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LC-MS/MS Quantification of Bioactive Angiotensin I-Converting Enzyme Inhibitory Peptides in Rye Malt Sourdoughs

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ABSTRACT: This study quantified antiotensin I-converting enzyme (ACE) inhibitory peptides in rye malt sourdoughs supplemented with gluten proteins and fermented with six strains of *Lactobacillus* spp. Bioinformatic analysis of prolamins from barley, rye, and wheat demonstrated that the ACE inhibitory peptides LQP, LLP, VPP, and IPP are frequently encrypted in their primary sequence. These tripeptides were quantified by liquid chromatography−tandem mass spectrometry. Tripeptide levels in sourdoughs were generally higher as compared to the chemically acidified controls. Sourdoughs fermented with different strains showed different concentrations of LQP and LLP. These differences corresponded to strain-specific differences in PepO and PepN activities. The highest levels of peptides VPP, IPP, LQP, and LLP, 0.23, 0.71, 1.09, and 0.09 mmol (kg DM)$^{-1}$, respectively, were observed in rye malt: gluten sourdoughs fermented with *Lactobacillus reuteri* TMW 1.106 and added protease. These concentrations were 6−7 times higher as compared to sourdough without fungal protease and exceed the IC_{50} by 100−1000-fold.

KEYWORDS: Proteolysis, peptide, fermentation, quantification, lactobacillus

INTRODUCTION

Food proteins act as an important source of peptides with antihypertensive, immunomodulating, antioxidative, antimicrobial, antilipidemic, antithrombotic, hypocholesterolemic, opioid, and other biological activities.[1] Bioactive peptides are encrypted in the protein sequence and are released by enzymatic proteolysis. Among them, angiotensin I-converting enzyme (ACE) inhibitory peptides have been extensively studied due to their capacity to control hypertension. These peptides can be used as ingredients in functional foods as an alternative to ACE inhibitory drugs.[2] Peptides with an antihypertensive effect in vivo have to be resistant to further digestion, absorbed from the intestine, and reach the target cells in the blood vessels in substantial concentrations.[3] Di- and tripeptides are readily absorbed in the small intestine[4] and are more likely to reach the bloodstream intact than larger peptides. After ingestion of a gelatin hydrolysate, only free amino acids, di-, and tripeptides were recovered in the blood of humans.[5] Particularly the antihypertensive tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP)[6] have been shown to be resistant to digestion and were able to exert antihypertensive activity through the inhibition of ACE in the aorta.[7,8]

The seed storage proteins of wheat, barley, rye, and oats contain known ACE-inhibitory di- and tripeptides in their primary structures.[9] Cereal grains are staples of the human diet, and glutamine and proline are the most abundant amino acids in proteins from wheat, rye, and barley. Hydrophobic amino acids also occur frequently.[10] The majority of ACE inhibitory di- and tripeptides have Pro in the C-terminal position and a branched chain amino acid, I, L, or V, in the N-terminal position. Alternative amino acids at the C and N termini are Y or W and A or G, respectively.[8] Fermentation with proteolytic strains of lactic acid bacteria (LAB) is a successful strategy to produce ACE inhibitory peptides in dairy products.[11] In cereal fermentations, extensive proteolysis of proteins is achieved by a combination of malt or fungal enzymes and peptidases of sourdough lactobacilli.[12−14] In such sourdoughs, peptides, rather than amino acids, are the major product of gluten hydrolysis.[15]

Gluten has adverse health effects in celiac disease. Gluten fragments remain partially unhydrolyzed after gastrointestinal digestion. In the digestive tract of celiac patients, they bind to human leukocyte antigen DQ2 or DQ8 molecules[16] and elicit an autoimmune response known as celiac disease.[17] Peptides that are toxic to celiac patients are mainly attributed to the repetitive N-terminal domain of α-gliadins and are rich in Q, P, and aromatic amino acids. However, the shortest peptides with known toxicity in celiac disease are nonapeptides. Extensive proteolysis in sourdough fermentation has been shown to eliminate the toxicity of gluten to celiac patients.[13] Therefore, cereal protein hydrolysates containing ACE inhibitory tripeptides may be nontoxic for celiac patients.

