis; celiac immunoreactivity

In food industry, various enzyme preparations are used for hydrolysis of harmful or immunogenic food proteins, or for preparation of protein hydrolysates with improved properties. Some microbial proteolytic enzymes can be a good technological tool during the food processing to cleave cereal proteins and peptides, thus reducing their immunogenic potential, which is important mainly for celiac or allergic patients [1].

Main cereal storage proteins, alcohol-soluble prolamins, can be characterized by consisting of large amounts of glutamine (35 %) and proline (15 %), and containing lots of repetitive sequences composed of these amino acids. Such proline-rich peptides are considered to be resistant to degradation by peptidases, including the wheat α2-gliadin fragment of 33-amino acids (LQLQPFPQPQLPYPQPQLPYPQPQPF), a trigger for development of celiac disease [2–4]. To hydrolyse peptide bonds between proline and other amino acids, it is necessary to use prolyl-specific peptidases. Prolyl endopeptidase (PEP), a serine peptidase, preferentially and effectively cleaves the peptide bond on the carboxyl side of proline residues [5], which makes PEP very suitable for degradation of prolamins. Prolyl-oligopeptidases can be found in most organisms, primarily in microorganisms and plants [6]. Specific PEP from *Aspergillus niger*, also marked as AN-PEP or Tolerase G (DSM, Heerlen, Netherlands) with pH optimum of 4.0–5.0, is able to hydrolyse intact gluten molecules and T-cell stimulatory epitopes from immunogenic gliadin and glutenin proteins. AN-PEP hydrolyses T-cell epitopes of a peptic/tryptic gluten digest and also intact gluten molecules, in contrast to prolyl oligopeptidases, which do not cleave

Barbara Mickowska, Małopolska Centre of Food Monitoring, Faculty of Food Technology, University of Agriculture in Krakow, Balicka 122, 30-149 Krakow, Poland.
Kvetoslava Romanová, Peter Socha, Dana Urminská, Department of Biochemistry and Biotechnology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.

Correspondence author:
Barbara Mickowska, e-mail: barbara.mickowska@urk.edu.pl

large peptides or intact molecules [7].

A group of enzymes widely used in many fields of industry are subtilisins [8]. They are characterized as a superfamily of bacterial alkaline peptidases produced by *Bacillus* strains, especially *Bacillus licheniformis* and *B. amyloliquefaciens*. Subtilisins are stable extracellular serine endopeptidases with a relatively low specifity for substrate, which make them an ideal component of detergents. Commercially available variants of subtilisins are known as Savinase (Subtilisin 309), Esperase (Subtilisin 147), Everlase (Novozymes, Basvaerd, Denmark), Properase or Purafect (Genencor, New York, New York, USA). One of the best known subtilisin enzyme preparations is Alcalase, called Subtilisin Carlsberg, too. Non-specific endoprotease preparation Alcalase 2.4 L FG (Novozymes, Basvaerd, Denmark) is widely used for improvement of nutritional and functional properties of protein hydrolysates. Alcalase is a complex of serine endopeptidases from *B. licheniformis* with a pH optimum of 8.0 and optimal temperature of 50–60°C [9].

A mixture of extracellular and intracellular enzymes from *Aspergillus oryzae* represents an enzyme coctail named Flavourzyme (Novozymes), used in food industry for protein hydrolysis [10, 11]. In this endopeptidases and exopeptidases complex, several major enzyme activities were identified, including three endopeptidases, two aminopeptidases, two dipeptidylpeptidases and α-amylase [12]. The optimum conditions for Flavourzyme activity are pH 5.5–7.5 and temperature 50–55 °C. The enzymatic reaction can be stopped by heat treatment for 10 min at 90 °C.

Flavourzyme is used in baking industry for hydrolytic modification of dough protein complex. Positive effect of the limited proteolysis is manifested by the reduction of dough mixing time and enlargement of loaf volume [10]. The enzyme complex is capable to hydrolyse wheat gluten proteins, the degree of hydrolysis being high [12]. Prolamins may be cleaved into smaller peptides, which may have a reduced celiac activity. Therefore, such hydrolysis can be considered as one of technological tools to eliminate immunogenic properties of gluten [1].

In the present study, the ability of Flavourzyme to hydrolyse and reduce immunoreactivity of alcohol-soluble fraction of wheat and rye proteins (prolamins) were examined by methods of Western blot and competitive enzyme-linked immunosorbent assay (ELISA), using polyclonal and monoclonal antibodies. To obtain accurate data, the evaluation of proteolysis was carried out by analysis of released free amino acids.

MATERIALS AND METHODS

Chemicals and equipment

Flavourzyme – peptidases and α-amylase mixture from *Aspergillus oryzae* (P6110; Sigma Aldrich, St. Louis, Missouri, USA), molecular weight protein markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE PageRuler Protein Ladder; Thermo Fisher Scientific, Waltham, Massachusetts, USA), Immobilon-P^{SQ} polyvinylidene fluoride (PVDF) transfer membrane (Millipore, Billerica, Massachusetts, USA), polyclonal anti-gliadin antibody (Sigma Aldrich), polyclonal anti-rabbit antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, California, USA), SigmaFAST 3,3'-diaminobenzidine tablets (Sigma Aldrich), RIDASCREEN Gliadin competitive ELISA kit (R-Biopharm, Darmstadt, Germany) were used. All other chemicals were of analytical grade. Electrophoresis and electrotransfer equipment was from Bio-Rad Laboratories, vacuum lyophylizer Christ Alpha1-2 LD Plus from Martin Christ (Osterode am Harz, Germany), chromatographic amino acid analyzer AAA400 from Ingos (Prague, Czech Republic), microplate spectrophotometric reader BioRad 680 from Bio-Rad Laboratories.

