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Synthesis of Angiotensin I-Converting Enzyme (ACE)-Inhibitory Peptides and γ-Aminobutyric Acid (GABA) during Sourdough Fermentation by Selected Lactic Acid Bacteria

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This article aimed at investigating the synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and γ-aminobutyric acid (GABA) during sourdough fermentation of white wheat, wholemeal wheat, and rye flours. Sourdough lactic acid bacteria, selected previously for proteinase and peptidase activities toward wheat proteins or for the capacity of synthesizing GABA, were used. The highest ACE-inhibitory activity was found by fermenting flour under semiliquid conditions (dough yield 330) and, especially, by using wholemeal wheat flour. Fourteen peptides, not previously reported as ACE-inhibitory, were identified from the water/salt-soluble extract of wholemeal wheat sourdough (IC_{50} 0.19−0.54 mg/mL). The major part of the identified peptides contained the well-known antihypertensive epitope VAP. The synthesis of GABA increased when the dough yield was decreased to 160. The highest synthesis of GABA (258.71 mg/kg) was found in wholemeal wheat sourdough.

KEYWORDS: Sourdough; lactic acid bacteria; ACE-inhibitory peptides; γ-aminobutyric acid

INTRODUCTION

Cereal foods are essential components of the daily human diet. Nutritionally, they are important sources of carbohydrates, proteins, dietary fibers (DF), vitamins, and of many other nonnutrients (e.g., food components not essential for growth but with potential biological functions) (*1*). Beyond the nutritional value, cereal foods have recently gained an increased interest also as functional foods.

Bioactive or biogenic peptides are components of functional foods, which may exert regulatory activities in the human organism, irrespective of their nutritive functions (*2*). Most of these bioactivities are encrypted within the primary structure of proteins, requiring proteolysis for the release from their precursors. Proteolysis may release biogenic peptides during gastrointestinal transit or during food processing. Some bioactive peptides have angiotensin I-converting enzyme (ACE)-inhibiting properties and may be used for preventing hypertension as well as for other therapeutic purposes (*3*). Although most of the ACE-inhibitory peptides were synthesized in functional dairy products, some of them have recently been identified and characterized from cereal proteins as well. The hydrolysis of the wheat germ by the alkaline protease from *Bacillus licheniformis* mainly produced the peptide IVY, which showed ACE-inhibitory activity, having an IC_{50} of 0.48 μM (*4*). The ACE-inhibitory peptide IAP (IC_{50} of 2.7 μM) was identified after the hydrolysis of gliadins with an acid protease (*5*). After intraperitoneal administration, it decreased the systolic blood pressure of spontaneously hypertensive rats (SHR). Six commercial proteases were used to hydrolyze corn flour. A complex of fungal endo- and exoproteases produced the hydrolyzate with the highest ACE-inhibitory activity (*6*). Rice proteins treated with the protease Alcalase showed an in vitro ACE-inhibitory activity (IC_{50} of 0.14 mg/mL), mainly due to the peptide TQVY. This preparation caused a significant decrease of the systolic blood pressure in SHR (*3*).

Besides foods enriched with ACE-inhibitory peptides, dietary materials and/or products, containing γ-aminobutyric acid (GABA) also showed the capacity to decrease blood pressure in SHR and hypertensive humans (*7, 8*). GABA, a nonprotein amino acid, possesses well-known physiological functions such as neurotransmission, induction of hypotension, and diuretic and tranquilizer effects (*9*). GABA is synthesized by glutamate decarboxylase (GAD) [EC 4.1.1.15], a pyridoxal 5′-phosphate (PLP)-dependent enzyme, that catalyzes the irreversible α-decarboxylation of L-glutamate to GABA. Several GABA-enriched cereal foods have been characterized: rice germ soaked in water, germinated brown rice treated by high-pressure, germinated wheat, and red-mold rice containing the *Monascus* fungus (*9*).

Sourdough fermentation has a well-known role in improving the flavor and structure of many cereal-based baked goods. More recently, the potential of sourdough fermentation for enhancing the nutritional properties of wheat, rye, and oat baked goods has been considered (*1*). Overall, sourdough fermentation improves the texture and palatability of whole grain, fiber rich, or gluten-free products (*10*); stabilizes or increases the level of various bioactive compounds; retards the starch bioavailability, thus decreasing the glyceamic index; and increases mineral

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bioavailability (*11, 12*). During long-time fermentation, pools of selected sourdough lactobacilli showed the capacity to completely degrade epitopes responsible for celiac disease and food allergies (*13–15*). