

Contribution of Sourdough Lactobacilli, Yeast, and Cereal Enzymes to the Generation of Amino Acids in Dough Relevant for Bread Flavor

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ABSTRACT

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The amino acid release was determined in wheat doughs supplied with salt, acid, dithiothreitol, or starter cultures to evaluate the relevance of the amino acid concentration on bread flavor. Wheat flour proteinases almost linearly released amino acids and the highest activity of wheat flour proteinases was found in acidified and reduced doughs. The effects of starter cultures on amino acid concentrations depended on their composition. Yeasts exhibited a high demand for amino acids, however, the total amino acid concentrations were not markedly affected by lactic acid bacteria. The individual amino acid contents were determined by the pH during fermentation and microbial metabolism. The formation of

proline was favored by values higher than pH 5.5, whereas release of phenylalanine, leucine and cysteine mainly occurred at lower pH. Ornithine was found only in doughs fermented with *Lactobacillus pontis*. To determine effects of the amino acid concentration on bread aroma, fermented doughs were evaluated in baking experiments. An increased intensity of bread flavor was obtained by preferments prepared with lactic acid bacteria. The roasty note of wheat bread crust could be markedly enhanced by *L. pontis*. This results support the assumption that flavor of wheat bread is enhanced by increasing the concentration of free amino acids and especially ornithine in dough.

Bread is prepared out of the essentially tasteless ingredients flour, water, salt and yeast. Almost all flavor active components are formed during dough fermentation and baking. The aroma compounds contributing to the typical bread flavor were identified in previous studies (Schieberle 1996). The roasty aroma of wheat bread depends on the formation of flavor active compounds in the crust during the baking process. 2-acetylpyrroline was identified as the character impact compound for the odor of wheat bread crust. In addition 13 more volatile compounds are important for the crumb aroma (Schieberle 1996). These compounds originate from the fatty acid oxidation (nonenal or decadienal), the thermal degradation of sugars (4-hydroxy-2,5-dimethyl-3(2H)-furanone), the thermal degradation of amino acids (methylbutanal, methional, and acetylpyrroline) or are products of microbial metabolism of amino acids (methylbutanol or phenylethanol). Amino acids in dough are reduced to 10–20% of their initial value during baking of sourdough breads (Gobbetti et al 1995). Increased concentrations of ornithine, leucine, and phenylalanine in dough resulted in an increased conversion to the flavor volatiles during bread production (Schieberle 1990; Gassenmeier and Schieberle 1995). Increased amounts of free amino acids in wheat dough may improve the flavor of wheat bread because of the significant role of amino acids for bread aroma. An enhanced proteolysis brought about by sourdough fermentation may account for the characteristic sensory properties of sourdough breads compared with breads produced from chemically acidified or yeasted doughs (Spicher et al 1980; Hansen et al 1989).

The preparation of preferments or the utilization of sourdough fermentation increased proteolysis and amino acid liberation in wheat and rye doughs (Spicher and Nierle 1984; Gobbetti et al 1994; Collar et al 1992). Generally, sourdough fermentations with lactic acid bacteria resulted in an increase of amino acid concentrations during the fermentation time, whereas dough fermentation with yeasts reduced the concentration of free amino acids. This enhanced proteolysis during sourdough fermentation may be attributed either to the proteolytic activity of sourdough lactic acid bacteria, or to an enhanced proteolysis by cereal enzymes under the conditions of the sourdough fermentation.

Microflora of sourdough depends strongly on fermentation conditions. The microflora of sourdoughs sustained by repeated inoculation at ambient temperature consists mainly of strains of *Lacto-*

bacillus sanfranciscensis (type I doughs). Strains of *L. reuteri*, *L. fermentum*, *L. pontis*, and *L. panis* are most frequently isolated from sourdoughs with longer fermentation times, or those doughs fermented at elevated temperature (type II doughs) (Vogel et al 1999). Lactic acid bacteria exhibit proteinase and peptidase activities, which are mainly bound to the cell wall (for review, see Christensen et al 1999).

The proteolytic system of *L. sanfranciscensis* CB1 was characterized and includes proteinase, dipeptidase, and aminopeptidase activities (Gobbetti et al 1996). However, a screening of several strains of *L. sanfranciscensis* for proteolytic activity toward gluten indicated that they only weakly hydrolyze wheat proteins (Wehrle et al 1999).

Kratochvil and Holas (1984) characterized the proteolytic activity of rye sour dough during the first 3 hr subject to temperature, pH, and sour dough type. Sterile doughs exhibited the same proteolytic activity as fermentations started with pure lactobacilli. Yeasted doughs were characterized by lower amino acid contents. The proteolytic activity of wheat doughs were determined by the increase of trichloroacetic acid soluble nitrogen or changes in rheological properties (Wang and Grant 1969; Wu and Hosney 1989). Recently, proteolytic enzymes associated with wheat gluten have been purified and characterized (Bleukx et al 1997; Bleukx and Delcour 2000).

Based on the data available on the proteolytic activity of wheat (sour) doughs, it remains unclear whether the proteolysis during sourdough fermentation is attributed to the proteolytic activity of starter cultures or cereal enzymes. A more detailed analysis of proteolysis during sourdough fermentation is required for the deliberate selection and combination of raw materials and starter cultures with defined enzymatic activities for improved bread flavor. It must further be taken into account that even limited proteolytic degradation of wheat proteins affects the physical properties of gluten and has a major effect on bread firmness and staling (Corsetti et al 2000). It was the aim of the present work to determine the contribution of cereal and microbial enzymes to the proteolytic liberation of amino acids during sourdough fermentation as well as the relevance for sensory attributes of bread. The effect of acidification and reducing agents on proteolysis in sterile wheat doughs was compared with the proteolytic activities of sourdoughs fermented with defined starter cultures. To assess the effect of proteolysis on bread flavor, the concentrations of individual amino acids relevant as precursor compounds for flavor volatiles (ornithine, phenylalanine, leucine, isoleucine, and methionine) was determined and baking experiments were conducted using sterile or lactic fermented doughs with known amino acid content. The relevance of the dough amino acid levels for bread flavor was determined by the sensory analysis of bread.

