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Proteolysis and Bioconversion of Cereal Proteins to Glutamate and γ-Aminobutyrate (GABA) in Rye Malt Sourdoughs

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ABSTRACT: This study aimed to achieve the conversion of cereal proteins to the alternative end products glutamate or γ-aminobutyrate (GABA). Rye malt, fungal proteases, and lactobacilli were employed to convert wheat gluten or barley proteins. Glutamate and GABA formations were strain-dependent. *Lactobacillus reuteri* TMW1.106 and *Lactobacillus rossiae* 34J accumulated glutamate; *L. reuteri* LTH5448 and LTH5795 accumulated GABA. Glutamate and GABA accumulation by *L. reuteri* TMW1.106 and LTH5448 increased throughout fermentation time over 96 h, respectively. Peptides rather than amino acids were the main products of proteolysis in all doughs, and barley proteins were more resistant to degradation by rye malt proteases than wheat gluten. However, addition of fungal protease resulted in comparable degradation of both substrates. Glutamate and GABA accumulated to concentrations up to 63 and 90 mmol kg^{-1} DM, respectively. Glutamate levels obtained through bioconversion of cereal proteins enable the use of hydrolyzed cereal protein as condiment.

KEYWORDS: Proteolysis, glutamate, γ-aminobutyrate, sourdough, *Lactobacillus*

INTRODUCTION

Prolamins and glutelins are the primary storage proteins for nitrogen in seeds of wheat, rye, and barley. Prolamins and glutelins have a high content of glutamine to allow efficient nitrogen storage, a high proline content to allow a tight packing of proteins, and a high content of hydrophobic amino acids, which reduces their solubility in water.[1] Glutamine and proline levels in prolamins of wheat, rye, and barley range from 35 to 37% and from 17 to 23%, respectively.[1,2] Cereal proteins are therefore highly suitable as raw material for bioconversion of glutamine to glutamate and γ-aminobutyrate (GABA), bacterial metabolites of glutamine.[3]

Glutamate imparts the savory taste of foods.[4] Glutamate is accumulated by strain-specific glutaminase activity of lactic acid bacteria during food fermentations.[5,6] Dietary GABA possesses multiple physiological functions,[7] including antihypertensive properties.[8,9] Glutamate conversion to GABA in food fermentations through glutamate decarboxylase activity of lactic acid bacteria was previously reported for cheese,[10] fruit juice,[11] spent distiller's grain,[12] and different sourdoughs.[13,14]

Proteolysis during cereal fermentations was studied extensively to improve flavor formation in baking[15] and to reduce protein and peptide concentrations to levels that are safe for celiac patients.[16,17] The poor solubility of prolamins and glutelins in water and their high content of proline also impede their proteolytic degradation to liberate glutamine.[18] Proteolytic degradation of gluten proteins from ungerminated cereals requires the use of fungal enzyme preparations[19] or enzyme-active cereal malt in combination with lactic fermentation.[17,18] Germinated rye was the most potent source of enzymes for the elimination of cereal proteins in acidic malt suspensions when compared to wheat and barley malt.[20] The metabolic activity of heterofermentative lactobacilli is required for complete proteolysis to achieve acidification of the substrate to match the pH optimum of proteases and reductive depolymerization of gluten proteins to improve their solubility and to provide proline−peptidase activities.[18]

Glutamate-enriched protein hydrolysates are currently used in food production to replace the use of glutamate as additive and to enable the reduction of salt in processed foods.[21] Cereal proteins with their exceptionally high content of glutamine are a suitable substrate for the production of hydrolysates rich in glutamate or GABA. Current processes achieve the quantitative degradation of cereal proteins through a combination of enzyme addition and lactic fermentation;[19] however, peptides rather than amino acids are the main products of hydrolysis.[16,18] Moreover, kinetic data on glutamine turnover by lactobacilli in cereal substrates are missing, and degradation of purified protein preparations has not been studied.

It was therefore the aim of this study to investigate whether bacterial conversion of glutamine to glutamate or GABA can be achieved during simultaneous fermentation and proteolysis of cereal proteins. Experiments were carried out with wheat gluten or barley hordeins as substrate and rye malt flour and fungal protease as source of proteases. The selection of lactic acid

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bacteria aimed to achieve accumulation of glutamate or GABA as alternative end products from glutamine.

MATERIALS AND METHODS

Strains and Growth Conditions. *Lactobacillus reuteri* TMW1.106, *L. reuteri* LTH5448, *L. reuteri* LTH5795, *L. reuteri* 100-23, *Lactobacillus rossiae* 34J, and *Lactobacillus plantarum* FUA3002 were grown in modified Man, Rogosa, and Sharp medium (mMRS) broth with the following composition per liter: 10 g of maltose, 5 g of glucose, 5 g of fructose, 10 g of peptone, 5 g of yeast extract, 5 g of beef extract, 4 g of $K_2HPO_4 \cdot 3H_2O$, 2.6 g of KH_2PO_4, 3 g of NH_4Cl, 0.5 g of L-Cys $HCl \cdot H_2O$, 1 g of Tween80, 0.05 g of $MnSO_4 \cdot H_2O$, and 0.2 g of $MgSO_4 \cdot 7H_2O$. The pH of media was adjusted to 6.2. A stock solution was added after autoclaving to achieve a concentration of 0.2 mg L^{-1} of the following compounds: biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin, and panthothenic acid. Agar (15 g L^{-1}) was added for solid media. *L. reuteri* was grown at 37 °C, and the other strains were grown at 30 °C, and incubated under modified atmosphere (4% O_2, 20% CO_2, and 76% N_2).

DNA Isolation and PCR Amplification of Genes Coding for Glutamate Decarboxylases. DNA was isolated from 1.5 mL overnight cultures of *L. reuteri* grown in mMRS using a DNeasy Blood and Tissue kit according to the instructions of the manufacturer (Qiagen, Mississauga, Canada). PCR was used to amplify an 864 bp sequence of *L. reuteri* glutamate decarboxylases genes (NCBI reference sequence: NZ-AAPZ 02000002.1). PCR reactions were performed with an annealing temperature of 55 °C on a GeneAmp PCR System 9700 (Applied Biosystems, Streetsville, Canada) in a volume of 50 μL containing 1 μL of template DNA, 5 μL of 10× PCR reaction buffer, 1.5 μL of 25 mM $MgCl_2$ (Invitrogen), 0.25 μL of primers GluDecar-50-F, CCCTATT-TTCGGTTCATACA) and GluDecar-1029-R, TTGGCTTTGA-AGGGTATAAA (IDT, Coralville, IA), 1 μL of dNTP, 40.7 μL of deionized water, and 0.3 μL (1.5 U) of Taq-Polymerase (Invitrogen). Genomic DNA of *L. reuteri* 100-23 was used as positive control. The PCR amplicons were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

Preparation and Characterization of Cereal Substrates. Germinated rye was obtained from Laihian Mallas (Laihia, Finland) and milled in a Retsch ZM-200 ultracentrifugal mill equipped with a 0.5 mm sieve. Wheat gluten was obtained from Sigma-Aldrich (St. Louis, MO). A barley hordein preparation was obtained from barley grain cultivar Falcon (harvest 2007) essentially as described.[22] In brief, defatted barley flour from pearled grains was extracted with alkaline solution (pH 11), the insoluble solids were removed by centrifugation (8500*g* for 15 min at 23 °C), and proteins in the supernatant were precipitated with 0.5 M HCl, recovered by centrifugation, and lyophilized. The protein content of the isolated barley protein was 85% as determined by combustion with a nitrogen analyzer (FP-428, Leco Corp., St. Joseph, MI) calibrated with analytical reagent grade EDTA and a protein calculation factor of 6.25.

To calculate the maximum theoretical yields of amino acids from the various cereal substrates, approximately 70 mg of rye malt flour, 25 mg of wheat gluten, or 25 mg of barley proteins was mixed with 3.0 mL of 6 M HCl and incubated at 110 °C for 24 h. After cooling, samples were diluted and mixed with internal standard, and the amino acid concentration was determined as described below.

Fermentation of Sourdoughs. Cultures were harvested from overnight cultures in mMRS by centrifugation, washed with autoclaved tap water, and resuspended in autoclaved tap water to their initial volume. Sourdoughs were prepared in 50 mL screw-cap tubes by mixing 5 mL of autoclaved tap water, 10 mL of culture, and 10 g of rye malt flour. Two levels of protein addition were evaluated. Doughs contained 5 g of rye malt flour, 5 g of wheat gluten or barley hordeins, 10 mL of culture, and 30 mL of tap water to achieve a protein to rye malt ratio of 1:1 and a dry matter content of 20% or 5 g of rye malt flour, 10 g of wheat gluten or barley hordeins, 15 mL of culture, and 35 mL of tap water to achieve a protein to rye malt ratio of 2:1 and a dry matter content of 23%. The sourdoughs were fermented at 30, 34, or 37 °C as indicated. A protease preparation of *Aspergillus oryzae* (Sigma-Aldrich) was added at a level of 2.31 μL g^{-1} dry matter where indicated. Chemically acidified dough without addition of cultures was used as control. Dough was acidified to a pH of 3.66 ± 0.23 with 0.27% of a mixture of acetic acid (100%w/w) and lactic acid (85% w/v) in a ratio of 1:5 and incubated under the same conditions as the sourdoughs. The pH and the cell counts were analyzed immediately after sampling; other analyses were performed with freeze-dried samples. Fermentations of sourdoughs and chemically acidified controls were carried out in duplicate independent experiments, and results are reported as the mean ± standard deviation.

Determination of pH and Cell Counts. For determination of the pH, sourdough samples were mixed with deionized water and the pH was measured with a glass electrode. Cell counts were determined by plating serial 10-fold dilutions of sourdough samples on mMRS agar. The colony morphology was observed to verify the identity of the inoculum with the fermentation microflora.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Lyophilized samples were extracted for 2 min at 100 °C with SDS extraction buffer (2% SDS, 1% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 5% bromophenol blue) in an extraction ratio of 1:11 (w/v) for unfermented samples and 1:5 (w/v) for fermented samples. Solids were removed by centrifugation at 10000*g* for 10 min, and 50 μL of the supernatant was mixed with 12.5 μL of SDS-PAGE sample buffer containing 8% SDS, 5% β-mercaptoethanol, 40% glycerol, 0.24 M Tris-HCl, pH 6.8, and 5% bromophenol blue. Samples were heated for 2 min at 100 °C and analyzed by SDS-PAGE using 11% Tris-HCl SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA). The gel electrophoresis was conducted at 130 V for about 120 min, and gels were stained with colloidal Coomassie blue. A prestained SDS-PAGE standard (Bio-Rad Laboratories, Inc.) was used to indicate the molecular weights.

Size Exclusion Chromatography (SEC). SEC analysis was carried out as described[17] with lyophilized samples. Briefly, dough samples were extracted with SDS solution (1.5% SDS, 50 mM sodium phosphate; pH 6.9) in an extraction ratio of 1:10 (w/v) for 1 h at room temperature with shaking. Solids were removed by centrifugation at 10000*g* for 10 min. Supernatant was mixed with an equal volume of eluent (0.1% SDS, 20% acetonitrile in 50 mM sodium phosphate; pH 6.9). Samples were analyzed by an HPLC system with Superdex 200 and Superdex Peptide columns in series (both columns from GE Healthcare, Chalfont St. Giles, U.K.). The flow rate was 0.4 mL min^{-1} and the injection volume was 50 μL, and proteins were detected at 280 nm. The HPLC system was calibrated to quantify the amount of proteins, peptides, and small peptides and amino acids.[18] Glycine (M_R 75) (Merck, Darmstadt, Germany), bacitracin (M_R 1400) (Sigma-Aldrich), and aprotinin (M_R 6500) (Sigma-Aldrich) were used as molecular weight standards to verify the calibration. Results are reported as peak area corresponding to proteins with a relative molecular weight (M_R) of >15000, polypeptides with M_R of 1400−15000, peptides with M_R of <1400, and amino acids.

