Reduction of immunoreactivity of rye and wheat prolamins by lactobacilli and Flavourzyme proteolysis during sourdough fermentation – a way to obtain low-gluten bread

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Summary

The aim of our work was to check the ability of selected strains of lactobacilli (*Lactobacillus plantarum* CCM 3626, *Lb. plantarum* CCM 3627, *Lb. zymae* CCM 7241 and *Lb. fermentum* CCM 7192) to reduce immunoreactivity of rye and wheat prolamins in fermented sourdoughs and, consequently, gluten content in bread. The proteolysis was increased by the addition of fungal proteases from *Aspergillus oryzae* (Flavourzyme; Novozymes, Basvaerd, Denmark). The proteolysis was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis, immunoreactivity by Western blot and enzyme-linked immunosorbent assay (sandwich and competitive ELISA). Amino acid analysis was performed to quantify the amount of free amino acids released during the sourdough fermentation. The most effective proteolysis and reduction of immunoreactivity was achieved in sourdoughs with Flavourzyme and with lactobacilli-Flavourzyme mixture, where only 1.6–2.3 % of initial immunoreactivity remained. Fungal proteases of Flavourzyme were responsible for the most of protein degradation and immunoreactivity decrease. Prepared pre-treated sourdoughs were used for bread baking and the gluten content was reduced to the level of 65–123 mg·kg^{-1} of fresh mass of bread, so bread with a low gluten content was obtained.

Keywords

lactobacilli; Flavourzyme; low-gluten bread; coeliac immunoreactivity

Sourdough fermentation is a traditional biotechnological process for production of cereal food, especially bread. In bakery technology, sourdough fermentation impacts the dough rheology, increases the retention of gas, causes gluten swelling and improves the dough texture [1]. Sourdough is a mixture of cereal flours and water, which is spontaneously fermented by autochthonous microflora composed of lactic acid bacteria and yeasts. The main genera isolated from sourdough are *Lactobacillus, Leuconostoc, Pediococcus* and *Weissella*, with dominance of heterofermentative *Lactobacillus* strains. Yeasts are present in minority in sourdough, the occurrence ratio of yeasts and lactobacilli is approximately 1 : 100 [2, 3].

In recent years, sourdough lactic acid bacteria are studied because of the proteolytic activity related to the degradation of coeliac-active proteins [4–7]. Coeliac disease is a chronical autoimmune disorder with inflammatory processes in the small intestine's mucosa in genetically predisposed individuals with human leucocyte antigens HLA-DQ2 and/or HLA-DQ8. The inflammation is caused by ingestion of prolamin proteins from wheat (gliadins), rye (secalins) or barley (hordeins), which are characterized by a high content of proline and glutamine. The high proline content provides the prolamin resistance to cleavage by gastrointestinal peptidases [8].

Lactobacilli produce intracellular aminopeptidases, dipeptidases, tripeptidases and peptidases capable to hydrolyse peptide bonds with proline, such as prolinase, prolidase, iminopeptidase and aminopeptidase P, but most of them cannot produce extracellular proteolytic enzymes and they require primary hydrolysis of proteins by endo-

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genous cereal proteases during the sourdough fermentation. Rye and wheat peptidases, in particular carboxypeptidases and aspartic proteinases active in acidic pH, promote proteolysis and liberation of free amino acids in sourdough. Thus, the composition of lactic acid microflora of dough may strongly affect the peptide composition in dough after the fermentation process [9].

An addition of peptidases of different origin or application of lactic acid bacteria with a specific set of peptidases may be a new alternative biotechnological approach for the production of low-gluten wheat and rye foods [10]. Several studies confirmed the effectiveness of bacterial and enzymatic proteolysis during the sourdough fermentation to cleave a considerable amount of coeliac-active proteins. Various strains of lactobacilli were used for gluten hydrolysis during wheat sourdough fermentation [11]. Sourdough proteolysis of rye by *Lactobacillus alimentarius* 15M, *Lb. brevis* 14G, *Lb. sanfranciscensis* 7A and *Lb. hilgardii* 51B was studied [5]. A lactobacillar starter culture with an addition of fungal enzyme preparations from *Aspergillus niger* and *A. oryzae* was used for the production of wheat bread with an intermediate gluten content [7, 12]. There were some experiments with *Lb. sanfranciscensis* TMW 1.53, *Lb. plantarum* TMW 1.468, *Lb. sakei* TMW 1.22 in combination with *Entorococcus faecalis* TMW 2.630 from a fermented sausage for wheat gluten proteolysis [13]. A probiotic preparation consisting of lactic acid bacteria and bifidobacteria (*Lb. casei, Lb. acidophilus, Lb. plantarum, Lb. delbrueckii* subsp. *bulgaricus, Bifidobacterium longum, B. infantis* and *B. breve*) was also used as a starter culture for the wheat sourdough preparation and found to cause significant degradation of wheat proteins [14].

The aim of this research was to study the effects of some selected *Lactobacillus* strains on the proteolytic modification of cereal prolamins in rye-wheat sourdoughs during the fermentation process. The bacterial proteolysis was assisted by a mixture of endo- and exopeptidases of *Aspergillus oryzae* called Flavourzyme (Novozymes, Basvaerd, Denmark), which is a mixture of α-amylase, two aminopeptidases, three endopeptidases and two dipeptidyl peptidases [15]. The potential of that fermentation process for the elimination of coeliac-active peptides in sourdoughs was checked and followed by an attempt to obtain low-gluten bread.

MATERIALS AND METHODS

Chemicals and equipment

Fungal enzyme complex from *Aspergillus oryzae*, Flavourzyme was from Novozymes, de Man, Rogosa and Sharpe (MRS) broth and MRS agar were from HiMedia (Mumbai, India), malt extract agar was from Conda (Madrid, Spain), molecular weight protein markers for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE PageRuler Protein Ladder) were from Thermo Fisher Scientific (Waltham, Massachusetts, USA), ImmobilonP^{SQ} PVDF transfer membrane was from Millipore (Billerica, Massachusetts, USA), polyclonal anti-gliadin antibody was from Sigma-Aldrich (St. Louis, Missouri, USA), polyclonal anti-rabbit antibody conjugated with HRP was from Bio-Rad Laboratories (Hercules, California, USA), SigmaFAST 3,3'-diaminobenzidine tablets and amino acid standard solution were from Sigma-Aldrich, RidaScreen Gliadin and RidaScreen Gliadin competitive enzyme-linked immunosorbent assay (ELISA) kits were from R-Biopharm (Darmstadt, Germany). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich.

Microplate spectrophotometric reader iMark as well as electrophoresis and electrotransfer equipment were from Bio-Rad Laboratories, vacuum lyophilizers Christ Alpha1-2 LD Plus and Alpha 2-4 LSC plus were from Martin Christ (Osterode am Harz, Germany), chromatographic amino acid analyzer AAA400 was from Ingos (Prague, Czech Republic).

Materials

Commercially available rye bread flour (PRO-BIO, Staré Město, Czech Republic) and wheat flour T-650 (Vitaflóra, Kolárovo, Slovakia) were used for sourdough preparation.

Bacterial cultures

Lyophylized *Lb. plantarum* CCM 3626, *Lb. plantarum* CCM 3627, *Lb. zymae* CCM 7241 and *Lb. fermentum* CCM 7192 were from Czech Collection of Microorganisms (Brno, Czech Republic). For sourdough fermentations, *Lactobacillus* strains were cultured in MRS broth until the late exponential phase of growth [16, 17].

Lactobacilli were harvested by centrifugation (9000 ×*g*, 10 min at 4 °C), washed with sterile physiological saline (pH 7.0), lyophilized and stored at 4 °C until use. For a short-time preservation of strains, MRS agar, pH 5.6, was used.