Samples

Commercially available wheat flour type 512 (Vitaflóra, Kolárovo, Slovakia) and whole grain rye flour (marked as "bio-product"; Bioharmonie, Vyškov, Czech Republic) were used as a source of alcohol-soluble proteins, prolamins.

Extraction of prolamins

Alcohol-soluble fraction of wheat and rye grain proteins were extracted according to Osborne's procedure [13] with modifications. Extraction was performed at magnetic stirring with 15 ml of solvent per 1 g of flour for 1 h at laboratory temperature. Extraction was carried out with the following series of solvents: 0.5 mol·l^{-1} NaCl to extract salt-soluble albumins and globulins (twice), distilled water to remove NaCl, and 70% (v/v) ethanol to extract alcohol-soluble proteins. Extracted proteins were lyophilized and used for further analysis. The protein content was calculated on the basis of amino acid analysis performed according to Moore and Stein [14].

Proteolysis

Lyophilized wheat and rye prolamins (1.5 mg) were dissolved in 150 μl of 0.1 mol·l^{-1} acetate and phosphate buffer pH 5.0 and 7.0, respectively.

Flavourzyme was diluted in 0.1 mol·l^{-1} Tris-HCl buffer pH 9.5 (Applichem, Darmstadt, Germany) in a concentration of 1 mg·ml^{-1}. Proteolysis was carried out in enzyme/prolamins ratios of 1:250; 1:500, 1:2000 and 1:5000 (w/w), in temperature conditions increasing from 37 °C to 50 °C and 60 °C. Proteolysis was stopped by addition of synthetic inhibitor PefablocSC (Sigma Aldrich) at final concentration of 0.5 mg·ml^{-1} after 1 h and 2 h at 37 °C and after reaching the mentioned temperatures.

SDS-PAGE

Prolamins and products of their hydrolysis were analysed by Tris-Tricine SDS-PAGE electrophoresis under reducing conditions according to SCHÄGGER and VON JAGOW [15].

Western blot

After SDS-PAGE, electrotransfer onto PVDF membrane Immobilon-P^{SQ} was performed during 1.5 h at 170 mA using 10 mmol·l^{-1} 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) with 10% methanol, pH 11 transfer buffer, according to the protocol of the manufacturer (Millipore). The membrane was blocked in 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer, pH 7.6, overnight. Incubation with the primary antibody (anti-gliadin antibody, concentration 2 μg·ml^{-1} in TBS containing 1% BSA and 0.5 g·l^{-1} Tween 20) was carried out for 1.5 h, followed by washing with TBS buffer containing 0.5 g·l^{-1} Tween 20 four times for 10 min. After that, the membrane was subjected to incubation with secondary antibody (anti-rabbit horseradish peroxidase-conjugated antibody, concentration 0.5 μg·ml^{-1} in TBS containing 1% BSA and 0.5 g·l^{-1} Tween 20) for 1 h, followed by washing with TBS buffer containing 0.5 g·l^{-1} Tween 20 five times for 10 min. Finally, blots were developed by chromogenic detection using SigmaFAST 3,3'-diaminobenzidine tablets according to protocol of manufacturer (D4293).

ELISA analysis

Prolamins were dissolved in buffers pH 5.0 and pH 7.0 (10 mg of lyophylized protein per 1·ml) and proteolysis was performed for 2 h at 37 °C at 1:250 (w/w) enzyme/protein ratio. Reaction was stopped by PefablocSC.

For analysis of immunoreactivity of prolamins and proteolysis products, a commercial ELISA kit RIDASCREEN Gliadin competitive based on the monoclonal antibody R5 was used, according to the instructions of the manufacturer (R-Biopharm).

Amino acids analysis

Lyophilized prolamins were hydrolysed in liquid 6 mol·l^{-1} HCl containing 0.5% phenol at 110 °C for 24 h under an argon atmosphere. The hydrolysates were lyophilized, dissolved in an appropriate volume of dilution buffer (sodium citrate buffer, pH 2.2), filtered through a syringe filter with PVDF membrane (pore size 0.45 μm) and applied to the amino acid analyser. Amino acids were determined by ion-exchange chromatography using an automatic amino acid analyser, with strong cation ion-exchanger and sodium-citrate elution buffer system followed by post-column derivatization with ninhydrin and spectrophotometric detection at wavelengths od 570 nm and 440 nm, according to the standard protocol of the manufacturer (Ingos). A standard solution of amino acids (AA-S-18, Sigma Aldrich) was used for calibration. Sulphur amino acids and tryptophan were not determined as they are destroyed during acid hydrolysis. Asparagine and glutamine transform into aspartic and glutamic acids and in these forms were determined.

