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Prolamin Hydrolysis and Pentosan Solubilization in Germinated-Rye Sourdoughs Determined by Chromatographic and Immunological Methods

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The assortment and quality of bakery products designed for celiac patients may be improved by designing whole grain ingredients with low residual prolamin contents. The main objective of this study was to evaluate the extent of prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs (GRSDs). Size-exclusion chromatography analyses, the fate of fluorescent prolamin, and immunological analyses determined the extent of prolamin hydrolysis and pentosan solubilization. Hydrolysis of rye prolamins was extensive in GRSDs, and according to enzyme-linked immunosorbent assay analyses, more than 99.5% of the prolamins were hydrolyzed. Pentosan solubilization occurred in native-rye sourdoughs, whereas in GRSDs, pentosans were partially hydrolyzed to monosaccharides. Test baking showed that the use of GRSD improved the overall quality of oat bread and that an estimated daily gluten intake from 100 g of bread would be less than 10 mg. However, the clinical safety must be assured before making any recommendations for celiac patients to use such products.

KEYWORDS: Prolamin; rye; sourdough; hydrolysis; germination; peptides; pentosan; size-exclusion chromatography; ELISA; gluten-free; baking; celiac disease

INTRODUCTION

Prolamin proteins of wheat, barley, and rye contain structures that trigger celiac disease, an autoimmune disorder also known as gluten intolerance. The prevalence of celiac disease is increasing, and recent evidence suggests its total prevalence among Finnish children, adults, and the elderly as 1.5, 2.0, and 2.5%, respectively (*1*−*3*). Moreover, the celiac disease iceberg model predicts the existence of a large population with silent or latent celiac disease (*4*). Celiac disease develops only in genetically susceptible individuals, and the onset of the disease is related to the intake of gluten-containing foods (*5*); however, it is unknown whether the onset could be delayed or prevented by reducing the gluten intake. Currently, the only treatment for celiac disease is a diet free of prolamins from wheat, rye, barley, and other Triticeae cereals. Food products containing less than 20 mg/kg gluten are generally considered safe for celiac patients and can be labeled "gluten-free". The latest draft of a Codex standard (*6*) also defines the term "very low-gluten", which refers to specially processed food products with reduced gluten contents between 20 and 100 mg/kg (*6*). Nevertheless, the daily intake of gluten is considered the most critical threshold for the health of celiac patients, and among celiac sufferers, a safe amount of gluten lies between 10 and 100 mg daily intake (*7, 8*).

Whole grain bread is an important source of dietary fiber and minerals. For instance, dark rye bread is part of the everyday diet in Finland, and Finnish celiac patients often miss the flavor of rye bread. The substitution of wheat and rye with refined starch and protein preparations in gluten-free baking frequently results in products with a low mineral and fiber content. Moreover, the texture and flavor of current gluten-free breads are often inferior when compared to conventional products (*9*).

The use of wheat, rye, or barley in gluten-free baking, after a quantitative hydrolysis of their prolamins, could be an alternative to the use of gluten-free cereals. Extensive prolamin hydrolysis is achievable through the combined use of sourdoughs and germinated grains or added fungal proteases (*10, 11*). In such dough systems, more than 95% of wheat prolamins are hydrolyzed during fermentation; however, an extensive hydrolysis of wheat prolamins (gluten proteins) is arguable because the hydrolysis destroys their technological functionality to form gluten structures, the unique and essential property of wheat proteins. As opposed to wheat baking, the dough hydration and gas retention in rye baking are based on pentosans rather than proteins. Pentosans of rye are a group of nonstarch polysaccharides that account for 6−12% of the dry matter content of rye grains and consist mainly of arabinoxylans. Water-soluble arabinoxylans positively affect loaf volume due to their high water-binding capacity and their ability to undergo oxidative

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gelation (*12*, *13*). Cereal hemicellulases increase the soluble arabinoxylan contents in rye sourdoughs at the expense of water-insoluble arabinoxylans (*13*, *14*). Moreover, rye prolamins, secalins, are unable to form elastic dough structures, but instead, their hydrolysis in sourdoughs contributes to flavor formation through the liberation of amino acids (*15*). A rye-based baking improver thus could improve the flavor, texture, and dietary fiber composition of gluten-free products, provided that appropriate fermentation protocols ensure the quantitative removal of prolamins.

The hydrolysis of prolamins requires specific enzyme activities. Cereal grains naturally contain proteases that hydrolyze prolamins, and most of these are de novo synthesized during grain germination (*16*−*20*). The proteolytic activity of germinated rye is mainly from aspartic and cysteine proteinases (*21*), which, along with carboxypeptidases, operate under mildly acidic conditions, whereas the serine and metallo proteinases of germinated grains operate at neutral pH. The slow acidification by lactic acid bacteria gradually shifts the pH from neutral to acidic during sourdough fermentation, which principally allows the different protease groups to operate. Moreover, peptidases of lactic acid bacteria contribute to the overall hydrolysis (*22*). Thus, the use of germinated rye in combination with biological acidification may be an efficient tool to eliminate prolamins in rye flour and to accumulate amino acids as flavor precursors.

This study aimed to determine the hydrolysis of prolamins, the accumulation of amino acids, and the solubilization of pentosans in germinated-rye sourdoughs (GRSDs) and sourdoughs prepared from native rye. A combination of chromatographic and immunological methods was used to evaluate the hydrolysis and solubilization of proteins and pentosans. Four different strains of lactobacilli were used as starter cultures for biologically acidified sourdoughs and were compared to chemically acidified sourdoughs.

MATERIALS AND METHODS

Rye Materials. Germinated rye (malted rye grain) and native rye (ungerminated) were provided by Laihian Mallas (Laihia, Finland). The germinated rye is used in baking applications as such or for the production of dark malts for a Finnish Easter pudding Mämmi. The germinated rye was manufactured in industrial scale using conventional malting practices with gentle drying, which guaranteed a raw material with high enzyme activity. Grains were finely ground with a Retsch ZM-200 ultracentrifugal mill (Retsch, Haan, Germany) equipped with a 0.5 mm sieve, and the obtained flours were used in experiments. The protease profile of germinated rye was determined by an azocasein method (*10*), which confirmed that the aspartic and cysteine proteases were the predominant protease groups in germinated rye as reported earlier (*21*).