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MATERIALS AND METHODS

Media, Microorganisms, and Growth Conditions

The microorganisms used in this study are shown in Table I. All strains are sourdough isolates. *Lactobacillus pontis* was selected because of its ability to convert arginine to ornithine (Vogel et al 1994). *Lactobacillus sanfranciscensis* LTH2581, previously designated *Lactobacillus brevis* var. *lindneri* II, is a constituent of a commercially available sourdough starter (Böcker et al 1990). The yeasts were grown on modified mMRS4 (Stolz et al 1993) at room temperature. The lactic acid bacteria were cultured at 30°C on modified Homiochii media containing 7.0 g of glucose × H₂O, 7.0 g of fructose, 7.0 g of maltose, 10.0 g of pepton from casein, 2.0 g of meat extract, 7.0 g of yeast extract, 5.0 g of sodium-acetate trihydrate, 5.0 g of (NH₃)₂ citrate, 5.0 g of KH₂PO₄, 2.0 g of Na-gluconate, 0.5 g of L-cysteine HCl × H₂O, 0.2 g of MgSO₄ × 7 H₂O, 0.1 g of MnSO₄ × H₂O, 0.05 g of FeSO₄ × 7 H₂O, 1.0 g of Tween 80. The media was adjusted to pH 5.4. Solid media additionally contained 17.0 g/L of agar.

TABLE I
Microorganisms and Culture Conditions

| Organism | Strain | Reference | Culture |
|---------------------------------|-----------|----------------------|----------------------------|
| <i>Lactobacillus pontis</i> | DSM 8475 | Vogel et al. (1994) | Homiochii, anaerobic 30 °C |
| <i>L. sanfranciscensis</i> | LTH2581 | Böcker et al. (1990) | Homiochii, anaerobic 30 °C |
| <i>Candida milleri</i> | TMW 3.139 | | MRS, aerobic 20°C |
| <i>Saccharomyces cerevisiae</i> | NCYC 1200 | | MRS, aerobic 20°C |

Analytical Procedures

Cell counts were determined by plating appropriate dilutions of dough on MRS4 agar and Homiochii agar. The media contained 20 mg/L of chloramphenicol or 75 mg/L of cycloheximid for selective enumeration of yeasts and lactic acid bacteria, respectively.

To determine the concentration of organic acids, alcohols, and carbohydrates, dough samples (500 mg) were thoroughly mixed with 500 µL of 7% perchloric acid and stored overnight at 4°C. The precipitate was removed by centrifugation at 15,000 × g for 10 min, and the clear supernatant was used for analysis by HPLC. Samples (20 µL) were injected on a Polyspher OACK column (300 × 7.8 mm, Merck, Darmstadt) connected to a refractive index detector and eluted with 5 mM H₂SO₄ at a flow of 0.4 mL/min. The column temperature was maintained at 70°C.

A modified ninhydrin method was used for determination of total amino nitrogen (Drawert 1987). Dough samples were clarified with perchloric acid as described above. Clear supernatant (100 µL) was mixed with 20 µL of 3M KCl to precipitate the perchloric acid. After 1 hr at room temperature, the precipitate was removed by centrifugation (10 min at 15,000 × g). Reagent 1 (100 µL, 5.0 g of Na₂HPO₄ × 2 H₂O, 6.0 g of KH₂PO₄, 0.5 g of ninhydrin and 0.3 g of fructose in 100 mL of H₂O_{bidest.}, pH 6.7) was mixed with 10 µL of sample and 190 µL of H₂O_{bidest.} heated for 16 min at 100°C. Reagent 2 (500 µL, 0.2 g of KIO₃ dissolved in 60 mL of bidistilled water and 40 mL of 96% ethanol) were added. The sample was mixed thoroughly and the absorbance of the solution was measured at 570 nm. A calibration curve was prepared with each measurement using glycine as standard. Results were expressed as mmol of glycine/L. The coefficient of variation of the assay was generally <5%; samples from duplicate sourdough fermentation were reproducible with a coefficient of variation of ≤10%.

TABLE II
Dough Formulas for Preparation of Sourdoughs

| | Sterile | Sterile Salt | Sterile Acid | Sterile DTT | Yeast | Mixed Culture | Lab |
|--|---------|--------------|--------------|-------------|-------|---------------|------|
| Wheat flour | 10 g | 10 g | 10 g | 10 g | 10 g | 10 g | 10 g |
| Tap water | 30 g | 30 g | 30 g | 30 g | 26 g | 22 g | 26 g |
| Chloramphenicol | 4 µg | 4 µg | 4 µg | 4 µg | 4 µg | - | - |
| Erythromycin | 4 µg | 4 µg | 4 µg | 4 µg | - | - | - |
| Cycloheximide | 6 µg | 6 µg | 6 µg | 6 µg | - | - | - |
| NaCl | - | 0.4 g | - | - | - | - | - |
| Lactic and acetic acids ^a | - | - | 180 µL | - | - | - | - |
| DTT | - | - | - | 77 mg | - | - | - |
| <i>Candida milleri</i> or <i>Saccharomyces cerevisiae</i> | - | - | - | - | 4 mL | 4 mL | - |
| <i>Lactobacillus sanfranciscensis</i> or <i>L. pontis</i> | - | - | - | - | - | 4 mL | 4 mL |

^a Mixture of 4 volumes of lactic acid (90%) and 1 volume of acetic acid (98%).

TABLE III
Preparation of Preferments for Baking Experiments

| Dough | Starter Culture | Fermentation Time (hr) | % Preferment Addition ^a | Additives |
|-------|--|------------------------|------------------------------------|--------------------------|
| D1 | None (control) | - | - | - |
| D2 | None (control, low amino acids) | - | - | Amino acids ^b |
| D3 | None (control, high amino acids) | - | - | Amino acids ^c |
| D4 | none (chemically acidified) | 40 | 13 | Lactic and acetic acids |
| D5 | <i>Saccharomyces cerevisiae</i> | 24 | 13 | |
| D6 | <i>Candida milleri</i> | 24 | 13 | |
| D7 | <i>Lactobacillus sanfranciscensis</i> | 40 | 13 | |
| D8 | <i>L. sanfranciscensis</i> + ornithine | 40 | 13 | Ornithine |
| D9 | <i>L. pontis</i> | 40 | 13 | |
| D10 | <i>L. pontis</i> | 20 | 7 | |
| D11 | <i>L. pontis</i> | 40 | 7 | |
| D12 | <i>L. pontis</i> | 40 | 3.5 | |
| D13 | <i>L. pontis</i> | 40 | 13 | |

^a % Flour used for preparation of preferment.