Quantification of Amino Acids and GABA Levels by High-Performance Liquid Chromatography (HPLC). Amino acids were extracted from lyophilized samples with water in an extraction ratio of 1:10 (w/v), and solids were removed by centrifugation at 10000*g* for 10 min. Amino acids were quantified after derivatization with *o*-phthaldialdehyde as described.[23] The sum of the concentrations of all free amino acids is reported as "total amino acids". β-Aminobutyric acid was used as internal standard.

Table 1. General Sourdough Parameters

	fermentation time (h)	temperature (°C)	CFU[a] (mL^{-1})	final pH[a]
control	24	37	0[b]	3.76 ± 0.12
L. reuteri TMW1.106	24	37	$(1.6 \pm 0.7) \times 10^9$	3.59 ± 0.04
	48	37	$(9 \pm 7) \times 10^8$	3.60 ± 0.05
	72	37	$(8.0 \pm 0.0) \times 10^8$	3.61 ± 0.01
	96	37	$(4 \pm 1) \times 10^7$	3.63 ± 0.01
L. reuteri LTH5448	24	37	$(2.3 \pm 1) \times 10^9$	3.60 ± 0.02
	48	37	$(3.3 \pm 0.7) \times 10^9$	3.64 ± 0.01
	72	37	$(6.0 \pm 0.0) \times 10^8$	3.71 ± 0.01
	96	37	$(3.0 \pm 0.0) \times 10^8$	3.73 ± 0.01
L. reuteri LTH5795	24	37	$(2.3 \pm 0.3) \times 10^9$	3.62 ± 0.03
L. rossiae 34J	24	30	$(2.0 \pm 1.0) \times 10^9$	3.62 ± 0.01
L. plantarum FUA3002	24	30	$(1.4 \pm 0.1) \times 10^9$	3.58 ± 0.05

[a] Average cell count and pH value for two separate fermentation. [b] No bacterial growth.

RESULTS

General Sourdough Parameters. Cell counts and pH of rye malt sourdoughs are shown in Table 1. Chemically acidified sample as control had no bacterial growth, and cell counts of sourdoughs after 24 h of fermentation ranged from 1×10^9 to 3×10^9 cfu mL^{-1}. The initial pH values for all sourdough fermentations were 6.2; the pH after 24 h of fermentation ranged from 3.5 to 3.9. With the increase of fermentation time from 24 to 96 h, the pH value increased by 0.04 and 0.13 in sourdoughs fermented with *L. reuteri* TMW1.106 and *L. reuteri* LTH5448, respectively. Cell counts of the two strains decreased about 100 and 10 times, respectively, over 4 days of fermentation.

Selection of Strains for Production of Glutamate and GABA. The conversion of glutamine to glutamate or GABA in the genus *Lactobacillus* is strain specific; therefore, five strains were initially screened for their ability to convert glutamine. A fermentation temperature of 34 °C was chosen to enable growth of mesophilic and thermophilic organisms, and Figure 1 depicts the contents of glutamine, glutamate, and GABA after 48 h of fermentation. The contents of glutamine were similar in all sourdoughs except dough fermented with *L. reuteri* LTH5448. However, the concentrations of glutamate and GABA were strain dependent. *L. reuteri* TMW1.106 and *L. rossiae* 34J accumulated glutamate. *L. reuteri* LTH5448 and *L. reuteri* LTH5795 reduced glutamate concentrations relative to the control but accumulated GABA, indicating glutamate decarboxylase activity. Glutamine, glutamate, and GABA concentrations in sourdoughs fermented with *L. plantarum* FUA3002 were comparable to the control. Concentrations of total amino acids in sourdoughs fermented with heterofermentative lactobacilli (*L. reuteri* and *L. rossiae*) ranged from 215 ± 5 to 231 ± 6 mmol kg^{-1} DM and were substantially higher than in sourdough fermented with *L. plantarum* or the control (168 ± 4 and 173 ± 6 mmol kg^{-1} DM, respectively). *L. reuteri* TMW1.106 and *L. reuteri* LTH5448 were selected for subsequent experiments as strains accumulating glutamate and GABA, respectively.

PCR with primers specific for the *L. reuteri* glutamate decarboxylase gene was used to determine whether the ability to convert glutamate to GABA correlates to the presence of the gene. *L. reuteri* 100-23, which harbors a glutamate decarboxylase gene and converts glutamate to GABA (data not shown), was used as reference. In accord with the phenotype of the strains, a PCR product of the expected size, 864 bp, was obtained with DNA from *L. reuteri* LTH5448 and LTH5795 as template but not with DNA from *L. reuteri* TMW1.106.

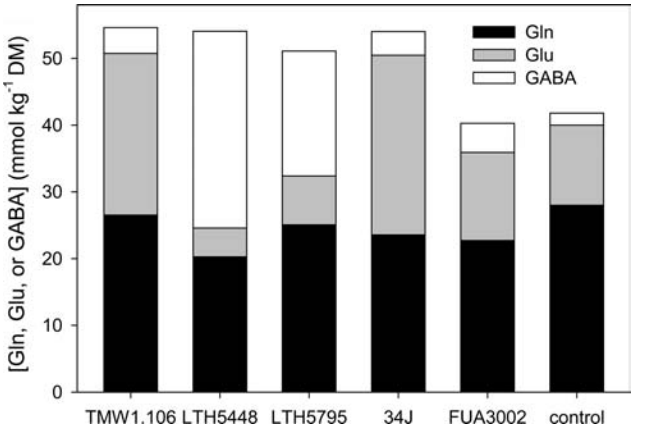

Figure 1. Concentrations of glutamine (Gln), glutamate (Glu), and GABA in rye malt sourdoughs and chemically acidified rye malt doughs (control) after 48 h of fermentation at 34 °C. Data are presented as means of two independent fermentations, and the experimental error was ≤7%.

