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Gluten Hydrolysis and Depolymerization during Sourdough Fermentation

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Hydrolysis and depolymerization of gluten proteins during sourdough fermentation were determined. Neutral and acidified doughs in which microbial growth and metabolism were inhibited were used as controls to take into account the proteolytic activity of cereal enzymes. Doughs were characterized with respect to cell counts, pH, and amino nitrogen concentrations as well as the quantity and size distribution of SDS-soluble proteins. Furthermore, sequential extractions of proteins and analysis by HPLC and SDS-PAGE were carried out. Sourdough fermentation resulted in a solubilization and depolymerization of the gluten macropolymer. This depolymerization of gluten proteins was also observed in acid aseptic doughs, but not in neutral aseptic doughs. Hydrolysis of glutenins and occurrence of hydrolysis products upon sourdough fermentation were observed by electrophoretic analysis. Comparison of sourdoughs with acid control doughs demonstrated that glutenin hydrolysis and gluten depolymerization in sourdough were mainly caused by pH-dependent activation of cereal enzymes.

KEYWORDS: Gluten macropolymer; *Lactobacillus sanfranciscensis*; *Lactobacillus pontis*; wheat sourdough; proteolysis; depolymerization

INTRODUCTION

Sourdough fermentation is a traditional process in bread production that retained its importance in wheat baking because it improves bread quality. Beneficial effects of sourdough fermentation on bread quality include, but are not limited to, a prolonged shelf life through inhibition of spoilage microorganisms (*1*, *2*), increased loaf volume and delayed staling (*3*, *4*), improved bread flavor (*5*), and improved nutritional quality based on a lowered glycemic index (*6*). The optimization of the sourdough process for industrial applications in wheat baking requires insight into the biochemical mechanisms responsible for the quality of sourdough fermented bread. Evidence for the impact of specific metabolic activities on bread quality was provided, for instance, concerning the generation of flavor precursors and flavor volatiles (*5*). The formation in dough of expolysaccharides by *Lactobacillus sanfranciscensis* improves wheat bread texture (*7*), but few data are available on other factors that affect the volume and texture of sourdough fermented wheat breads. Proteolytic events during sourdough fermentation liberate amino acids, which improve bread flavor (*5*); however, proteolytic degradation of gluten proteins may adversely affect rheological characteristics of wheat doughs and bread texture.

Gluten proteins are a major determinant for the rheological properties of wheat doughs and the texture of wheat breads attained by straight dough processes. A high correlation is observed between the glutenin subunits and the glutenin macropolymer (GMP) content of flours and their bread-making quality (*8*, *9*). Glutenin subunits link by disulfide bonding to large polymers that may then aggregate to the glutenin macropolymer, with molecular masses up to several million (*9*, *10*). It was proposed that the GMP partially depolymerizes into smaller aggregates during dough mixing, followed by incorporation of high molecular weight glutenin subunits into the GMP during resting time (*11*, *12*). It has also been proposed that gluten strength is furthermore influenced during dough mixing by disulfide interchange of intermolecular glutenin SS bonds with low molecular weight sulfhydryl compounds, for instance, cysteine or glutathione (*13*). Rheological studies on wheat doughs acidified with lactic and acetic acids and on sourdoughs demonstrated that the rheological characteristics of doughs changed entirely with fermentation. Major developments included an overall decrease in viscosity, which was related to acidification by the sourdough microflora (*14*). The loss of extensibility of cracker sponges during fermentation was most pronounced at pH values ranging from 3.8 to 4.1, corresponding to the optimum pH of wheat flour proteinases (*15*, *16*). Substantial hydrolysis of gliadin and glutenin proteins occurs during sourdough fermentation; proteolysis in sourdoughs and the rheological consequences of gluten degradation are mainly based on the pH-mediated activation of cereal enzymes (*17*−*19*). Additionally, lactic acid bacteria for use in sourdough fermentation may exhibit proteolytic activity, but their activities

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Table 1. Dough Formulas for Preparation of Control Doughs and Sourdoughs

	control neutral	control acid	LAB[a]	LAB, buffer
flour (g)	20	20	20	20
NaCl (g)	0.4	0.4	0.4	0.4
water (g)	20	20	18	16
erythromycin (mg)	2			
lactic and acetic acids[b] (mg)		0.360		
preculture (g)			2	2
phosphate buffer[c] (g)				2

[a] *L. sanfranciscensis* LTH2581 or *L. pontis* LTH2587. Cells were harvested from 5 mL of preculture and washed twice with tap water. [b] Mixture of 4 vol of lactic acid (90%) and 1 volume of acetic acid (98%). [c] 1 M sodium phosphate, pH adjusted to 6.3 with NaOH.

are strain specific (*20*, *21*) and appear to play only a minor role in overall proteolytic events during fermentation (*5*, *17*, *19*).

Whereas increasing knowledge has been attained on the proteolytic events during sourdough fermentation, few data are available on the consequences of proteolysis with repect to the quantity and composition of the GMP. It was the aim of this work to analyze qualitative and quantitative changes of the GMP during sourdough fermentation. Two strains of lactobacilli were employed, *L. sanfranciscensis* LTH2581 and *Lactobacillus pontis* TMW1.397, representing the microflora of traditionally prepared sourdoughs (*22*). To differentiate between effects of mere acidification, and specific metabolic activities of the lactic acid bacteria, sourdoughs were compared to acidified (pH 3.6) and neutral (pH 6.3) control doughs in which microbial metabolism was inhibited.