^b 0.23 mmol of ornithine, 0.53 mmol of leucine, 0.30 mmol of phenylalanine, 0.23 mmol of isoleucine, and 0.13 mmol of methionine per kg of flour.

^c Amino acids (10 mmol/kg of flour).

Amino Acid Analysis

To determine the amino acid concentration, dough (500 mg) was mixed with 500 μL of 96% ethanol. The sample was incubated for 1 hr at 4°C to prevent proteolysis during sample extraction. After centrifugation, 500 μL of supernatant was freeze-dried. It was verified by extraction of dough samples and cultures of *L. sanfranciscensis* and *Saccharomyces cerevisiae* in mMRS4 that this extraction procedure provided the same amino acid recovery from doughs as the extraction procedure previously used by Collar et al (1991). Additionally, it recovered amino acids accumulated intracellularly by the fermentative microorganisms (data not shown). The sample was analyzed on a cation exchange column by an automated amino acid analyzer (LC3000, Eppendorf Biotronik, Germany) using ninhydrin postcolumn derivatization essentially according to Spackmann et al (1958). Sample (20 μL) was injected on a BTC. 2410 column (125 \times 4.6 mm, 4- μm particle size, 10% cross-linked) and eluted at 0.25 mL/min with the following solvents and column temperatures: buffer A (8.2 g/L of Na-acetate, 75 mL/L of methanol, 3 mL/L of formic acid, 15 mL/L of acetic acid, 100 $\mu\text{L/L}$ of octanoic acid, pH 3.3) 10 min, 47°C; buffer B (8.2 g/L of Na-acetate, 3 mL/L of formic acid, 20 mL/L of acetic acid, 100 $\mu\text{L/L}$ of octanoic acid, pH 3.6) 6 min, 48°C; buffer C (8.2 g/L of Na-acetate, 2 mL/L of formic acid, 1.5 mL/L of acetic acid, 100 $\mu\text{L/L}$ of octanoic acid, pH 4.5) 9 min, 49°C; buffer C, 14 min, 50°C min; buffer D (8.2 g/L of Na-acetate, 1.2 mL/L of formic acid, 5 mL/L of acetic acid, 2 g/L of boric acid, Na₂ EDTA 0.5 g/L, NaOH 6 g/L, 100 $\mu\text{L/L}$ of octanoic acid, pH 11.0) 15 min, 52°C; buffer E (as buffer D, pH 11.6) 10 min, 56°C, buffer E, 18 min, 60°C. Derivatization with ninhydrine was achieved with a flow of 0.25 mL/min of ninhydrin reagent (20 g of ninhydrin, 0.6 g of hydrindantin dihydrate, 50 mL of tetrahydrofuran, 500 mL of ethyleneglycol, 450 mL of 5.1 molar K-acetate, pH 5.59) at 125°C. Proline was detected by UV-absorption at 440 nm and all other amino acids at 570 nm. The coefficient of variation of the analysis was generally <2% and samples from duplicate sourdough fermentations were reproducible with a coefficient of variation $\leq 6\%$.

Sourdough Fermentation

Wheat flour was obtained at a local mill and contained 13.6% moisture, 34% wet gluten, and 0.98% ash. Starter cultures were grown overnight, harvested at 15,000 \times g for 10 min, washed once with sterile tap water, and then resuspended in the same volume of sterile tap water. Doughs with a dough yield of 400 were prepared according to the formulas shown in Table II in 50-mL glass beakers, stirred to homogeneity with a spatula, covered, and incubated at 30°C. Samples were taken at appropriate intervals to determine viable cell counts, pH level, and concentrations of organic acids, ethanol, and amino acids. The absence of contaminants in sourdough fermentation was verified by determination of the colony morphology and microscopy of selected colonies for the determination of the cell morphology. Each fermentation was conducted in duplicate or quadruplicate.

Baking Experiments

Preferments were prepared with 480 g of wheat flour, 120 mL of cell suspension, and 600 g of tap water, mixed for 2 min at medium speed, and 1 min at high speed with a Hobart kneader and incubated at 30°C, and 80% rh. Bread dough was prepared with 3,000 g of flour, 1,740 g of tap water, 150 g of pressed yeast, 60 g of salt, 16.2 g of glucose, and 27 g of of a commercially available baking aid devoid of protease activities (composition according to supplier: 30% soy lecithin, 30% guar flour, 30% gelatinized flour 8% vegetable fat, 1% ascorbic acid, 0.25% α -amylase, and 1% hemicellulase preparation). Preferments were prepared as indicated in Table III, and the dough formulas were corrected for the amounts of flour and water added with the preferment. Bread doughs were kneaded with a Diosna spiral kneader, 2 min at low speed and 5 min at high speed. First proofing of the doughs lasted 20 min at 25°C, second proofing of the formed rolls took 45 min at 30°C

and 80% rh. The rolls were baked for 17 min at 240°C at three different places in the oven to rule out possible effects of heat and moisture variations within the oven. After 1 hr of cooling at room temperature, sensory evaluation was performed by an expert panel consisting of three to eight persons. Three batches of dough and the control dough (straight-dough process without preferment or additives) were prepared on a single day. The panelists were asked to score the aroma intensity of these four samples and to describe the crust odor in comparison with that of the control rolls.

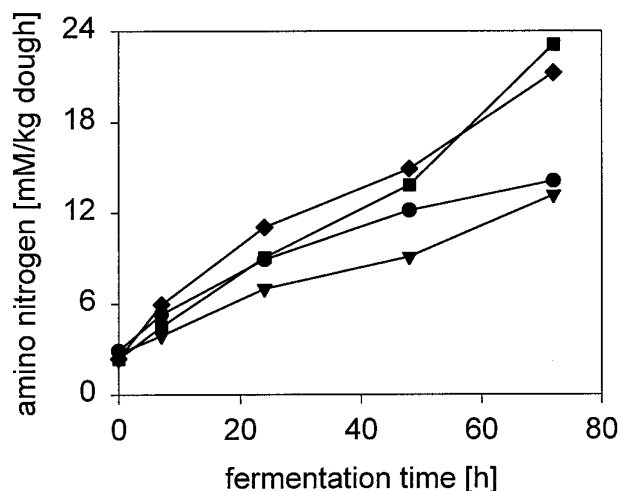


Fig. 1. Concentration of total amino nitrogen in sterile fermented doughs. Amino nitrogen concentration in dough without additives (●, $n = 4$); dough containing 4% NaCl (▼, $n = 2$), dough acidified to pH 3.8 (■, $n = 4$), and dough containing 0.7% DTT (◆, $n = 2$). Mean values of n fermentations and coefficient of variation <10%.

