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Prolamin Hydrolysis in Wheat Sourdoughs with Differing Proteolytic Activities

JUSSI LOPONEN,*,† TUULA SONTAG-STROHM,† JARKKO VENÄLÄINEN,§ AND HANNU SALOVAARA†

Cereal Technology Group, Department of Food Technology, University of Helsinki, Finland, and Department of Pharmacology and Toxicology, University of Kuopio, Finland

The prolamins of wheat, rye, and barley contain structures that are harmful to gluten-sensitive people, and an extensive degradation of these prolamins during food processing might eliminate this problem. Sourdough fermentation is a cereal food process during which some protein degradation occurs. In this study, the prolamin hydrolysis that occurred in a high-proteolytic-activity germinated-wheat sourdough (GWSD) was compared with that of wheat sourdough systems which contained moderate or no proteolytic activities. Virtually all of the wheat prolamins (gliadins and glutenins) were degraded during the GWSD fermentation. Quantification of its prolamin levels confirmed that extensive prolamin hydrolysis had occurred in the GWSD. This hydrolysis was attributed to the cysteine proteinase activities of the germinated wheat. The use of high-proteolytic sourdoughs in baking could make it possible to prepare new low-prolamin cereal-based products for use by gluten-sensitive people, who could then diversify their diets by including these whole grain containing products into their every day diets.

KEYWORDS: Prolamin; hydrolysis; wheat; sourdough; proteolysis; gluten sensitivity; germination; proteases

INTRODUCTION

Prolamins are the causal factor in celiac disease (CD). In CD the ingestion of prolamin-containing food leads to the atrophy of the small intestinal villi. The current opinion is that certain polypeptide structures present in the prolamins of wheat (gliadins, glutenins), rye (secalins), and barley (hordeins) are the causal epitopes in CD. These prolamins are, in the context of CD, included together under the term gluten, and celiac patients are often referred to as gluten-sensitive people. Certain of the polypeptide types that occur in the primary structures of wheat gliadins apparently possess celiac-toxic properties (*1*−*3*). It has been proposed that some of these harmful gluten proteins could be detoxified by enhancing their hydrolysis during food processing (*4*−*6*).

Previously, we monitored and explained the degradation of high molecular weight (HMW) glutenins and secalins in traditional wheat and rye sourdoughs, respectively (*7*, *8*). In these systems protein degradation was evident, and the endogenous aspartic proteinases of the component cereals were responsible for this proteolytic breakdown. A critical interpretation of our previous results with wheat sourdoughs, however, revealed that only little hydrolysis of the gliadin proteins occurred in these sourdoughs (*7*). This was probably due to the inability of the aspartic proteinases to extensively hydrolyze gliadins (*9*, *10*). Evidence from wheat germination studies, however, has indicated that the cysteine proteinases of germinating wheats can efficiently degrade the gliadins (*11*, *12*). In gliadin hydrolysis, the wheat cysteine proteinases operated between pH 3.5 and 5.5 (*12*), a pH range that would be ideal for their hydrolyzing the proteins in sourdoughs. The germination of cereal grains also induces the synthesis of numerous other proteinases (*13*) and peptidases, including those that specifically hydrolyze proline-containing substrates (*14*−*16*). Sourdoughs are pH-dynamic systems and thus offer a wide pH range over which diverse proteolytic enzymes that have different pH optima can operate effectively. These premises encouraged us to test the utilization of germinated wheat, with its high and diverse proteolytic activities, in sourdoughs. It seemed likely that this might result in an extensive hydrolysis of the wheat prolamins, and especially of the gliadins. To our knowledge no studies using germinated wheat as a raw material in sourdough fermentations had been carried out previously. This study aimed to evaluate the extent of prolamin hydrolysis during a germinated-wheat sourdough (GWSD) fermentation.

MATERIALS AND METHODS

Wheat Grain Germination and Drying. To select the cereal raw material for the GWSD fermentation, two batches of germinated wheat grains were prepared and their proteolytic activities determined. The

* Address correspondence to this author at the Department of Food Technology, P.O. Box 66, FI-00014 University of Helsinki, Finland (e-mail jussi.loponen@helsinki.fi).
† University of Helsinki.
§ Universityof Kuopio.

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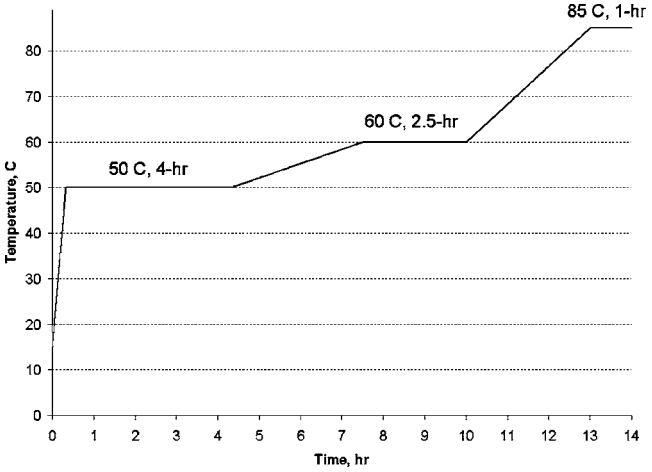

Figure 1. Germinated wheat grains were dried with a typical pilsner-drying procedure in which the temperature was sequentially raised to 85 °C during a 14 h drying period.

term "germination" here refers to the process used by the brewing industry, rather than to the true biological germination.

Wheat grains (*Triticum aestivum* L. cv. Kruunu) were obtained from Boreal Plant Breeding Ltd. (Jokioinen, Finland). The grains were germinated in 6 × 800 g batches in commercial pilot malting equipment (Joe White Malting Systems, Melbourne, Australia) in darkness at the Technical Research Centre of Finland (VTT, Espoo, Finland). The final germination process was selected according to the results of preliminary trials, and its details were as follows. The grains were steeped at 15 °C for 48 h (16 h wet steep, 8 h air rest, 8 h wet, 15 h air, 1 h wet); this resulted in grains with a moisture content of ∼47% (w/w). The steeped grains were germinated at 15 °C for 3, 5, or 8 days. Each of these samples was divided into two portions, of which one underwent pilsner-drying (PD) and the other was freeze-dried (FD). In PD the temperature of the malt was sequentially raised from 50 to 85 °C during a drying period of 14 h (**Figure 1**). Air was passed through the sample throughout the drying.

Proteolytic Activities. To select the raw material that had high levels of diverse proteolytic activities for the GWSD fermentation, the general proteolytic activities of the collected germination samples were determined using a modified azogelatin method (*17*). In addition, the activities of two proline-specific enzymes, prolyl oligopeptidase (POP) and dipeptidyl-peptidase IV (DPP IV), were measured by using specific 7-amino-4-methylcoumarin (AMC) substrates: succinyl (SUC)-Gly-Pro-AMC for POP and H-Gly-Pro-AMC for DPP IV. On the basis of the results from these protease assays, a mixture of 8-day-germinated grains was used in the GWSD fermentations. The grain mixture contained equal proportions (volumes) of PD and FD samples of 8-day-germinated grains. A proteinase enzyme extract was prepared from this grain mixture (*18*), and its component protease profile was determined by using class-specific proteinase inhibitors as described earlier (*7*).

Preparation of Enzyme Extracts. The enzyme extracts used for the azogelatin and azocasein protease assays (see below) were prepared with a modified extraction procedure (*18*). The tested wheat grains were ground with a cyclone sample mill (Tecator Cyclotec 1093, Höganäs, Sweden) fitted with a 0.5 mm screen. Eight grams of the meal was suspended in 24 mL of chilled 0.1 M sodium acetate, pH 4.7, buffer in a 50 mL centrifuge tube. The suspension was incubated at 5 °C in a shaker for 35 min and centrifuged at 5 °C (12000*g*, 20 min). The supernatant was filtered through a 0.45 μm syringe filter (HPF Millex filter with PVDF membrane, Millipore, Billerica, MA), and the filtrate obtained was dialyzed (Spectra/Pro 3, Spectrum Laboratories Inc., Rancho Dominguez, CA) overnight against 5 L of 5 mM sodium acetate, pH 4.7, buffer at 5 °C. The dialyzed enzyme extracts were collected and stored at −20 °C. Immediately before it was used for analyses, the thawed enzyme extracts were centrifuged (11000*g*, 2 min) to remove the residual haziness. The protein contents of the enzyme extracts were determined with a protein assay (Bio-Rad Labs, Hercules, CA) that was based on the Bradford method (*19*), and bovine serum albumin was used as a standard.

Table 1. Classification of the GWSD Proteinases[a]

inhibitor	inhibition target	reaction concentration[b]	inhibition %[c]
	none		0
PEP-A	aspartic proteinases	20 μM	3
PMSF	serine proteinases	10 mM	14
E-64	cysteine proteinases	10 μM	70
O-FEN	metalloproteinases	1 mM	7

[a] Inhibitor studies with the mixture of germinated grains that was used for the GWSD fermentations. Azocasein was hydrolyzed with the enzymes in the presence of class-specific proteinase inhibitors. [b] Concentrations are based on the previous work of Zhang and Jones (*20*). The concentrations of the inhibitor stock solutions used were 2, 500, 1, and 100 mM for PEP-A, PMSF, E-64, and *O*-FEN, respectively. [c] Inhibition % = 100% − ($A440_{sample}/A440_{no\ sample}$) × 100%.