Sourdough fermentation

The composition of sourdough samples and formulas of sourdough preparations are presented in Tab. 1. The lactic acid fermentation in semi-liquid conditions was executed at 37 °C for

12 h and 24 h, followed by freezing inactivation at –30 °C. Samples were lyophilized and stored at 4 °C.

Each sourdough type was prepared in following variants:

- control sourdough - only flour mixture;
- chemically acidified sourdough - acidified to pH 4.0 by addition of a mixture of acetic acid (99 %) and lactic acid (80 %) in a ratio of 1 : 4 (v/v);
- lactobacilli sourdough - lyophilized bacterial cultures were added before the fermentation, the pool of mixed four strains contained approximately 10^8 CFU of each *Lactobacillus* strain resuspended in 375 ml of tap water used for the sourdough preparation;
- Flavourzyme sourdough - enzyme was added before the fermentation in a dose of 1 g of enzyme per 10 g of cereal protein (maximal recommended dose for an extensive proteolysis);
- lactobacilli-Flavourzyme sourdough - enzyme and mixed bacterial cultures were added together before the fermentation.

Yeast fermentation

For dough making, aliquots of 150 g of rye-wheat sourdough and 300 g of buckwheat flour with maize starch mixture (70 % and 30 %, respectively) were used. In a further step, a dose of 23.2 g of bakery *Saccharomyces cerevisiae* (FALA, Lesaffre, France) dissolved in 89.2 ml of tap water (25 °C) was added, with 5.8 g of sugar and 10.4 g of salt. Doughs were mixed at room temperature in 3 stages: 20 Hz for 10 min, 25 Hz for 2 min and 50 Hz for 5 min (Diosna SP12; Diekers and Söhne, Osnabrück, Germany). The alcohol fermentation by bakery yeasts took 40 min at 30 °C.

Bread baking

Breads were baked at 240 °C for 10 min and subsequently at 220 °C for 25 min (MIWE Condo, Arnstein, Germany). For further analysis, breads were milled and lyophylized.

Determination of pH and total titratable acidity of sourdoughs

Samples were collected and measured during 24 h of fermentation. Measurement of total titratable acidity (*TTA*) was preformed according to AACC International Method 02-31.01 [18]. The *TTA* value was determined as the percentage of lactic acid.

Protein extraction

For SDS-PAGE and Western blot analysis, proteins from raw flours, lyophilized sourdoughs and lyophilized breads were extracted directly with electrophoresis loading buffer (125 mmol·l^{-1} Tris-HCl pH 6.8, 4 % sodium dodecyl sulphate (SDS), 20 % glycerol, 10 % 2-mercaptoethanol, 0.05 % Coomassie Brilliant Blue G-250) in a 10-fold excess of buffer to sample weight. Samples were shaken vigorously for 1 h at room temperature and centrifuged at 15 000 ×*g* for 15 min at 4 °C. Aliquots of supernatants were frozen at –20 °C.

Tab. 1. Ingredients for sourdough preparation.

Sourdough type	Rye flour [%]	Wheat flour [%]	Flour mixture : water (w/w)
1	90	10	1 : 2.5
2	80	20	1 : 2.5
3	70	30	1 : 2.5

SDS-PAGE

Proteins extracted from flours, sourdoughs and breads were analysed by Tris-Tricine SDS-PAGE under reducing conditions according to the method of SCHÄGGER and VON JAGOW [19].

Western blot

After SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane ImmobilonP^{SQ} under conditions of 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). Membranes were blocked in 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer, pH 7.6, overnight and incubated with the primary antibody (polyclonal anti-gliadin antibody, concentration 1 μg·ml^{-1} in blocking solution containing 1 ml·l^{-1} Tween 20) for 1 h, followed by washing with TBS buffer containing 1 ml·l^{-1} Tween 20 (four times for 10 min). After that, the membrane was subjected to incubation with secondary antibody (polyclonal anti-rabbit horseradish peroxidase-conjugated antibody, concentration 0.2 μg·ml^{-1} in blocking solution containing 1 ml·l^{-1} Tween 20) for 1 h, followed by washing with TBS buffer containing 1 ml·l^{-1} Tween 20 (five times for 10 min). Blots were developed by chromogenic detection using diaminobenzidine tablets according to the protocol of manufacturer (Sigma-Aldrich).

ELISA analysis

Quantitative analysis of gliadin and gluten content in lyophylized sourdoughs and breads was accomplished by ELISA based on R5 monoclo-

nal antibody. Specifically, competitive ELISA for fermented and hydrolysed samples (Flavourzyme sourdough, lactobacilli-Flavourzyme sourdough and bread samples) and sandwich ELISA for samples without intensive hydrolysis of prolamins (control sourdough, acidified sourdough and lactobacilli sourdough). Protein extraction for ELISA was carried out according to the method by MENA et al. [20] with some modifications, specifically, samples were incubated at 50 °C for 60 min and, after addition of 80% ethanol, they were shaken vigorously for 10 min. Analysis by ELISA was performed according to the instructions of the manufacturer (R-Biopharm). The results were presented as residual immunochemical reactivity, which was immunoreactivity of samples remaining after fermentation related to the initial immunoreactivity of control samples, and expressed as percentage.

Amino acid analysis

Lyophilized samples 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) and filtered through a syringe filter (pore size 0.45 μm). Amino acids were determined by ion-exchange chromatography using an automatic amino acid analyser, with a strong cation-exchanger and sodium-citrate elution buffer system followed by post-column derivatization with ninhydrin and spectrophotometric detection at 570 nm and 440 nm, according to the standard protocol of the manufacturer (Ingos). Sulphur-containing amino acids were analysed as oxidation products (cysteic acid and methionine sulfone) obtained by performic acid oxidation followed by standard hydrolysis procedure with HCl. Tryptophan was not determined as it is destroyed during acid hydrolysis. Asparagine and glutamine turn to aspartic acid and glutamic acid and in these forms are determined.

Analysis of free amino acids

An amount of 1 g of sample was extracted with shaking for 1 h in 10 ml of 0.1 mol·l^{-1} HCl, followed by precipitation of proteins by addition of solid sulfosalicylic acid (30 mg·ml^{-1}). After incubation for 1 h at 4 °C, samples were centrifuged at 15000 ×*g* for 15 min and lyophilized. After dissolving in an appropriate volume of dilution buffer and filtering through a syringe filter (pore size 0.45 μm), chromatographic analysis of amino acids was performed.

Statistical analysis

Statistical analysis (Student's t-tests) was per-

Tab. 2. Changes in pH and total titrable acidity values of sourdoughs during the fermentation.

Sourdough	pH			*TTA* [%]		
	0 h	12 h	24 h	0 h	12 h	24 h
Control	6.5	5.9–6.0	4.1–4.3	0.1	0.3	0.7
Acidified	4.0	4.0	4.1–4.2	0.6	0.6	0.6
Lactobacilli	6.0	3.3–3.4	3.6	0.1	0.9 -1.0	1.1–1.2
Flavourzyme	6.3	6.1–6.2	4.7–4.8	0.2	0.5–0.6	0.8–0.9
Lactobacilli + Flavourzyme	6.2	3.5–3.7	3.6	0.3	1.4–1.6	1.8–1.9

Results for all sourdough types are presented, values represent ranges of results (minimum and maximum).
TTA – total titrable acidity, expressed as percentage of lactic acid.

Tab. 3. Counts of lactic acid bacteria of sourdoughs during the fermentation.