Preparation of Fluorescent-Labeled Secalin. Native-rye flour (80 g) was extracted with 800 mL of 1 M NaCl and 50 mM Tris-HCl, pH 8.0, at room temperature for 1 h and centrifuged (10000*g*, 20 min), and the supernatant containing albumins and globulins was discarded. The pellet was extracted with 50% of 2-propanol at 50 °C for 1 h and centrifuged. The supernatant containing the secalins was collected, and to precipitate the secalins, the supernatant was dialyzed against an excess of deionized water at +5 °C using a Spectra/Pro 3 dialysis membrane with cutoff 12−14 kilodaltons (kDa) (Spectrum Laboratories, Rancho Dominguez, CA). After 60 h of dialysis with four water exchanges, the contents of the dialysis membrane were centrifuged at +2 °C (10000*g*, 30 min) and the obtained pellet containing isolated secalins was lyophilized. The procedure for fluorescence labeling was modified from previous works (*23*, *24*). Of the lyophilized secalin, 30 mg was dissolved in 3 mL of 50% 2-propanol and 0.1 M sodium carbonate, pH 9.0, and 3 mL of dimethyl sulfoxide containing 30 mg of fluorescein isothiocyanate (FITC; Fluka, Buchs, Switzerland) was added. The reaction mixture was agitated in the dark for 2 h after which 1 mL of the mixture was suspended in 1.5 mL of 1.5% sodium dodecyl sulfate (SDS) and 50 mM sodium phosphate, pH 6.9. Unbound FITC was removed by filtering the suspension through a PD-10 Sephadex G-25 desalting column (GE Healthcare Bio-Sciences, Piscataway, NJ). The column was equilibrated with 25 mL of 1.5% SDS and 50 mM sodium phosphate, pH 6.9, the 2.5 mL sample was allowed to penetrate the column, and the fraction containing the fluorescent-labeled secalin was eluted with 3.5 mL of 1.5% SDS and 50 mM sodium phosphate, pH 6.9. The eluted fraction was substantially free of unbound FITC and contained fluorescent-labeled rye prolamins corresponding to HMW secalins (100 kDa), γ-75 secalins (75 kDa), ω-secalins (50 kDa), and γ-40 secalins (40 kDa) (**Figure 1**). One milliliter of this FITC-secalin was used freshly in sourdough preparations.

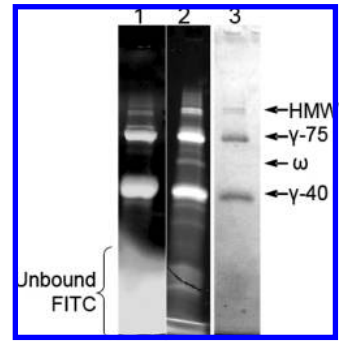

Figure 1. FITC-labeled secalin analyzed prior to the gel filtration with transluminator (lane 1), after removal of unbound FITC by the gel filtration (lane 2), and with Coomassie staining (lane 3). The unbound FITC is indicated on the left. The major secalin groups HMW, γ-75, ω, and γ-40 secalins, with respective M_r values of 100, 75, 50, and 40 kDa, are indicated on the right.

Strains and Growth Conditions. The lactobacilli strains used in this study were *Lactobacillus sakei* LS8, *Lactobacillus sanfranciscensis* DSM20451^T, a mutant strain of *L. sanfranciscensis* DSM20451 lacking the glutathione reductase gene (*25*), and *Lactobacillus reuteri* TMW1.103. The strains were grown in a modified de Man, Rogosa, Sharpe medium (mMRS) broth containing the following ingredients per liter: 10 g of maltose, 5 g of glucose, 5 g of fructose, 10 g of peptone, 5 g of yeast extract, 5 g of beef extract, 4 g of $K_2HPO_4 \cdot 3H_2O$, 2.6 g of KH_2PO_4, 3 g of NH_4Cl, 0.5 g of L-Cys $HCl \cdot H_2O$, 1 g of Tween 80, 0.05 g of $MnSO_4 \cdot H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.2 mg each of biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin, and panthothenic acid. The pH of media was adjusted to 6.2, and 15 g of agar was added to the recipe for solid media. Additionally, 10 mg of erythromycin was added to the media used for the mutant strain. All of the strains except *L. reuteri* (grown at 37 °C) were grown at 30 °C, and agar−plate incubations took place under modified atmosphere (4% O_2, 20% CO_2, and 76% N_2).

Sourdough Preparation. GRSDs and native-rye sourdoughs (RSDs) were prepared in 50 mL test tubes (with screw caps) by mixing 5.0 g of flour with 2.5 mL of tap water and 5 mL of starter culture. The starter culture was prepared by harvesting cells from overnight cultures in 5 mL of mMRS by centrifugation at 2800*g* at 4 °C for 10 min, washing with tap water, and resuspension in 5 mL of tap water. The chemical control doughs were prepared by mixing 5.0 g of flour with 7.5 mL of tap water containing 200 μL of acid mixture of 100% acetic acid and 85% lactic acid at a ratio of 1:5. In sourdoughs that were supplemented with FITC-secalin, 1 mL of water was replaced with freshly prepared FITC-secalin. The sourdoughs were fermented at the growth temperatures of the starter organisms used, and chemical controls were correspondingly incubated at 30 and 37 °C. After 24 h of fermentation, the pH and total titratable acidity (TTA) were determined as described previously (*10*). Cell counts were determined by plating the sourdough samples on mMRS agar. Sourdough samples were lyophilized for other analyses.

Analysis of Amino Nitrogen. Of the lyophilized sourdoughs, 50 mg was suspended in 1.0 mL of 200 mM sodium phosphate (pH 8.0), extracted at room temperature for 1 h, and centrifuged (10000*g*, 10

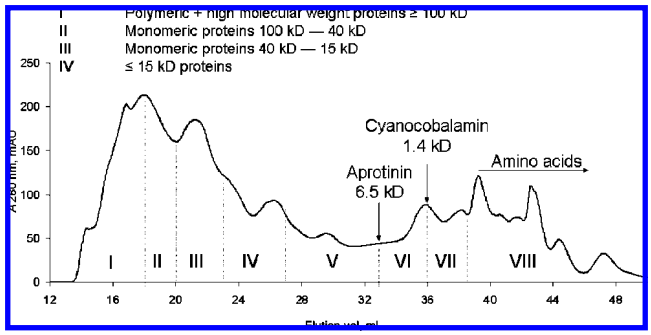

Figure 2. SEC-HPLC system calibration resulted in eight fractions (I−VIII). The chromatogram illustrates elution of proteins present in a secalin isolate. Molecular masses of fractions I−IV are based on their analysis with SDS-PAGE. Arrows show the elution of peptide standards and amino acids.

min). The amino nitrogen concentrations of supernatants were analyzed with a ninhydrin method (*26*). The ninhydrin solution contained 5.0 g of $Na_2HPO_4 \cdot 2H_2O$, 6.0 g of KH_2PO_4, 0.3 g of fructose, and 0.5 g of ninhydrin (Sigma, Oakville, Canada) in 100 mL of deionized water. One hundred microliters of the ninhydrin solution was mixed with 200 μL of the diluted supernatant and incubated at 100 °C for 16 min. After 20 min of cooling at room temperature, 0.5 mL of KIO_3 solution [2.0 g/L of 40% (v/v) ethanol] was added, and the absorbance was measured at 570 nm. Glycine was used as a standard. The statistical significance between each sourdough and the corresponding chemical control dough was determined using Student's *t* test with SigmaPlot software using data from duplicate analyses of two independent fermentations.

Size-Exclusion Chromatography (SEC)−High-Performance Liquid Chromatography (HPLC) Analysis of Sourdough Proteins. The lyophilized sourdoughs were extracted 1:10 with 1.5% SDS and 50 mM sodium phosphate, pH 6.9, at room temperature for 1 h and centrifuged (5000*g*, 20 min). One volume (2.0 mL) of the supernatant was mixed with 1 volume of the elution buffer (0.1% SDS, 20% acetonitrile, and 50 mM sodium phosphate, pH 6.9), filtered (0.45 μm), and analyzed with a SEC system. To separate the proteins and peptides in wide size ranges, SEC columns Superdex Peptide 10/300 GL and Superdex 200 10/300 GL (GE Healthcare Biosciences AB, Uppsala, Sweden) were coupled in series in a model 1200 HPLC system equipped with multiple wavelength and fluorescence detectors (Agilent Technologies, Waldbronn, Germany). The setup was calibrated by analyzing the elution fractions of the isolated secalin with SDS-polyacrylamide gel electrophoresis (PAGE) and by using low molecular mass peptide and amino acid standards: 0.5 mM aprotinin (6.5 kDa), 6.5 mM cyanocobalamin (1.4 kDa), and 1.5 M glycine (75 Da). Calibration distinguished eight fractions (I−VIII) (**Figure 2**) that were grouped based on their molecular masses: proteins >15 kDa (I−III), polypeptides 1.4−15 kDa (IV−VI), and peptides <1.4 kDa and amino acids (VII−VIII). For the sourdough extracts, the injection volume was 50 μL, and the flow rate was 0.5 mL/min. The analysis was performed at room temperature, and proteins were detected at 280 nm. With the FITC-secalin-supplemented sourdough samples, the injection volume was 100 μL, the flow rate was 0.3 mL/min, and the fluorescence was detected at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Prolamin Determination and Quantification. *Immunoblotting.* Sourdough proteins were extracted and analyzed with a Western blot technique using a prolamin specific R5 antibody (*27*). Lyophilized sourdoughs of 100 mg were extracted with a SDS buffer [2% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM tris(hydroxymethyl)aminomethane, 1% (w/v) dithiothreitol, and bromophenol blue, pH 8.5] in a boiling water bath for 2 min and centrifuged. Proteins were separated in NuPage (12%) Bis-Tris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane HCl] gel (Invitrogen, Carlsbad, CA). NP MES SDS running buffer was used with NuPAGE antioxidant. The running conditions were 150 V for 70 min, and a Novex Sharp LC5800 prestained standard (Invitrogen) was used as a molecular marker. After electrophoretic separation, the proteins were transferred (30 V, 60 min) to polyvinylidene fluoride membranes in 25 mM tris(hydroxymethyl)aminomethane, 192 mM glycine, and 20% (v/v) methanol. The membrane was blocked with bovine serum albumin (BSA) and then incubated in the solution of Ridascreen Gliadin horseradish peroxidase-conjugated R5 antibody (R-Biopharm, Darmstadt, Germany) in the dilution buffer of the gliadin assay. Sodium azide and BSA were added to the buffer. After incubation, the membranes were washed and stained with tetramethylbenzidine (Promega, Madison, WI). The extended staining time assured that all of the prolamins were detected.

Sandwich R5 Enzyme-Linked Immunosorbent Assay (ELISA). The prolamin contents of sourdough samples were determined with a commercial kit using the extraction with the Ridascreen Gliadin cocktail solution (R-Biopharm). Of each lyophilized sourdough, 125 mg was incubated with 1.25 mL of the solution at 50 °C for 40 min. After the mixture was cooled at room temperature, 3.75 mL of 80% (v/v) ethanol was added, and the suspension was extracted for 60 min at room temperature. After centrifugation (10000*g*, 10 min), the prolamin concentration of supernatant was determined by following the manufacturer's instructions with the exception that the result was not multiplied by two, because the majority of rye sourdough prolamins are soluble in the extraction conditions. Two different dilutions of sourdough samples from two independent fermentations were each analyzed in duplicate.

Competitive R5 ELISA. The quantification of prolamins was also investigated with a Ridascreen Gliadin Competitive immunoassay (R-Biopharm). The method is based on the same R5 antibody as the sandwich method, but it is developed for the quantitative analysis of protein hydrolysates and peptides. Lyophilized samples (125 mg) were extracted with 1.25 mL of 60% ethanol (v/v), and the obtained peptide concentrations were divided by 250 to convert them to prolamin concentrations as stated in the manufacturer's instructions. Two different dilutions of sourdough samples from two independent fermentations were each analyzed in duplicate.

Pentosan Analysis. Water-soluble polysaccharides were extracted and analyzed. Two grams of lyophilized sourdoughs was extracted with 8 mL of 200 mM Tris-HCl, pH 8.0, at room temperature for 30 min and centrifuged (5100*g*, 20 min). Polysaccharides were precipitated by mixing 4 mL of the supernatant with 9 mL of ice-cold 100% ethanol and incubating the suspension at +4 °C for 3 h. The precipitate was collected by centrifugation (5100*g*, +4 °C, 10 min) and washed with 70% (v/v) ethanol. The pellet was resuspended in 10 mL of deionized water by vortexing and an overnight incubation at +4 °C. The resulting suspension was dialyzed overnight against deionized water using the Spectra/Pro 3 dialysis membrane. The retentate contained the water-soluble polysaccharides and was analyzed on Superdex Peptide 10/300 GL and Superdex 200 10/300 GL SEC columns. The eluent was deionized water at a flow rate of 0.5 mL/min, and polysaccharides were detected with an Agilent 1200 series refractive index (RI) detector. Analyses were performed with samples from two independent fermentations, and the analytical error was 18% or less. For quantification of free arabinose and xylose in the sourdough extracts (200 mM Tris-HCl, pH 8.0), monosaccharides were analyzed with an Aminex HP 87P column (BioRad, Hercules, CA) coupled to the RI detector. The column was eluted with water at a flow rate of 0.4 mL/min and a column temperature of 80 °C. Arabinose and xylose were used as external standards.

Baking Test. To test whether the highly enzymatic sourdoughs have practical relevance in gluten-free baking, an oat baking procedure was tested. An obvious prerequisite for successful baking with germinated rye was to control its high amylase activity by ensuring that the pH of the final dough was lower than 4.5. This was achievable through adjustment of sourdough dosage and by using a second acidification stage. Two hundred grams of germinated-rye or native-rye flour was each mixed with 300 g of tap water and 0.1 g of Florapan L62 *L. brevis* starter culture (Lallemand, Blagnac Cedex, France). Sourdoughs were fermented at 37 °C for 24 h. For baking, 100 g of ripe sourdough or 100 g of appropriately diluted sourdough was mixed with 70 g of Mylly-Matti organic oat flour (Helsingin Mylly, Järvenpää, Finland), 1.5 g of table salt, and 3.0 g of dry baker's yeast; the yeast was fully suspended in 20 mL of ~42 °C tap water. The dough was bulk-fermented for 3 h at 32 °C, 85% relative humidity (RH), molded in a baking pan, proofed for 2 h (32 °C, 85% RH), baked at 200 °C for 20 min, and cooled at

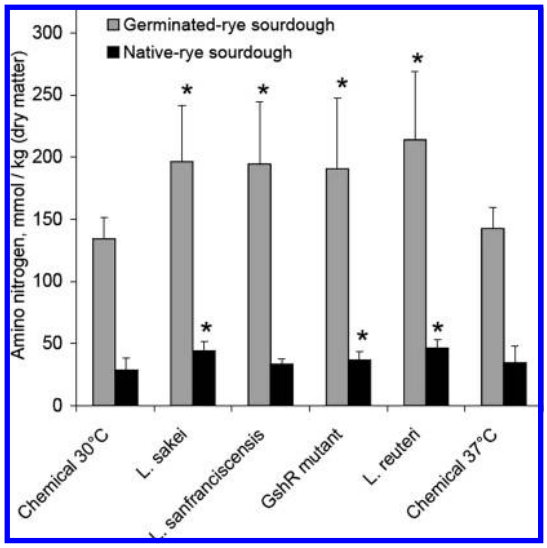

Figure 3. Amino nitrogen levels in sourdoughs determined by a ninhydrin method. Statistically significant differences between the sourdough and the corresponding chemically acidified control (CA) are indicated with an asterisk ($p < 0.05$).

room temperature for 1 h. The observations for crust color and the evaluation of sensory properties were committed subjectively by a panel of five people familiar with rye and gluten-free products.

RESULTS

General Sourdough Parameters. The initial pH values for RSDs and GRSDs were 6.2 and 5.6, respectively. After 24 h of fermentation, the sourdoughs had pH values of 3.7 ± 0.2 and TTA values of 17 ± 2.5 (RSD) and 26 ± 3.5 (GRSD), and the cell counts were (5.0 ± 3.5) × 10^9 cfu/g sourdough. A uniform colony morphology that matched the colony morphology of the inoculum was observed in all sourdough samples, indicating that the inoculated strains dominated the microflora throughout fermentation. The chemically acidified doughs maintained a constant pH of 3.4−3.6 throughout the incubations. The native and germinated grains had initial cell counts of 10^4 and 10^5 cfu/g on mMRS, respectively, and no microbial growth was detected during incubation of chemically acidified doughs.

Amino Nitrogen. The amino nitrogen content is a quantitative measure for the extent of proteolysis in sourdough. The amino nitrogen content of native-rye flour was 13 mmol/kg. In the fermented RSDs and GRSDs, the amino nitrogen contents were 30−50 and 130−210 mmol/kg, respectively (**Figure 3**). The amino nitrogen contents were generally significantly higher in lactobacilli-fermented sourdoughs as compared to the corresponding chemically acidified doughs, and in sourdoughs prepared from germinated rye, the amino nitrogen contents were, on average, 5-fold greater as compared to the corresponding levels in RSDs (**Figure 3**).

SEC Analysis of Rye Proteins. To determine the size distribution of rye proteins and peptides in sourdoughs, SDS-soluble fractions of sourdoughs were analyzed with a SEC-HPLC system and compared to SDS-soluble proteins of unfermented rye. The chromatograms of *L. reuteri* sourdoughs and the corresponding chemically acidified doughs represent typical chromatograms for RSD and GRSD samples (**Figure 4**). Protein elution profiles showed that during the sourdough fermentations the amount of proteins (>15 kDa) substantially decreased, whereas the amount of small hydrolysis products (peptides <1.4 kDa and amino acids) increased (**Figures 4** and **5**). Analysis of the peak areas corresponding to proteins,

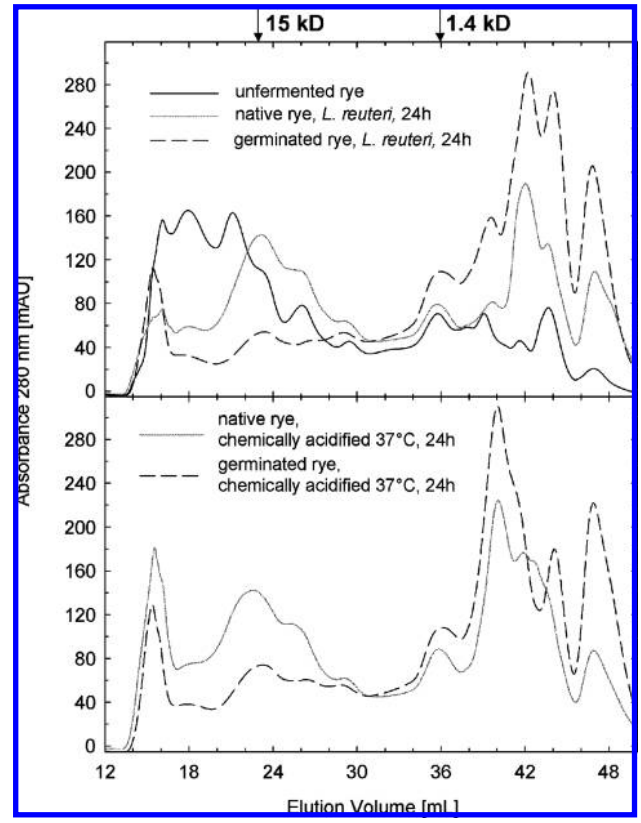

Figure 4. SEC analysis of SDS-soluble proteins. Chromatograms representing unfermented rye and *L. reuteri*-fermented RSD and GRSD are shown in the upper panel, and the corresponding chemically acidified doughs are shown in the lower panel. The results are representative for two independent fermentations.

polypeptides, as well as small peptides and amino acids verified that the hydrolysis of proteins and the formation of hydrolysis products were more extensive in GRSDs as compared to RSDs (**Figure 5**). In unfermented rye, the proteins comprised 46%, whereas in RSD and GRSD, the proportion of proteins was 23−31 and 13−17%, respectively. Corresponding proportions for small hydrolysis products were 25 (unfermented rye), 36−46 (RSD), and 56−66% (GRSD).

Hydrolysis of FITC-Secalin in Sourdoughs. To study the hydrolysis of rye prolamins in sourdoughs, a secalin isolate was prepared and labeled with FITC. SDS-PAGE analyses showed that the labeled and gel-filtered FITC-secalin contained all of the major secalin groups and was substantially free of unbound FITC (**Figure 1**). FITC-secalin was added to sourdough fermentations that were carried out with *L. reuteri* or chemical acidification at 37 °C using flours from native and germinated rye. SDS-soluble extracts of doughs were analyzed with the SEC-HPLC using fluorescence detection (**Figure 6**). The degradation of FITC-secalin was apparent in the RSDs as a shift of fluorescent proteins to lower molecular mass area. The major peak of hydrolysis products eluted at the elution volume of 24 mL, which corresponds to secalin polypetides of 15 kDa. In GRSDs, the majority of fluorescent compounds eluted after 36 mL, corresponding to amino acids and small peptides (<1.4 kDa).

Prolamin Analysis with Immunoblotting. The existence of prolamins in SDS-soluble sourdough fractions was further evaluated by Western blot analyses using a prolamin specific antibody. The R5 antibody identified all of the major secalin groups of unfermented rye (**Figure 7**). RSD fermentations resulted in modified secalin patterns, and smaller prolamin

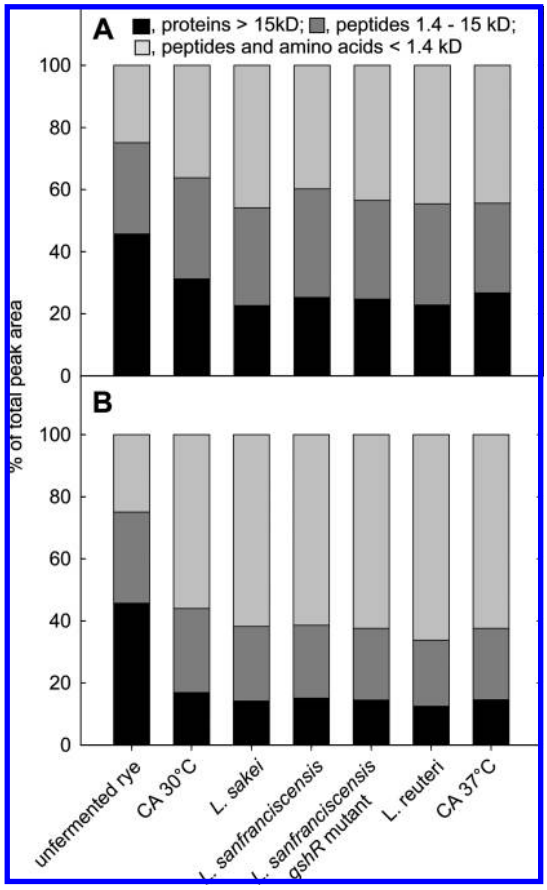

Figure 5. Proportional size distribution of proteins (>15 kDa, black), polypeptides (1.4−15 kDa, dark gray), and small hydrolysis products (peptides <1.4 kDa and amino acids, light gray) in SDS extracts of (**A**) native-rye and (**B**) GRSDs. The results are expressed as percent proportions as compared to the total area. The results are based on SEC results of each sourdough type and are representative for two independent fermentations.

polypeptides appeared. This indicated that the secalin degradation occurred, but still, most of the hydrolysis products cross-reacted with the antibody. In contrast, the secalin hydrolysis appeared highly more extensive in GRSD fermentations, as only a faint protein area was observable in the 70 kDa region.

Prolamin Quantification by Immunoassays. Prolamin contents of sourdoughs were quantified with two enzyme immunoassays designed for prolamin analysis. The prolamin concentrations in fermented RSD samples determined using sandwich and competative assays were 61−95 and 56−98 mg/g, respectively (**Table 1**). The respective prolamin concentrations in fermented GRSD samples were in the ranges of 0.24−0.48 and 0.28−0.43 mg/g. The prolamin content of rye flour determined by the official prolamin method, the sandwich method, was 106 mg/g.

Analysis of Water-Soluble Polysaccharides. The size distribution of water-soluble polysaccharides in sourdoughs was analyzed by SEC (**Figure 8**). RSD fermentation increased the levels of high molecular mass polysaccharides by about 50−70% (**Figure 8A** and data not shown). In contrast, levels of water-soluble polysaccharides increased only slightly during GRSD fermentations (**Figure 8B**). In both sourdough types, the levels of water-soluble polysaccharides were comparable in chemically acidified doughs and sourdoughs (**Figure 8** and data not shown). To verify that the lower levels of water-soluble polysaccharides in GRSDs were attributable to pentosan hydrolysis, the levels of pentose monosaccharides, xylose and

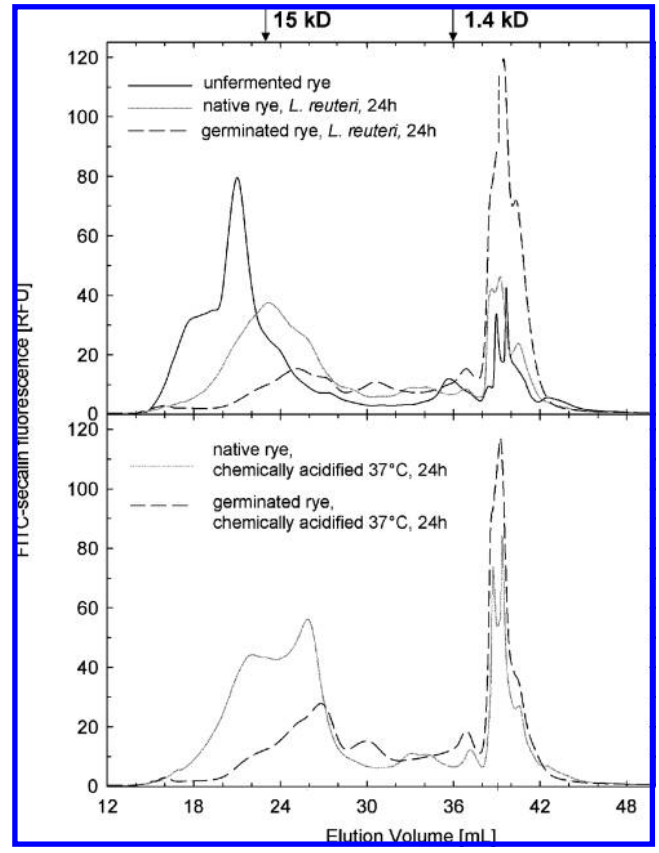

Figure 6. SEC analysis of FITC-secalin-supplemented sourdoughs. SDS extracts from unfermented rye and *L. reuteri*-fermented RSD and GRSD are shown in the upper panel, and the corresponding chemically acidified doughs are shown in the lower panel. Fluorescence was detected at an excitation wavelength of 488 nm and an emission of 530 nm.

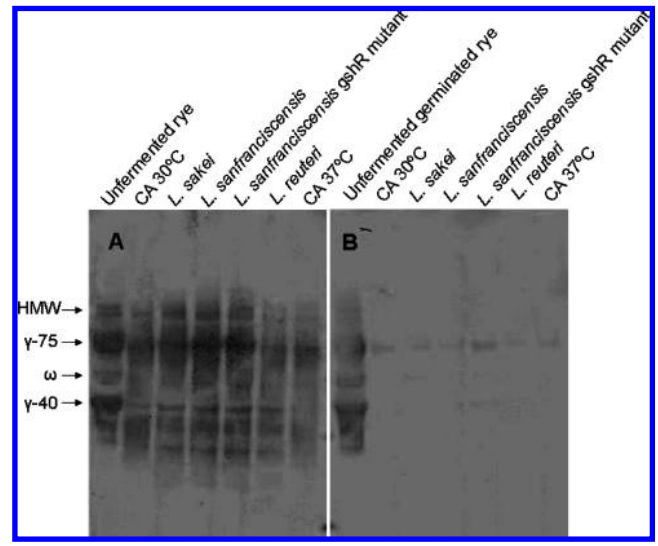

Figure 7. Western blotting analysis of total protein extracts from (**A**) RSD and (**B**) GRSD. Proteins from sourdough samples were blotted against a commercial prolamin R5 antibody. The major secalin groups are indicated on the left.

arabinose, were measured in chemically acidified doughs fermented at 30 °C. Xylose and arabinose levels were below the detection limit (0.25 mM/kg) in unfermented flour and chemically acidified rye dough, but the chemically acidified GRSD contained 5 and 7 mM/kg arabinose and xylose, respectively, corresponding to hydrolysis of 3 g arabinoxylan

Table 1. Prolamin Concentrations of Sourdoughs as Determined by Two Immunoassays

	prolamin concentration (mg/g lyophilized dough)			
	rye sourdough		GRSD	
	sandwich	competitive	sandwich	competitive
unfermented	106 ± 28	85 ± 18	58 ± 15	49 ± 2
chemically acidified, 30 °C	75 ± 11	98 ± 17	0.28 ± 0.21	0.35 ± 0.11
L. sakei	72 ± 13	56 ± 6	0.43 ± 0.34	0.38 ± 0.07
L. sanfranciscensis	95 ± 15	83 ± 17	0.48 ± 0.23	0.38 ± 0.09
L. sanfranciscensis Δ*gshR*	92 ± 22	91 ± 15	0.44 ± 0.17	0.43 ± 0.08
L. reuteri	65 ± 27	69 ± 13	0.24 ± 0.11	0.28 ± 0.05
chemically acidified, 37 °C	61 ± 6	86 ± 18	0.26 ± 0.06	0.32 ± 0.13

per kg flour to monomers. The analysis was not carried out in sourdoughs, as pentoses liberated during fermentation are metabolized by the starter cultures.

Test Baking. The applicability of GRSD in oat baking was tested, and the resulting bread was compared to oat bread prepared using RSD. The specific volumes of breads containing 25% fermented rye of total flour were 1.8 mL/g with both bread types, whereas the baking losses for RSD- and GRSD-based oat breads were 16 and 17%, respectively. The development of brownish color was favored by the use of GRSD (**Figure 9**), which also resulted in softer dough and less gummy bread crumbs as compared to RSD-based oat breads. The use of GRSD added more flavor to bread as compared to RSD, and the panelists described the flavor as being "sour" and "mild rye like". The intensity of brownish color, as well as rye flavor, increased as dosages of GRSD were elevated in the recipes from 15 to 35%.

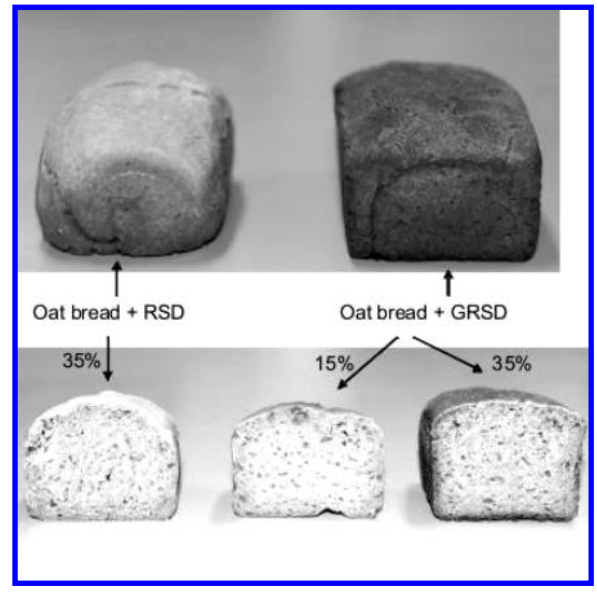

Figure 9. Oat bread was prepared using either rye sourdough or GRSD in the recipe. The percent value indicates the proportion of fermented rye flour of total flour used in the recipes.

DISCUSSION

The rye prolamins were efficiently hydrolyzed in sourdoughs that were prepared using germinated rye. Lyophilized GRSD samples had prolamin concentrations between 0.24−0.48 and 0.28−0.43 mg/g as analyzed by the sandwich-ELISA and competitive-ELISA, respectively (**Table 1**). A comparison of these prolamin concentrations to that of unfermented rye flour (106 mg/g) indicated that more than 99.5% of the rye prolamins were hydrolyzed in GRSDs. As the dry matter content of sourdoughs with dough yield of 250 is ∼35%, the corresponding prolamin concentrations of fresh sourdoughs are 80−170 mg/kg (ppm), and this further dilutes when sourdough is used in baking at varying dosage levels. For instance, ingestion of 100 g of bread with a prolamin content of 50 mg/kg corresponds to a daily intake of 5 mg of prolamin. Generally, the daily gluten amount considered safe for celiac patients lies between 10 and 100 mg (*7*, *8*). Thus, the results from this study indicate that the preparation of breads with low gluten contents is possible when using GRSDs in gluten-free recipes. Whether these "analytically safe" bakery products will prove "clinically safe" as a part of celiac patients' every day diets remains to be solved.

SEC-HPLC analysis of total sourdough proteins and the fate of the fluorescent secalin as well as immunological analyses of prolamins confirmed that in GRSDs the hydrolysis of rye prolamins to peptides and amino acids was extensive. The use of peptide standards and the analysis of elution fractions with SDS-PAGE allowed the estimation of the molecular masses of hydrolysis products. Proteolysis in RSDs produced protein fragments with M_r values around 15 kDa as well as small peptides and amino acids. In GRSDs, the majority of hydrolysis

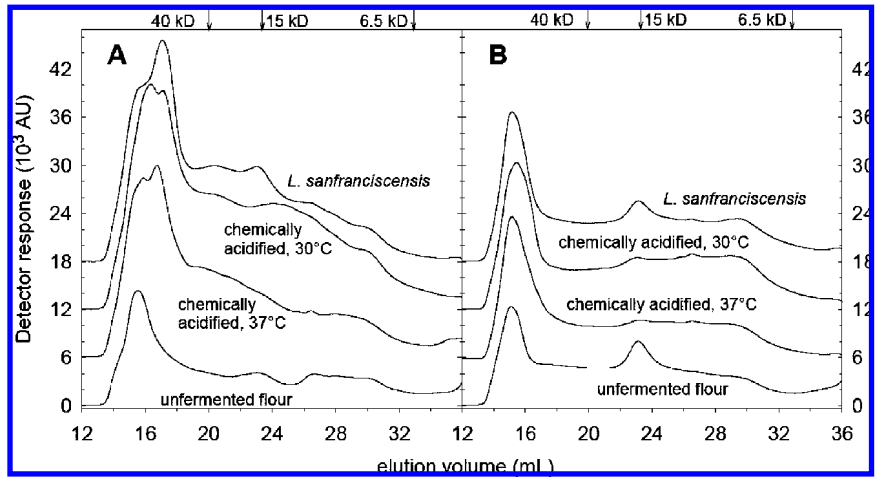

Figure 8. SEC analysis of water-soluble polysaccharides in rye flour and rye sourdoughs. (**A**) Unfermented rye flour, chemically acidified rye doughs incubated at 30 and 37 °C, and rye sourdough fermented with *L. sanfranciscensis*. (**B**) Germinated-rye flour, chemically acidified GRSDs incubated at 30 and 37 °C, and GRSD fermented with *L. sanfranciscensis*. Results are representative for analyses of two independent fermentations, and the analytical error was 18% or less.

products eluted after the 1.4 kDa polypeptide standard, corresponding to small peptides and amino acids. The smallest reported prolamin peptide that has elicited T-cell proliferation is a 10-mer, a peptide composed of 10 amino acids and having a M_r value of 1.2 kDa (*28*), whereas recent studies suggest the immunodominancy of larger polypeptides such as 33-mer (*29*). Thus, on the basis of these considerations, hydrolysis products from prolamin hydrolysis that elute after the 1.4 kDa standard have a good possibility of being nonimmunogenic.

The amino nitrogen and the SEC analyses indicated that sourdoughs acidified with lactobacilli had higher amino nitrogen contents as compared to chemically acidified doughs; this is in agreement with previous observations in wheat sourdough (*30*). On the other hand, the prolamin quantification by immunoassays indicated that the chemical and biological acidification resulted in comparable prolamin contents. The heterofermentative lactobacilli influence the overall proteolysis in wheat sourdoughs by providing reductive activity into the system (*25*, *31*, *32*). In the present study, the lactobacilli had no clear effect on proteolysis, which can be explained by the endogenous protein reduction systems of germinated rye that probably masked the effect of lactobacilli. The fermentation temperature clearly affected proteolysis in chemically and biologically acidified doughs because the activity of proteolytic enzymes is likely to increase when the temperature is elevated from 30 to 37 °C. *L. reuteri* also grows at elevated fermentation temperatures between 40 and 45 °C, which could further accelerate enzyme activity and proteolysis and result in even lower prolamin concentrations.

Arabinoxylans are the predominant water-soluble polysaccharides in rye doughs. This study confirmed that rye arabinoxylans solubilize during RSD fermentations (*13*, *14*). A slight increase of water-soluble polysaccharides was also observed in GRSDs; however, enzyme activities in germinated rye degraded arabinoxylans during fermentation to arabinose and xylose. Comparable to the pentosan levels in RSDs, the lactic starter cultures had little influence on the arabinoxylan levels in GRSDs when compared to the chemically acidified controls. Water-soluble pentosan is a major contributor to the water-binding and gas-retention capacity of rye doughs, and pentosan degradation to monosaccharides thus may decrease the technological functionality of GRSDs as baking improvers. This effect may be offset, however, in baking applications with oats or gluten-free cereals such as sorghum where hemicellulases from germinated rye may contribute to the solubilization of arabinoxylans and other insoluble nonstarch polysaccharides at the dough stage.

Overall, germinated grains are a natural and cheap source of hydrolytic enzymes. The enzyme arsenal present in grains is, by its nature, dedicated to hydrolyze the storage proteins of grain and its cell wall structures. Endogenous enzymes of cereals are among the most effective enzymes also for the hydrolysis of prolamins. This was also recognized by pharmaceutical research as a recombinant barley cysteine proteinase effectively hydrolyzed the 33-mer (*33*). Combining the same recombinant protease with a proline specific enzyme and gastrointestinal proteases elicited an effective hydrolysis of wheat gluten (*34*). This demonstrates that using a pool of enzymes instead of a single enzyme increases hydrolysis. In germinated cereals, a natural pool of enzymes exists. Cysteine and aspartic proteinases are the predominant proteases in germinated rye (*21*), but germinated cereal grains also contain proline specific peptidases (*16*,*35*). This study demonstrated that the endogenous protease pool of germinated rye efficiently hydrolyzed rye prolamins, secalins, in sourdoughs to an extent that may have rendered rye into celiac-safe mode.

In conclusion, this study provides a proof of concept that a combination of germinated rye and sourdough fermentation can be used to hydrolyze prolamins in rye flour to levels that might be tolerated by celiac patients. The use of rye flour with low prolamin content in gluten-free baking has several advantages over other approaches to improve the quality of gluten-free bread: Amino acid accumulation improves flavor and color development in gluten-free baking, rye pentosans and germinated-rye enzymes improve the texture of gluten-free bread, and detoxified rye flour imparts the typical rye flavor to gluten-free breads. The use of GRSD in oat baking confirmed its suitability as a baking improver. The use of whole grain rye with low prolamin contents as a part of bread recipes could improve and diversify the flavor and nutritional properties of low-gluten breads and increase the assortment of cereal products that are designed for gluten-sensitive people. However, because the accuracy of prolamin analyses is unclear in terms of celiac safety, evidence on the clinical safety of products from such technologies is necessary before using such baking improvers in industrial practice.

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GRSD, germinated-rye sourdough; HPLC, high-performance liquid chromatography; kDa, kilodalton; mMRS, modified de Man, Rogosa, and Sharpe; RSD, native-rye sourdough; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; TTA, total titratable acidity.

LITERATURE CITED

(1) Mäki, M.; Mustalahti, K.; Kokkonen, J.; Kulmala, P.; Haapalahti, M.; Karttunen, T.; Ilonen, J.; Laurila, K.; Dahlbom, I.; Hansson, T.; Hopfl, P.; Knip, M. Prevalence of celiac disease among children in Finland. *N. Engl. J. Med.* **2003**, *348*, 2517–2524.

(2) Lohi, S.; Mustalahti, K.; Kaukinen, K.; Laurila, K.; Collin, P.; Rissanen, H.; Lohi, O.; Bravi, E.; Gasparin, M.; Reunanen, A.; Mäki, M. Increasing prevalence of coeliac disease over time. *Aliment. Pharmacol. Ther.* **2007**, *26*, 1217–1225.

(3) Vilppula, A.; Collin, P.; Mäki, M.; Valve, R.; Luostarinen, M.; Krekelä, I.; Patrikainen, H.; Kaukinen, K.; Luostarinen, L. Undetected coeliac disease in the elderly. A biopsy-proven population-based study. *Dig. Liver Dis.* **2008**, *40*, 809–813.

(4) Mäki, M.; Collin, P. Coeliac disease. *Lancet* **1997**, *349*, 1755–1759.

(5) Ivarsson, A.; Persson, L. A.; Nyström, L.; Ascher, H.; Cavell, B.; Danielsson, L.; Dannaeus, A.; Lindberg, T.; Lindquist, B.; Stenhammar, L.; Hernell, O. Epidemic of coeliac disease in Swedish children. *Acta Paediatr.* **2000**, *89*, 165–171.

(6) Codex Alimentarius Commission. Draft Revised Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten; Rome, Italy, ALINORM 08/31/26; Appendix III, pp 50−51.

(7) Hischenhuber, C.; Crevel, R.; Jarry, B.; Mäki, M.; Moneret-Vautrin, D. A.; Romano, A.; Troncone, R.; Ward, R. Review article: Safe amounts of gluten for patients with wheat allergy or coeliac disease. *Aliment. Pharmacol. Ther.* **2006**, *23*, 559–575.