Free amino acids analysis

Lyophilised prolamins were dissolved in buffers of pH 5.0 and pH 7.0 (10 mg of lyophylized protein per 1 ml) and proteolysis was performed for 2 h at 37 °C at 1:250 (w/w) enzyme/proteins ratio. Reaction was stopped by PefablocSC and proteins together with peptides were precipitated by solid sulfosalicylic acid (30 mg·ml^{-1}). After 1 h at 4 °C, samples were centrifuged at 15000 ×g for 15 min, filtered through a syringe filter (pore size 0.45 μm) and a chromatographic amino acids analysis was performed.

RESULTS AND DISCUSSION

In our previous study [16], we examined enzymatic cleavage of prolamins extracted from wheat, barley, rye, triticale and oat. The proteolysis was carried out by microbial proteases from *Bacillus stearothermophilus*, *B. licheniformis*, *B. thermoproteolyticus* and *Streptomyces griseus*. Most of the prolamins were susceptible to proteolysis and their immunoreactivity decreased.

The aim of this paper was to study the ability of fungal protease mixture from *Aspergillus oryzae* named Flavourzyme to hydrolyse alcohol-soluble fraction of wheat and rye grain proteins and possibility to reduce celiac immunoreactivity of prolamins by this proteolytic reaction. Proteolysis of wheat gliadins and rye secalins was performed at pH 5.0 and 7.0 and at various enzyme/prolamins

ratios and the reaction was effective not only at highest enzyme concentration, but also at lowest amounts of the enzyme. Limited proteolysis was carried out at 37 °C for 2 h and continued finally to 60 °C. Parameters of proteolysis could simulate the changing conditions during fermentation of sourdough and the initial baking phase of cereal products.

The results of SDS-PAGE analysis of hydrolysis of prolamins are shown in Fig. 1 and Fig. 2. Flavourzyme activity caused nearly complete degradation of wheat gliadins with the molecular weight between 25 kDa and 70 kDa after first 60 min of the processing time at pH 7.0. Proteolysis of wheat prolamins was effective at each applied enzyme/proteins ratio, although in case of lower enzyme concentrations some residues of gliadins still remained present. Similar results were obtained for rye secalins, as almost complete degradation of proteins with molecular weight between 35 kDa and 70 kDa was achieved at pH 5.0 (Fig. 2). The results of SDS-PAGE suggest that more effective degradation of wheat prolamins was achieved at pH 7.0, in contrast to hydrolysis of rye secalins, which was more intensive at pH 5.0 and proteins seem to be slightly more resistant to proteolysis at other pH values, where formation of new peptides with molecular size between 3.4 kDa and 15 kDa

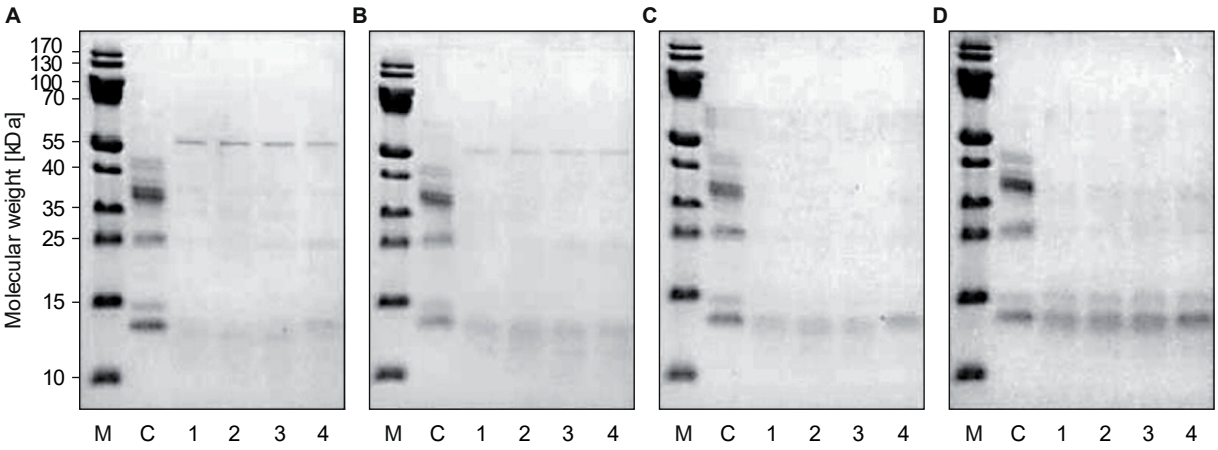

Fig. 1. SDS-PAGE separation of products of proteolysis of wheat gliadins by Flavourzyme at pH 7.0.

A – proteolysis at enzyme/prolamins ratio of 1 : 250 (w/w), B – proteolysis at enzyme/prolamins ratio of 1 : 500 (w/w), C – proteolysis at enzyme/prolamins ratio of 1 : 2000 (w/w), D – proteolysis at enzyme/prolamins ratio of 1 : 5000 (w/w).
Lanes: M – molecular weight marker; C – control sample (untreated prolamins); 1 – proteolysis for 60 min at 37 °C; 2 – proteolysis for 120 min at 37 °C; 3 – proteolysis for additional 15 min at 50 °C; 4 – proteolysis for additional 15 min at 60 °C.