For the DPP IV and POP activity assays, grains were homogenized (3 × 20 s) in 4 volumes of 0.05 M sodium acetate, pH 5.0, buffer by using a Polytron PT 1200 homogenizer at a speed of 25000 rpm (Kinematica AG, Littau-Lucerne, Switzerland). The homogenates were centrifuged at 4 °C (10000g, 20 min), and aliquots of the supernatants were stored at −70 °C.

Azogelatin Hydrolysis. To compare the general proteolytic activities of germinated seed samples, azogelatin was hydrolyzed with the enzyme extracts at pH values of 4.2, 4.6, and 5.4. In this modified method (*17*) we prepared a 2% (w/v) substrate solution by dissolving lyophilized azogelatin in 100 mM sodium acetate buffers. The substrate solution (stored at 5 °C) was warmed to 40 °C before being used in the experiments. Each reaction mixture included 100 μL of appropriate acetate buffer (100 mM, pH 3.5−5.0) and 100 μL of the enzyme extract being studied. The mixtures were incubated for 5 min at 40 °C before the reactions were started by adding 300 μL of the substrate solution. The reaction took place at 40 °C in a water bath for 30 min. After this incubation, the reaction was terminated by adding 700 μL of 25% trichloroacetic acid (TCA) to the reaction mixture. The stopped reaction mixture was incubated in an ice−water bath for 15 min before being centrifuged at 5 °C (11000*g*, 10 min), and the absorbance of each supernatant was read at 440 nm against a reaction blank. The reaction blanks were prepared by adding the TCA to the enzyme extract immediately before the azogelatin was added.

Azocasein Hydrolysis in the Presence of Class-Specific Proteinase Inhibitors. The proteinase activity of the grain mixture that was used in the GWSD preparation (see below) was profiled by adding class-specific proteinase inhibitors to azocasein hydrolyses as described earlier (*7*). The azocasein substrate solution was prepared by following the manufacturer's instructions (Megazyme International Ireland Ltd., Co. Wicklow, Ireland), and 100 mM sodium acetate, pH 4.5, buffer was used. Appropriate volumes (5 or 10 μL) of stock solutions of inhibitors were mixed with 200 μL of the enzyme extracts 5 min prior to the start of the reactions. The reactions were initiated by mixing 200 μL of the azocasein solution with the enzyme−inhibitor mixtures; in the reactions the effective pH was 4.9. The inhibitors used and their reaction concentrations were selected on the basis of previous work (*20*) (**Table 1**). The reaction mixtures were incubated at 40 °C for 60 min, after which 1.2 mL of 5% TCA was added to terminate the reactions. The linearity of the absorbance development in these reactions was checked and was linear throughout the 60 min incubations. After 5 min of cooling, the terminated reaction mixture was centrifuged (11000*g*, 10 min), and the absorbance of the supernatant was measured against the reaction blank at 440 nm. The inhibitory effect of methanol (a solvent used with inhibitors other than water-dissolved E-64) was also tested, and no effect was found.

Determination of POP and DPP IV Activities. The POP and DPP IV activities were measured using the specific substrates SUC-Gly-Pro-AMC (*21*) and H-Gly-Pro-AMC (*22*), respectively. In both cases the linearity of the 2 h reactions was confirmed before the hydrolytic activities were measured. The POP activities were determined using a modified method reported previously (*23*). Briefly, 20 μL of the grain

homogenate was preincubated with 455 μL of 0.1 M sodium−potassium phosphate, pH 7, buffer for 30 min at 30 °C. The reaction was initiated by adding 25 μL of 4 mM SUC-Gly-Pro-AMC, and the samples were incubated at 30 °C for 2 h. The reaction was terminated by the addition of 500 μL of 1 M sodium acetate, pH 4.2, buffer and the formation of AMC was measured with a Victor2 fluorescence plate reader (Perkin-Elmer Inc., Wellesley, MA) at excitation and emission wavelengths of 355 and 460 nm, respectively.

The DPP IV activities were determined using the same procedure as for POP, but the DPP IV assay used 25 μL of 2 mM H-Gly-Pro-AMC as the substrate.

Sourdough Fermentations. *Sourdough Recipes and Fermentations.* Two separate sourdough fermentations were conducted: a control sourdough (CSD) and a germinated-wheat sourdough (GWSD). In addition, an inactive sourdough-like ferment (ISD) was prepared as a negative control. Nongerminated wheat grains (*Triticum aestivum* L. cv. Kruunu) were used in the preparation of CSD. For the preparation of GWSD, equal proportions (by volume) of freeze-dried and pilsner-dried samples of 8-day-germinated grains were pooled. The ISD was prepared with whole-meal wheat flakes. All grains and flakes were ground by using the cyclone sample mill equipped with a 0.5 mm screen (see above). To prepare the CSD and GWSD, 100 g of the respective meals was carefully mixed with 160 g of tap water. In the case of ISD, 100 g of the ground flakes was put into boiling water (∼500 mL) to which 2 mL of heat-stable α-amylase (Termamyl 120L, Novozymes A/S, Bagsvaerd, Denmark) had previously been added. The purpose of the boiling was to ensure that the wheat suspension contained no cereal-derived proteolytic activities, and the amylase-treatment was aimed at producing a sourdough structure that was comparable to those of the other two sourdoughs (the hydrolysis of the starch prevented its gelatinization). The ISD mixture was then boiled for 30 min to liquefy the starch, after which the excess water was evaporated by boiling until the weight of the mixture corresponded to those of the CSD and GWSD (260 g). Ten grams of commercial fructose (Fruisana, Danisco Sweeteners Ltd., Kotka, Finland) was then added to each meal−water suspension.

To start the fermentations, 5 mL of an overnight culture of *Lactobacillus brevis* L62 (Florapan L62, Lallemand SA, Blagnac Cedex, France) was added. This culture was prepared by cultivating 20 mg of a freeze-dried starter culture in 300 mL of MRS broth (de Man, Rogosa, Sharpe, Merck, Darmstadt, Germany) at 37 °C for 20 h. This suspension was then centrifuged (1000*g*, 10 min), and the harvested cells were washed with tap water and again harvested by centrifugation. Finally, the washed and reharvested cells were suspended in 40 mL of tap water, and 5 mL of this suspension was used to inoculate each sourdough. The inoculated sourdoughs were incubated in a water bath at 34 °C for 24 h. Samples to be used for later protein extractions were collected from the CSD and GWSD after 0, 6, 12, and 24 h of fermentation, and from ISD at the beginning and the end of the fermentation. All samples were immediately frozen and stored at −20 °C until used for protein extractions.

Sourdough Fermentation Parameters (pH, TTA, cfu). The progress of the sourdough fermentations was followed to confirm that they proceeded as expected. The total titratable acidity (TTA) and pH values of collected sourdough samples were determined from a separate 10 g sourdough sample that was suspended in sterile water, homogenized for 30 s with a bar blender, and titrated with 0.1 N NaOH to pH 8.5 (the TTA value is the milliliters of NaOH that is consumed while the suspension is stabilized to pH 8.5). The concentrations of the lactic acid bacteria in 24 h fermented sourdoughs were determined by cultivating the diluted sourdough samples on MRS agar (Merck, Darmstadt, Germany) plates for 72 h under anaerobic conditions (BBL GasPak Kit, BD Diagnostic Systems, Sparks, MD).

Protein Extraction and Analysis. To follow the progression of the protein degradation in sourdoughs, a sequential protein extraction procedure based on Osborne's classification system (*24*) was performed on the sourdough samples, and the extracts obtained were analyzed with SDS-PAGE (*25*). The extent of protein hydrolysis was evaluated by measuring the free amino nitrogen (FAN) contents of the sourdough extracts by a ninhydrin method (*26*). For more precise determinations, the extent of prolamin hydrolysis during the GWSD and CSD fermentations was monitored by using an ELISA method that was designed for measuring prolamins (*27*).

SDS-PAGE. A sequential protein extraction consisting of three steps was conducted on the sourdough samples. In the first step, each 5 g sourdough sample was neutralized with 15 mL of appropriately diluted 0.1 N NaOH; the amount of 0.1 N NaOH required was calculated as being half of the TTA value of the sample (the TTA was determined for a 10 g sample). To the neutralized suspension was added 20 mL of 0.1 M disodium hydrogen phosphate, pH 8.0, buffer, and the resulting suspension was incubated for 1 h with continuous shaking at room temperature (RT = 21 °C). After centrifugation at RT (12000*g*, 15 min), the supernatant containing the albumins was frozen. For the second extraction step, 35 mL of 55% 1-propanol was poured onto the sediment and mixed into a suspension. The suspension was incubated for 1 h at 50 °C (with continuous shaking) and centrifuged as above. The supernatant obtained contained the alcohol-soluble proteins and was stored at 5 °C. In the third extraction step the sediment was extracted with SDS sample buffer containing 1% dithiothreitol for 1 h at 50 °C with continuous shaking. After centrifugation, the supernatant that contained the SDS-soluble proteins was frozen.

FAN. The FAN contents of the albumin extracts were measured by using the ninhydrin method (*26*). The albumin extract samples were diluted, and their dilution factors varied from 5 to 100, depending on their FAN contents. Of each of the diluted samples, 400 μL was heated with the ninhydrin color reagent (200 μL) for 16 min at 100 °C. After 20 min of cooling in a water bath at 20 °C, 1.0 mL of the dilution solution was added, and the absorbance of the solution was read at 570 nm against a reaction blank. Glycine solution (2 mg of FAN/L) was used as a standard, and deionized water was used in preparing the reaction blank. The average absorbance values obtained on measuring triplicate reaction samples were used in all calculations.