Bioinformatic analysis of cereal proteins sequences revealed that particularly four tripeptides with known ACE inhibitory activity, LQP, VPP, IPP, and LLP, are frequently encrypted in the

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Table 1. Tripeptides with ACE Inhibitory Activity (IC_{50}, μM) Encrypted in the Primary Sequence of Proteins from Rye, Wheat, and Barley

	NCBI accession number	VPP[a]	IPP	LQP	LLP
ACE IC_{50} (μM)[b]		9	5	2	57
	rye secalin				
γ-75k-secalin	ABO32294	0	0	0	1
ω-secalin	ACQ83625	0	0	6	0
	wheat gluten				
γ-gliadin	AAA34289	1	0	1	0
ω-gliadin	AAT01617	0	4	2	0
α-gliadin	ABQ52121	0	1	3	0
HMW glutenin subunit	CAC40686	1	0	1	0
	barley hordein				
D-hordein	BAA11642	0	0	1	0
γ -hordein	1604464A	1	0	1	2
C-hordein	AAA92333	0	0	9	0

[a] The one letter notation for amino acids is used. [b] IC_{50}, half maximal inhibitory concentration.

primary structure of rye secalin, wheat gluten, and barley hordein (Table 1).[10,18−20] However, the release of ACE inhibitory tripeptides in cereal fermentations has not yet been demonstrated. It was therefore the aim of this study to determine whether ACE inhibitory peptides are accumulated during cereal fermentations. The proteolysis of wheat and barley protein isolates was achieved by fermentation of rye malt sourdough alone or in combination with a fungal protease.[15] Chemically acidified doughs served as controls. Strains were characterized with respect to peptidase activities, and liquid chromatography−tandem mass spectrometry (LC-MS/MS) was used to quantify the tripeptides LQP, LLP, VPP, and IPP. The prolamin contents of sourdoughs were quantified with an enzyme immunoassay to determine the hydrolysis of rye secalin or added cereal prolamins.

MATERIALS AND METHODS

Strains and Growth Conditions. The sourdough isolates *Lactobacillus reuteri* TMW 1.106, *L. reuteri* LTH 5448, *Lactobacillus rossiae* 34J, *Lactobacillus hammesii* DSM 16381, and *Lactobacillus plantarum* FUA 3002 were used in this study. The strains were grown in modified de Man, Rogosa, and Sharpe medium (mMRS).[15] All strains except *L. reuteri* (grown at 37 °C) were grown at 30 °C, and agar plate incubations took place under modified atmosphere (4% O_2, 20% CO_2, and 76% N_2). Inocula for sourdough fermentation were prepared by centrifugation of overnight cultures for 10 min at 4 °C and 2800*g*. The cell pellet was washed once with autoclaved tap water and resuspended in tap water to the original volume.

Materials and Fermentation Condition. This study employed freeze-dried sourdough samples that were prepared in a previous study.[15] Rye malt flour was obtained from Laihian Mallas (Laihia, Finland); wheat gluten was purchased from Sigma Co. (St. Louis, MO). A barley hordein preparation was obtained from barley grains cultivar Falcon (harvest 2007) as previously described.[15]

Standards and Chemicals. The external standards (IPP, VPP, LQP, and LLP) were purchased from United Peptide Corp. (Bethesda, MD). The synthetic substrates Gly-Pro-*p*-nitroanilide (*p*-NA) and Z-Val-Pro hydrochloride that were used were purchased from Sigma. The synthetic substrates Leu-*p*NA and Z-Gly-Pro-*p*NA that were used were purchased from Bachem Inc. (Torrance, CA).

Table 2. LC-MS/MS Parameters for the Determination of Four Tripeptides in Water-Soluble Extracts of Sourdoughs or Chemically Acidified Doughs[a]

		V			
peptide	transition (m/z)	DP	CE	CXP	retention time (min)
VPP	312 → 213	31	25	12	20.8
IPP	326 → 213	36	25	12	24.3
LLP	342 → 116	36	19	8	33.7
LQP	357 → 242	31	19	6	25.2

[a] DP, declustering potential; CE, collition energy; and CXP, collision exit potential.

Determination of Free Amino Nitrogen (FAN). FAN contents of sodium dodecyl sulfate (SDS)-soluble fractions in different sourdough samples were measured by the ninhydrin method. Fifty milligrams of lyophilized sourdough was suspended in 1.0 mL of 200 mmol L^{-1} sodium phosphate. It was extracted for 1 h at room temperature on a shaking plate. After centrifugation for 10 min at 10000*g*, the supernatant was diluted 1:201 with deionized water. Diluted supernatant (200 μL) was mixed with 100 μL of ninhydrin solution (50.0 g L^{-1} $Na_2HPO_4 \cdot H_2O$, 60.0 g L^{-1} KH_2PO_4, 3.0 g L^{-1} D-(−)-fructose, and 0.5 g L^{-1} ninhydrin (Sigma) and incubated at 100 °C for 16 min. After it was cooled at room temperature for 20 min, the mixture was diluted with 0.5 mL of KIO_3 solution (per 100 mL: 0.2 g of KIO_3, 60 mL of deionized water, and 40 mL of ethanol) mixed, and the absorbance was measured at 570 nm (Jasco, Gross-Umstadt; Germany). The glycine solution with a concentration from 5.0 to 20.0 mg L^{-1} was used to establish a calibration curve.