MATERIALS AND METHODS

Strains and Culture Conditions. The sourdough isolates *L. sanfranciscensis* LTH2581 (23) and *L. pontis* TMW1.397 (= DSM 8475[T], *24*) were grown in de Man, Rogosa, Sharpe (MRS) medium modified according to the method of Stolz et al. (*25*) (mMRS) containing 10 g L^{-1} maltose and 5 g L^{-1} each of glucose and fructose. Agar–agar (15 g L^{-1}) was added to obtain solid media. *L. sanfranciscensis* was grown at 30 °C, *L. pontis* was grown at 37 °C, and plates were incubated under modified atmosphere (4% O_2, 20% CO_2, and 76% N_2). To obtain inocula for sourdoughs, $\sim 10^9$ cells from an overnight culture were washed in sterile tap water, resuspended in 2 mL of sterile tap water, and used immediately to inoculate the doughs.

Sourdough Fermentation. A commercial wheat flour with an ash content of 0.55%, 13.4% moisture, and 12.3% crude protein (N × 5.70) was obtained at a local mill. Farinograms were performed according to ICC standard method 115/1; the flour was characterized by a water absorption of 61%, a dough development time of 2.8 min, a dough stability of 7.1 min, and a degree of softening of 41 Brabender units after 12 min. Doughs were prepared according to the dough formulas shown in **Table 1**, mixed to homogeneity with a spatula (3 min of mixing time), and incubated in glass beakers at 30 °C. Samples were taken at 0, 6, and 24 h for the subsequent analyses. Two independent fermentations were carried out.

Determination of Cell Counts, pH, and Amino Nitrogen. Cell counts were determined by plating appropriate dilutions of dough samples on mMRS agar. To determine the dough pH, 1 g of dough was mixed with 9 mL of water and vortexed for 5 min, and the pH was measured with a glass electrode. A modified ninhydrin method was used for determination of total amino nitrogen as described (*5*). Dough samples were mixed with an equal volume of 7% perchloric acid and stored for 1 h at 4 °C. The supernatant was collected by centrifugation and neutralized with 1.5 vol of 0.43 M KOH to precipitate perchloric acid. To 10 μL of sample were added 100 μL of reagent 1 (1 L of H_2O, 50 g of $Na_2HPO_4 \cdot 2H_2O$, 60 g of KH_2PO_4, 0.5 g of ninhydrin, 3 g of fructose, pH 6.7) and 190 μL of H_2O, and the mixture was heated for 16 min at 100 °C. Upon rapid cooling to ambient temperature in a water bath, 500 μL of reagent 2 (600 mL of H_2O, 400 mL of 96% ethanol, 2 g of KIO_3) was added, and the absorbance of the samples was measured at 570 nm. A calibration curve was prepared with each measurement using glycine as standard, and results were expressed as millimoles of glycine per kilogram of dough.

Size Exclusion Chromatography (SEC) of Sodium Dodecyl Sulfate (SDS)-Soluble Proteins and SDS–Dithiothreitol (DTT)-Soluble Proteins. Dough samples were characterized by SEC with respect to the amount of SDS-soluble proteins as well as their size distribution as described (*16*). One gram of dough was extracted at ambient temperature by vortexing the sample, followed by overhead shaking for 15 min with 4 mL of 1.5% SDS in 50 mM sodium phosphate buffer, pH 6.9. Proteins in the residue of this first extraction were subsequently solubilized in 1.5% SDS in 50 mM phosphate buffer, pH 6.9, with 4% DTT. SDS extracts were applied on a Superdex 200 column coupled to a Superdex Peptide column (both from Amersham Bioscienes, Uppsala, Sweden) to achieve fractionation in the relative molecular weight (M_r) range from 100 to 5 × 10^6. SDS–DTT extracts were applied to a Superdex 200 column to achieve fractionation in the M_r range from 10^4 to 5 × 10^6. Samples were eluted at ambient temperature with 50 mM sodium phosphate buffer, pH 7.7, containing 0.1% SDS and 20% acetonitrile at a flow of 0.4 mL/min. The UV detector was set to 210 and 280 nm. The areas under the peaks representing the polymeric, monomeric, and low molecular weight (LMW) components were determined using the 280 nm trace rather than the 210 nm trace to avoid interference with lactic acid and other nonprotein carboxyl compounds. The column(s) were calibrated using the HMS and LMW GPC calibration kits (Amersham Bioscienes). Polymeric proteins eluted at 12 mL (void volume) to 18 mL, monomeric proteins eluted at 18–30 mL, and amino acids and peptides with a molecular weight of <5000 eluted at 30–44 mL (total column volume). The experimental error of dough extraction, chromatographic separation, and peak integration was <10%, and two independent fermentations yielded qualitative and quantitative consistent results.

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE analysis of flour proteins was performed in a Bio-Rad Mini-Protean vertical electrophoresis cell (Bio-Rad, Munich, Germany) using the Laemmli buffer system according to the instructions of the manufacturer. Dough extracts were mixed with 3 vol of sample buffer and heated to 90 °C for 5 min in the presence of 5% mercaptoethanol, separated in 12% acrylamide, 2.67% cross-linked gels, and stained with Coomassie Blue. Four independent fermentations yielded qualitative consistent results.

Extraction of Doughs and Reversed-Phase (RP)-HPLC Analysis of Dough Extracts. Sequential extraction of dough samples was performed essentially according to the method of Kruger et al. (*26*) with the solvents (1) 0.5 M NaCl and 150 mM sodium phosphate, pH 6.8, (2) 50% 1-propanol in H_2O, and (3) 50% 1-propanol in H_2O, 1% acetic acid, and 4% DTT. Proteins soluble in propanol and propanol–acetic acid–DTT were designated fractions P1 and P2, respectively. To 2 g of dough was added 6 mL of solvent, and the suspension was thoroughly mixed and incubated for 30 min at ambient temperature in an overhead mixer. The extraction protocol of Kruger et al. (*26*) was changed to compensate for pH differences in the dough samples. It was verified that polymeric proteins were fully solubilized by this protocol and that the buffering capacity of solvent 1 sufficed to compensate for differences in pH of the various neutral and acidic dough samples. A 4 mL water wash was included between solvents 1 and 2 to remove salt and residual organic acids. Dough extracts were stored at −20 °C in the dark until analysis by SDS-PAGE or HPLC.

For quantitative analysis of the protein content in fractions P1 and P2, RP-HPLC was performed according to the method of Wieser et al. (*27*). Twenty microliter samples were injected on a C18 RP column (250 × 4.6 mm, 300 Å, 10 μm particle size, Phenomenex, Torrence, CA) coupled to a UV detector set to 210 nm, the flow was 1 mL/min, and the column temperature was 70 °C. Solvents A and B consisted of 0.1% trifluoroacetic acid in water and acetonitrile, respectively, and samples were eluted with the following solvent gradient: 0–2 min: 24% B, 2–32 min, gradient from 24 to 56% B. The relative amounts of protein in the extracts was determined through integration of the

Table 2. Cell Counts, pH, and Amino Nitrogen Contents of Control Doughs and Sourdoughs

fermentation time	neutral	acid	LTH2581	TMW 1.397	LTH2581, buffer
0 h					
cell count[a]	–[b]	–	2 × 10^6	6 × 10^6	2 × 10^6
pH	6.21	3.56	6.25	6.18	6.51
amino N[c]	4.0 ± 0.3	3.6 ± 0.5	4.1 ± 0.5	4.1 ± 0 3	4.3 ± 0.3
6 h					
cell count	–	–	4 × 10^7	1 × 10^8	2 × 10^7
pH	6.23	3.69	5.28[d]	4.52[d]	6.03
amino N	5.5 ± 0.2	5.2 ± 0	5.9 ± 0.6	5.0 ± 0.2	5.5 ± 0.2
24 h					
cell count	–	–	1 × 10^9	7 × 10^8	1 × 10^9
pH	6.51	3.67	3.62	3.60	3.76
amino N	8.8 ± 0.8	7.9 ± 0.6	7.4 ± 0.2	9.1 ± 0.2	6.6 ± 0 3

[a] Cell counts is expressed in cfu g^{-1} of dough. [b] Cell counts of control neutral and acid doughs were <10^4 cfu g^{-1} throughout fermentation. [c] Amino nitrogen is expressed in mmol of glycine kg^{-1} of dough. [d] The lag times of *L. sanfrancsicensis* and *L. pontis* differed considerably when two independent fermentations were compared. Therefore, the pH of the doughs after 6 h varied between 4.5 and 5.3.

210 nm trace. The experimental error of dough extraction, chromatographic separation, and integration was <5%, and two independent fermentations yielded qualitative and quantitative consistent results.

RESULTS

Microbial Growth and Proteolysis during Dough Fermentation. Sourdough fermentations were carried out using two strains of lactobacilli, and control fermentations were carried out aseptically at neutral and acid conditions (pH 6.3 and 3.5, respectively) to account for proteolytic events and modifications of the glutenin macropolymer in the absence of microbial metabolic activity. Additionally, sourdough fermentations were carried out in the presence of phosphate buffer to further differentiate between effects of microbial metabolism and a decreased pH. Cell counts, pH values, and the concentration of amino nitrogen for the various doughs are shown in **Table 2**. *L. sanfranciscensis* and *L. pontis* grew to cell counts of 10^9 colony-forming units (cfu)/g as typically observed in sourdough fermentations. The cell counts of control doughs were <10^4 cfu/g throughout fermentation, excluding a contribution of microbial metabolism to any of the analytical parameters observed. Samples were taken at the following times: unfermented dough (0 h), 6 h of fermentation corresponding to a pH of 4.5−5.5 and exponentially growing cells in sourdoughs, and 24 h of fermentation corresponding to a pH of 3.6−3.8. Growth and metabolism of lactobacilli ceased when the dough pH was reduced to 3.6−3.7, in agreement with the observation that growth of lactobacilli in sourdough is limited by dough pH (*28*). Addition of phosphate buffer to doughs fermented with *L. sanfranciscensis* LTH2581 delayed acidification without interfering with microbial growth or metabolism.