Prolamin Quantification with an ELISA Method. To obtain more specific information about the course of the prolamin degradation that occurred during the sourdough fermentations, the prolamin contents of sourdough samples were determined with a commercial ELISA kit following the manufacturer's instructions (Transia Plate *Prolamins*, Raisio Diagnostics, Finland). For the assay, a separate propanol extract was prepared by extracting the straight sourdoughs with 55% 1-propanol. The separate extraction was necessary because some of the prolamin hydrolysis products might have been rendered more soluble during the hydrolysis. Using the straight extraction ensured that these were also included in the analyzed propanol extract. One gram of each sourdough sample was extracted with 7.0 mL of propanol (1 h, 50 °C). The extracts were analyzed immediately, and calculations were made following the manufacturer's instructions.

RESULTS

Proteolytic Activities of the Germinated Samples. To select the raw material to be used in the GWSD fermentation, the proteolytic activities of germinated grain samples were determined (**Figures 2** and **3**). The azogelatin hydrolyses determined the general proteolytic activities at three pH values. The 8-day-PD sample had the highest proteolytic activity under each of the tested conditions (**Figure 2**). Despite the 8-day-FD sample having a lower azogelatin hydrolytic activity than the 8-day-PD sample, its activities with the POP and DPP IV substrates were higher (**Figure 3**). On the basis of these results, we prepared a grain mixture that included equal portions (by volume) of the 8-day-PD and 8-day-FD germination samples. The proteolytic profiling of this mixture showed that members of the cysteine proteinase group were the predominant proteinases in the grain mixture, as they accounted for roughly 70% of the total proteinase activity (**Table 1**). In addition, the mixture contained some serine, aspartic, and metalloproteinase activities (**Table 1**). On the basis of these results we decided to use this mixture of germinated wheat grains as the raw material for the GWSD.

Sourdough Fermentation. Sourdough Parameters (TTA, pH, cfu). All three fermentations proceeded as expected. The

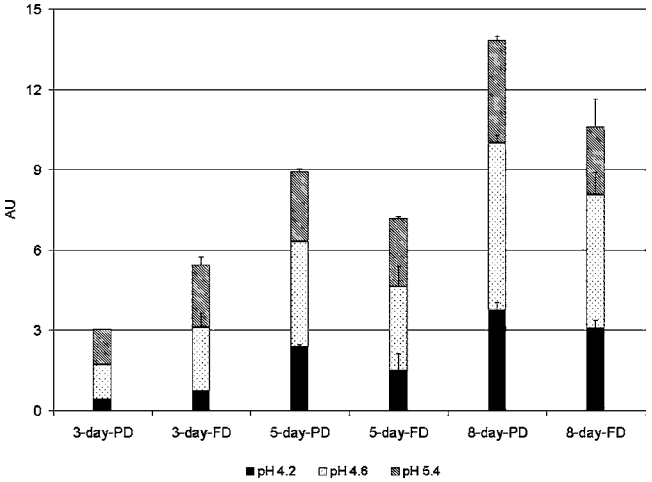

Figure 2. Azogelatin hydrolysis by pilsner-dried and freeze-dried germinated-wheat extracts at pH values of 4.2, 4.6, and 5.4. A cumulative presentation of the increase in the general proteolytic activity of the germinated grain samples versus 0-day samples (PD, pilsner-dried; FD, freeze-dried). The arbitrary proteolytic activity units (AU) were calculated from the equation AU = $[(A440_{sample} - A440_{0-day-sample})/30\ min \times$ protein content (mg/mL)$] \times 1000$. The standard deviation calculations are for the two germination batches.

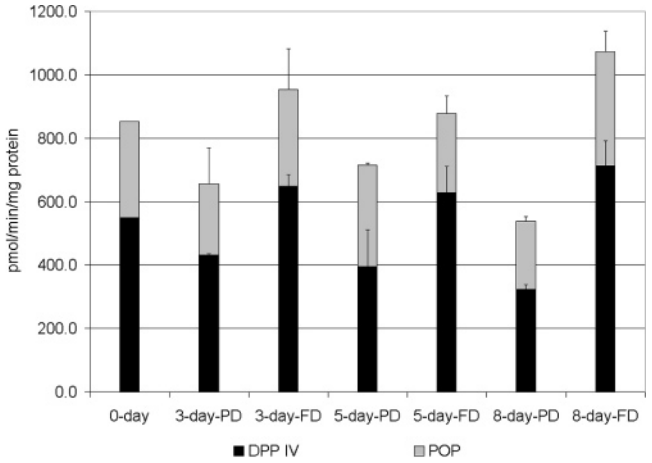

Figure 3. DPP IV and POP activities of the germinated grain samples (PD, pilsner-dried; FD, freeze-dried). The standard deviation calculations are for the two germination batches.