Quantification of Tripeptides by LC-MS/MS Analysis. Quantification was performed using a 1200 series HPLC unit and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA) connected to a 4000 Q TRAP LC-MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). Peptides were separated on a Luna C18 RP-HPLC column (5 μm, 250 mm × 4.6 mm, Phenomenex, Torrance, CA) and detected at 205, 210, 220, and 280 nm. Mobile phase A consisted of 0.1% formic acid in Milli-Q water. Mobile phase B consisted of 0.1% formic acid in acetonitrile. Samples were eluted at a flow of 0.5 mL min^{-1} at 40 °C with the following gradient: 4 min, 0% B; 4−16 min, 0−7% B; 16−44 min, 7−30% B; 44−51.5 min, 30−45% B; and 51.5−57 min, 45−0% B; and re-equilibration time of 10 min. LC-MS/MS analysis was performed using atmospheric pressure electrospray ionization in positive mode. After optimizing individually on the protonated precursor ions and the dominant fragments of each of the four tripeptides, analytes were detected and quantified using multiple reaction monitoring (MRM). LC-MS/MS parameters for quantification of four tripeptides are shown in Table 2. The values for optimum ion source parameters were as follows: spray voltage, 4 kV; source temperature, 600 °C; nebulizing gas, 50 psi; heating gas, 50 psi; and curtain gas, 20 psi.

For confirmatory analysis, an information-dependent acquisition (IDA) experiment was performed using MRM scan and an IDA threshold set at 100 cps, above which enhanced product ion (EPI) spectra were collected from the eight most intense signals. The MRM scan used both Q1 and Q3 at unit mass resolution. The product ion spectra were obtained over a range from m/z 50 to 500 in 1 s. The LIT fill time was set at 20 ms. The EPI scan rate was 4000 amu s^{-1}. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas under collision energy of 22 eV. Other MS parameters were as

follows: declustering potential (DP), 31 V; entrance potential (EP), 10 V; and collision exit potential (CXP), 6 V. Data acquisition was interfaced to a computer workstation running Analyst 1.5 (Applied Biosystems), which also served as the controlling software for LC/UV system.

External calibration standards of LQP, LLP, VPP, and IPP were prepared in 30% (v/v) methanol in 0.1% aqueous formic acid. Calibration curves consisted of seven concentrations ranging from 0.001 to 1.00 mg L^{-1}. Lyophilized sourdoughs were extracted with 30% (v/v) methanol in 0.1% aqueous formic acid, solids were removed by centrifugation at 10000*g* for 10 min, and samples were stored at −20 °C until analysis. Data are reported as means ± one standard deviation of duplicate independent fermentations analyzed in duplicate. To ensure differentiation of peptides with identical molecular masses and mass spectra by means of the retention time (LQP and IQP; LLP and IIP, ILP, or LIP; and IPP or LPP), selected samples were additionally analyzed in duplicate after internal standard addition.

Prolamin Determination by Competitive R5 ELISA. Prolamins were quantified with a Ridascreen Gliadin Competitive immunoassay employing the R5 antibody (RBiopharm, Darmstadt, Germany). Lyophilized samples (125 mg) were extracted with 1.25 mL of 60% ethanol (v/v), and the obtained peptide concentrations were divided by 250 to convert them to prolamin concentrations as stated in the manufacturer's instructions. Two different dilutions of sourdough samples from two independent fermentations were analyzed in duplicate.

Preparation of Crude Enzyme Fractions. Overnight cultures (50 mL) were harvested by centrifugation (10000*g* for 10 min at 4 °C) and washed twice with 50 mmol L^{-1} sodium acetate buffer (pH 6.4). Cells were recovered by centrifugation (4000*g* for 30 min at 4 °C), washed, and resuspended in 0.1 mol L^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 7.0). Cells were disrupted at 4 °C with an ultrasound probe. Cytoplasmic proteins were separated from cell debris by centrifugation for 30 min at 4000*g* and 4 °C. Supernatants (cell-free lysate) were stored in aliquots at −20 °C. The protein concentrations were determined using a Bio-Rad Assay Kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) using bovine serum albumin (BSA) as a standard. Peptidase activities were analyzed from cell free lysates prepared from three independent cultures for each strain.

Quantification of Peptidase Activity of Lactobacilli. General aminopeptidase type N (EC 3.4.11.11; PepN), X-prolyl dipeptidyl aminopeptidase (EC 3.4.14.5; PepX), and endopeptidase (EC 3.4.23; PepO) activities were determined as described[13,21] using Leu-*p*NA, Gly-Pro-*p*NA, and Z-Gly-Pro-*p*NA as substrates. Standard curves were established with *p*NA (Sigma). One unit (U) of PepN, PepX, and PepO activity was defined as the amount of enzyme required to release 1 nmol of *p*NA per min. The prolidase (EC 3.4.13.9; PepQ) activity was determined with the dipeptide VP by the Cd-ninhydrin method. One unit of activity was defined as the amount of enzyme required to release 1 nmol amino acid per min.[22] Specific enzyme activities were expressed as U (mg protein)$^{-1}$.