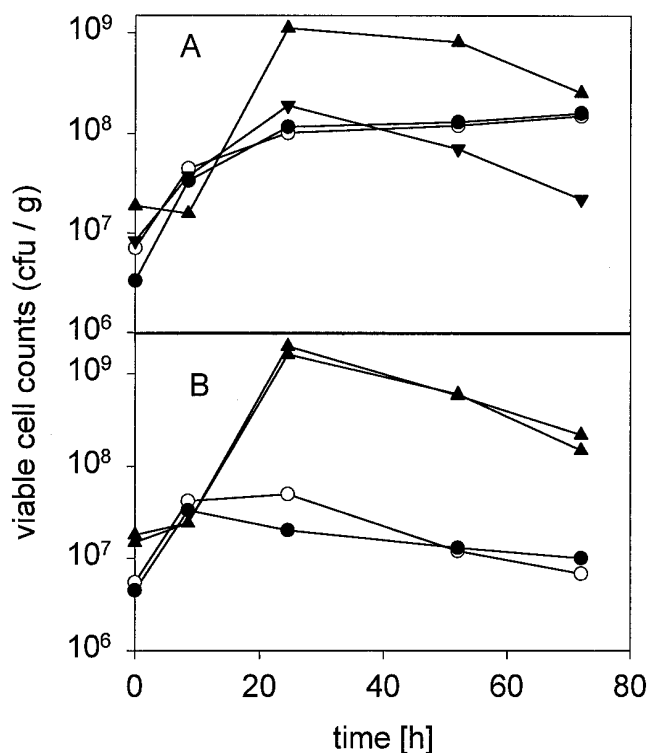


Fig. 2. Cell counts of lactic acid bacteria and yeasts during dough fermentation. **A.** Pure cultures. **B.** Mixed cultures of *Lactobacillus sanfranciscensis* (▼), *L. pontis* (▲), *Saccharomyces cerevisiae* (○), *Candida milleri* (●). Data represents duplicate fermentations.

RESULTS

Effect of Salt, Acid and Dithiothreitol (DTT) on Total Amino Nitrogen Content in Sterile Wheat Doughs

To take into account pH and rH effects on the activity of wheat flour proteolytic enzymes during the sourdough fermentation, the amino nitrogen release in sterile doughs was determined. Wheat doughs with different additives were incubated for three days at 30°C. Microbial growth was inhibited by addition of erythromycin, chloramphenicol, and cycloheximide and cell counts of sterile doughs did not exceed 10⁴ cfu/g, excluding an influence of microbial proteases. The amino nitrogen concentrations of sterile wheat doughs are shown in Fig. 1. In all doughs, the amino nitrogen content increased during fermentation. After 24 hr, between 4.3 and 8.3 mM of amino nitrogen is released. The addition of salt reduced the release of amino nitrogen. In contrast, addition of lactic and acetic acids and DTT increased the rate of proteolysis. Whereas after 24 hr, the highest amino nitrogen levels were found in sterile DTT doughs, amino nitrogen levels of reduced and acidified doughs were virtually identical after 72 hr of incubation.

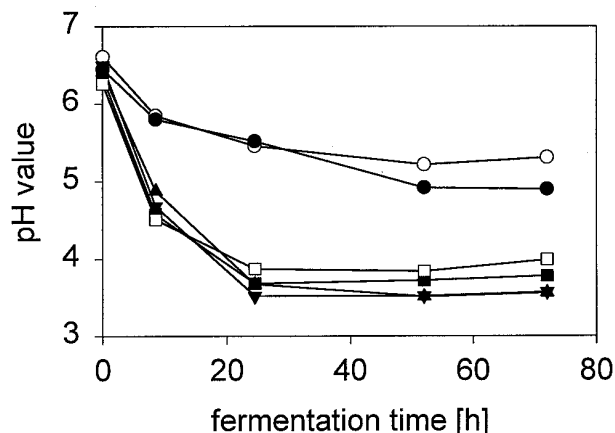


Fig. 3. Evolution of pH in doughs fermented with lactobacilli and yeasts. pH value of doughs fermented with *Saccharomyces cerevisiae* (○), *Candida milleri* (●), *Lactobacillus sanfranciscensis* (▼), *L. pontis* (▲), *L. pontis* and *S. cerevisiae* (□), and *L. pontis* and *C. milleri* (■). Data represents duplicate fermentations.

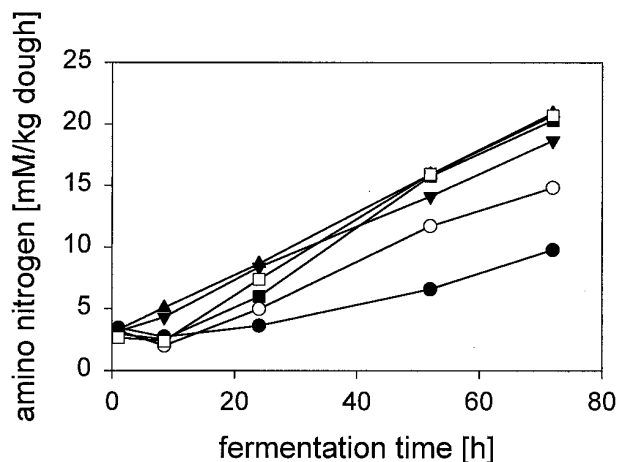


Fig. 4. Concentration of total amino nitrogen in doughs fermented with lactobacilli and yeasts. Amino nitrogen concentration in dough fermented with *Saccharomyces cerevisiae* (○), *Candida milleri* (●), *Lactobacillus sanfranciscensis* (▼), *L. pontis* (▲), *L. pontis* and *S. cerevisiae* (□), and *L. pontis* and *C. milleri* (■). Mean values of duplicate fermentations and coefficient of variation <10 %.