(8) Akobeng, A. K.; Thomas, A. G. Systematic review: Tolerable amount of gluten for people with coeliac disease. *Aliment. Pharmacol. Ther.* **2008**, *27*, 1044–1052.

(9) Gallagher, E.; Gormley, T. R.; Arendt, E. K. Recent advances in the formulation of gluten-free cereal-based products. *Trends Food Sci. Technol.* **2004**, *15*, 143–152.

(10) Loponen, J.; Sontag-Strohm, T.; Venäläinen, J.; Salovaara, H. Prolamin hydrolysis in wheat sourdoughs with differing proteolytic activities. *J. Agric. Food Chem.* **2007**, *55*, 978–984.
(11) Rizello, C. G.; De Angelis, M.; Di Cagno, R.; Camarca, A.; Silano, M.; Losito, I.; De Vincenzi, M.; De Bari, M. D.; Palmisano, F.; Maurano, F.; Gainfrani, C.; Gobbetti, M. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: New perspectives for celiac disease. *Appl. Environ. Microbiol.* **2007**, *73*, 4499–4507.
(12) Vinkx, C. J. A.; Delcour, J. A. Rye (*Secale cereale* L.) arabinoxylans: A critical review. *J. Cereal Sci* **1996**, *24*, 1–14.
(13) Boskov Hansen, H.; Andreasen, M. F.; Nielsen, M. M.; Larsen, L. M.; Back Knudsen, K. E.; Meyer, A. S.; Christensen, L. P.; Hansen, Å. Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread-making. *Eur. Food Res. Technol.* **2002**, *214*, 33–42.
(14) Korakli, M.; Rossmann, A.; Gänzle, M. G.; Vogel, R. F. Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *Lactobacillus sanfranciscensis*. *J. Agric. Food Chem.* **2001**, *49*, 5194–5200.
(15) Hansen, Å.; Schieberle, P. Generation of aroma compounds during sourdough fermentation: Applied and fundamental aspects. *Trends Food Sci. Technol.* **2005**, *16*, 85–94.
(16) Mikola, L. Acid carboxypeptidases in grains and leaves of wheat, *Triticum aestivum* L. *Plant Physiol.* **1986**, *81*, 823–829.
(17) Dunaevsky, Y. E.; Sarbakanova, S. T.; Belozersky, M. A. Wheat seed carboxypeptidase and joint action on gliadin of proteases from dry and germinating seeds. *J. Exp. Bot.* **1989**, *40*, 1323–1329.
(18) Dominguez, F.; Cejudo, F. J. Pattern of endoproteolysis following wheat grain germination. *Physiol. Plant.* **1995**, *95*, 253–259.
(19) Mikola, M.; Brinck, O.; Jones, B. L. Characterization of at endoproteinases that hydrolyze oat avenins. *Cereal Chem.* **2001**, *78*, 55–58.
(20) Jones, B. L. Endoproteases of barley and malt. *J. Cereal Sci.* **2005**, *42*, 139–156.
(21) Brijs, K.; Trogh, I.; Jones, B. L.; Delcour, J. A. Proteolytic enzymes in germinating rye grains. *Cereal Chem.* **2002**, *79*, 423–428.
(22) Gänzle, M. G.; Loponen, J.; Gobbetti, M. Proteolysis in sourdough fermentations: Mechanisms and potential for improved bread quality. *Trends Food Sci. Technol.* **2008**, *19*, 513–521.
(23) Lindsay, M. P.; Tamas, L.; Appels, R.; Skerritt, J. H. Direct evidence that the number and location of cysteine residues affect glutenin polymer structure. *J. Cereal Sci.* **2000**, *31*, 321–333.
(24) Thiele, C.; Gänzle, M. G.; Vogel, R. F. Fluorescence labeling of wheat proteins for determination of gluten hydrolysis and depolymerization during dough processing and sourdough fermentation. *J. Agric. Food Chem.* **2003**, *51*, 2745–2752.
(25) Jänsch, A.; Korakli, M.; Vogel, R. F.; Gänzle, M. G. Glutathione reductase from *Lactobacillus sanfranciscensis* DSM20451^T: Contribution to oxygen tolerance and thiol exchange reactions in wheat sourdoughs. *Appl. Environ. Microbiol.* **2007**, *73*, 4469–4476.
(26) ASBC. Free amino nitrogen (international method). In *Methods of Analysis of the American Society of Brewing Chemists*, 8th ed.; Technical and Editorial Committees of the ASBC; American Society of Brewing Chemists: St. Paul, MN, 1992; Wort-12.
(27) Osman, A. A.; Uhlig, H. H.; Valdes, I.; Amin, M.; Méndez, E.; Mothes, T. A monoclonal antibody that recognizes a potential coeliac-toxic repetitive pentapeptide epitope in gliadins. *Eur. J. Gastroenterol. Hepatol.* **2001**, *13*, 1–5.
(28) Sjöström, H.; Lundin, K. E.; Molberg, O.; Ḱorner, R.; McAdam, S. N.; Anthonsen, D.; Quarsten, H.; Norén, O.; Roepstorff, P.; Thorsby, E.; Sollid, L. M. Identification of a gliadin T-cell epitope in coeliac disease: General importance of gliadin deamidation for intestinal T-cell recognition. *Scand. J. Immunol.* **1998**, *48*, 111–115.
(29) Shan, L.; 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* **2002**, *297*, 2275–2279.
(30) Vermeulen, N.; Pavlovic, M.; Ehrmann, M. A.; Gänzle, M. G.; Vogel, R. F. Functional characterization of the proteolytic system of *Lactobacillus sanfranciscensis* DSM 20451^T during growth in sourdough. *Appl. Environ. Microbiol.* **2005**, *71*, 6260–6266.
(31) Vermeulen, N.; Kretzer, J.; Machalitza, H.; Vogel, R. F.; Gänzle, M. G. Influence of redox-reactions catalysed by homo- and heterofermentative lactobacilli on gluten in wheat sourdoughs. *J. Cereal Sci.* **2006**, *43*, 137–143.
(32) Loponen, J.; König, K.; Wu, J.; Gänzle, M. G. Influence of thiol metabolism of lactobacilli on egg white proteins in wheat sourdoughs. *J. Agric. Food Chem.* **2008**, *56*, 3357–3362.
(33) Bethune, M. T.; Strop, P.; Tang, Y.; Sollid, L. M.; Khosla, C. Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endoprotease. *Chem. Biol.* **2006**, *13*, 637–647.
(34) Siegel, M.; Bethune, M. T.; Gass, J.; Ehren, J.; Xia, J.; Johannsen, A.; Stuge, T. B.; Gray, G. M.; Lee, P. P.; Khosla, C. Rational design of combination enzyme therapy for celiac sprue. *Chem. Biol.* **2006**, *13*, 649–658.
(35) Simpson, D. J. Proteolytic degradation of cereal prolamins—The problem with proline. *Plant Sci.* **2001**, *161*, 825–838.

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