TTA values of 28 for GWSD and 20 for CSD indicated that there was a strong production of acid during the fermentations, whereas the corresponding ISD TTA value was only 8 (**Figure 4**). The pH values showed that the GWSD and CSD fermentations acidified normally, whereas the ISD acidification was slower (**Figure 4**). The concentrations of lactobacilli (determined by MRS-agar plates) in the CSD, GWSD, and ISD were 1×10^9, 2×10^9, and 4×10^8 cfu/g of sourdough, respectively, after 24 h of fermentation.

Protein Degradation during Sourdough Fermentations. *FAN.* To generally evaluate the extent of protein hydrolysis during the sourdough fermentations, we measured the development of their FAN concentrations in their albumin fractions (**Figure 5**). The FAN value is a parameter that is commonly used in brewing technology to measure the concentration of small proteinaceous compounds (peptides and free amino acids) that are important for yeast growth and that are formed during protein hydrolysis. It is assumed that the FAN value gives a good indication of the extent of the overall proteolysis that has occurred, not only during mashing but also during the sourdough

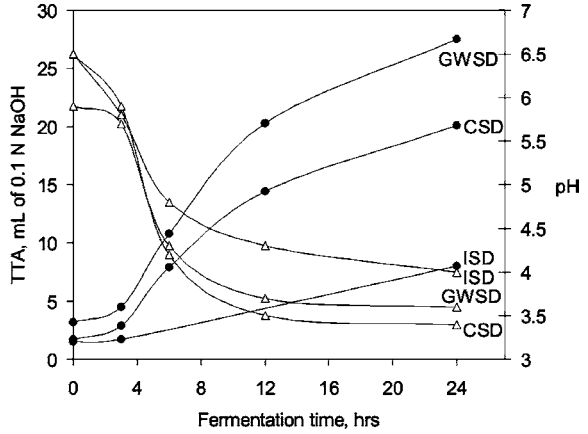

Figure 4. Development of the TTA (●) and pH (△) values during the 24 h fermentations of GWSD, CSD, and ISD.

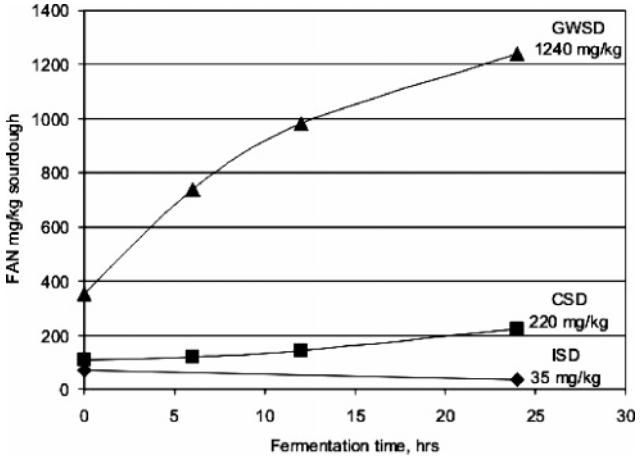

Figure 5. Development of the free amino nitrogen concentrations of the albumin fractions during the three sourdough fermentations. The label at the right-hand end of each curve shows the FAN values of the corresponding 24 h samples. Each sample had a standard deviation of ≤2% within triplicate measurements.

fermentation. The formation of soluble protein hydrolysis products was superior in the GWSD fermentation, as its FAN value was >1200 mg/kg of sourdough, whereas during the 24 h fermentation of CSD the FAN level only doubled, from 110 to 220 mg/kg, and in the ISD the amount of FAN actually decreased (**Figure 5**).

SDS-PAGE. SDS-PAGE analysis of protein extracts showed that the hydrolysis of wheat proteins was extensive in the GWSD (**Figure 6**). The proteins that were present in the alcohol-soluble and SDS-soluble fractions at the beginning of the GWSD fermentation underwent an especially extensive hydrolysis, resulting in the virtual disappearance of all of the proteins from their respective gels (**Figure 6B**,**C**). This extensive hydrolysis took place soon after the beginning of the GWSD fermentation, with no protein bands being observable in the alcohol-soluble fraction after 6 h of GWSD fermentation (**Figure 6B**). The only wheat proteins that were detectable at the end of the GWSD fermentation were those in the albumin fraction (**Figure 6A**; horizontal arrows).