Effect of Fermentation Time on the Accumulation of Glutamate, GABA, and Amino Acids. To determine the influence of fermentation time on accumulation of amino acids, rye malt sourdoughs were fermented with *L. reuteri* TMW1.106 and *L. reuteri* LTH5448 for 4 days and glutamine, glutamate, and GABA concentrations were analyzed (Figure 2). In sourdoughs fermented with *L. reuteri* TMW1.106, concentrations of glutamate and total amino acids increased throughout fermentation, whereas glutamine concentrations decreased slightly. The level of GABA remained unchanged and low throughout fermentation. The total amino acid concentrations in sourdoughs fermented with *L. reuteri* LTH5448 were comparable to those of *L. reuteri* TMW1.106; however, concentrations of glutamine and glutamate decreased over time, and GABA accumulated to 44 mmol kg^{-1} DM. Different from the sourdoughs, the total amino acid concentrations in chemically acidified doughs remained constant after 24 h of fermentation, indicating that microbial peptidases rather than malt enzymes are responsible for the liberation of amino acids after 24 h of fermentation.

Glutamine Metabolism in Sourdoughs Supplemented with Cereal Proteins. Sourdoughs were supplemented with wheat gluten or barley hordein to determine whether an

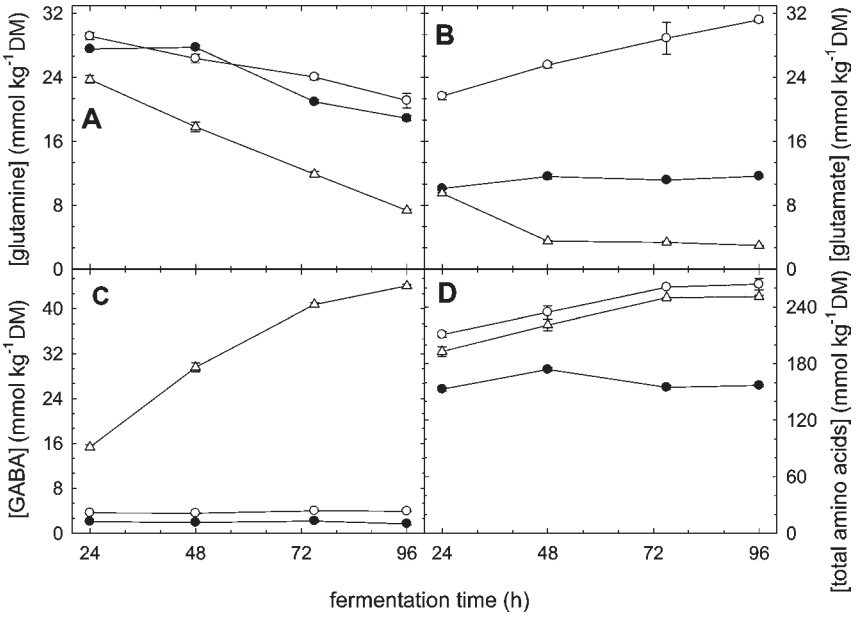

Figure 2. Concentrations of glutamine (A), glutamate (B), GABA (C), and total amino acids (D) in rye malt sourdoughs fermented with *L. reuteri* TMW1.106 (○) or LTH5448 (△) for 24−96 h at 37 °C. Chemically acidified doughs served as control (●). Data are presented as the mean ± standard deviation of two independent fermentations.

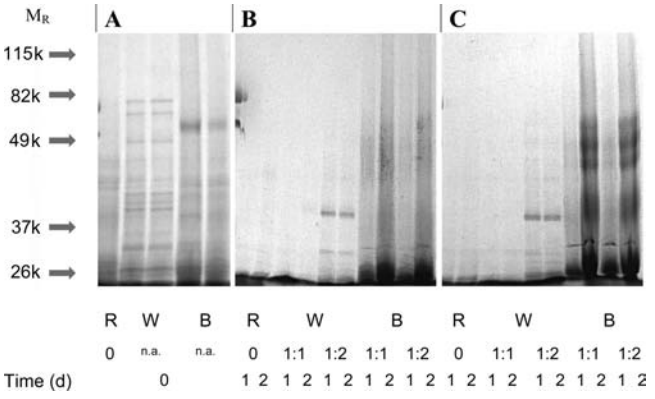

Figure 3. SDS-PAGE analysis of proteins extracted from unfermented doughs (A), sourdoughs fermented with *L. reuteri* TMW1.106 (B), and chemically acidified doughs (C). R, rye malt doughs; W, rye malt doughs with addition of wheat gluten; B, rye malt doughs with addition of hordein. The level of protein addition was 1:1 or 1:2 as indicated. Doughs were fermented for 0, 1, or 2 days at 37 °C as indicated. Samples were loaded on the gel to represent equal dough weights for all samples on a gel. The extraction ratio for unfermented samples (A) was 1:5; the extraction ratio of fermented samples (B, C) was 1:11. Data are representative of two independent fermentations. n.a., not applicable.

increased availability of proteins for hydrolysis increases the yield of glutamate or GABA. Proteolysis was initially assessed by SDS-PAGE (Figure 3). Proteins with a relative molecular weight ranging from 25000 to 45000 were extracted from rye malt sourdoughs; proteins with a M_R of >45K were extracted from doughs containing wheat gluten or barley hordeins (Figure 3A). Cereal proteins were hydrolyzed in sourdoughs fermented with *L. reuteri* TMW1.106 when mixed with rye malt in a ratio of 1:1 (Figure 3B); however, undigested proteins remained in rye malt sourdoughs with protein in a ratio of 1:2 (Figure 3B). In rye malt/barley sourdoughs with rye malt to protein ratios of 1:1 and 1:2, the distinct bands visible in unfermented samples disappeared but continuous bands were observed, particularly after 48 h of fermentation. This indicates that the solubility of hordeins increased with the fermentation time and that hordeins were not completely hydrolyzed during 48 h of incubation. Hydrolysis of rye malt proteins and wheat gluten in chemically acidified doughs was comparable to that of sourdoughs (Figure 3B,C); however, compared to sourdoughs, chemically acidified doughs with hordein contained proteins with a higher molecular weight (Figure 3C).