Total Amino Nitrogen Content in Fermented Wheat Doughs

Wheat doughs were fermented with starter cultures: *Saccharomyces cerevisiae*, *Candida milleri*, *Lactobacillus sanfranciscensis*, *Lactobacillus pontis*, and mixtures of *L. pontis* with *S. cerevisiae* or *C. milleri*. Because the demand of microorganisms for amino acids during growth, as well as the pH drop during fermentation, are likely to affect amino acid concentrations, the growth of microorganisms as well as the dough pH were determined. The growth of the microorganisms in doughs is shown in Fig. 2. In all fermentations, contaminants were not detectable, that is, they accounted for <0.1% of the total viable cell counts. The yeasts and *L. sanfranciscensis* started to grow essentially without lag and reached the stationary growth phase after 24 hr of incubation. *L. pontis* exhibited a lag phase of ≈4 hr and grew exponentially at 10–20 hr. The cell counts of lactic acid bacteria decreased by ≈10% in the stationary phase of growth. Whereas cell counts of *L. pontis* were unaffected by the presence or absence of yeasts, the cell counts of *S. cerevisiae* and *C. milleri* were considerably reduced with *L. pontis*.

The pH values of fermented doughs are shown in Fig. 3. In doughs fermented with lactobacilli, the pH decreases from ≈6.5 to 4.5–5.0 within 8 hr. After 24 hr, when the lactobacilli reached the stationary growth phase, acid production ceased and the pH remained stable. The final pH of doughs fermented with lactobacilli only was ≈0.2 units lower than the pH of doughs fermented with mixed cultures of yeasts and lactobacilli. In doughs fermented with pure cultures of *S. cerevisiae* and *C. milleri*, the pH decreased to 5.3 and 4.9, respectively, within 52 hr and remained stable afterward.

The total amino nitrogen of fermented doughs during fermentation is shown in Fig. 4. The release of amino nitrogen is clearly related to the growth of the organisms. In all doughs fermented with yeasts, a decrease in amino acid concentration during the exponential growth phase was detected. Amino acid concentrations started to increase after the yeasts entered the stationary phase of growth. In doughs fermented with lactobacilli only, amino acid concentrations linearly increased throughout the fermentation. The final amino acid concentration was highest in doughs fermented with *L. pontis*, with or without yeasts. Fermentation with *L. sanfranciscensis* resulted in slightly lower amino acid concentrations. The lowest amino nitrogen concentrations were found in doughs fermented with yeasts only. Whereas 20 mmol/kg of amino nitrogen were detected in doughs fermented with *L. pontis*, only 14 and 10 mmol/kg were determined in doughs fermented with *C. milleri* and *S. cerevisiae*, respectively.

A comparison of sterile fermented doughs with doughs inoculated with lactobacilli or yeasts allows us to estimate the contribution of microbial proteases to proteolysis in wheat doughs. In doughs fermented with yeasts, the amino nitrogen concentration during the first 24 hr was lower than that of any sterile doughs, indicating that the amino acid consumption of yeasts is greater than any proteolytic activity the organisms may exhibit. The release of amino nitrogen in doughs fermented with lactobacilli did not exceed the amino nitrogen concentrations in acidified or reduced doughs, indicating that the proteolytic activity of lactobacilli is negligible compared with the proteolytic activity of wheat flour. The reduction of the pH and rH values brought about by the lactic fermentation however, greatly enhanced proteolysis in dough compared with neutral, sterile doughs.

Changes in Individual Amino Acid Concentration

The concentration of amino acids such as ornithine, methionine, phenylalanine, leucine, isoleucine, and valine in dough is of prime importance for bread flavor. We therefore determined the concentration of individual amino acids in dough by HPLC. To compare the amino acid composition of doughs with different absolute levels of total amino acid concentration, the content of individual amino acids relative to the total amino acid concentration was calculated. The

amino acid concentration determined by HPLC agreed well with the total amino nitrogen determined with the ninhydrin method ($r^2 \geq 0.95$). The relative contents of sterile and fermented doughs of arginine and ornithine are shown in Fig. 5A and B. Because ornithine, one of the precursors for acetylpyrroline, is not a proteinogenic amino acid, its presence in dough is the result of microbial metabolism. Ornithine was found only in doughs fermented with *L. pontis*. Arginine was released by proteolytic enzymes from wheat proteins and the relative content of arginine was largely independent of the presence of acid, DTT, or salt. Arginine was not found in doughs fermented with *L. pontis*. However, the ornithine levels in these doughs corresponded well to the arginine levels in sterile doughs or doughs fermented with *L. sanfranciscensis* or yeasts.

The relative proline content of sterile and fermented doughs is shown in Fig. 5C. The highest proline contents were found in yeasted doughs and sterile neutral doughs. In contrast, relative proline contents remained low in sterile acidified doughs. In doughs fermented with lactobacilli, proline levels corresponded to those of neutral doughs in the first hours of fermentation and a decrease of the relative proline content was observed after the dough was lowered to pH 5.5 or less. After 72 hr, the relative content of proline was <33% of the neutral and yeasted doughs. These results show that the release of proline depends strongly on the dough pH. The high relative proline content of doughs fermented with *S. cerevisiae* indicates that proline, in contrast to most other amino acids, is not metabolized by the yeast.

The relative levels of phenylalanine and leucine in sterile and fermented doughs are shown in Fig. 5D and E. The release of these amino acids was low in sterile neutral doughs, and the highest levels of hydrophobic amino acids were found in chemically acidified and lactic fermented doughs. Both amino acids were metabolized by *S. cerevisiae* during exponential growth. *L. sanfranciscensis* and *L. pontis* did not exhibit any demand for these amino acids. During the first 10 hr the relative content of phenylalanine and leucine compared well with that of neutral sterile doughs. When the dough reached a pH 4.0, phenylalanine and leucine levels were comparable with that of sterile acidified dough.