During the 24 h CSD fermentation the HMW glutenins were evidently effectively degraded, whereas some gliadins and low molecular weight (LMW) glutenins were still observable (**Figure 6B**,**C**; horizontal arrows). Prior to their degradation, the HMW and, apparently, LMW glutenins underwent depolymerization, as these glutenin proteins had shifted from the

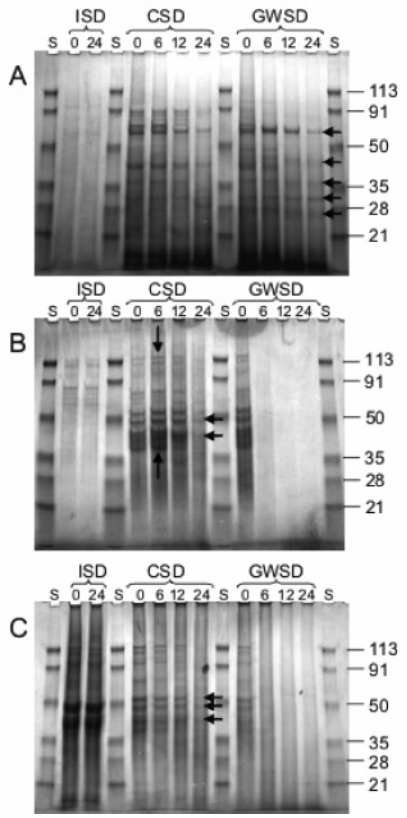

Figure 6. SDS-PAGE analyses of the protein components of the three fermentations. Three protein solubility fractions were analyzed: the albumin (**A**), the alcohol-soluble (**B**), and the SDS-soluble (**C**) fractions. The numbers above the gels indicate the fermentation times. The molecular weight marker lanes are labeled "S", and the molecular masses (kDa) of the standard proteins are listed on the right-hand sides of the gels.

SDS-soluble fraction into the alcohol-soluble fraction. This is visible as an increase in their intensities in the 6 h CSD sample (**Figure 6B**; vertical arrows). No clear degradation occurred during the ISD fermentation. Instead, it was seen that the solubilities of the wheat albumin and alcohol-soluble proteins were lowered, presumably due to the boiling treatment that was used in preparing the ISD fermentation.

Prolamin Quantification. Quantification of the prolamin contents of the sourdoughs using the R5-ELISA method confirmed the observations from the SDS-PAGE analyses. The prolamin concentration of the GWSD fermentation decreased drastically, dropping from 27000 to 3700 ppm during the first 6 h of fermentation (**Figure 7**). After 24 h of GWSD fermentation, the prolamin content was only 1200 ppm, whereas in the CSD the prolamin concentration remained at 24000 ppm (**Figure 7**). In the ISD the prolamin contents remained practically the same throughout the fermentation.

DISCUSSION

This study showed that during the GWSD fermentation an extensive hydrolysis of wheat prolamins occurred. The hydrolysis of both the gliadins and glutenins took place at the beginning of the 24 h fermentation. This was observed in the 6 h fermentation sample as a disappearance of the prolamins (**Figure 6**). Further quantification of the sourdough prolamin contents verified that the hydrolysis was very extensive and that the final concentration of prolamins was <5% of what was initially present (**Figure 7**). The amount of FAN in the GWSD (1240 mg/kg) was much larger than that of the CSD (220 mg/kg),

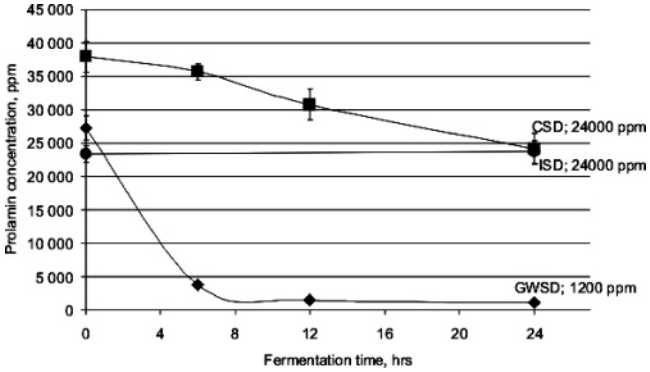

Figure 7. Quantification of the prolamin concentrations of the three sourdoughs during their fermentations by ELISA. The labels on the right-hand side of each curve indicate the prolamin concentration of the corresponding 24 h sample, in ppm. When the error bars are not shown, they are smaller than the symbol that was used.

which indicated that very effective proteolysis had occurred (**Figure 5**). The protein changes observed in the control sourdough (CSD) matched well those that we reported previously; the HMW glutenins were effectively degraded (*7*). Even the depolymerization of the HMW glutenins was evident, as they had already shifted into the alcohol-soluble fraction when the 6 h WSD sample was taken (**Figure 6B**; vertical arrow). This depolymerization is a well-known phenomenon in wheat sourdoughs (*7*, *28*). In ISD the FAN values decreased, probably as a consequence of the uptake of peptides by microbes. The pH development of the ISD was slow, and its fermentation resulted in low TTA values (**Figure 4**) and small lactobacilli counts, compared to those of the GWSD and CSD fermentations. These findings indicate that the absence of cereal proteases in the ISD fermentation probably led to its deficient microbial growth.

Germination induces the production of an arsenal of endogenous cereal enzymes and provides a method for food technologists to exploit these natural enzymes in food processing. Traditionally, the germination-induced enzyme activities have been exploited in beer production to ensure good yeast growth, among other things. During the mashing stage of the brewing process, the cysteine proteinases are the most important proteases present, but the metalloproteinases also apparently play an important role in hydrolyzing proteins during this process (*29*).