Statistical Analysis. Statistical analysis of peptidase activities and peptide concentrations was carried out by analysis of variance (ANOVA) followed by Turkey's test. Evaluation was carried out with the procedure PROC GLM using the Statistical Analysis System (SAS) v.9.2 (SAS Institute Inc. Cary, NC). *P* values of less than 0.05 were defined as significant.

RESULTS

Quantification of Tripeptides by LC-MS/MS Analysis. Liquid chromatography coupled with linear ion trap tandem mass spectrometry was used for the selective and sensitive quantification of peptides. The precursor−product ion transitions that were detected in MRM mode are shown in Table 2. The

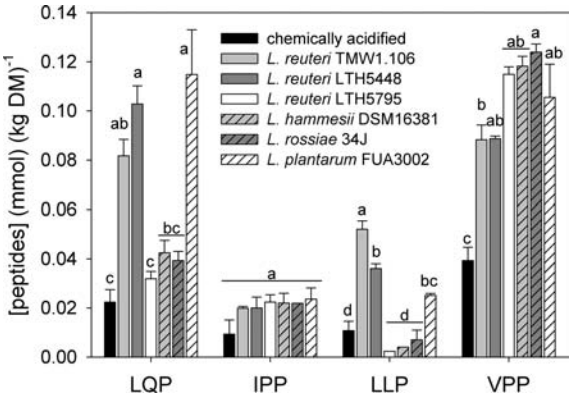

Figure 1. Concentration of bioactive peptides in rye sourdoughs fermented at 34 °C with different strains after 24 h of fermentation. Data are shown as means ± standard deviations of two independent fermentations analyzed in duplicate. Concentrations of the same peptide in different samples are significantly different ($p < 0.05$) if the bars do not share a common superscript.

calibration curves for the four tripeptides were linear in the concentration range of 0.001−1.0 mg L^{-1} with correlation coefficients of 0.999 or higher. Consistent with the presence of tripeptides with identical molecular masses and mass spectra, one peak only was detected in the MRM mode specific for VPP. Two peaks with identical mass spectra were observed for LQP and IPP, respectively, representing LQP or IQP and IPP or LPP, respectively. Four peaks were observed in the MRM mode specific for LLP (LLP, IIP, ILP, or LIP). The separation of isobaric peptides by RP-HPLC and comparison with retention times of internal standards allowed correct identification and quantification of the four tripeptides despite the presence of isobaric peptides in the extract (data not shown).

In all raw materials used for sourdough fermentation, LQP and LLP concentrations were below the detection limit. IPP and VPP concentrations were below the detection limit of 1 μg L^{-1} in wheat gluten and barley hordein but were present in rye malt flour at 0.007 and 0.013 mmol (kg DM)$^{-1}$, respectively.

Production of Tripeptides in Rye Malt Sourdoughs with Different Starter Cultures. The effect of different starter cultures on tripeptide levels was assessed by quantification of LQP, LLP, IPP, and VPP in rye malt sourdoughs fermented with six different strains of *Lactobacillus* spp. Chemically acidified doughs served as the control (Figure 1). Tripeptides were essentially absent in unfermented doughs but accumulated to 0.01−0.13 mmol (kg DM)$^{-1}$ in sourdoughs after 24 h of fermentation. The highest levels were found for VPP and LQP. LQP and LLP levels in chemically acidified doughs were significantly lower as compared to sourdoughs fermented with *L. reuteri* TMW1.106 and LTH5448 and *L. plantarum* FUA3002; VPP levels in chemically acidified doughs were significantly lower as compared to all sourdoughs. These results indicate that microbial peptidases contribute to the release of tripeptides. Individual strains accumulated different concentrations of LQP, LLP, and VPP, but the concentrations of IPP in different sourdoughs were generally similar. LQP and LLP concentrations were highest in sourdoughs fermented with *L. reuteri* TMW1.106 and LTH5448 and *L. plantarum* FUA3002. VPP concentrations in sourdoughs fermented with *L. reuteri* TMW1.106 were lower as compared to sourdoughs fermented with *L. rossiae* 34J.

Table 3. Peptidase Activities [U (mg Protein)$^{-1}$] of *Lactobacillus* spp. Employed in Rye Malt Sourdough Fermentations[a]

	enzyme and substrate			
	Pep N	Pep X	Pep O	Pep Q
strain	Leu-*p*NA	Gly-Pro-*p*NA	Z-Gly-Pro-*p*NA	Z-Val-Pro
L. reuteri TMW1.106	24.0 ± 4.6 C	77.0 ± 3.5 D	57.0 ± 4.8 C	1290 ± 70 A
L. reuteri LTH5448	33.0 ± 8.0 C	113.0 ± 7.5 C	36.0 ± 1.7 C	1290 ± 20 A
L. rossiae 34J	156.0 ± 7.8 A	305 ± 8.5 B	526 ± 26 A	937 ± 43 C
L. plantarum FUA3002	57.0 ± 12 B	671 ± 27 A	261.0 ± 8.0 B	1020 ± 62 B

[a] Peptidase activities in the same column with different letters differ significantly ($P < 0.05$).