Sourdough	Counts of lactic acid bacteria [CFU·g^{-1}]					
	Sourdough type 1 (rye-wheat flour ratio 9:1)		Sourdough type 2 (rye-wheat flour ratio 8:2)		Sourdough type 3 (rye-wheat flour ratio 7:3)	
	12 h	24 h	12 h	24 h	12 h	24 h
Control	1.4×10^8	4.2×10^9	2.7×10^8	3.9×10^9	2.7×10^8	2.9×10^9
Acidified	3.2×10^6	1.5×10^6	2.0×10^6	3.0×10^5	2.2×10^6	1.1×10^5
Lactobacilli	1.4×10^{11}	8.2×10^{10}	1.0×10^{11}	7.2×10^{10}	9.6×10^{10}	7.6×10^{10}
Flavourzyme	2.0×10^{10}	3.0×10^{10}	1.3×10^{10}	2.0×10^{10}	6.0×10^9	4.0×10^9
Lactobacilli + Flavourzyme	3.0×10^{10}	1.9×10^{11}	1.0×10^{11}	1.7×10^{11}	3.3×10^{11}	1.8×10^{11}

Initial count of lactic bacteria in control sourdough was 10^5 CFU·g^{-1}.

formed using MS Excel software (Microsoft, Redmond, Washington, USA), the significance level was established at $p < 0.05$.

RESULTS AND DISCUSSION

Sourdough is a naturally fermented mixture of flour, water and some other ingredients (e. g. NaCl), where the fermentation is performed by naturally occurring lactic acid bacteria and yeasts. In mature sourdoughs, dominating microorganisms are lactic acid bacteria (> 10^8 CFU·g^{-1}). One of the sourdough properties is the proteolysis and liberation of free amino acids (and their derivatives) by the lactic acid bacteria and their proteinase system. Some of sourdough lactobacilli strains are capable to hydrolyse the proline-reach coeliac-active peptides. This ability was previously achieved when the mixture of strains was used, which together had complementary proteinase activities. Selected sourdough lactic bacteria (at approximately 10^9 CFU·g^{-1} in dough) showed the ability to degrade prolamins of wheat and rye, in contrast to those of buckwheat, oats and millet, which were not affected [21].

The aim of our work was to study the ability of selected strains of lactobacilli (*Lb. plantarum* CCM 3626, *Lb. plantarum* CCM 3627, *Lb. zymae* CCM 7241 and *Lb. fermentum* CCM 7192) to reduce the immunoreactivity of prolamin or gluten in fermented sourdoughs and breads and to increase the proteolysis by the addition of fungal proteases from *A. oryzae* (Flavourzyme preparation), whose ability to decrease wheat and rye prolamins immunoreactivity was studied earlier [22]. Flavourzyme was used in a dose of 10 % of protein content, which is the recommended dose for extensive protein proteolysis.

The determined values of pH and acidity of sourdoughs are presented in Tab. 2. Regarding these values, differences between sourdough type 1 (rye/wheat flour ratio 9:1), type 2 (rye/wheat flour ratio 8:2) and type 3 (rye/wheat flour ratio 7:3) were minimal. The initial pH values decreased, which was correlated with an increasing content of lactic acid (determined as *TTA*).

The initial cell count of lactic acid bacteria at the beginning of the fermentation of control sourdough was 10^5 CFU·g^{-1}. Values of colony forming units after fermentation of sourdoughs are shown in Tab. 3. Natural flour microflora growth in sourdoughs reached 10^8–10^9 CFU·g^{-1}, in sourdoughs with lactobacilli and/or Flavourzyme it reached 10^{10}–10^{11} CFU·g^{-1}, which indicated that the addition of enzymes did not influence bacterial growth significantly. The chemical acidification strongly inhibited growth of lactobacilli. No significant differences were observed in the numbers of bacteria in sourdoughs prepared from rye and wheat flours of different ratios.

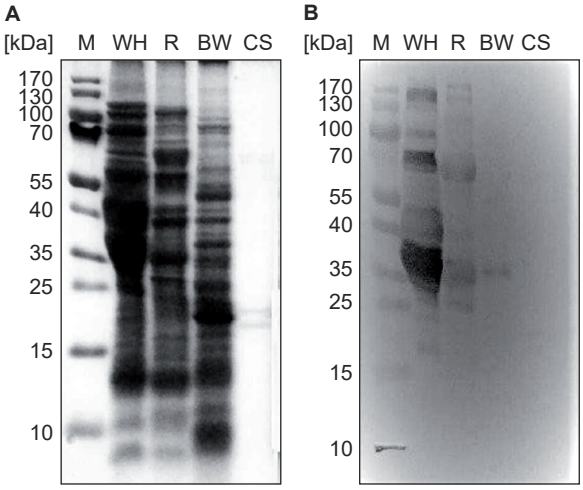

Fig. 1. Protein profiles and immunogenicity of proteins extracted from flours used for making sourdoughs and breads.

A – SDS-PAGE, B – Western blot.
M – molecular weight marker, WH – wheat proteins, R – rye proteins, BW– buckwheat proteins, CS – maize starch.

The protein profiles of flours, sourdoughs and breads were analysed by SDS-PAGE and their immunogenicity by Western blot with polyclonal anti-gliadin antibody. Control SDS-PAGE analysis and corresponding Western blot of proteins of raw materials used for the sourdough preparation are shown on Fig. 1. The strongest immunoreactivity was observed for wheat and rye proteins, as expected. Buckwheat proteins are almost free from immunoreactive proteins, a weak signal can come from contaminants. Maize starch is almost protein-free.

The same analysis was performed for sourdoughs and breads. The comparison of protein profiles of sourdoughs prepared from mixtures of rye and wheat flours after 12 h and 24 h of fermentation are shown in Fig. 2. In all samples, the most effective protein degradation was visible in case of sourdoughs prepared by the combination of lactobacilli and Flavourzyme (in all rye-wheat ratios). Proteolysis by Flavourzyme alone was very extensive and only remains of initial proteins were visible after it. The loss of immunoreactivity in these samples was confirmed by Western blot. The comparison of SDS-PAGE and Western blot results suggested that lactobacilli alone were also

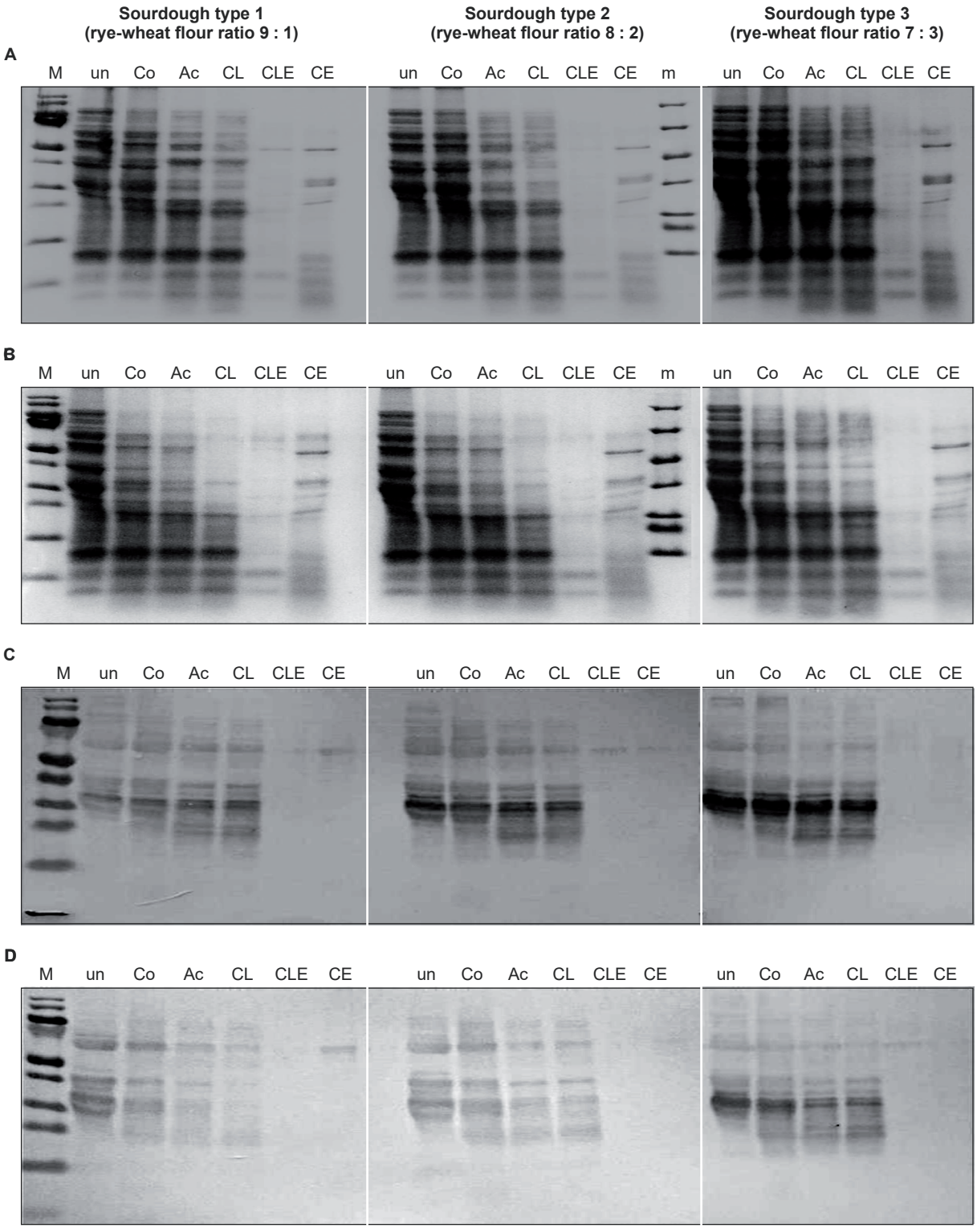

Fig. 2. Protein profiles and immunogenicity of sourdough proteins.

A, B – SDS-PAGE of sourdoughs fermented for 12 h and 24 h, respectively; C, D – Western blot of sourdoughs fermented for 12 h and 24 h, respectively.
M – molecular weight marker (170, 130, 100, 70, 55, 40, 35, 25, 15, 10 kDa), un – unfermented, initial flour mixture (rye and wheat), Co – control sourdough, Ac – acidified sourdough, CL – sourdough prepared with addition of lactobacillar starter culture, CLE – sourdough prepared with addition of lactobacillar starter culture and Flavourzyme, CE – sourdough prepared with addition of Flavourzyme, m – molecular weight marker (116, 66.2, 45, 35, 25, 18.4, 14.4 kDa).

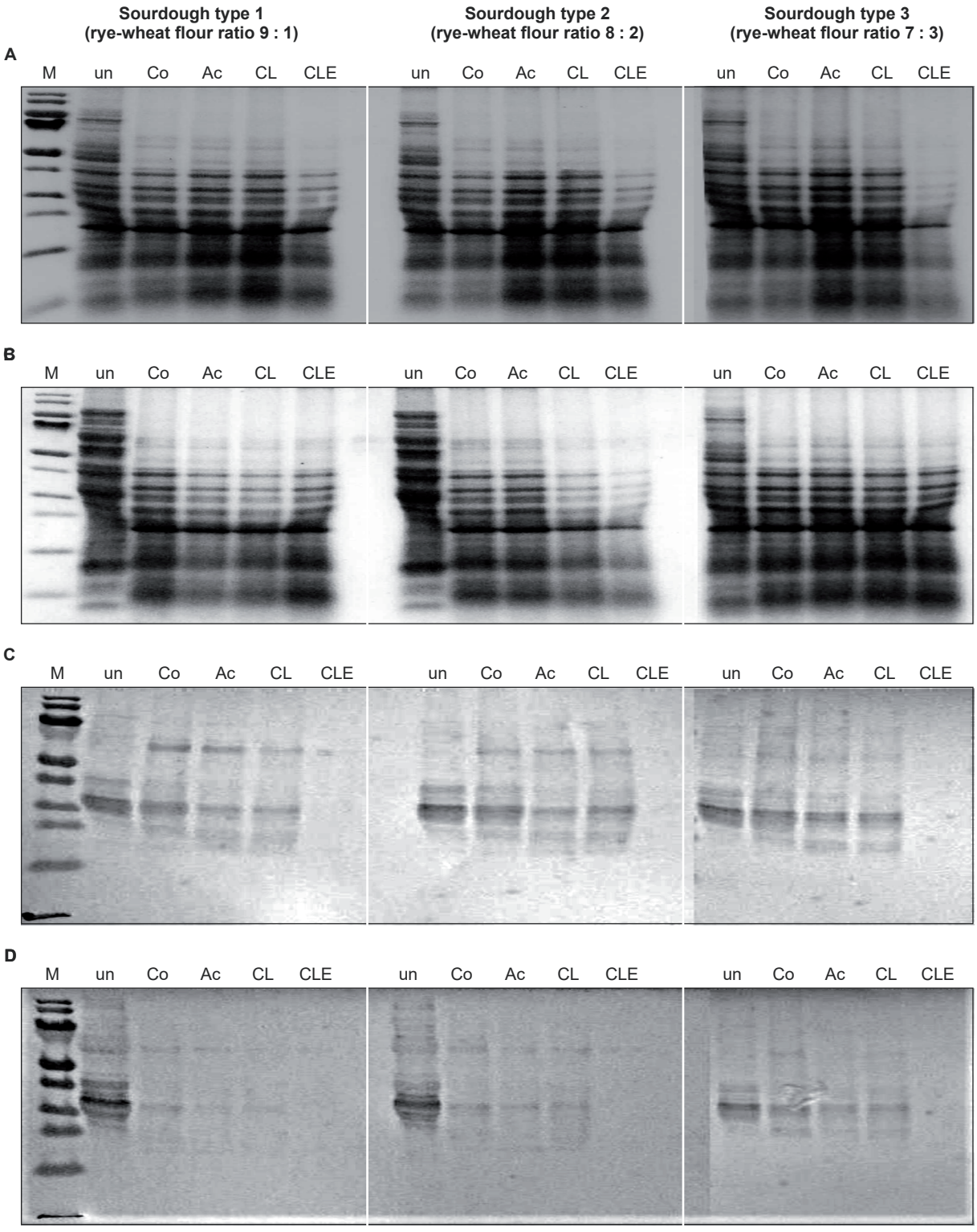

Fig. 3. Protein profiles and immunogenicity of bread proteins.

A, B – SDS-PAGE of breads baked from sourdoughs fermented for 12 h and 24 h, respectively; C, D – Western blot of breads baked from sourdoughs fermented for 12 h and 24 h, respectively.
M – molecular weight marker (170, 130, 100, 70, 55, 40, 35, 25, 15, 10 kDa), un – unfermented mixture of rye-wheat sourdough, buckwheat flour and maize starch, Co – bread baked from control sourdough, Ac – bread baked from acidified sourdough, CL – bread baked from sourdough prepared with addition of lactobacillar starter culture, CLE – bread baked from sourdough with addition of lactobacillar starter culture and Flavourzyme.

involved in the protein cleavage, but not in a significant reduction of immunoreactivity. Prolonged time of fermentation increased the extent of protein degradation.