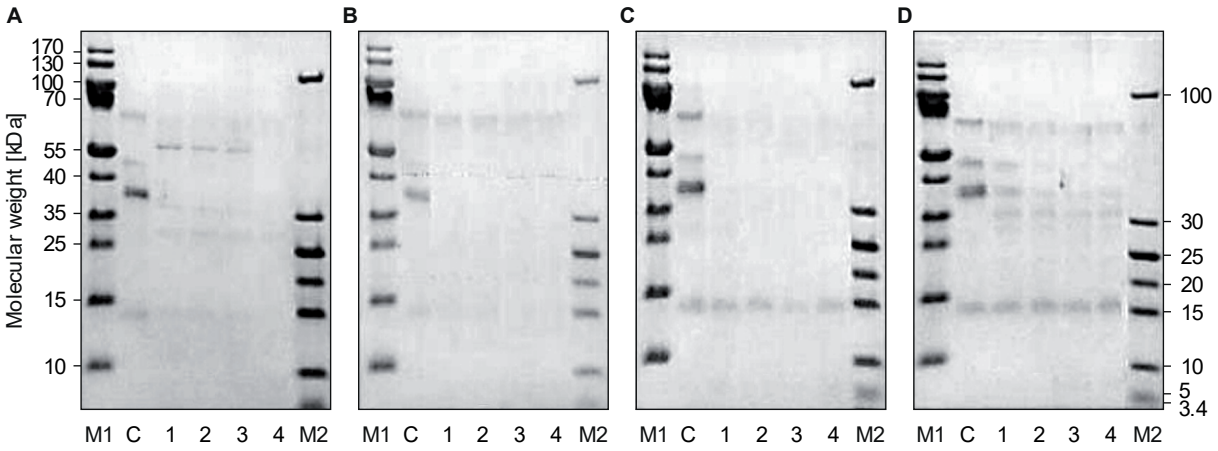

Fig. 2. SDS-PAGE separation of products of proteolysis of rye secalins by Flavourzyme at pH 5.0.

A – proteolysis at enzyme/prolamins ratio of 1 : 250 (w/w), B – proteolysis at enzyme/prolamins ratio of 1 : 500 (w/w), C – proteolysis at enzyme/prolamins ratio of 1 : 2000 (w/w), D – proteolysis at enzyme/prolamins ratio of 1 : 5000 (w/w).
Lanes: M1 – molecular weight marker (10–170 kDa); C – control sample (untreated prolamins); 1 – proteolysis for 60 min at 37 °C; 2 – proteolysis for 120 min at 37 °C; 3 – proteolysis for additional 15 min at 50 °C; 4 – proteolysis for additional 15 min at 60 °C; M2 – low range molecular weight marker (3.4–100 kDa).

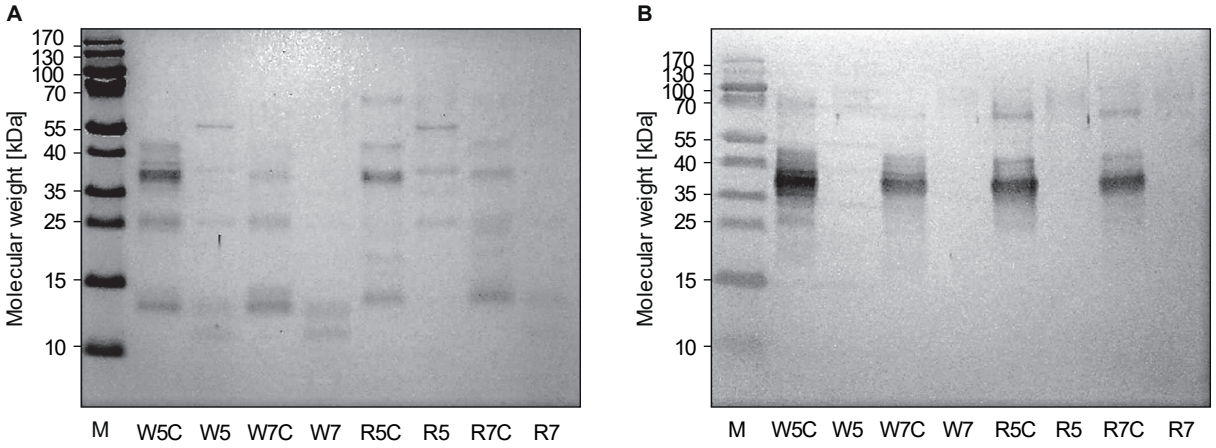

Fig. 3. Immunoreactivity of prolamins before and after proteolysis by Flavourzyme.

A – SDS-PAGE, B – Western blot.
Proteolysis was performed at enzyme/prolamins ratio of 1 : 250 (w/w) for 120 min at 37 °C.
M – molecular weight marker; W5C, W5 – wheat gliadins before and after hydrolysis at pH 5.0; W7C, W7 – wheat gliadins before and after hydrolysis at pH 7.0; R5C, R5 – rye secalins before and after hydrolysis at pH 5.0; R7C, R7 – rye secalins before and after hydrolysis at pH 7.0.

could be observed (data not shown). The results of hydrolysis could be additionally affected by solubility of prolamins, because they are hydrophobic and usually not dissolve well in aqueous solutions.

Western blot analysis with polyclonal anti-gliadin antibody proved that proteolysis with Flavourzyme resulted in complete disappearance of immunoreactive proteins of wheat and rye. The antibody recognized only uncleaved prolamins with molecular weights ranging from 25 kDa to 55 kDa (Fig. 3).

The quantitative analysis of immunoreactivity of prolamins and products of their proteolysis was performed by competitive ELISA based on the monoclonal R5 antibody. ELISA measurements were done before proteolysis and after the proteolytic reaction. Relative residual immunochemical reactivity of cleaved prolamins detected by R5 antibody is summarized in Fig. 4. The results were complementary with SDS-PAGE and Western blot analysis and confirmed high efficiency of proteolysis, only 2.3–5.3 % residual activity being observed for wheat and 2.8–6.2 % in case of rye. The reduction of immunoreactivity of wheat gliadins determined by ELISA was slightly stronger at pH 7.0 and in case of rye secalins at pH 5.0.

The proteolytic cleavage of wheat and rye prolamins was evaluated additionally by measure of released free amino acids in comparison to amino acids composition of native prolamins (Tab. 1, Tab. 2). Although amino acid composition of native prolamins of wheat and rye is similar, wheat gliadins seem to be more susceptible to proteolysis by Flavourzyme. This was suggested by the fact that after hydrolysis at pH 5.0, seven from thirteen of determined amino acids were released in a free form at a level higher than 15 %, with a maximum of 58 % for serine. The efficiency of cleavage was lower at pH 7.0, where only five from thirteen amino acids were released at a level higher than 10 %, with a maximum of 56 % for serine again. Totally, 9.2 % of amino acids of wheat prolamin molecules was released to free form at pH 5.0

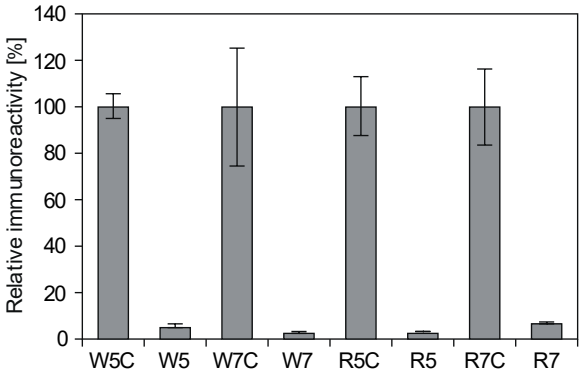

Fig. 4. Relative immunoreactivity of prolamins before and after proteolysis by Flavourzyme.

Proteolysis was performed at enzyme/prolamins ratio of 1 : 250 (w/w) for 120 min at 37 °C.
W5C, W5 – wheat gliadins before and after hydrolysis at pH 5.0; W7C, W7 – wheat gliadins before and after hydrolysis at pH 7.0; R5C, R5 – rye secalins before and after hydrolysis at pH 5.0; R7C, R7 – rye secalins before and after hydrolysis at pH 7.0.

Tab. 1. Free amino acids released from wheat gliadins by Flavourzyme proteolysis.

Amino acid	Native protein [mol%]	Amino acids released [%]	
		at pH 5.0	at pH 7.0
Asp	2.5 ± 0.1	5. 9 ± 0.0	2.8 ± 0.0
Thr	1.8 ± 0.0	IC	IC
Ser	5.1 ± 0.0	58.4 ± 1.8	56.7 ± 0.7
Glu	39.8 ± 1.4	1.0 ± 0.0	0.3 ± 0.0
Pro	19.6 ± 0.5	0.7 ± 0.1	0.3 ± 0.0
Gly	3.0 ± 0.0	6.5 ± 0.3	8.1 ± 0.0
Ala	2.6 ± 0.0	9.9 ± 0.1	9.2 ± 0.1
Val	3.9 ± 0.0	17.4 ± 0.1	10.5 ± 0.0
Ile	3.8 ± 0.0	10.8 ± 0.1	8.2 ± 0.0
Leu	6.7 ± 0.2	25.1 ± 0.7	12.7 ± 0.0
Tyr	2.0 ± 0.1	IC	IC
Phe	4.8 ± 0.1	17.4 ± 0.2	7.1 ± 0.1
His	2.1 ± 0.0	25.6 ± 0.1	13.1 ± 0.1
Lys	0.5 ± 0.0	16.5 ± 0.1	12.9 ± 0.1
Arg	1.9 ± 0.2	21.3 ± 0.6	8.7 ± 0.0

Proteolysis was performed at enzyme/protein ratio of 1 : 250 (w/w).
Values represent mean ± standard deviation. Released free amino acids are compared to total amino acids composition of unhydrolysed proteins.
IC – inconclusive.

Tab. 2. Free amino acids released from rye secalins by Flavourzyme proteolysis.