Different LQP and LLP concentrations in sourdough may result from a different pattern of peptidase activities in the strains. Therefore, specific activities of peptidases were compared in *L. reuteri* TMW1.106 and LTH5448, *L. rossiae* 34J, and *L. plantarum* FUA3002 (Table 3). The activities of the endopeptidase PepO, general aminopeptidase PepN, proline-specific peptidases PepX, and PepQ were quantified. *L. rossiae* 34J exhibited high PepO and PepN activities and moderate PepX activity. *L. plantarum* FUA3002 showed high Pep X activity and intermediate PepQ, PepN, and PepO activities. *L. reuteri* TMW1.106 and LTH5448 exhibited high PepQ and low PepN, PepX, and PepO activities. Thus, high LQP and LLP levels in sourdough were correlated to low PepO and high PepN activities.

Kinetics of Accumulation of Tripeptides. To determine the influence of fermentation time on accumulation of four tripeptides, rye malt sourdoughs were fermented with *L. reuteri* TMW1.106 for 96 h. The four tripeptides were quantified in sourdoughs after 24−96 h of fermentation, and concentrations were compared to chemically acidified doughs. In sourdoughs, levels of tripeptides after 24 h of fermentation [116 ± 16, 25 ± 5, 26 ± 4, and 135 ± 18 μmol (kg DM)$^{-1}$ for LQP, IPP, LLP, and VPP, respectively] were generally higher when compared to the chemically acidified control [40 ± 5, 26 ± 2, 13 ± 1, and 66 ± 6 μmol (kg DM)$^{-1}$ for LQP, IPP, LLP, and VPP, respectively]. Levels of LQP and LLP in sourdoughs decreased over time. After 96 h, the concentrations in chemically acidified doughs [35 ± 4 and 16 ± 2 μmol (kg DM)$^{-1}$ for LQP and LLP, respectively] were higher than in sourdoughs [12 ± 2 and 3.0 ± 0.5 μmol (kg DM)$^{-1}$ for LQP and LLP, respectively]. These results indicate that LQP and LLP are accumulated as intermediates prior to hydrolysis to amino acids by peptidases of lactobacilli. To confirm hydrolysis of peptides containing V, I, or L, amino acids levels were quantified as previously described.[15] The concentration of leucine, isoleucine, and valine increased from 20.4 ± 0.2, 9.6 ± 0, and 16.2 ± 0.2 after 24 h to 24.5 ± 0.4, 13.1 ± 0.4, and 21.6 ± 0.6 after 96 h. The V, I, and L concentrations in chemically acidified doughs remained essentially unchanged at 16.4, 6.4, and 11.0 mmol (kg DM)$^{-1}$.

Effect of Protein Supplementation on Production of Tripeptides. The effect of protein supplementation on the release of tripeptides was assessed by analysis of sourdough samples fermented with the addition of wheat gluten. Moreover, the tripeptides IPP and LQP are encrypted more frequently in wheat gluten proteins than in rye secalins (Table 1). After 96 h of fermentation, levels of all tripeptides except VPP were substantially higher in rye malt: gluten sourdoughs [258 ± 18, 131 ± 55, 21 ± 15, and 39 ± 15 μmol (kg DM)$^{-1}$ for LQP, IPP, LLP, and VPP, respectively] as compared to rye malt sourdoughs

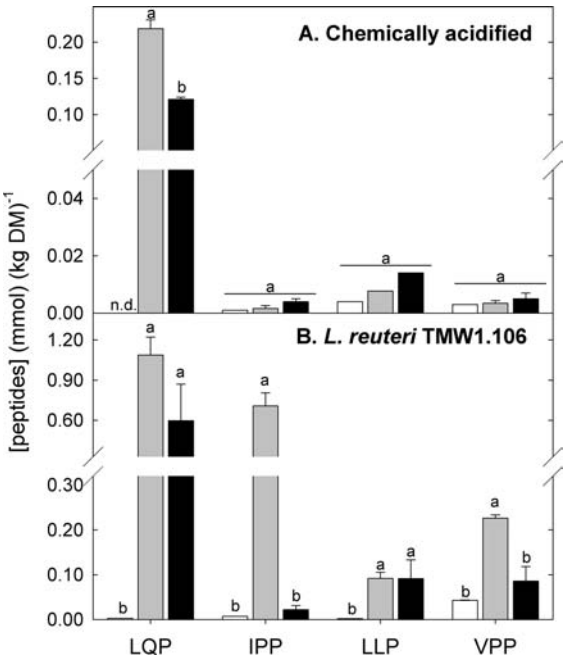

Figure 2. Concentration of bioactive peptides in chemically acidified rye malt doughs (A) or in rye malt sourdoughs (B) after fermentation with added fungal protease at 37 °C for 48 h. Bars indicate rye malt doughs (white); rye malt:wheat gluten doughs (light gray); and rye malt: hordein doughs (black). Data are shown as means ± standard deviations of two independent fermentations analyzed in duplicate. Concentrations of the same peptide in different samples are significantly different ($p < 0.05$) if the bars do not share a common superscript.