Glutamine, glutamate, GABA, and amino acid concentrations in rye malt sourdoughs and rye malt sourdoughs supplemented with wheat gluten are depicted in Table 2. Samples were fermented with *L. reuteri* TMW1.106 or *L. reuteri* LTH5448 for 96 h. The analysis focused on rye malt sourdoughs supplemented with wheat gluten in a ratio of 1:1 because extensive protein hydrolysis was observed only in these doughs (Figure 3). Rye malt sourdoughs and chemically acidified doughs are included in Table 2 for comparison. In rye malt/wheat gluten sourdoughs, amino acid concentrations increased about 2-fold compared to rye malt sourdoughs and the concentration of (Gln + Glu + GABA) increased from about 50 to >130 mmol kg^{-1} DM. The increase in total amino acids and (Gln + Glu + GABA) was much less pronounced in chemically acidified doughs. In keeping with the results from rye malt sourdoughs, *L. reuteri* TMW1.106 partially converted glutamine to glutamate, whereas *L. reuteri* LTH5448 accumulated GABA. In sourdoughs fermented with *L. reuteri* LTH5448, glutamate and particularly GABA concentrations were substantially higher than the concentration of glutamine. However, in rye malt/wheat gluten sourdoughs fermented with *L. reuteri* TMW1.106, glutamine levels remained higher than glutamate after 96 h of fermentation, indicating that microbial glutaminase activity may limit the yield of glutamate from wheat gluten if glutamate is not further converted to GABA.

Glutamine Metabolism in Sourdoughs Supplemented with Cereal Proteins and Fungal Protease. To evaluate whether the addition of a fungal protease improves the solubilization

Table 2. Glutamine, Glutamate, GABA, and Amino Acid Concentration in Rye Malt Sourdough or Rye Malt Sourdough with the Addition of Wheat Gluten after 96 h of Fermentation at 37 °C[a]

	glutamine (mmol kg^{-1} DM)	glutamate (mmol kg^{-1} DM)	GABA (mmol kg^{-1} DM)	total amino acids (mmol kg^{-1} DM)
Chemically Acidified				
rye	18.9 ± 0.4	11.7 ± 0.2	1.7 ± 0.2	156 ± 1
rye/wheat (1:1)	23.7 ± 2.5	9.6 ± 0.9	1.0 ± 0.1	200 ± 14
***L. reuteri* TMW1.106**				
rye	21.1 ± 1.3	29.8 ± 1.2	4.0 ± 0.1	264 ± 6
rye/wheat (1:1)	73.1 ± 0.4	63.1 ± 0.3	2.1 ± 0.3	533 ± 2
***L. reuteri* LTH5448**				
rye	7.3 ± 0.2	2.9 ± 0.1	44.1 ± 0.2	251 ± 2
rye/wheat (1:1)	13.1 ± 0.7	33.5 ± 0.1	89.7 ± 6.8	447 ± 30

[a] Chemically acidified dough served as control. Data are presented as the mean ± standard deviation of two independent fermentations.

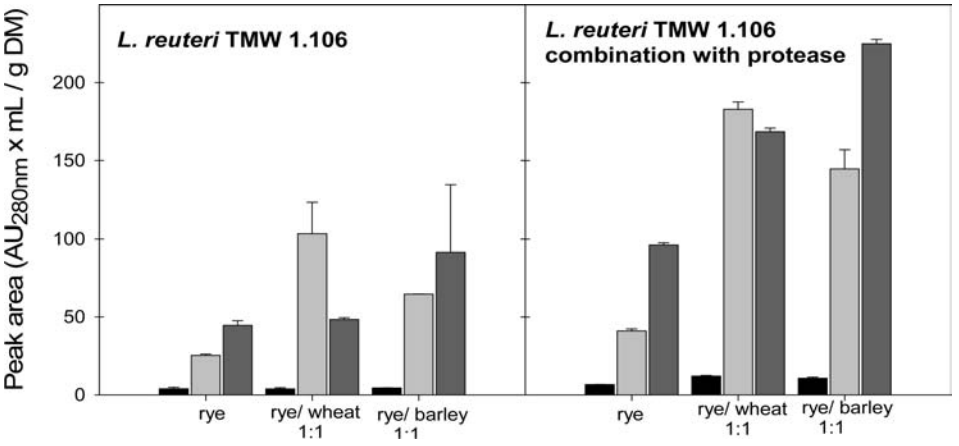

Figure 4. Contents of proteins, peptides, and amino acids in rye malt sourdoughs supplemented with cereal proteins or fungal protease after 48 h of fermentation with *L. reuteri* TMW1.106. Cereal proteins and hydrolysis products were separated by size exclution chromatography and detected by the absorption at 280 nm. Black bars depict the peak area corresponding to proteins with M_R of >15K, light gray bars the peak area corresponding to polypeptides with M_R of 1.4−15K, and dark gray bars the peak area corresponding to peptides with M_R of <1.4K and amino acids. Data are presented as the mean ± standard deviation of two independent fermentations.

and proteolysis of cereal proteins, rye malt sourdoughs were supplemented with wheat gluten or barley hordeins and a protease preparation from *Aspergillus oryzae*. The effect of the protease on proteolytic events in the sourdough was initially assessed by SEC analysis of SDS-soluble proteins, peptides, and amino acids (Figure 4). The addition of protease increased the overall peak area of all sourdoughs, indicating improved solubilization of proteins and their degradation products. Moreover, protease addition increased the ratio of solutes with M_R of <1400 to proteins and peptides with M_R of >1400 in all doughs, indicating improved conversion of soluble proteins to small peptides and amino acids.