In protein profiles of breads prepared from sourdoughs fermented for 12 h and 24 h, visible degradation of proteins was achieved only in breads prepared with addition of the lactobacillar starter culture in combination with Flavourzyme in case of all rye-wheat ratios (Fig. 3). Buckwheat proteins were predominant in electrophoretic profiles of all breads, which suggested that they were more resistant to bacterial proteolysis than wheat or rye proteins. Disappearance of proteins of molecular weight in range 55–100 kDa present in unfermented flour mixtures could be observed. In Western blot analysis, no immunoreactivity was detected only for breads prepared from lactobacilli-Flavourzyme sourdough. For other samples, the signal was weaker in comparison to unfermented samples.

Quantitative analysis of the reduction of immunoreactivity of sourdoughs during their fermentation and of the corresponding breads was performed by ELISA based on monoclonal R5 antibody. Fermented and hydrolysed samples were analysed by competitive ELISA (Flavourzyme sourdough, lactobacilli-Flavourzyme sourdough and bread samples), as only a single epitope in a peptide fragment could be detected in this format. Samples without any intensive hydrolysis of prolamins (unfermented and control sourdoughs, acidified sourdough and lactobacilli sourdough) were analysed by sandwich ELISA, which demands at least two epitopes [23]. Relative residual immunochemical reactivities of sour-

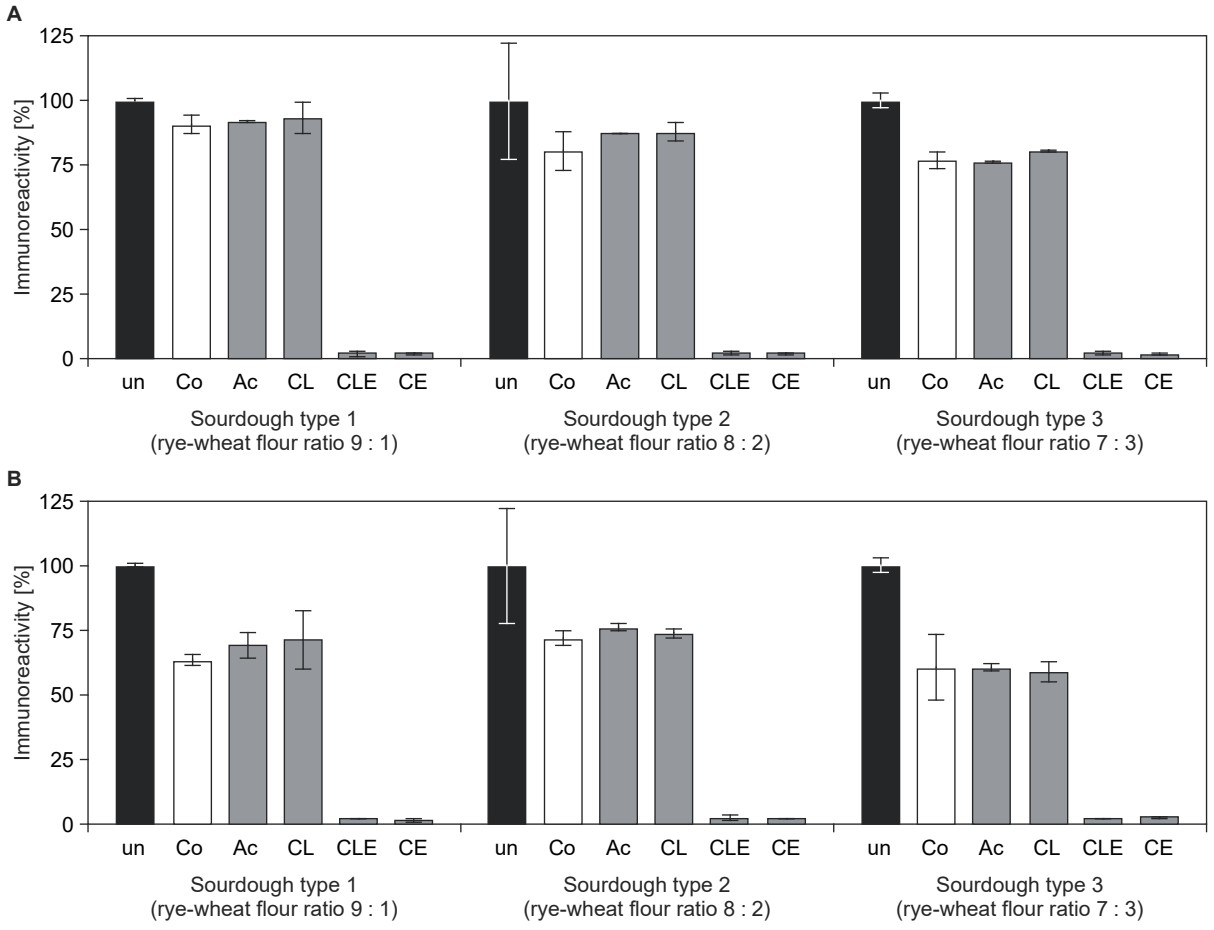

Fig. 4. Relative residual immunoreactivity of sourdoughs.

A – sourdoughs fermented for 12 h, B – sourdoughs fermented for 24 h.
Error bars represent confidence intervals (95%).
un – unfermented initial flour mixture (rye and wheat), Co – control sourdough, Ac – acidified sourdough, CL – sourdough prepared with addition of lactobacillar starter culture, CLE – sourdough prepared with addition of lactobacillar starter culture and Flavourzyme, CE – sourdough prepared with addition of Flavourzyme.