(see above). In contrast to rye malt sourdoughs, the concentrations of LQP, IPP, LLP, and VPP tended to increase with fermentation time in rye malt sourdoughs supplemented with gluten (data not shown).

Effect of Protease Addition on Production of Tripeptides. To determine whether the addition of fungal protease increases the accumulation of tripeptides, rye malt sourdoughs, rye malt sourdoughs supplemented with wheat gluten, or rye malt sourdoughs supplemented with hordein were fermented with added fungal protease. Peptide levels after 24 and 48 h of fermentation with *L. reuteri* TMW1.106 were compared to chemically acidified doughs (Figure 2 and data not shown). Tripeptide levels were less than 0.05 mmol (kg DM)$^{-1}$ in all chemically acidified doughs; only LQP accumulated to more than 0.1 (kg DM)$^{-1}$ in chemically acidified doughs supplemented with wheat gluten or hordeins. LQP levels were different ($p < 0.05$) in chemically

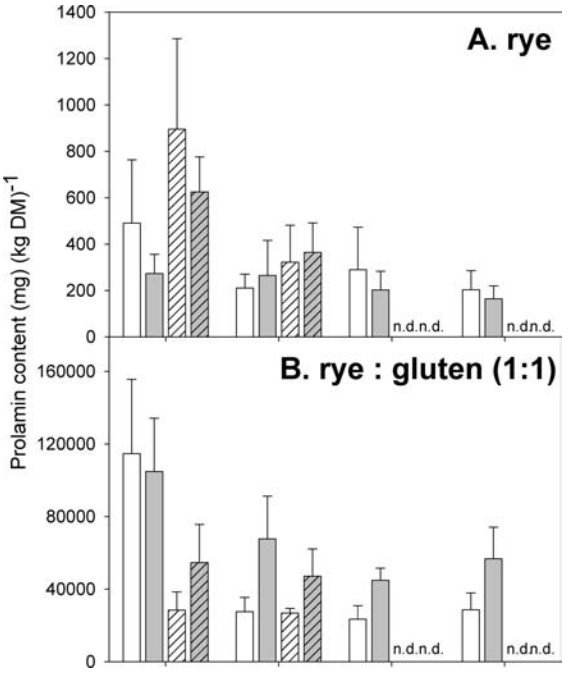

Figure 3. Prolamin contents of sourdoughs fermented by *L. reuteri* TMW1.106 (white) and chemical acidification (gray) in addition with fungal protease (hatched) in rye malt (A) and rye/wheat 1:1 (B) doughs or sourdoughs. Data are shown as means ± standard deviations of two independent fermentations analyzed in quadruplicate; n.d., not determined.

acidified doughs supplemented with gluten or hordein. In rye malt sourdoughs with protein supplementation and added fungal protease, LQP, IPP, and VPP levels ranged from 0.025 to 1.2 mmol (kg DM)$^{-1}$. The gluten addition significantly ($p < 0.05$) increased all tripeptide levels as compared to rye malt sourdoughs; addition or hordein to sourdoughs significantly ($p < 0.05$) increased LQP and LLP concentrations (Figure 2). In rye malt:gluten sourdoughs, LQP was the most abundant tripeptide, followed by IPP, VPP, and LLP. In rye malt:hordein sourdoughs, LQP also was the most abundant tripeptide. The concentration of IPP and VPP in rye malt:hordein sourdoughs was low in comparison to rye malt:wheat gluten sourdoughs.

Prolamin Quantification by Immunoassays. Prolamins in sourdoughs and chemically acidified doughs were quantified to determine whether proteolysis during dough incubations eliminates prolamins to a level that is safe for celiac patients. In rye malt flour without fungal proteases, the prolamin concentrations decreased from 56000 to 98000 to 200−400 mg (kg DM)$^{-1}$ (Figure 3A). Prolamin concentrations in chemically acidified dough and sourdough fermented with *L. reuteri* TMW1.106 were essentially comparable. The addition of fungal protease did not affect prolamin concentrations in rye malt sourdoughs (Figure 3B). However, prolamin levels in rye malt:wheat gluten sourdoughs were lower as compared to control doughs, and protease addition decreased prolamin levels after 24 and 48 h of fermentation. These results confirm earlier observations that the protease activity of rye malt is sufficient for extensive prolamin hydrolysis in rye malt sourdoughs. However, proteolysis in rye malt supplemented with prolamins from ungerminated cereals is dependent on the presence of reductive protein depolymerization potential of lactobacilli and protease addition.[14,15]