In keeping with the results of SEC, the effect of fungal protease on the amino acid concentrations in sourdoughs measured by HPLC was least pronounced in rye malt sourdoughs, where amino acid levels increased by about 15% after protease addition (Table 3). However, in sourdoughs supplemented with wheat gluten and hordeins, the addition of fungal protease increased amino acid levels by 50 and 100%, respectively (Table 3). Correspondingly, protease addition had only minor effects on glutamine and glutamate concentrations in rye malt sourdoughs, but the levels of both amino acids were substantially increased in rye malt/wheat gluten and rye malt/hordein sourdoughs (Table 3). Similar glutamate concentrations were observed in rye malt/gluten and rye malt/hordein sourdoughs; however, glutamine concentrations in rye malt/gluten sourdoughs exceeded those in rye malt/hordein sourdoughs almost 3-fold.

Production of Other Amino Acids and Their Conversion. All strains of *L. reuteri* and *L. rossiae* 34J quantitatively converted arginine to ornithine (data not shown), in keeping with the conversion of arginine via the arginine-deiminase (ADI) pathway in these two species. The highest level or ornithine, 32 ± 1 mmol kg^{-1} DM, was observed in rye malt/hordein sourdoughs fermented for 48 h in the presence of fungal protease. The concentrations of the substrate arginine and the intermediate citrulline were generally <5% of the ornithine concentration. Ornithine was not accumulated in chemically acidified doughs or sourdoughs fermented with *L. plantarum* FUA3002; in these doughs, arginine was accumulated. The amino acid analyses provided no evidence for conversion of asparagine, leucine, isoleucine, tyrosine, histidine, or phenylalanine by any of the strains.

DISCUSSION

This study aimed to convert wheat gluten and barley hordeins to the alternative end products glutamate or GABA, to characterize

Table 3. Effect of Fungal Protease on Glutamine, Glutamate, GABA, and Amino Acid Concentration in Rye Malt Sourdough, Rye Malt Sourdough with the Addition of Wheat Gluten, or Rye Malt Sourdough with the Addition of Hordein[a]

	protease	Gln (mmol kg^{-1} DM)	glutamate (mmol kg^{-1} DM)	GABA (mmol kg^{-1} DM)	total amino acids (mmol kg^{-1} DM)
rye malt	−	26.3 ± 0.7	25.6 ± 0.5	3.6 ± 0.1	235 ± 10
rye malt	+	27.7 ± 0.5	29.6 ± 0.7	4.0 ± 0.1	270 ± 4
rye/wheat (1:1)	−	38.2 ± 14	45.7 ± 3.6	2.0 ± 0.4	401 ± 35
rye/wheat (1:1)	+	114 ± 2	58.1 ± 0.8	3.3 ± 0.1	586 ± 16
rye/barley (1:1)	−	17.6 ± 7.4	38.9 ± 11.1	1.7 ± 0.5	286 ± 86
rye/barley (1:1)	+	38.3 ± 0.6	62.2 ± 0.7	2.7 ± 0.4	556 ± 9

[a] Sourdoughs were fermented for 48 h at 37 °C with *L. reuteri* TMW1.106. Chemically acidified dough served as control (data not shown). Data are presented as the mean ± standard deviation of two independent fermentations.

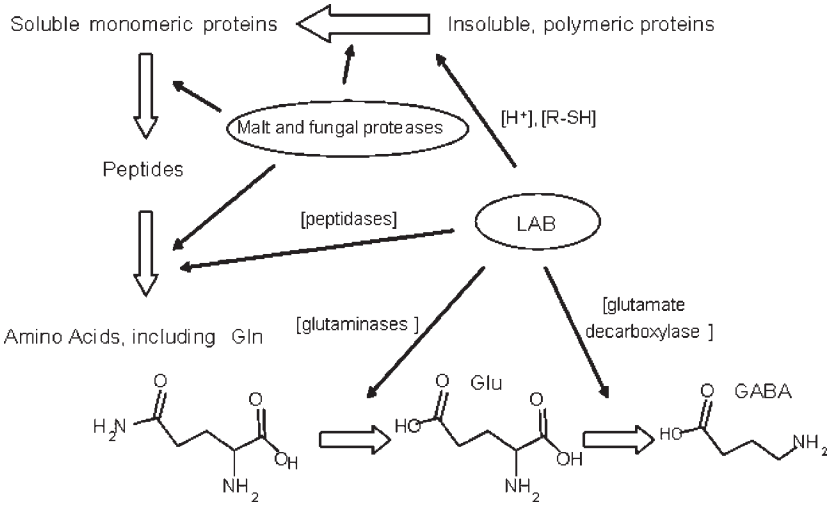

Figure 5. Proteolysis and bioconversion of cereal proteins to glutamate and GABA during fermentation of rye malt sourdoughs. The contributions of malt and fungal proteases and of metabolic activities of lactic acid bacteria (LAB) are indicated by arrows.

glutamine metabolism of lactobacilli in cereal substrates, and to identify factors limiting the quantitative conversion of glutamine from cereal proteins to glutamate or GABA. The conversion proceeds through the steps protein solubilization, primary proteolysis, peptide hydrolysis, glutaminase activity for conversion to glutamate, and glutamate decarboxylase activity for conversion of glutamate to GABA (Figure 5).

Acidification in combination with glutathione reductase or cystathionine lyase activities of heterofermentative lactobacilli leads to depolymerization and solubilization of gluten proteins.[18,24] In keeping with previous papers,[20] proteolysis of rye malt proteins was not dependent on reductive depolymerization by heterofermentative lactobacilli because the proteins of the resting grain are mobilized during germination. However, proteolysis was enhanced by the heterofermentative *L. reuteri* when protein preparations from ungerminated cereals were present, indicating that thiol accumulation contributes to protein solubilization and proteolysis.[24]

Germinated rye has a high level of diverse proteolytic enzymes[25] and hydrolyzes cereal prolamins and glutelins more effectively than germinated barley or wheat.[20] The protease activity in rye malt was sufficient to hydrolyze all rye malt proteins after 24 h of fermentation (this study and ref 18), but proteolytic activity was limiting protein turnover in samples with added protein. In these samples, the addition of a fungal protease preparation from *A. oryzae* was necessary.