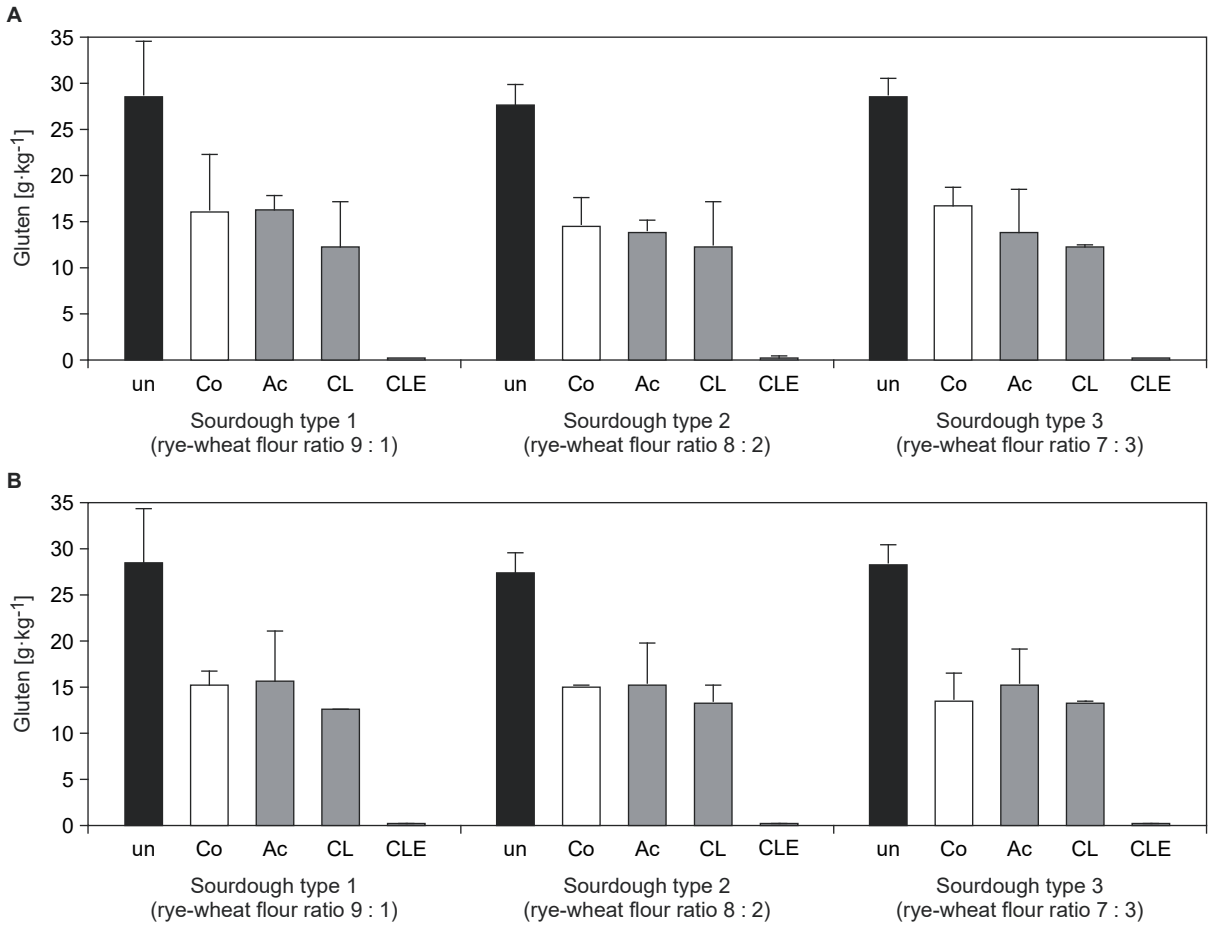

Fig. 5. Gluten content in breads.

A – breads baked from sourdoughs fermented for 12 h, B – breads baked from sourdoughs fermented for 24 h.
Gluten content is expressed per kilogram of fresh mass of bread. Error bars represent confidence intervals (95%).
un – unfermented mixture of rye-wheat sourdough, buckwheat flour and maize starch, Co – bread baked from control sourdough, Ac – bread baked from acidified sourdough, CL – bread baked from sourdough prepared with addition of lactobacillar starter culture, CLE – bread baked from sourdough with addition of lactobacillar starter culture and Flavourzyme.

doughs are summarized in Fig. 4. The results are complementary with SDS-PAGE and Western blot analysis and confirm the high efficiency of proteolysis especially in Flavourzyme and lactobacilli-Flavourzyme sourdoughs, where only 1.6–2.3 % of the initial immunoreactivity remained. Prolonged time of fermentation led to considerable reduction of immunoreactivity in other sourdoughs, the residual immunoreactivity decreased from the range 76–93 % after 12 h of fermentation to the range 59–76 % after 24 h of fermentation.

In case of breads, the gluten content in fresh mass was estimated, the results are presented on Fig. 5. For all sourdough breads, immunoreactivity decreased by approximately 50% in comparison with unfermented flours, with no particular influence of duration of fermentation. The main difference between sourdough and bread was the additional time for yeast fermentation, followed by a gradual increase in temperature till 240 °C, which can momentarily increase the kinetics of various enzymatic reactions in dough. Gluten contents in fresh mass of breads baked with lactobacilli and Flavourzyme sourdoughs were lowered to the level of 106.8 ± 0.0 mg·kg^{-1} and 123.1 ± 54.5 mg·kg^{-1} for sourdough type 1, 105.8 ± 58.1 mg·kg^{-1} and 71.4 ± 20.5 mg·kg^{-1} for sourdough type 2, 65.3 ± 6.0 mg·kg^{-1} and 80.2 ± 11.2 mg·kg^{-1} for sourdough type 3 (for 12 h and 24 h of sourdough fermentation, respectively).

The general problem with ELISA analysis of gluten is overestimation. The sources of inaccuracies are mainly the antibody specificity and the standard material. R5 is a monoclonal antibody raised against rye secalins (60% ethanolic extract from rye flour), it recognizes the epitopes QQPFP,

Tab. 4. Amino acid composition of the mixture of rye-wheat flours and free amino acids release in sourdough type 1 during fermentation.

Amino acid	Initial amino acid composition		Free amino acids released by Flavourzyme [%]		Free amino acids released by Flavourzyme and lactobacilli [%]	
	In mixture [g·kg^{-1}]	In protein [%]	12 h	24 h	12 h	24 h
Asp	4.3 ± 0.4	6.7 ± 0.2	IC	IC	IC	IC
Thr	1.5 ± 0.1	2.4 ± 0.0	IC	IC	IC	IC
Ser	2.3 ± 0.1	3.6 ± 0.0	IC	IC	IC	IC
Glu	20.0 ± 1.0	31.4 ± 0.5	7.2 ± 0.0	ND	7.9 ± 0.2^{b}	7.1 ± 0.4^{b}
Pro	7.1 ± 0.5	11.1 ± 0.1	34.2 ± 5.3^{A}	65.7 ± 2.8	22.6 ± 0.3^{Ab}	20.0 ± 2.9^{b}
Gly	2.6 ± 0.1	4.1 ± 0.1	33.3 ± 1.0^{A}	51.9 ± 2.0	35.1 ± 0.1^{Ab}	35.6 ± 1.3^{b}
Ala	2.3 ± 0.1	3.7 ± 0.1	52.7 ± 2.2^{A}	82.5 ± 3.4	51.5 ± 0.4^{Ab}	48.2 ± 2.6^{b}
Cys	1.4 ± 0.0	2.2 ± 0.1	23.1 ± 3.8^{a}	21.3 ± 0.9^{a}	13.7 ± 1.8^{b}	13.1 ± 0.6^{b}
Val	3.4 ± 0.2	5.3 ± 0.1	52.2 ± 10.0^{Aa}	63.2 ± 3.0^{a}	49.9 ± 0.1^{A}	46.5 ± 0.6
Met	1.0 ± 0.0	1.6 ± 0.1	40.0 ± 2.1^{a}	42.9 ± 0.1^{a}	33.1 ± 0.8^{b}	29.6 ± 7.6^{b}
Ile	2.3 ± 0.2	3.7 ± 0.0	55.5 ± 1.3^{A}	74.0 ± 1.7	54.5 ± 0.2^{Ab}	50.9 ± 2.0^{b}
Leu	4.2 ± 0.2	6.7 ± 0.1	48.8 ± 1.5	66.2 ± 0.4	55.2 ± 0.3^{b}	53.4 ± 2.6^{b}
Tyr	1.3 ± 0.2	2.1 ± 0.2	IC	IC	IC	IC
Phe	3.2 ± 0.3	5.0 ± 0.1	46.2 ± 0.4^{A}	66.4 ± 2.8	45.5 ± 0.1^{Ab}	44.0 ± 2.3^{b}
His	1.5 ± 0.1	2.4 ± 0.1	51.5 ± 0.3	18.8 ± 1.0	73.2 ± 0.2^{b}	78.0 ± 14.6^{b}
Lys	2.2 ± 0.1	3.4 ± 0.0	41.1 ± 0.4	26.9 ± 1.1	59.1 ± 0.5^{b}	61.3 ± 3.6^{b}
Arg	3.0 ± 0.6	4.8 ± 0.6	46.6 ± 7.3	2.9 ± 0.5	18.6 ± 0.2	25.8 ± 1.5
Total	63.6 ± 0.2	100	> 25.6 ± 0.7^{A}	> 33.3 ± 1.2	> 24.3 ± 0.2^{Ab}	> 22.7 ± 1.0^{b}

Rye-wheat flour ratio 9 : 1 (w/w). Released free amino acids are given as weight percentage of composition of the initial sample. Values represent mean ± confidence interval. The same letters in a row indicate no statistically significant differences at $p > 0.05$. IC – inconclusive, ND – not detected.