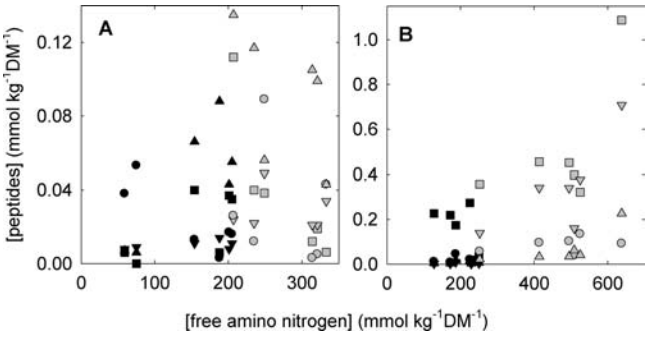

Figure 4. Comparison of biopeptide levels to FAN levels in rye malt doughs (A) and rye malt/wheat gluten doughs (B). Black symbols, chemically acidified doughs; gray symbols, sourdoughs fermented with *L. reuteri* TMW1.106. Concentrations of LQP (■), IPP (▼), LLP (●), and VPP (▲) are indicated as means of two independent fermentations analyzed in duplicate.

Biopeptides and FAN Levels. Peptides are intermediates of protein hydrolysis to amino acids. Amino acids and peptides were major products of protein hydrolysis in rye malt sourdoughs and rye malt sourdoughs supplemented with protein from ungerminated cereals, respectively.[15] To assess the relationship between the tripeptide levels and the extent of proteolysis, FAN levels in chemical acidified doughs and sourdoughs were compared to tripeptide levels (Figure 4). FAN measurement provides a direct quantification of proteolysis as cleavage of one peptide bond generates one FAN. In rye malt sourdoughs, maximum peptide levels were observed in doughs with a FAN concentration of about 200 mmol (kg DM)$^{-1}$. Increasing proteolysis to achieve FAN levels higher than 200 mmol (kg DM)$^{-1}$ decreased tripeptide concentrations (Figure 4A). This decrease was most noticeable for LQP and LLP and least pronounced for IPP and VPP. In rye malt:wheat gluten doughs, increasing FAN concentrations corresponded to elevated tripeptide levels (Figure 4B). In rye malt:hordein doughs, maximum peptide levels were observed at FAN levels of about 300−500 mmol (kg DM)$^{-1}$ (data not shown). Taken together, these results demonstrate that the optimization of the yield of ACE inhibitory tripeptides requires careful control of proteolysis. In industrial applications, the FAN content may be a convenient measure for batchwise control of proteolysis. Moreover, IPP and VPP are more resistant to hydrolysis by cereal or microbial enzymes than LQP and LLP.

DISCUSSION

This study quantified the concentrations of four tripeptides with ACE inhibitory activity in rye malt sourdoughs and elucidated factors contributing to their release from rye secalins, wheat gluten, or barley hordeins. The accumulation of ACE inhibitory peptides in dairy fermentations has been extensively studied,[1,2,7,11,28] but only few studies identified ACE inhibitory peptides in wheat, rye, or barley fermentations.[23] This study targeted tripeptides with known biological activity rather than an approach based on bioassay-guided fractionation on the basis of the following considerations: First, current knowledge about structure−function relationships of ACE inhibitory peptides in combination with sequence information on their occurrence in proteins from wheat, barley, or rye allows the identification of active peptides that can be released by proteolysis. Second, clinical evidence for antihypertensive activity is essentially limited to

di- and tripeptides, particularly IPP and VPP. This approach has the advantage of allowing a preliminary assessment of in vivo activity on the basis of clinical studies with the same peptides in other food systems. However, ACE inhibitory peptides other than those quantified by use of external standards were likely present but not accounted.

LC-MS/MS with MRM allows specific and accurate peptide quantification by selecting the m/z of the precursor ion for MS/MS fragmentation and monitoring only one of the fragment ions produced. LC-MRM-MS with isotope-labeled internal standard was employed for quantification of 17 ACE-inhibitory peptides in human blood plasma samples.[24] LC with subsequent MS^3 was used for confirmatory analysis of VPP and IPP in traditional cheeses.[25] This study quantified tripeptides by LC-MS/MS with MRM mode in a new and simplified manner without sample pretreatment. Because the presence of I or L in a peptide sequence is not differentiated by MS methods, the unambiguous identification of IPP, LLP, and LQP required reproducible retention times, which were verified by internal standards.