In this study we showed that when germinated wheat grains, with their high and diverse proteolytic activities, were used as a raw material in sourdough fermentations, extensive proteolysis occurred. This proteolysis efficiently degraded the gliadins, as well as the glutenins. Presumably, the cysteine proteinases were mainly responsible for the gliadin degradations, because they were the predominant protease group in the germinated wheat grains that were used to prepare the GWSD. In addition, previous studies have shown that the cysteine proteinases of wheat grain were evidently capable of hydrolyzing both gliadins and glutenins (*11*, *12*, *30*), whereas the wheat aspartic proteinases predominantly degraded only the glutenins (*10*).

The pH conditions that occur in sourdough fermentations offer an ideal environment for the cysteine proteinases to operate, and they probably remain active throughout the sourdough fermentation (*12*). The pH dynamic nature of sourdoughs, however, also enables the activation of other proteinases that have different pH optima. At the beginning of the fermentations the pH is around 6, which enables enzymes with neutral pH

optima to be active, and as the acidification process proceeds, those proteinases that have acidic pH optima can become active.

This study, to our knowledge, is the first to report the use of germinated grains as a raw material in sourdough fermentations. The study aimed to answer the question of whether the wheat prolamins can be extensively degraded during such highly proteolytic activity sourdough fermentations. The results presented here show that the wheat prolamins, including gliadins, were extensively hydrolyzed during the GWSD fermentation. These results are very consistent with those obtained in our preliminary experiments (data not shown), which indicates that the reproducibility of the experiments is good. We also observed a very similar degradation of wheat prolamins when we used commercial wheat malt (Laihian Mallas, Laihia, Finland) in one of our preliminary experiments (data not shown). The cysteine class proteases were also the predominant proteinases present in this commercial wheat malt. Despite this study showing that the wheat prolamins were extensively hydrolyzed during the fermentations, it is worth emphasizing that we did not look for any evidence that the CD toxicity of wheat prolamins or their hydrolysis products is in any way reduced by using germinated material in sourdoughs.

However, a very recent study showed that the pool of proteases present in germinating grains, including wheat, hydrolyzed typical gliadin peptides into fragments that were apparently no longer harmful for celiac patients (*31*). This finding supports our thinking that sourdoughs made with germinated grains could, in the future, be used to manufacture cereal products that would be acceptable to people with gluten sensitivities. Before using such food technologies, however, the safety of such production technologies and their products needs to be evaluated carefully and ensured. It is important that the safety of such products is confirmed using good clinical practices by professionals in the field of gluten sensitivity.

It is also obvious that when using GWSD-like fermentations for baking, it will be impossible to prepare doughs that are based on the formation of glutens, since practically all of the gluten proteins were extensively hydrolyzed during the fermentation. In terms of using different cereals, the use of germinated rye grains for such processes might be more feasible, because the rye proteins do not form gluten-like dough structures, so the extensive degradation of their proteins would not be particularly detrimental to the dough formation. Of course, for any baking that is aimed at producing products for gluten-sensitive people, all other ingredients used in the recipe will also need to be gluten-safe. In addition, one must be aware that germinated grains will always contain other hydrolytic enzymes besides the proteases. For instance, the inactivation of the germinated grain amylolytic enzymes would be necessary before any starch-based baking technologies could be used.

In addition to brewing and sourdough applications, other (semi) liquid cereal processing technologies could also utilize the enzymatic potential that is present in germinated grains. The use of pH dynamic processing technologies to ensure the activation of the entire protease battery of germinated grains also needs to be studied more in detail. Overall, high-proteolytic-activity sourdoughs and other cereal-processing technologies that provide effective proteolysis could be used to diversify the sensory properties of low-gluten or gluten-free products and, thereby, diversify the diets of gluten-sensitive people by offering them the option of adding health-promoting cereal-derived components to their everyday diets.

ABBREVIATIONS USED

AMC, 7-amino-4-methylcoumarin; CD, celiac disease; CSD, control wheat sourdough; DPP IV, dipeptidyl-peptidase IV (EC 3.4.14.5); E-64, epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; ELISA, enzyme-linked immunosorbent assay; FAN, free amino nitrogen; FD, freeze-dried; GWSD, germinated-wheat sourdough; HMW, high molecular weight; ISD, inactive sourdough-like ferment; LMW, low molecular weight; *O*-FEN, 1,10-phenanthroline; PD, pilsner-dried; PEP-A, pepstatin A; PMSF, phenylmethanesulfonyl fluoride; POP, prolyl oligopeptidase (EC 3.4.21.26); RT, room temperature; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SUC, succinyl; TCA, trichloroacetic acid; TTA, total titratable acidity.

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