Amino acid	Native protein [mol%]	Amino acids released [%]	
		at pH 5.0	at pH 7.0
Asp	2.1 ± 0.1	2.9 ± 0.1	2.2 ± 0.1
Thr	2.1 ± 0.1	IC	IC
Ser	5.5 ± 0.1	7.5 ± 0.0	32.7 ± 0.4
Glu	39.6 ± 0.9	0.2 ± 0.0	0.2 ± 0.0
Pro	21.2 ± 0.8	nd	0.3 ± 0.1
Gly	2.9 ± 0.0	3.0 ± 0.3	6.9 ± 0.1
Ala	2.6 ± 0.1	3.5 ± 0.1	7.4 ± 0.2
Val	4.5 ± 0.0	2.7 ± 0.1	7.1 ± 0.2
Ile	3.3 ± 0.3	2.3 ± 0.1	7.2 ± 0.0
Leu	5.9 ± 0.5	2.7 ± 0.0	8.7 ± 0.0
Tyr	1.5 ± 0.1	IC	IC
Phe	4.9 ± 0.2	1.4 ± 0.2	3.8 ± 0.2
His	1.9 ± 0.2	4.3 ± 0.1	10.9 ± 0.1
Lys	0.7 ± 0.0	7.9 ± 0.2	10.1 ± 0.0
Arg	1.5 ± 0.3	3.5 ± 0.6	5.0 ± 0.0

Proteolysis was performed at enzyme/protein ratio of 1 : 250 (w/w).
Values represent mean ± standard deviation. Released free amino acids are compared to total amino acids composition of unhydrolysed proteins.
IC – inconclusive, nd – not determined.

and 6.3 % at pH 7.0. In case of rye, the proteolytic reaction did not lead to release free amino acids in such a large extent. In this case, at pH 7.0 only three amino acids were released in a free form at a level higher than 10 %, with a maximum for serine at 32 % but, at pH 5.0, the maximum level of hydrolysis to free amino acids was only for two of them and approximately 7 %, which totally gives 4.2 % released amino acids to free form at pH 7.0 and only 1.4 % at pH 5.0. Opposite to high contents of proline and glutamine in native prolamins, these amino acids were released from polypeptide chains at the lowest degree. In case of the use of a smaller amount of enzyme to hydrolyse rye secalins, e. g. at enzyme/prolamins ratio of 1 : 1 000, the released free amino acids were below the limit of quantification, so rye secalins seemed to be more resistant to proteolysis.

Several other studies also reported a successful hydrolysis of plant proteins during limited proteolysis by microbial peptidases. The activity of peptidases was usually measured by the degree of hydrolysis, which was calculated by determination of free α-amino groups released from the protein molecules. Meinlschmidt et al. [17] performed a study with the hydrolysis of soya protein isolate by Flavourzyme and some other microbial enzyme preparations such as endoeptidase from *B. licheniformis* (Alcalase 2.4 L FG), endopeptidases from *B. subtilis* and *B. stearothermophilus* (Corolase 7089 and Corolase 2TS, respectively; AB Enzymes, Darmstadt, Germany), endopeptidase from *B. amyloliquefaciens* (Neutrase 0.8 L, Novozymes), endopeptidases from *B. licheniformis* and *B. amyloliquefaciens* (Protamex, Novozymes) or proteases from *Bacillus* sp. (Protease A-01 or Protease A-0; ASA Spezialenzym, Wolfenbüttel, Germany). Degree of hydrolysis of soya protein isolate by Flavourzyme was determined as 8.5 % after two hours of enzymatic reaction at pH 6.0 and 50 °C, although only slight changes in SDS-PAGE pattern were observed.

The ability of Flavourzyme to hydrolyse cereal proteins to peptides was confirmed in a study of Bamdad et al. [18]. Barley hordeins were hydrolysed at pH 7.0 and 50 °C with final hydrolysis degree of 2 % and 6 % after 1 h and 2 h of hydrolysis, respectively. The highest degree of hydrolysis of 16.4 % was observed after 4 h. Size-exclusion chromatography of the hordein hydrolysates showed extensive hydrolysis already after first 30 min of incubation with creation of peptides with a broad molecular size distribution.

The most exhaustive research on Flavourzyme and its enzymatic activities was performed by Merz et al. [12, 19, 20] where also wheat gluten hydroly-

sis was studied. The maximum degree of hydrolysis achieved in optimized conditions was approximately 64 % and 44 % at substrate concentrations of 2 $g \cdot l^{-1}$ and 10 $g \cdot l^{-1}$, respectively, at hydrolysis duration of 24 h. The degree of hydrolysis decreased with an increase in substrate concentration, which was explained by the product inhibition of the peptidases. Antigenicity was measured by self-made competitive ELISA based on polyclonal antibody and was shown to be dependent on the degree of hydrolysis as well as to decrease along with the enzymatic hydrolysis. Nevertheless, no gluten-free hydrolysates were obtained by sole enzymatic hydrolysis, but additional processing operations were necessary to further reduce the antigenicity. Our interests were focused mainly on the ability of Flavourzyme to reduce the immunoreactivity of proline-rich wheat and rye proteins from the point of view of celiac toxicity. Although none of Flavourzyme peptidases is classified as prolyl-specific, our study proved that this enzyme mixture is able to destroy celiac-active sequences in wheat and rye prolamins.