Overall proteolysis in doughs was quantified by determination of amino nitrogen. The concentration of amino nitrogen increased during fermentation and roughly doubled within 24 h. It can be estimated that the increase of amino nitrogen by 5 mM kg^{-1} corresponds to hydrolysis of <0.5% of the protein available in the doughs. No appreciable differences were observed between sourdoughs and neutral or acid control doughs. The levels of amino nitrogen in doughs fermented with *L. sanfranciscensis* were slightly lower compared to doughs fermented with *L. pontis*, and a noticeably lower proteolytic activity was observed in lactic fermented doughs with addition of phosphate buffer. These data are in overall accordance with

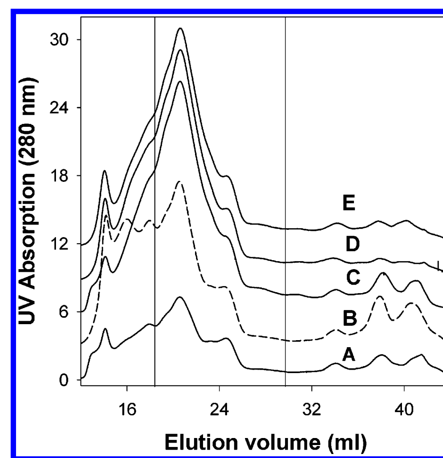

Figure 1. Separation of SDS-soluble proteins from aseptic control doughs and sourdoughs by SEC. UV-280 nm traces represent extractions from neutral control dough at 0 h (trace **A**) and extractions after 24 h of fermentation from neutral control dough (trace **B**), acid control dough (trace **C**), sourdough with *L. sanfranciscensis* LTH2581 (trace **D**), and sourdough with *L. pontis* TMW 1.397 (trace **E**) fermented doughs after 24 h as indicated. Chromatograms were offset by 3 AU. 12–18 mL elution volume, polymeric proteins with M_r >200000; 18–30 mL elution volume, monomeric proteins; 30–44 mL elution volume, peptides and amino acids with M_r <10000.

previous investigations using the same strains but a different wheat flour (*5*).

Quantification and Size Distribution of SDS-Soluble Proteins. SDS-soluble proteins were extracted from control doughs and sourdoughs, and the relative changes in protein concentrations and size distribution of SDS-soluble proteins were determined by SEC. The glutenin macropolymer is poorly soluble in SDS unless reducing agents or sonification is employed. Therefore, a depolymerization or disaggregation of the GMP is detectable by increased amounts of SDS-soluble proteins. The fractionation range of the SEC columns furthermore made it possible to estimate the amount and size distribution of peptides resulting from proteolytic hydrolysis of proteins. The relative protein contents of SDS extracts were estimated by integration of the 280 nm UV tract, and examples of chromatograms of SDS extracts are shown in **Figure 1**. In neutral control doughs the amount of soluble protein increased but the ratio of polymeric to monomeric proteins remained constant throughout fermentation. The amount of protein extracted from acid control doughs after 24 h increased greatly compared to the 0 h control, and the extracted proteins were mainly monomers. Extracts from sourdoughs fermented with *L. sanfranciscensis* and *L. pontis* for 24 h were highly similar to acid control doughs with respect to amount and size distribution of SDS-soluble proteins. However, as opposed to neutral and acid control doughs, the peak area representing low molecular weight peptides (M_r ~ 500−5000) was strongly reduced in sourdoughs.

To quantify the relative protein contents of SDS-soluble proteins in various doughs as well as the relative contents of monomeric and polymeric proteins, the peak areas corresponding to polymeric proteins, monomeric proteins, and LMW peptides and amino acids were determined (**Figure 2**). In neutral control doughs, total soluble protein, including soluble polymeric and monomeric proteins, increased during fermentation. At the start of the acid control fermentation, an increased amount of total SDS-soluble protein was apparent, even in unfermented doughs. During fermentation, the amount of polymers decreased and the amount of monomers increased. The amounts of SDS-