Cysteine affects dough rheology and methionine serves as precursor for the aroma compound 3-(methylthio)propanal. The relative contents of cysteine and methionine of sterile and fermented doughs are shown in Fig. 5F and G. Cysteine was released only in acidified doughs; in all other sterile samples, only traces were detected. Likewise, methionine levels in sterile acidified doughs were $\approx 50\%$ higher than that of neutral doughs. Whereas cysteine and methionine levels of doughs fermented with *L. sanfranciscensis* were comparable with that of chemically acidified doughs, fermentation of doughs with *L. pontis* reduced the levels of both amino acids. This indicates that *L. pontis*, in contrast to *L. sanfranciscensis*, degrades these amino acids.

Sensory Evaluation of Rolls

To establish the influence of different preferments on the taste of wheat bread rolls, baking experiments and subsequent flavor analysis were conducted. The results of the descriptive sensory evaluation and the ranking according to the aroma intensity are given

in Table IV. A total of three different preferments was prepared on a single day and compared with a control roll without addition of a preferment. Any bread prepared with a preferment had a more aromatic flavor than the control bread.

Doughs D1 to D3 were prepared to evaluate the effect of addition of amino acids to bread dough on bread flavor. Addition of ornithine, isoleucine, leucine, phenylalanine, and methionine at concentrations comparable to microbial preferments did not result in significant differences compared with the control bread. When 10 mmol of amino acids/kg of flour were added, an aromatic and roasty to slightly burnt odor was detectable. The taste was bitter, burnt, slightly cheesy, and rubberlike. Preferments inoculated with either *S. cerevisiae* or *C. milleri* improved the flavor of the rolls. A more aromatic and yeasty flavor was perceived (doughs D5 and D6) compared with the controls.

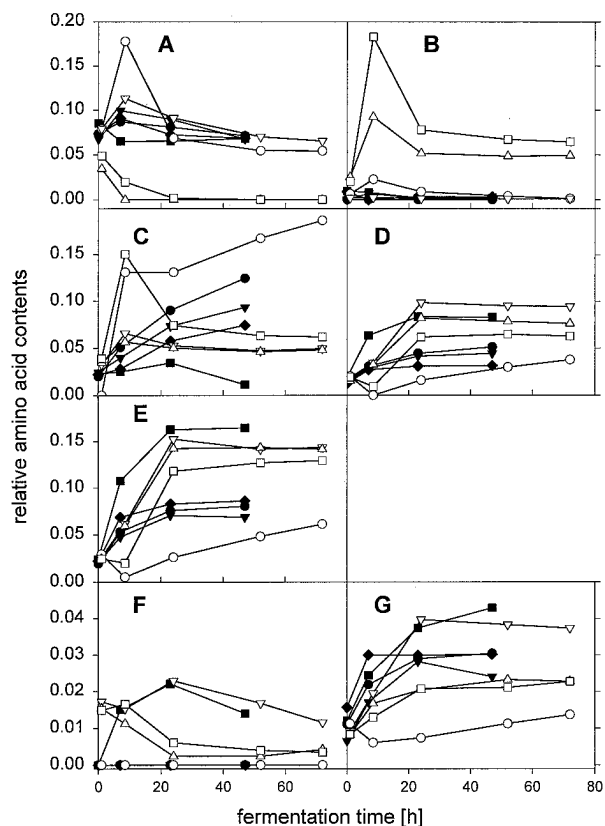


Fig. 5. Relative amino acid contents of sterile and fermented doughs during incubation at 30°C. Concentration of arginine (A), ornithine (B), proline (C), phenylalanine (D), leucine (E), cysteine (F) and methionine (G) relative to the total amino acid concentration as determined by HPLC. Sterile doughs without additives (●), sterile dough with 4% NaCl (▼), sterile dough acidified to pH 3.5 (■), sterile dough with 0.7% DTT (◆), dough fermented with *Saccharomyces cerevisiae* (○), *Lactobacillus sanfranciscensis* (∇), *L. pontis* (Δ) and *L. pontis* and *S. cerevisiae* (□). Mean values of duplicate fermentations and coefficient of variation <5 %.

TABLE IV
Sensory Analysis of Wheat Rolls

| Aroma Intensity ^a | | | |
|------------------------------|---|---|--|
| Lowest | Low | High | Highest |
| D1 mild, flat | D3 aromatic, bitter, burnt, cheesy, rubberlike | | |
| D1, D2 mild, flat | D4 not fresh, slightly more aromatic, slightly roasty | D5, D6 more aromatic, yeasty, mild sour | |
| D1 mild, flat | D7 more aromatic, mild sour, slightly fermented | D8 more aromatic, well rounded | D9 aromatic, roasty to burnt, bitter, sour |
| D1 mild, flat | D10 slightly aromatic, slightly roasty | D11 aromatic, roasty, mild sour | |
| D1 mild, flat | D12 slightly sour, mild | D13 aromatic very sour, very roasty | |

^a Doughs supplemented with different preferments as indicated in Table III and doughs within a row were compared with each other and ranked according to aroma intensity.

Chemically acidified preferments produced breads with a slightly roastier flavor than the control breads. In comparison, doughs fermented with *L. sanfranciscensis* exhibited an improved aroma despite comparable levels of acidity as well as organic acid and amino acids concentrations. When ornithine was added to the *L. sanfranciscensis* preferment before baking, the overall impression of the flavor improved. The flavor of breads prepared with preferments containing *L. sanfranciscensis* and *L. pontis* were clearly discernible by the more roasted flavor of the latter breads. Addition of 13% *L. pontis* preferment was perceived as burnt and bitter by several panelists.

To distinguish between the effect of microbial metabolism and amino acid accumulation, preferments with *L. pontis* were incubated for 20 and 40 hr. After 20 hr, growth and organic acid production by *L. pontis* ceased, but proteolytic release of amino acids and ornithine formation by *L. pontis* continued over 40 hr. The roasty note of bread crust was enhanced by increased fermentation times of the preferments. The flavor intensity of 40-hr fermented bread was so high that it was perceived as unpleasant by several panelists. The roasty note of bread could further be enhanced by increasing levels of *L. pontis* preferments added to the bread doughs (doughs D11, D12, and D13).

DISCUSSION

We have determined the effect of acidification, addition of DTT, and sourdough fermentation on amino acid levels in dough. The results show that the amino acid levels in wheat doughs depend mainly on the pH level of the dough, the fermentation time, and the consumption of amino acids by the fermentative microflora. It was demonstrated that increased levels of amino acids in doughs improved bread flavor. Furthermore, microbial formation of ornithine specifically enhanced the roasty note of bread crust odor.