Amino acid concentrations in sourdough from wheat or rye flour without added proteolytic enzymes generally do not exceed 2 g kg^{-1}, corresponding to about 0.02 mol kg^{-1}.[15,17] The amino acid content in rye malt sourdoughs was about 0.15 mol kg^{-1} DM for chemically acidified doughs and 0.25 mol kg^{-1} DM for sourdoughs fermented with *L. reuteri*, comparable to the amino nitrogen levels reported by Loponen et al.[18] using comparable experimental conditions. Different amino acid levels between sourdoughs and chemically acidified doughs support the notion that microbial peptidases contribute substantially to peptide hydrolysis.[18,26] However, these amino acid concentrations represent only 50% of the yield obtained after chemical hydrolysis of rye malt flour (Table 2 and data not shown). Amino acid concentrations in sourdoughs with added cereal proteins and fermented with the addition of fungal protease represent about 20% of the theoretical yield (Table 3 and data not shown), indicating that peptides rather than amino acids are the major products of proteolysis.

Only about 35 and 10% of the glutamine available in proteins of the substrate was detected as (glutamine + glutamate + GABA) in rye malt sourdoughs and rye malt sourdoughs with added cereal proteins, respectively. However, the highest level of glutamate observed in this study, 0.8%, obtained by fermentation of rye malt sourdoughs with the addition of cereal proteins, is comparable to glutamate levels in condiments such as soy sauce or fish sauce.[21] In this study, *L. reuteri* and *L. rossiae* but not *L. plantarum* converted glutamine to glutamate. The conversion of glutamine to glutamate by *L. reuteri* continued throughout 4 days of fermentation. It is remarkable that the increased availability of glutamine through the addition of fungal protease did not

substantially increase glutamate levels in rye malt sourdoughs with wheat gluten (Tables 2 and 3), indicating that product inhibition or product transport may limit the conversion. Genome sequence data are available for three strains of *L. reuteri*; all three strains harbor three different glutaminase genes. However, it remains unclear which of the three genes is responsible for glutamine conversion by stationary phase cells of *L. reuteri*.[27]

In *L. reuteri*, GABA formation was strain dependent and PCR detection of the *L. reuteri* glutamate decarboxylase gene corresponded to the ability of strains to produce GABA. GABA formation continued throughout 4 days of fermentation; different from glutamate formation, the accumulation of GABA from glutamate was not inhibited by high product concentrations. Glutamate decarboxylase is a key contributor to acid resistance in *Escherichia coli* and related organisms.[28] Stationary phase survival of the glutamate-decarboxylase positive *L. reuteri* LTH5448 was improved compared to the glutamate-decarboxylase negative *L. reuteri* TMW1.106, indicating that glutamate decarboxylase also contributes to acid tolerance in *L. reuteri*. In this study, GABA accumulated to 9 g kg^{-1} DM. Previous studies in food fermentations reported GABA concentrations of 0.26 g kg^{-1} or less;[10−13] Coda et al.[14] reported GABA concentrations of 1−1.2 g kg^{-1} in chickpea and amaranth sourdoughs.

Fermentation of rye malt sourdoughs supplemented with hordeins produced lower amino acid levels unless fungal protease was added. Moreover, the hordein preparation employed in this study was only partially solubilized even when SDS buffer under reducing conditions was employed. Resistance of hordeins to solubilization and proteolytic degradation may relate to the proline content of hordeins, which is 6 and 3% higher when compared to gliadins and glutenins.[2] However, the commercial wheat gluten employed in this study was obtained by aqueous treatment at neutral pH, whereas an alkaline extraction protocol was employed for extraction of barley proteins.

The metabolism of arginine by lactobacilli followed the pattern of ADI-positive and ADI-negative lactobacilli observed previously in sourdough fermentations.[15] Tyrosine, histidine, and phenylalanine, precursor amino acids to the biogenic amines tyramine, histamine, and phenylethylamine, were not metabolized by *L. reuteri* or *L. rossiae*, and these amines were not detected. Biogenic amine formation by strain-specific decarboxylase activity of lactobacilli contributes to pH homeostatis;[29] however, in contrast to GABA, biogenic amines have adverse biological activity.

In conclusion, this study achieved the enzymatic and bacterial conversion of cereal proteins to protein hydrolysates enriched with glutamate. The high glutamate concentrations after 4 days of fermentation enable the use of hydrolyzed cereal protein as condiment, which is functionally equivalent to soy sauce or hydrolyzed vegetable protein. GABA was accumulated as alternative end product of glutamine metabolism by lactobacilli. Because peptides rather than amino acids were the main product of proteolysis, peptides with biological activity may additionally be present in the protein hydrolysates. Despite the extensive literature on the use of cereal protein hydrolysates in baking, the occurrence of bitter peptides has not been observed;[15−19] however, peptides with angiotensin I-converting enzyme inhibitory were identified from cereal protein hydrolysates.[13]

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REFERENCES

(1) Wieser, H. Chemistry of gluten proteins. *Food Microbiol.* **2007**, *24*, 115–119.

(2) Belitz, H.-D.; Grosch, W.; Schieberle, P. *Food Chemistry*; Springer: Berlin, Germany, 2004.

(3) Gänzle, M. G. From gene to function: metabolic traits of starter cultures for improved quality of cereal foods. *Int. J. Food Microbiol.* **2009**, *134*, 29–36.

(4) Drake, S. L.; Carunchia Whetstine, M. E.; Drake, M. A.; Courtney, P.; Fligner, K.; Jenkins, J.; Pruitt, C. Sources of umami taste in Cheddar and Swiss cheeses. *J. Food Sci.* **2007**, *72*, S360–366.

(5) Weingand-Ziade, A.; Gerber-Decombaz, C.; Affolter, M. Functional characterization of a salt- and thermotolerant glutaminase from *Lactobacillus rhamnosus*. *Enzyme Microb. Technol.* **2003**, *32*, 862–867.

(6) Vermeulen, N.; Gänzle, M. G.; Vogel, R. F. Glutamine deamidation by cereal-associated lactic acid bacteria. *J. Appl. Microbiol.* **2007**, *13*, 1198–1205.