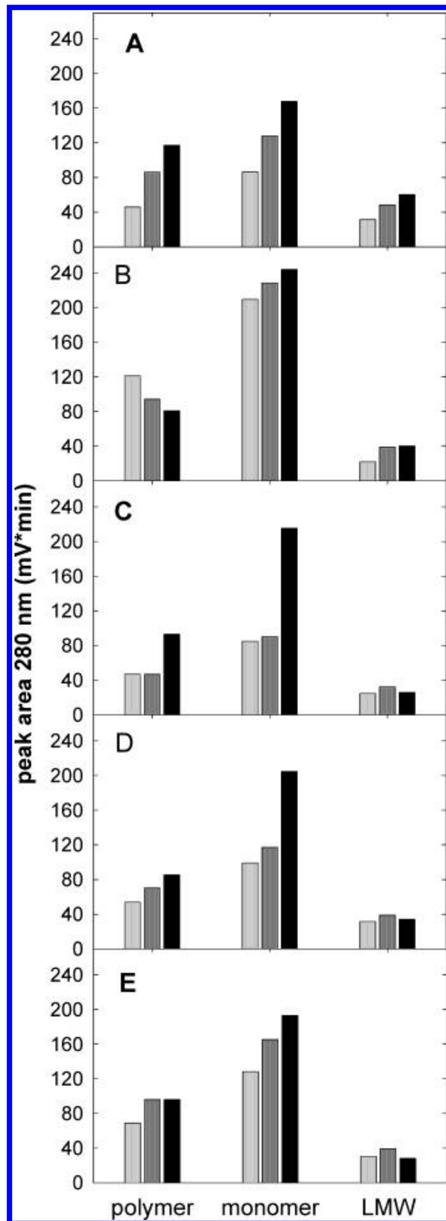

Figure 2. Amounts of polymeric and monomeric proteins and low molecular weight peptides extracted with SDS from unfermented aseptic control doughs and sourdoughs and after 6 and 24 h of fermentation: (**A**) neutral control dough; (**B**) acid control doughs; (**C**) sourdough fermented with *L. sanfranciscensis* LTH2581; (**D**) sourdough fermented with *L. pontis* TMW1.397; (**E**) phosphate-buffered dough fermented with *L. sanfranciscensis* LTH2581. Amounts of proteins are expressed as AU × mL (280 nm)/2 mg of flour. Bars indicate extractions at 0 h (light shading), after 6 h (medium shading), and after 24 h (heavy shading). The experimental error of dough extraction, chromatographic separation, and peak integration was generally <10%.

soluble proteins in unfermented sourdoughs were comparable to neutral doughs, and only after 24 h of fermentation were large amounts of SDS-soluble protein present in the sourdoughs. A decrease of peptides was observed in sourdoughs, whereas an increase was apparent in both control doughs. The addition of buffer to doughs fermented with *L. sanfranciscensis* increased the solubility of proteins at 0 and 6 h of fermentation (**Figure 2E**). After 24 h, no difference was observed between doughs fermented with *L. sanfranciscensis* in the presence or absence of phosphate buffer. Both the increase of SDS-soluble protein and the shift of peak areas from polymeric to monomeric

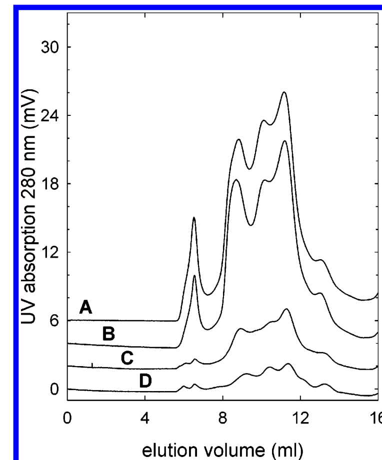

Figure 3. Separation of SDS-insoluble and SDS–DTT-soluble proteins from neutral control dough and sourdough by SEC. UV-280 nm traces represent extractions from neutral control dough at 0 h (trace **A**), unfermented sourdough ($t = 0$ h) with *L. sanfranciscensis* LTH2581 (trace **B**), after 24 h of fermentation from neutral control dough (trace **C**), and after 24 h of fermentation with *L. sanfranciscensis* LTH2581 (trace **D**). Chromatograms were offset by 2 AU.

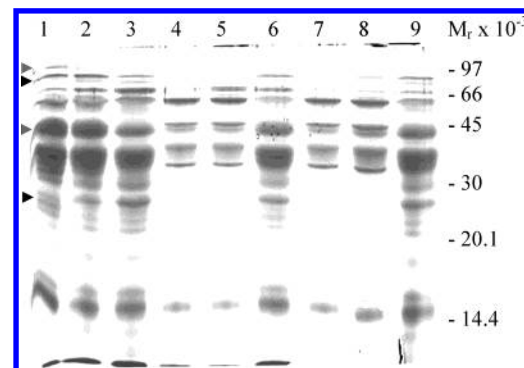

Figure 4. Separation of SDS-soluble proteins from aseptic control doughs and sourdoughs by SDS-PAGE: (lanes 1, 2, and 3) acid control doughs extracted after 0, 6, and 24 h, respectively; (lanes 4, 5, and 6) sourdough with *L. sanfranciscensis* LTH2581 extracted after 0, 6, and 24 h, respectively; (lanes 7, 8, and 9) sourdough with *L. pontis* TMW 1.397 dough extracted after 0, 6, and 24 h, respectively. Samples were applied to represent equal amounts of flour. Migration of the molecular weight markers in the gel is indicated. Black arrows indicate protein bands not detected in unfermented doughs or neutral aseptic doughs, and gray arrows indicate proteins bands degraded after fermentation at acid conditions.

proteins indicate a depolymerization of gluten proteins during fermentation at acidic conditions induced by the addition of lactic and acetic acids (acid control doughs) or by sourdough fermentation.

To verify that an increased protein content in SDS extracts corresponds to a decreased content of SDS-insoluble GMP in dough, the residue of the SDS extraction was extracted with SDS−DTT. Examples of SEC separations of SDS−DTT extracts are shown in **Figure 3**. Increased values for SDS-soluble protein corresponded to decreased SDS-insoluble/SDS−DTT-soluble proteins in all doughs at any time (**Figure 3** and data not shown).