The highest proteolytic activity was observed in acidified and reduced sterile doughs. Several mechanisms may account for the effect of salt, pH, and reducing agents on the proteolytic system of wheat doughs.

The additives may affect the activity of proteolytic enzymes. Endogenous wheat proteinases be optimum at pH 3.0–4.0 and the activity decreases toward higher pH (Kawamura 1982; Wu and Hoseney 1989; Bleux and Delcour 2000). Reducing agents such as DTT may further activate sulfhydryl proteases that are present in wheat flour (Kruger et al 1991).

The release of amino acids is also influenced by the level of accessible substrate. The insolubility of gluten in water may limit the substrate concentration for proteolytic enzymes. The reduction of interprotein disulfide bonds during dough mixing enhances the solubility of gliadins and glutelins. Lactic and acetic acids further swell gluten proteins. In our experiments, a reduced viscosity of reduced doughs was apparent immediately upon mixing, whereas weakening of acidified doughs was observed only after 24 hr of incubation. The addition of salt has an opposite effect on the solubility of gluten because of the high sensitivity of gluten to salts (Preston and Kruger 1976).

Dough fermentation with yeasts resulted in a decrease of free amino acids in the first fermentation phase due to microbial metabolism (Collar et al 1991). *S. cerevisiae* and sourdough yeasts convert leucine and phenylalanine to the flavor volatiles 3-methylbutanol and 2-phenylethanol during dough fermentation (Hansen and Hansen 1989; Damiani et al 1996; Schieberle 1996). Because of the high pH in the dough, the activity of flour proteinases is too low to replenish the amino acid pool, and amino acid levels increase only after yeasts reach the stationary growth phase.

The enhanced proteolysis during sourdough fermentation is in agreement with previous reports on the evolution of amino acid levels during sourdough fermentation (Collar et al 1991, 1992; Gobetti et al 1994). Proteolysis in sourdough was mainly attributed to

the proteolytic activity of *L. sanfranciscensis* and other sourdough lactobacilli (Gobbetti et al 1996a,b). In contrast, the comparison of amino acid levels in fermented and sterile acidified doughs presented in this work strongly indicates that the proteolytic activity of sourdough lactic acid bacteria does not exceed the demand of these organisms for amino acids during growth. Lactic acid bacteria require amino acids or peptides for growth (Juillard et al 1998) and several amino acids are metabolized to flavor volatiles (Gao et al 1998; Smacchi and Gobetti 1998; Juillard et al 1999; Smit et al 2000; Tammam et al 2000). The observation that the uptake of nitrogen by lactobacilli did not result in reduced amino acid levels in doughs is explained by the preferential uptake of peptides and intracellular hydrolysis to amino acids (Berg et al 1981; Aasen et al 2000).

The levels of individual amino acids were influenced by pH and starter culture. Ornithine formation was observed only in doughs fermented with *L. pontis* because of the ability of this organism to convert arginine to ornithine (Vogel et al 1994). Ograbek et al (*unpublished*) demonstrated that the arginine metabolism of *L. pontis* was responsible for ornithine formation in wheat doughs and contributed to a more roasty flavor of the bread crust. Our results concur with these observations that ornithine formation in doughs is greatly enhanced by arginine metabolism of lactobacilli. Schieberle (1990) reported that ornithine levels in wheat doughs and corresponding levels of 2-acetylpyrroline in wheat bread crust were enhanced by addition of baker's yeast. Ornithine levels in fresh baker's yeast range from 21 to 300 mg of ornithine/100 g of yeast dry weight (Schieberle 1990; Münch et al 1997, 1998). We found virtually no ornithine in doughs fermented with yeasts only. These large differences are explained by the fact that baker's yeast usually is contaminated by lactobacilli that may or may not be able to convert arginine to ornithine. Based on a 5% addition of baker's yeast to wheat doughs, we can estimate that ornithine from baker's yeast accounts for <2–20 mg of ornithine/kg of dough. Addition at a level of 20% of a preferment prepared with arginine-degrading *L. pontis* provides >25 mg of ornithine/kg of dough (Ograbek et al, *unpublished*).

Accumulation of proline was favored by fermentation at near neutral pH, whereas release of hydrophobic and sulfur containing amino acids was greatly enhanced by chemical or microbial acidification. The pH for optimum activity of proline aminopeptidases apparently differs from that of other wheat proteolytic enzymes, which are mainly active at low pH (Bleux et al 1997).

The effect of proteolysis during dough fermentation of bread aroma was evaluated by baking experiments. Any preferment improved bread flavor. Preferments that were chemically acidified or fermented with *L. sanfranciscensis* enhanced the roasty odor of the bread crust. However, overall bread taste and flavor of the latter breads was superior to sterile preferments. The roasty note of bread crust odor was most pronounced in breads fermented with *L. pontis*. Because dough fermentation with *L. pontis* and *L. sanfranciscensis* resulted in comparable amino acid levels of the preferments, with exception of arginine and ornithine levels, this roasty note may be attributed to the presence of ornithine in doughs fermented with *L. pontis*.

It must be emphasized that the preferment application chosen in our study (addition of 5–20% preferment to a yeasted bread dough) allows for two mechanisms for converting amino acids to flavor volatiles: 1) conversion of amino acids to volatiles by baker's yeast during proofing of the bread dough; and 2) thermal degradation during baking (Schieberle 1996).

A notable exception is ornithine, which is not metabolized by *S. cerevisiae* but is converted to 2-acetylpyrroline (roasty, cracker-like) during baking.

We anticipate that the differences between various preferments will be more pronounced in applications where the bread dough is leavened without addition of baker's yeast, such as in traditional sourdough fermentations.