(7) Oh, S. H.; Oh, C. H. Brown rice extracts with enhanced levels of GABA stimulate immune cells. *Food Sci. Biotechnol.* **2003**, *12*, 248–252.

(8) Inoue, K.; Shirai, T.; Ochiai, H.; Kasao, M.; Hayakawa, K.; Kimura, M.; Sansawa, H. Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. *Eur. J. Clin. Nutr.* **2003**, *57*, 490–495.

(9) Hayakawa, K.; Kimura, M.; Kasaha, K.; Matsumoto, K.; Sansawa, H.; Yamori, Y. Effect of a γ-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Br. J. Nutr.* **2004**, *92*, 411–417.

(10) Siragusa, S.; De Angelis, M.; Di Cagno, R.; Rizzello, C. G.; Coda, R.; Gobbetti, M. Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.* **2007**, *73*, 7283–7290.

(11) Kim, J. Y.; Lee, M. Y.; Ji, G. E.; Lee, Y. S.; Hwang, K. T. Production of γ-aminobutyric acid in black raspberry juice during fermentation with *Lactobacillus brevis* GABA100. *Int. J. Food Microbiol.* **2009**, *15*, 12–16.

(12) Yokoyama, S.; Hiramatsu, J.-I.; Hayakawa, K. Production of γ-aminobutyric acid from alcohol distillery lees by *Lactobacillus brevis* IFO-12005. *J. Biosci. Bioeng.* **2002**, *93*, 95–97.

(13) Rizzello, C. G.; Cassone, A.; Di Cagno, R. D.; Gobbetti, M. Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and γ-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria. *J. Agric. Food Chem.* **2008**, *56*, 6936–6943.

(14) Coda, R.; Rizzello, C. G.; Gobbetti, M. Use of sourdough fermentation and pseudo-cereals and leguminous flours for the making of a functional bread enriched of γ-aminobutyric acid (GABA). *Int. J. Food Microbiol.* **2010**, *137*, 236–245.

(15) Thiele, C.; Gänzle, M. G.; Vogel, R. F. Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. *Cereal Chem.* **2002**, *79*, 45–51.

(16) Di Cagno, R; De Angelis, M.; Lavermicocca, P.; De Vincenzi, M.; Giovannini, C.; Faccia, M.; Gobbetti, M. Proteolysis by sourdough

lactic acid bacteria: Effects on wheat flour protein fractions and gliadin peptides involved in human cereal intolerance. *Appl. Environ. Microbiol.* **2002**, *68*, 623–633.

(17) Loponen, J.; Kanerva, P.; Zhang, C.; Sontag-Strohm, T.; Salovaara, H.; Gänzle, M. G. Prolamin hydrolysis and pentosan solubilization in germinated-rye sourdoughs determined by chromatographic and immunological methods. *J. Agric. Food Chem.* **2009**, *57*, 746–753.

(18) Gänzle, M. G.; Loponen, J.; Gobbetti, M. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. *Trends Food Sci. Technol.* **2008**, *19*, 513–521.

(19) Rizzello, C. G.; De Angelis, M.; Di Cagno, R.; Camarca, A.; Silano, M.; Losito, I.; De Vincenzi, M.; De Bari, M. D.; Palmisano, F.; Maurano, F.; Fianfrani, C.; Gobbetti, M. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. *Appl. Environ. Microbiol.* **2007**, *73*, 4499–4507.

(20) Loponen, J.; Kanerva, P.; Gänzle, M. G.; Sontag-Strohm, T.; Salovaara, H. Endogenous cereal enzymes in the eliminaton of prolamins. In *Second International Symposium on Gluten-Free Cereal Products and Beverages*; Salovaara, H., Ed.; University of Helsinki: Helsinki, Finland, 2010; pp 81−82.

(21) Jinap, S.; Hajeb, P. Glutamate, its application in food and contribution to health. *Appetite* **2010**, *55*, 1–10.

(22) Wang, C.; Tian, Z.; Chen, L.; Temelli, F.; Liu, H.; Wang, Y. Functionality of barley proteins extracted and fractionated by alkaline and alcohol methods. *Cereal Chem.* **2010**, *87*, 597–606.

(23) Sedgewick, G. W.; Fenton, T. F.; Thompson, J. R. Effect of protein precipitating agents on the recovery of plasma free amino acids. *Can. J. Anim. Sci.* **1991**, *71*, 953–957.

(24) Jänsch, A.; Korakli, M.; Vogel, R. F.; Gänzle, M. G. Glutathione reductase from *Lactobacillus sanfranciscensis* DSM20451T: contribution to oxygen tolerance and thiol-exchange reactions in wheat sourdoughs. *Appl. Environ. Microbiol.* **2007**, *73*, 4469–4476.

(25) Brijs, K.; Trogh, I.; Jones, B. L.; Delcour, J. A. Proteolytic enzymes in germinating rye grains. *Cereal Chem.* **2002**, *79*, 423–428.

(26) De Angelis, M.; Cassone, A.; Rizzello, C. G.; Gagliardi, F.; Minervini, F.; Calasso, M.; Di Cagno, R.; Francavilla, R.; Gobbetti, M. Mechanisms of degradation of immunogenic gluten epitopes from *Triticum turgidum* L. var. *durum* by sourdough lactobacilli and fungal proteases. *Appl. Environ. Microbiol.* **2010**, *76*, 508–518.

(27) Jeon, J.-M.; Lee, H.-I.; Han, S.-H.; Chang, C.-S.; So, J.-S. Partial purification and characterization of glutaminase from *Lactobacillus reuteri* KCT3594. *Appl. Biochem. Biotechnol.* **2010**, *162*, 146–154.

(28) Foster, J. W. *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat. Rev. Microbiol.* **2004**, *2*, 898–907.

(29) Koning, W. N. The cell membrane and the struggle for life of lactic acid bacteria. *Antonie van Leeuwenhoek* **2002**, *82*, 3–27.