To determine the composition of proteins solubilized by sourdough fermentation, SDS-soluble proteins from doughs were analyzed by SDS-PAGE (**Figure 4**). In neutral control doughs and unfermented doughs, only small amounts of glutenins and gliadins were extracted with SDS (**Figure 4** and data not shown). In acid control doughs, glutenins and gliadins were SDS-soluble,

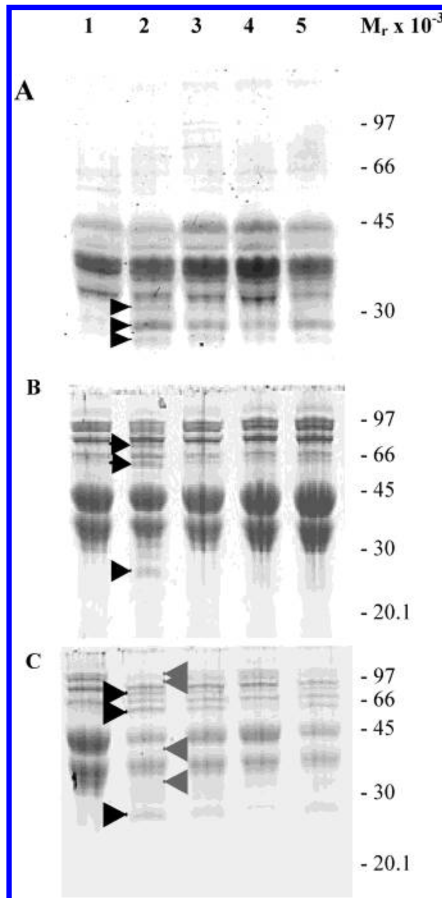

Figure 5. SDS-PAGE analysis of protein fractions P1 and P2 extracted from control doughs and sourdoughs: (**A**) fraction P1 extracted after 24 h [(lane 1) neutral control dough; (lane 2) acid control dough; (lane 3) sourdough (*L. sanfranciscensis* LTH2581); (lane 4) buffered sourdough (*L. sanfranciscensis* LTH2581); (lane 5) sourdough (*L. pontis* TMW1.397)]; (**B**) fraction P2 extracted after 6 h [(lane 1) neutral control dough; (lane 2) acid control dough; (lane 3) sourdough (*L. sanfranciscensis* LTH2581); (lane 4) buffered sourdough (*L. sanfranciscensis* LTH2581); (lane 5) sourdough (*L. pontis* TMW1.397)]; (**C**) fraction P2 extracted after 24 h (lanes as in panel **B**). Samples were applied on the gel to represent equal amounts of flour. Black arrows indicate protein bands not detected in unfermented doughs or neutral aseptic doughs, and gray arrows indicate proteins bands degraded after fermentation at acid conditions.

indicating that acidity inhibited the formation of the GMP. After 24 h of acid control fermentation, the band intensity of individual high molecular weight (high M_r) glutenins decreased and additional bands were detectable with a M_r of ~30000. The composition of SDS-soluble proteins extracted from sourdoughs at time 0 h was comparable to neutral control doughs, and after 24 h, the SDS-PAGE profile of sourdoughs was comparable to that of acid control doughs. Taken together, the qualitative and quantitative analyses of SDS-soluble protein suggest partial hydrolysis and depolymerization of the GMP during fermentation at acidic conditions.

Sequential Extraction of Gluten Proteins and Qualitative and Quantitative Analysis by SDS-PAGE and RP-HPLC. To enable analysis of proteins remaining in the GMP after fermentation, proteins from various dough samples were sequentially fractionated into water/salt-soluble proteins and fractions P1 and P2 and separated electrophoretically. No differences were detected between the different unfermented doughs in terms of the protein composition of fractions P1 and P2 (data not shown). The analysis by SDS-PAGE of fraction

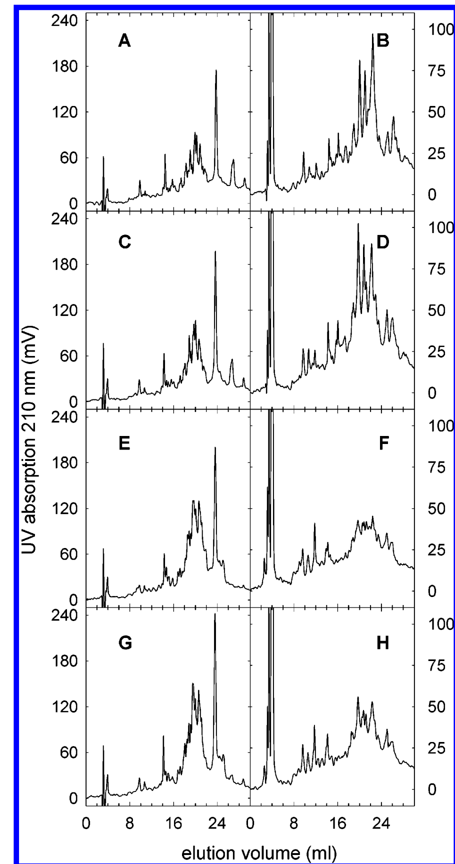

Figure 6. Separation of propanol-soluble proteins (**A**, **C**, **E**, **G**) and propanol–DTT-soluble proteins (**B**, **D**, **F**, **H**) extracted from control doughs and sourdoughs by RP-HPLC. Shown are chromatograms of extracts of unfermented neutral dough (**A**, **B**), neutral control dough after 24 h of fermentation (**C**, **D**), acid control dough after 24 h of fermentation (**E**, **F**), and sourdoughs fermented for 24 h with *L. sanfranciscensis* LTH2581 (**G**, **H**). The amount of protein applied to the column represented 3 mg of flour.