Western blot performed with polyclonal antibody did not detect any positive products of proteolysis, although native prolamins with molecular weights in the range of 25–55 kDa gave strong signal. In comparison to polyclonal anti-gliadin antibody, which recognizes many epitopes in the whole protein molecule, the specificity of monoclonal antibody R5 used in the performed ELISA analysis is narrowed to recognize only celiac-active epitopes QQPFP, QQQFP, LQPFP and QLPFP in wheat, rye and barley prolamins [21, 22]. The competitive ELISA was designated for hydrolysed samples, as already one recognized epitope is enough to detect even a short fragment of molecule containing the specific amino acid sequence. Our ELISA analysis confirmed that celiac-active sequences in prolamin molecules were destroyed by proteolysis.

Suprisingly Flavourzyme was able to inactivate the immunoreactive epitopes inside the polypeptide chain practically without cleavage peptide bonds at proline or glutamine residues and practically no release of free forms of these amino acids, as demonstrated by results of the amino acid analysis. The loss of immunoreactivity can be a result of a synergistic effect of activities of all endo- and exopeptidases in Flavourzyme, which can cleave the protein molecules inside polypeptide chain creating various peptides.

Results of our study proved that Flavourzyme is very efficient for cleavage of wheat and rye prolamins, being able to reduce their celiac-activity significantly, although it is mixture of several peptidases with different specificities. Elimination of immunogenic peptides by Flavourzyme can be potentially used in food processing as one of possible alternatives for production of gluten-free food by proteolytic gluten modification.

CONCLUSIONS

The aim of the study was to check the ability of Flavourzyme to hydrolyse wheat and rye prolamins, and to reduce their immunoreactivity as a result of proteolysis. Proteolysis was carried out under conditions simulating the sourdough fermentation and initial baking phase of cereal products (temperature 37–60 °C, pH 5.0 and 7.0). SDS-PAGE analysis proved that gliadins of molecular weight of 25–70 kDa and secalins of molecular weight of 35–70 kDa were almost completely degraded by proteolysis at pH 7.0 and 5.0, respectively. Western blot performed with polyclonal anti-gliadin antibody did not detect any immunoreactive proteins or peptides after proteolysis. Quantitative analysis by competitive ELISA, based on monoclonal R5 antibody, confirmed the reduction of immunoreactivity of prolamins as, after proteolysis, it amounted for gliadins 2.3–5.3 % (at pH 7.0 and 5.0, respectively) and for secalins 2.8–6.2 % (pH 5.0 and 7.0) of initial values. Amounts of free amino acids released from prolamins were 6.3–9.2 % for gliadins and 1.4–4.2 % for secalins. The effectiveness of proteolysis was better at pH 7.0 for wheat gliadins and at pH 5.0 for rye secalins. Proline and glutamine were released at the lowest degree, despite the highest content of these amino acids in native prolamins. Presented results proved that Flavourzyme effectively hydrolysed wheat and rye prolamins, and significantly reduced immunoreactivity of these proteins. This was probably a result of a synergistic action of all proteinases present in this preparation. Interestingly, the reduction of immunoreactivity was achieved almost without releasing free proline or glutamine from prolamin molecules. Proteolytic modification of prolamins and elimination of immunoreactive epitopes by Flavourzyme may find potential use in food processing as one of the alternatives for production of food with a reduced gluten content.

Acknowledgements

This research was financed by the Ministry of Science and Higher Education of the Republic of Poland and supported by the project KEGA 020SPU-4/2016.