The relative concentrations of LQP, IPP, LLP, and VPP in rye malt sourdoughs were generally in agreement with the frequency of these sequences in selected cereal proteins (Table 1).[9] LQP was the most abundant tripeptide in all samples. IPP was most abundant after supplementation with wheat gluten but virtually absent after supplementation with hordein (Table 1 and Figure 2). The simultaneous supplementation of rye malt with substrate and fungal proteases was a successful strategy to maximize the concentration of ACE inhibitory peptides. The strong influence of fermentation with heterofermentative lactobacilli on peptide concentrations in sourdoughs is partially explained by the higher levels of proteolysis in sourdoughs when compared to chemically acidified doughs.[12,13,15] This observation also confirms earlier observations that peptidases of LAB contribute to secondary proteolysis in sourdoughs[12,13] and extends these to the quantification of specific peptides. Because of the high level of proline in cereal prolamins, peptide hydrolysis by lactobacilli is dependent on the proline peptidases PepX, PepQ, PepR, PepI, and PepP.[12] Proline peptides are degraded by LAB through combined PepX and PepQ or PepP and PepI activities.[26] Peptidase activities of LAB are strain specific,[12] and lactobacilli with complementary peptidase activities were previously used to degrade gluten proteins.[13] VPP and IPP appeared more resistant to hydrolysis in rye malt sourdoughs or control doughs as compared to LQP and LLP. Peptidases of LAB degrade LQP or LLP, but peptidases that cleave imino bonds between two proline residues have not been described.[12,26] In keeping with the accumulation of IPP and VPP in fermented dairy products,[11,25] lactobacilli apparently contribute to their release but not their degradation. The suitability of proteases from *Aspergillus oryzae* to produce tripeptides (X-PP) and dipeptides (X-P) in dairy products was previously reported.[27]

Extensive hydrolysis of prolamins was achieved in rye malt sourdoughs.[14] Prolamin hydrolysis in rye malt sourdoughs fermented with *L. reuteri* TMW1.106 is well in agreement with prior observations obtained under comparable conditions.[14] Remarkably, the combination of rye malt with fungal protease did not further reduce prolamin concentrations. The tripeptide LQP is encrypted in the α-gliadin derived 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) and several related peptides with known toxicity in celiac disease.[10] Accumulation of ACE inhibitory tripeptides in sourdoughs is thus compatible with elimination of prolamins in rye, wheat, and barley. However, prolamin levels remained above 200 mg kg^{-1} in all doughs, well above the 20 mg kg^{-1} that is currently considered safe for celiac patients.

The peptides LQP, IPP, LLP, and VPP levels exhibit ACE inhibitory activity IC_{50} values of 2, 9, 57, and 5 μM,[19,20] respectively. In the present work, LQP, IPP, LLP, and VPP concentrations in rye malt:wheat gluten sourdoughs fermented with protease addition were 1000, 700, 140, and 225 μmol (kg DM)$^{-1}$, corresponding to about 360, 230, 50, and 70 mg (kg DM)$^{-1}$, respectively. These concentrations cumulatively exceed in vitro inhibitory activities 500−1000-fold. Moreover, the concentrations are at the upper limit of IPP concentrations (0−94.5 mg kg^{-1}) and VPP concentrations (0−224 mg kg^{-1}) reported in cheese.[25]

The active concentrations of VPP and IPP in vivo are difficult to estimate on the basis of current literature. Data for LQP and LLP are not available. Several clinical trials with hypertensive humans show a moderate but relatively consistent reduction of systolic blood pressure upon consumption of the fermented milk products Calpis and Evolus containing VPP and IPP.[8,28] The daily consumption of VPP and IPP in these studies was about 3.75 mg VPP and 2.25 mg IPP.[28] The peptide tablets Ameal S containing IPP and VPP reduced the systolic blood pressure in 131 patients at a dose equivalent to 3.6 mg peptides per day.[29] In comparison with the effective levels of bioactive peptides in dairy products, the concentration of ACE inhibitory peptides in rye malt sourdoughs likely exceeded in vivo active levels 10−100-fold.

The current industrial use of sourdough-based baking improvers aims to improve bread flavor through accumulation of organic acids and flavor-active amino acids, including glutamate and ornithine.[15,30,31] The rye malt sourdoughs analyzed in this study can be produced with only minor modifications to current processes. The concentration of ACE inhibitory peptides in the experimental sourdoughs may allow dosage levels of 1−10%, in keeping with the current use of sourdough-based baking improvers.[30,31] The biological activity of peptides may be enhanced by simultaneous accumulation of ACE inhibitory peptides not accounted for in this study, as well as γ-aminobutyrate.[15] In conclusion, cereal proteins are as suitable as dairy proteins for generation of bioactive peptides in fermented food. The combination of ACE inhibitory peptides with other bioactives and flavor-active amino acids may provide interesting perspectives for food product development.

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