P1 obtained after 24 h of fermentation is shown in **Figure 5A**, and the analysis of fraction P2 obtained after 6 and 24 h of fermentation are shown in **Figure 5B**,**C**, respectively. Protein patterns of fraction P1 extracted from neutral control doughs did not show appreciable differences over fermentation time. Extracts P1 from acid control doughs and sourdoughs were characterized by the presence of high M_r glutenin subunits after 6 and 24 h of fermentation and proteins with a M_r of $2-3 \times 10^4$ that were not present in unfermented doughs or in neutral control dough. Changes in fraction P2 extracted from neutral control doughs were not observed, and virtually identical protein patterns were detected in all unfermented doughs (**Figure 5B**,**C** and data not shown). After 6 h of acid aseptic fermentation, hydrolysis of individual high M_r subunits and an additional protein with a M_r of $\sim 6 \times 10^4$ as well as proteins in the M_r range of $(2-3) \times 10^4$ were detected. The protein patterns of sourdoughs were comparable to that of neutral control doughs after 6 h of fermentation. After 24 h of fermentation, both acid control doughs and sourdoughs were characterized by virtually quantitative hydrolysis of individual high M_r glutenin subunits and the presence of hydrolysis products with M_r of 6×10^4 and 2.5×10^4, respectively.

Quantification of the amounts of protein fractions P1 and P2 was performed by RP-HPLC separation of extracts and integration of the UV-210 trace. Chromatograms from selected doughs (neutral control, 0 h, neutral control, 24 h, acid control, 24 h, and sourdough *L. sanfranciscensis*, 24 h) are shown **Figure 6**.

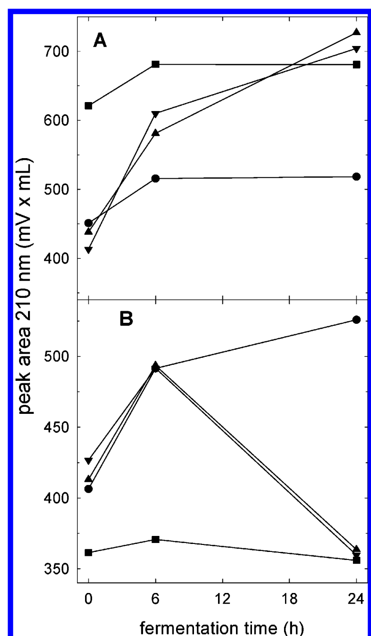

Figure 7. Quantification with reversed-phase HPLC of proteins extracted from control doughs and sourdoughs. The amounts of proteins are expressed as AU × mL (210 nm)/3 mg of flour. Shown are propanol–water-soluble proteins (**A**) and propanol–acetic acid–DTT-soluble proteins (**B**). Symbols indicate extracts from neutral control (●) and acid control doughs (■) and sourdoughs fermented with *L. sanfranciscensis* LTH2581 (▲) or *L. pontis* TMW1.397 (▼). Experimental error is smaller than the symbol size.

The peak areas correlating to total protein content of all extracts are shown in **Figure 7**. The peaks were not assigned to individual gluten subunits because, after fermentation at low pH, most glutenin subunits were present in fraction P2 and new peaks were observed in fraction P2 upon fermentation mainly in the hydrophilic part of the gradient (6−16 min), which presumably arise from partial hydrolysis of glutenin subunits as also observed by SDS-PAGE.

In neutral control doughs, an increase in protein concentration was observed in fraction P1 over time, whereas a strong increase was observed in fraction P2. In contrast, a strong decrease in proteins in fraction P2 was observed after 24 h of fermentation of *L. sanfranciscensis* and *L. pontis* doughs. Correspondingly, a strong increase of the protein content in fraction P2 was detected, indicating glutenin depolymerization. In acid control doughs, low protein levels were found in fraction P2 already in unfermented doughs and no major changes were observed during fermentation. To determine whether gluten proteins are degraded to water-soluble degradation products, the sum of proteins in fractions P1 and P2 was calculated. The overall protein levels in fractions (P1 + P2) increased in all doughs during fermentation by about 20% (neutral control, *L. sanfranciscensis*, and *L. pontis* doughs) and 10% (acid control). After 24 h of fermentations, no differences concerning the amounts of protein in fractions (P1 + P2) were found among the various doughs. This result indicates the solubility of gluten proteins was altered strongly through sourdough fermentation, but proteolytic degradation of gluten proteins to water soluble amino acids or peptides did not play a major role in any of the doughs.

DISCUSSION

In this study was shown that fermentation of wheat doughs in the presence of organic acids results in virtually quantitative depolymerization of the GMP and partial hydrolysis of glutenin subunits. Differences observed between neutral and acid control doughs with respect to glutenin depolymerization and proteolytic hydrolysis of individual high M_r glutenins were more pronounced than those differences between doughs fermented with *L. sanfranciscensis* or *L. pontis*. Major effects are therefore attributable to the acidity and proteolytic enzymes of wheat flour rather than proteolytic activities of sourdough lactobacilli.