REFERENCES

1. Mazzeo, M. – Bonavita, R. – Maurano, F. – Bergamo, P. – Siciliano, R. – Rossi, M.: Biochemical modifications of gliadins induced by microbial transglutaminase on wheat flour. Biochimica et Biophysica Acta (BBA) - General Subjects, *1830*, 2013, pp. 5166–5167. DOI: 10.1016/j.bbagen.2013.07.021.
2. Shan, M. – Molberg, Ø. – Parrot, I. – Hausch, F. – Filiz, F. – Gray, G. M. – Sollid, L. M. – Khosla, C.: Structural basis for gluten intolerance in celiac sprue. Science, *13*, 2002, pp. 2275–2279. DOI: 10.1126/science.1074129.
3. Siegel, M. – Bethune, M T. – Gass, J. – Ehren, J. – Xia, J. – Johannsen, T. B. – Stuge, T. B. – Gray, G. M. – Lee, P. P. – Khosla, C.: Rational design of combination enzyme therapy for celiac sprue. Chemistry and Biology, *13*, 2006, pp. 649–658. DOI: 10.1016/j.chembiol.2006.04.009.
4. Caputo, I. – Lepretti, M. – Martuciello, S. – Esposito, C.: Enzymatic strategies to detoxify gluten: implications for celiac disease. Enzyme Research, *2010*, 2010, Article ID 174354. DOI: 10.4061/2010/174354.
5. Linke, D.: Enzymatic prevention of health risks in food. Current Opinion in Food Science, *1*, 2015, pp. 21–27. DOI: 10.1016/j.cofs.2014.09.004.
6. M'hir, S. – Ziadi, M. – Chammem, N. – Hamdi, M.: Gluten proteolysis as alternative teraphy for celiac patients: A mini-review. African Journal of Biotechnology, *11*, 2012, pp. 7323–7330. DOI: 10.5897/AJB11.726.
7. Stepniak, D. – Spaenij-Dekking, L. – Mitea, C. – Moeser, M. – de Ru, A. – Baak-Pablo, R. – van Veelen, P. – Edens, L. – Konig, F.: Highly efficient gluten degradation with a newly identified prolyl endopeptidase: implication for celiac disease. American Journal of Physiology – Gastrointestinal and Liver Physiology, *291*, 2006, pp. G621–G628. DOI: 10.1152/ajpgi.00034.2006.
8. Maurer, K. H.: Detergent proteases. Current Opinion in Biotechnology, *15*, 2004, pp. 330–334. DOI: 10.1016/j.copbio.2004.06.005.
9. Yang, A. – Long, C. – Xia, J. – Tong, P. – Cheng, Y. – Wang, Y. – Chen, H.: Enzymatic characterisation of the immobilised Alcalase to hydrolyse egg white protein for potential allergenicity reduction. Journal of the Science of Food and Agriculture, *97*, 2016, pp. 199–203. DOI: 10.1002/jsfa.7712.
10. Aguilar, C. N. – Gutiérrez-Sánchez, G. – Rado-Barragán, P. – Rodríguez-Herrera, R. – Martínez-Hernandez, J. – Contreras-Esquivel, J.: Perspectives of solid state fermentation for production of food enzymes. American Journal of Biochemistry and Biotechnology, *4*, 2008, pp. 354–366. DOI: 10.3844/ajbbsp.2008.354.366.
11. Tavano, O. L.: Protein hydrolysis using proteases: An important tool for food biotechnology. Journal of Molecular Catalysis B: Enzymatic, *90*, 2013, pp. 1–11. DOI: 10.1016/j.molcatb.2013.01.011.
12. Merz, M. – Eisele, T. – Berends, P. – Appel, D. – Rabe, S. – Blank, I. – Stressler, T. – Fischer, L.: Flavourzyme, an enzyme preparation with industrial relevance – Automated nine-step purification and partial characterization of eight enzymes. Journal of Agricultural and Food Chemistry, *62*, 2015, pp. 5682–5693. DOI: 10.1021/acs.jafc.5b01665.
13. Osborne, T. B. (Ed.): The vegetable proteins. 2nd edition. London : Longmans, Green and Co., 1924.
14. Smith A. J. Post column amino acid analysis. In: Smith B. J. (Ed.): Protein sequencing protocols. Methods in Molecular Biology, Volume 211. 2nd edition. Totowa, New Jersey : Humana Press, 2003, pp. 133–142. ISBN: 9780896039759.
15. Schägger, H. – von Jagow, G.: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry, *166*, 1987, pp. 368–379. DOI: 10.1016/0003-2697(87)90587-2.
16. Mickowska, B. – Socha, P. – Urminská, D.: Immunochemical evaluation of proteolysis of cereal proteins causing celiac disease by microbial proteases. Food and Agricultural Immunology, *27*, 2016, pp. 743–757. DOI: 10.1080/09540105.2016.1148665.
17. Meinlschmidt, P. – Sussmann, D. – Schweiggert-Weisz, U. – Eisner, P.: Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionalicity, and sensory properties. Food Science and Nutrition, *4*, 2016, pp. 11–18. DOI: 10.1002/fsn3.253.
18. Bamdad, F. – Wu, J. – Chen, L.: Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. Journal of Cereal Science, *54*, 2011, pp. 20–28. DOI: 10.1016/j.jcs.2011.01.006.
19. Merz, M. – Ewert, J. – Baur, C. – Appel, D. – Blank, I. – Stressler, T. – Fischer, L.: Wheat gluten hydrolysis using isolated Flavourzyme peptidases: Product inhibition and determination of synergistic effects using response surface methodology. Journal of Molecular Catalysis B: Enzymatic, *122*, 2015, pp. 218–226. DOI: 10.1016/j.molcatb.2015.09.010.
20. Merz, M. – Kettner, L. – Langolf, E. – Appel, D. – Blank, I. – Stressler, T. – Fischer, L.: Production of wheat gluten hydrolysates with reduced antigenicity employing enzymatic hydrolysis combined with downstream unit operations. Journal of the Science of Food and Agriculture, *96*, 2016, pp. 3358–3364. DOI: 10.1002/jsfa.7515.
21. Thompson, T. – Méndez, E.: Commercial assays to assess gluten content of gluten-free foods: Why they are not created equal. Journal of the American Dietetic Association, *108*, 2008 pp. 1682–1687. DOI: 10.1016/j.jada.2008.07.012.
22. van Eckert, R. – Bond, J. – Rawson, P. – Klein, Ch. L. – Stern, M. – Jordan, T. W.: Reactivity of gluten detecting monoclonal antibodies to a gliadin reference material. Journal of Cereal Science, *51*, 2010, pp. 198–204. DOI: 10.1016/j.jcs.2009.11.012.

Received 6 February 2018; 1st revised 12 April 2018; accepted 16 April 2018; published online 4 September 2018.