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LITERATURE CITED

- Aasen, I. L., Moreto, T., Katla, T., Axelsson, L., and Storro, I. 2000. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Appl. Microbiol. Biotechnol.* 53:159-166.
- Berg, R. W., Sandine, W. E., and Anderson, A. W. 1981. Identification of a growth stimulant for *Lactobacillus sanfrancisco*. *Appl. Environ. Microbiol.* 42:6, 786-788.
- Bleukx, W., and Delcour, J. A. 2000. A second aspartic proteinase associated with wheat gluten. *J. Cereal Sci.* 32:31-42.
- Bleukx, W., Roels, S. P., and Delcour, J. A. 1997. On the presence and activities of proteolytic enzymes in vital wheat gluten. *J. Cereal Sci.* 26:183-193.
- Böcker, G., Vogel, R. F., and Hammes, W. P. 1990. *Lactobacillus sanfrancisco* als stabiles Element in einem Reinzucht-Sauerteig-Präparat. *Getr. Mehl Brot* 44:269-274.
- Collar, C., Mascaros, A. F., Prieto, J. A., and Benedito de Barber, C. 1991. Changes in free amino acids during fermentation of wheat doughs started with pure culture of lactic acid bacteria. *Cereal Chem.* 68:66-72.
- Collar, C., Mascaros, A. F., and Benedito de Barber, C. 1992. Amino acid metabolism by yeast and lactic acid bacteria during bread dough fermentation. *J. Food Sci.* 57:1423-1427.
- Drawert, F. 1987. *Brautechnische Analysenmethoden (MEBAK)*. Band 2. Selbstverlag der MEBAK Freising.
- Gao, S., Mooberry, E. S., and Steele, J. L. 1998. Use of ¹³C nuclear magnetic resonance and gas chromatography to examine methionine catabolism by lactococci. *Appl. Environ. Microbiol.* 64:4670-4675.
- Gassenmeier, K., and Schieberle, P. 1995. Potent aromatic compounds in the crumb of wheat bread (French-type)-influence of pre-ferments and studies on the formation of key odorants during dough processing. *Z. Lebensm. Unters. Forsch.* 201:241-248.
- Gobbetti, M., Simonetti, M. S., Rossi, J., Cossignani, L., Corsetti, A., and Damiani, P. 1994. Free D- and L-amino acid evolution during sourdough fermentation and baking. *J. Food Sci.* 59:881-884.
- Gobbetti, M., Smacchi, E., and Corsetti, A. 1996a. The proteolytic system of *Lactobacillus sanfrancisco* CB1. Purification and characterization of a proteinase, a dipeptidase and an aminopeptidase. *Appl. Environ. Microbiol.* 62:3220-3226.
- Gobbetti, M., Smacchi, E., Fox, P., Stepaniak, L., and Corsetti, A. 1996b. The sourdough microflora. Cellular localization and characterization of proteolytic enzymes in lactic acid bacteria. *Lebens. Wiss. Technol.* 29:561-569.
- Hansen, A., Lund, B., and Lewis, M. J. 1989. Flavour of sourdough rye bread crumb. *Lebens. Wiss. Technol.* 22:141-144.
- Juillard, V., Guillot, A., Le Bars, D., and Gripon, J. C. 1998. Specificity of milk peptide utilization by *Lactococcus lactis*. *Appl. Environmental Microbiol.* 64:1230-1236.
- Kawamura, Y., and Yonezawa, D. 1982. Wheat flour proteinases and their action on gluten proteins in diluted acetic acid. *Agric. Biol. Chem.* 46:767-773.
- Kratochvil, J., and Holas, J. 1984. Untersuchung über proteolytische Vorgänge im Roggensauerteig. *Getr. Mehl Brot* 38:330-332.
- Kruger, J. E., MacGregor, A., and Marchylo, B. 1991. Endogenous cereal enzymes. Pages 11-46 in: *Food Enzymology*. P. F. Fox, ed. Elsevier: New York.
- Münch, P., and Schieberle, P. 1998. Quantitative studies on the formation of key odorants in thermally treated yeast extracts using stable isotope dilution assays. *J. Agric. Food Chem.* 46:4695-4701.
- Münch, P., Hofmann, R., and Schieberle, P. 1997. Comparison of key odorants generated by thermal treatment of commercial and self-prepared yeast extracts: Influence of the amino acid composition on odorant formation. *J. Agric. Food Chem.* 45:1338-1344.
- Preston, K. R., and Kruger, J. E. 1976. Purification and properties of two proteolytic enzymes with carboxypeptidase activity in germinated wheat. *Plant Physiol.* 58:516-520.
- Schieberle, P. 1990. The role of free amino acids present in yeast as precursors of the odorants 2-acetyl-L-pyrroline and 2-acetyltetrahydropyridine in wheat bread crust. *Lebensm. Unters. Forsch.* 191:206-209.
- Schieberle, P. 1996. Intense aroma compounds—Useful tools to monitor the influence of processing and storage on bread aroma. *Adv. Food Sci.* 18:237-244.
- Smacchi, E., and Gobbetti, M. 1998. Purification and characterization of cystathionine gamma-lyase from *Lactobacillus fermentum* DT41. *FEMS Microbiol. Lett.* 166:197-202.
- Spackmann, D. H., Stein, W. H., and Moore, S. 1958. Automatic recording apparatus for use on the chromatography of amino acids. *Anal. Chem.* 30:1190-1206.
- Stolz, P., Böcker, G., and Hammes, W. P. 1993. Utilisation of maltose and glucose by lactobacilli isolated from sourdough. *FEMS Microbiol. Lett.* 109:237-242.
- Tammam, J. D., Williams, A. G., Novle, J., and Lloyd, D. 2000. Amino acid fermentation in non-starter *Lactobacillus* spp. isolated from cheddar cheese. *Lett. Appl. Microbiol.* 2000:370-374.
- Vogel, R. F., Böcker, G., Stolz, P., Ehrmann, M., Fanta, D., Ludwig, W., Pot, B., Kersters K., and Schleifer, K. H. 1994. Identification of *Lactobacilli* from sourdough and description of *Lactobacillus pontis* sp. nov. *Int. J. Sys. Bacteriol.* 44:223-229.
- Vogel, R. F., Knorr, R., Müller, M. R. A., Steudel, U., Gänzle, M. G., and Ehrmann, M. 1999. Non-dairy lactic fermentations: the cereal world. *Antonie van Leeuwenhoek* 76:403-411.
- Wang, C. C., and Grant, D. R. 1969. The proteolytic enzymes of wheat flour. *Cereal Chem.* 46:537-544.
- Wehrle, K., Crowe, N., van Boeijen, I., and Arendt, E. K. 1999. Screening methods for the proteolytic breakdown of gluten by lactic acid bacteria and enzyme preparations. *Eur. Food Res. Technol.* 209:428-433.

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