Tab. 5. Amino acid composition of the mixture of rye-wheat flours and free amino acids release in sourdough type 2 during fermentation.

Amino acid	Initial amino acid composition		Free amino acids released by Flavourzyme [%]		Free amino acids released by Flavourzyme and lactobacilli [%]	
	In mixture [g·kg^{-1}]	In protein [%]	12 h	24 h	12 h	24 h
Asp	4.4 ± 0.2	6.1 ± 0.1	IC	IC	IC	IC
Thr	1.7 ± 0.1	2.4 ± 0.0	IC	IC	IC	IC
Ser	2.9 ± 0.2	4.1 ± 0.0	IC	IC	IC	IC
Glu	24.4 ± 1.9	33.9 ± 0.5	10.3 ± 4.3^{A}	ND	12.8 ± 1.3^{A}	7.9 ± 1.5
Pro	7.8 ± 0.6	10.9 ± 0.1	40.9 ± 18.0^{A}	68.0 ± 1.6	23.2 ± 3.4^{Ab}	25.1 ± 6.7^{b}
Gly	2.8 ± 0.2	3.8 ± 0.0	41.0 ± 9.8^{A}	53.4 ± 1.3	35.8 ± 2.5^{Ab}	39.3 ± 5.1^{b}
Ala	2.4 ± 0.3	3.3 ± 0.2	66.5 ± 16.9^{Aa}	79.6 ± 2.8^{a}	56.8 ± 4.0^{Ab}	54.8 ± 7.1^{b}
Cys	1.5 ± 0.2	2.1 ± 0.1	27.0 ± 9.1^{Aa}	26.4 ± 0.5^{a}	9.1 ± 1.9^{A}	13.4 ± 1.4
Val	3.7 ± 0.1	5.1 ± 0.1	62.6 ± 24.2^{Aa}	64.9 ± 2.0^{a}	49.5 ± 4.2^{Ab}	51.1 ± 6.9^{b}
Met	1.0 ± 0.1	1.5 ± 0.1	45.3 ± 1.7^{a}	48.1 ± 3.1^{a}	40.9 ± 1.4^{b}	41.8 ± 2.4^{b}
Ile	2.6 ± 0.1	3.6 ± 0.0	66.8 ± 15.7^{Aa}	75.6 ± 1.3^{a}	54.6 ± 3.6^{Ab}	55.5 ± 6.4^{b}
Leu	4.8 ± 0.2	6.7 ± 0.1	58.7 ± 15.1^{Aa}	67.9 ± 1.7^{a}	58.3 ± 4.3^{Ab}	56.8 ± 6.4^{b}
Tyr	1.5 ± 0.0	2.1 ± 0.2	IC	IC	IC	IC
Phe	3.5 ± 0.2	4.8 ± 0.1	57.3 ± 12.6^{Aa}	66.0 ± 4.0^{a}	50.3 ± 4.4^{Ab}	49.7 ± 6.4^{b}
His	1.7 ± 0.1	2.3 ± 0.0	65.3 ± 15.3^{A}	23.1 ± 0.4	76.3 ± 4.6^{Ab}	77.7 ± 9.4^{b}
Lys	2.3 ± 0.1	3.1 ± 0.0	4.2 ± 0.6	40.0 ± 1.9	63.1 ± 4.6^{b}	63.9 ± 8.0^{b}
Arg	2.9 ± 0.1	4.1 ± 0.1	26.8 ± 3.4^{A}	4.9 ± 0.8	29.2 ± 2.1^{Ab}	33.0 ± 4.1^{b}
Total	71.8 ± 0.3	100	> 28.0 ± 8.4	> 33.4 ± 0.3	> 26.7 ± 2.1	> 25.6 ± 3.6

Rye-wheat flour ratio 8 : 2 (w/w). Released free amino acids are given as weight percentage of composition of the initial sample. Values represent mean ± confidence interval. The same letters in a row indicate no statistically significant differences at $p > 0.05$. IC – inconclusive, ND – not detected.

Tab. 6. Amino acid composition of the mixture of rye-wheat flours and free amino acids release in sourdough type 3 during fermentation.

Amino acid	Initial amino acid composition		Free amino acids released by Flavourzyme [%]		Free amino acids released by Flavourzyme and lactobacilli [%]	
	In mixture [g·kg^{-1}]	In protein [%]	12 h	24 h	12 h	24 h
Asp	4.4 ± 0.2	5.7 ± 0.1	IC	IC	IC	IC
Thr	1.9 ± 0.3	2.4 ± 0.3	IC	IC	IC	IC
Ser	3.2 ± 0.1	4.1 ± 0.2	IC	IC	IC	IC
Glu	26.8 ± 1.7	34.8 ± 1.4	10.0 ± 2.3	ND	7.1 ± 0.5^{b}	7.8 ± 0.4^{b}
Pro	7.9 ± 0.6	10.3 ± 1.0	58.7 ± 9.1^{a}	68.5 ± 9.1^{a}	16.2 ± 0.2^{b}	17.5 ± 1.7^{b}
Gly	2.9 ± 0.1	3.7 ± 0.0	49.0 ± 7.8^{a}	50.6 ± 6.1^{a}	29.6 ± 2.2	37.6 ± 1.0
Ala	2.4 ± 0.0	3.1 ± 0.0	75.0 ± 12.0^{a}	85.1 ± 10.2^{a}	47.4 ± 3.8^{b}	55.5 ± 1.3^{b}
Cys	1.6 ± 0.0	2.1 ± 0.1	25.0 ± 4.9^{a}	23.2 ± 4.3^{a}	11.3 ± 0.9	14.7 ± 0.1
Val	3.8 ± 0.1	5.0 ± 0.0	64.6 ± 12.8^{a}	63.0 ± 8.1^{a}	43.6 ± 1.9	50.1 ± 2.3
Met	1.1 ± 0.1	1.4 ± 0.1	52.3 ± 6.9^{a}	47.7 ± 4.6^{a}	29.6 ± 3.8^{b}	35.3 ± 3.2^{b}
Ile	2.8 ± 0.1	3.6 ± 0.1	73.2 ± 10.9^{a}	75.0 ± 8.9^{a}	51.4 ± 3.4	58.1 ± 2.3
Leu	5.1 ± 0.3	6.6 ± 0.2	65.2 ± 10.3^{Aa}	69.0 ± 8.4^{aC}	58.4 ± 3.7^{Ab}	63.4 ± 2.2^{bC}
Tyr	2.0 ± 0.4	2.6 ± 0.6	IC	IC	IC	IC
Phe	3.7 ± 0.1	4.8 ± 0.1	65.5 ± 9.7^{Aa}	68.6 ± 8.4^{a}	49.5 ± 4.1^{Ab}	53.3 ± 2.6^{b}
His	1.9 ± 0.1	2.4 ± 0.1	57.9 ± 8.0^{A}	9.5 ± 1.2	70.0 ± 5.1^{Ab}	74.2 ± 2.3^{b}
Lys	2.2 ± 0.1	2.9 ± 0.0	40.3 ± 5.6	58.0 ± 6.1	56.6 ± 4.1	64.8 ± 1.8
Arg	3.3 ± 0.1	4.3 ± 0.2	57.8 ± 5.6	4.2 ± 0.4	35.2 ± 3.6	25.6 ± 1.0
Total	76.9 ± 0.3	100	> 32.6 ± 5.7	> 33.1 ± 4.3	> 22.5 ± 1.5	> 24.6 ± 0.7

Rye-wheat flour ratio 7 : 3 (w/w). Released free amino acids are given as weight percentage of composition of the initial sample. Values represent mean ± confidence interval. The same letters in a row indicate no statistically significant differences at $p > 0.05$. IC – inconclusive, ND – not detected.