The fermentation conditions had no major effect on proteolytic events as determined by the release of amino nitrogen. Using the same strains but a different flour, comparable levels of amino nitrogen were found after 24 h of fermentation at acid or neutral conditions or in sourdoughs started with lactobacilli (*5*). However, sourdoughs and acid control doughs showed increased amino nitrogen levels compared to neutral doughs, and the concentration of individual amino acids indicated that different substrates are hydrolyzed under neutral and acidic conditions (*5*). Lactobacilli have multiple amino acid auxotrophies, and oligopeptide transport is considered the main route for nitrogen entry into the bacterial cells (*29*). It was previously shown that *L. sanfranciscensis* requires peptides for growth (*30*), and the study presented here provides evidence in situ that *L. sanfranciscensis* and *L. pontis* utilize mainly peptides during sourdough fermentation to meet their requirements with respect to amino acids.

During sourdough fermentation, glutenin subunits were hydrolyzed and the resulting peptides, with lower molecular weights, remained associated with the gluten macropolymer. Nearly all high M_r glutenin subunits were digested in doughs fermented at a pH of <4.0. Substantial proteolysis of gliadin and glutenin proteins during sourdough fermentation was demonstrated previously through the use of fluorescent substrates in situ (*16*). Autodigestion of Osborne fractionated gluten at acidic conditions resulted in glutenin hydrolysis and the generation of degradation products (*16*). Protein patterns similar to those reported by Bleukx et al. (*16*) were observed by electrophoretic analysis of Osborne fractions obtained from fermented doughs. Thus, hydrolysis of glutenins is mainly dependent on the pH and was not related to specific proteinases of lactic acid bacteria, in accordance with previous observations that wheat proteinases that degrade gluten proteins have their optimum activity at pH values of ≤4.0 (*15*, *16*, *31*).

Quantification of gliadin and glutenin proteins by HPLC in this study indicated that glutenin polymers were depolymerized during fermentation of acid control doughs and doughs fermented with *L. sanfranciscensis* or *L. pontis*. Increased amounts of protein were extracted with SDS or 50% 1-propanol; correspondingly, decreased amounts of proteins were recovered in the second extraction using SDS−DTT or propanol−acetic acid−DTT. Solubilization of the glutenin polymers was also revealed by SEC analysis of SDS-soluble proteins, which increased during fermentation and were mainly monomers. Whereas >50% of gluten proteins occurred as soluble or insoluble polymers in unfermented doughs, the majority of gluten proteins were recovered as SDS-soluble monomeric proteins after 24 h of fermentation at pH values of <4.0. SDS-soluble and-insoluble glutenin polymers are thought to differ in their molecular conformation in addition to their molecular weight (*32*). Several factors may account for the gluten depolymerization: an enhanced solubility of glutenins at low pH in the presence of organic acids, proteolytic hydrolysis of glutenin subunits, and inhibition of glutathione dehydrogenase by low pH.

The concentrations of lactic and acetic acids in wheat sourdoughs after 24 h of fermentation typically range between

150 and 250 mM and between 20 and 50 mM per kilogram of dough, respectively, and these concentrations are known to enhance the solubility of glutenins. The pH affects non-covalent interactions between glutenin subunits and thus may increase their solubility in dilute SDS. However, the buffering capacity of extraction solvents used in this work compensated for differences in pH of the various dough samples during extraction.

In sourdoughs and acid control doughs, high M_r glutenin subunits were hydrolyzed during fermentation, and this proteolytic degradation of gluten proteins is expected to contribute to the depolymerization of the GMP. However, proteolytic degradation of glutenins does not fully account for the gluten depolymerization in unfermented acid control doughs. In these doughs, a depolymerization of gluten proteins was already apparent in unfermented doughs, but proteolytic degradation of glutenin subunits was observed only after 6 h and was more pronounced after 24 h of fermentation. The high level of monomeric proteins in unfermented acid control doughs could arise indirectly from the inhibition of GSH dehydrogenase by low pH (*33*). Thus, GSH may remain available for glutenin depolymerization through disulfide interchange in those doughs acidified already during dough mixing. Sulfhydryl/disulfide (SH/SS) interchange reactions of low molecular weight sulfhydryls into flour proteins weaken the gluten by SS interchange of intermolecular SS bonds (*13*, *34*). Lactic acid bacteria generate GSH through the activity of glutathione dehydrogenase during fermentation (*35*) and may thus further contribute to depolymerization of disulfide-bonded glutenins.

Data in the literature on loaf volumes of sourdough fermented wheat breads demonstrate that increased volumes are obtained when compared to straight dough processes under otherwise identical conditions (*3*, *36*−*38*). In wheat sourdough processes typical for the bakery practice, only 10−20% of the flour used for baking is fermented with lactobacilli to a dough pH of <4.0. Inclusion of a sourdough preferment in which gluten proteins were degraded to bread dough at a 20% level results in a weakened gluten network in bread doughs as well. (*18*). The conclusion that, of all flour components, protein or protein-related parameters determine bread-making quality to the greatest extent (*9*) may therefore not apply for sourdough fermented wheat breads. Our study indicates that gluten properties of wheat flours are of decreased importance in sourdough baking, whereas other factors are expected to be more relevant. Wheat arabinoxylans are solubilized during sourdough fermentation, and sourdough lactobacilli produce substantial amounts of exopolysaccharides during fermentation (*7*). The increased contents of water-soluble polysaccharides in sourdough fermented bread doughs compared to straight doughs contribute to the water absorption and gas retention capacities of the doughs as observed in rye baking. Changes in the solubility and cross-linking of wheat polysaccharides rather than changes in wheat proteins may contribute to the beneficial effect of sourdough fermentation on the loaf volume of wheat breads.

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