QQQFP, LQPFP, QLPFP and the antibody is designated for prolamin detection. For sandwich ELISA, the standard material is gliadin, for competitive ELISA it is a mixture of peptide fragments of gliadins, secalins and hordeins, so the antibody specificity and the standard material are different, followed by diverse protein composition in samples of different origin, not always compatible with the standard material [24]. Additionally, the protein content in standards (in case of competitive ELISA, is determined according to the Dumas method based on nitrogen determination (R-Biopharm data), so the conversion to protein can be inaccurate. Converting prolamin content to gluten by factor 2 also leads to inaccuracy. Generally, the gluten content is overestimated when the prolamin content is multiplied by factor 2. The real factors for the calculations of gluten for wheat are in a range of 1.32–1.66 and for rye in a range of 1.12–1.16 [25]. In our calculations, we decided to use the average value for wheat and rye. Moreover, according to a recent study on reactivities of gluten proteins with sandwich ELISA kits, R5 ELISA overestimated gluten in wheat, rye and barley due to the high affinity to ω1,2-gliadins, ω-secalins and D-hordeins [26]. Our observations are in accordance with these results, as the comparison of protein content calculated form amino acid composition with gluten protein determined by ELISA confirmed overestimation of gluten content by ELISA.

Proteolysis during sourdough fermentation was also evaluated by analysis of released free amino acids in comparison to the initial samples, and on the basis of total protein content. The total protein content of flour mixtures was in the range of 63–77 g·kg^{-1} and it increased with the increase in wheat flour share (Tab. 4–6). The majority of amino acids in the analysed samples were glutamic acid and proline, characteristic for prolamins, which are the main proteins of the cereal grain. During fermentation and proteolysis, amino acids are released not only from prolamins, but also from albumins, globulins and glutelins. The comparison of total amino acid composition and the amount of free amino acids present in all non-fermented flour mixtures showed that more than 99.3 % of amino acids were bound in polypeptide chains of proteins, as the total content of free amino acids in flours was only approximately 0.4 g·kg^{-1}. During sourdough fermentation with an addition of Flavourzyme, more than 25 % of

total amino acids of proteins were released to a free form (Tab. 4–6) and efficiency of that process correlated with prolongation of reaction time as well as with the content of wheat flour. Finally, the amount of released free amino acids reached more than 33 %. In case of the combination of Flavourzyme with lactobacilli, the level of released free amino acids was lower, with values in the range of 22.5–26.7 % and with no significant influence of prolongation of reaction time on the results. This suggests that some kind of state of equilibrium was achieved, as lactic acid bacteria can take up available free amino acids or peptides that are produced during the proteolysis and fermentation.

For ion-exchange chromatography of amino acids, appropriate pH and ionic strength of the samples are extremely important to achieve the most effective high resolution and separation. During elution, when ionic strength and pH of the eluent increase, amino acids are eluted in the order of their isoelectric point values. In case of free amino acid analysis of sourdoughs, samples not only contained lactic acid, but the acidity of samples was increased additionally by 5-sulfosalicylic acid, a non-volatile compound that is impossible to be removed by lyophilization. That influenced mainly the initial phase of the chromatographic separation, as some peaks were co-eluted or misshaped or retention times changed, so identification and quantitative analysis of these amino acids was unsure or even impossible (in tables indicated as inconclusive). Interestingly, in all samples containing Flavourzyme and long-term fermentation (24 h), complete disappearance of glutamic acid was observed.

Other authors' studies proved the ability to degrade rye prolamins during the sourdough fermentation by selected lactobacilli, namely, *Lb. sanfranciscensis* 7A, *Lb. brevis* 14G, *Lb. alimentarius* 15M and *Lb. hilgardii* 51B. Western blot analysis based on R5 antibody showed an almost complete degradation of proteins after 24 h of fermentation followed by no polypeptide recognition by R5 antibody after 48 h [5]. The possibility of gluten hydrolysis in wheat flour using specific bacterial strains in combination with fungal proteases during the fermentation process was also examined. For the wheat sourdough fermentation, selected lactobacillar strains were used: *Lb. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38, LS47, *Lb. brevis* 14G, *Lb. alimentarius* 15M and *Lb. hilgardii* 51B. Proteolysis was supplemented with two proteases routinely used in bakeries, the enzymes originating from *A. oryzae* and *A. niger* (Bio-Cat, Troy, Virginia, USA). Sandwich and competitive R5-based ELISA tests showed the reduction of gluten content from approximately 103 $g \cdot kg^{-1}$ in unfermented wheat flour to below 0.01 $g \cdot kg^{-1}$ after fermentation. The result was confirmed also by R5 antibody-based Western blot and supported by the measurement of the total free amino acids content, a 15-fold increase after fermentation being determined [27]. A similar study was performed with the addition of the same *Lactobacillus* species and both fungal proteases, the obtained sourdoughs being used for bread preparation. Gluten contents were determined by sandwich R5-based ELISA with untreated flour being found to contain 82 $g \cdot kg^{-1}$ of gluten and bread prepared from the experimental sourdough to contain the residual gluten content of approximately 58 $g \cdot kg^{-1}$. The degree of gluten degradation was approximately 28 %. Amount of total free amino acids increased about 9-fold in sourdough in comparison to flour [12].

In our experiment, during the natural fermentation of sourdoughs, the prolamin immunoreactivity was reduced to approximately 76–90 %, and to 60–72 % in case of a long-term fermentation. In case of sourdoughs with added lactobacilli, these values were 80–93 % and 59–74 %, respectively. Amino acid analysis results suggested that Flavourzyme only released more free amino acids from cleaved proteins than Flavourzyme combined with lactobacilli. Free amino acids increased up to 62.5-fold and 45-fold, respectively. On the other hand, SDS-PAGE showed that the combination of Flavourzyme and lactobaccilli was the most effective in case of degradation of complete protein molecules during fermentation. Immunoreactivity measured by ELISA did not show significant differences between these two variants, their relative residual immunoreactivity being in the range of 1.5–2.3 %.

According to Codex Alimentarius [28] and EU regulation [29], two categories for labelling of food according to the gluten content exist: „gluten-free“ products, which contain less than 20 $mg \cdot kg^{-1}$, and „very low gluten“ products, whose gluten content is above 20 $mg \cdot kg^{-1}$ and up to 100 $mg \cdot kg^{-1}$. As determined by R5-based ELISA, we achieved the reduction of gluten content in breads baked from pre-treated sourdoughs to the level of 65–123 $mg \cdot kg^{-1}$ of fresh mass of bread, therefore, low-gluten bread was obtained. Moreover, on the basis of the comparison of amino acid analysis as the most accurate method of protein content determination and on the basis of the calculated R5-based ELISA results, we can suspect that gluten content determined by ELISA was still a bit overestimated.

CONCLUSIONS

Fermented rye-wheat sourdoughs as materials for obtaining bread with a low gluten content were studied in this work. The sourdoughs were fermented naturally and with the addition of selected *Lactobacillus* spp. strains. The mixture of fungal proteases Flavourzyme was also added. The observed synergistic effect of the proteolytic activity of lactobacilli and Flavourzyme was weaker than expected. In fact, Flavourzyme was responsible for the most of proteolysis, although degradation of proteins was more complete when lactobacilli were added, too. The prepared sourdoughs were used for bread baking and low-gluten bread was obtained, with gluten content in the range of 65–123 mg·kg^{-1}.

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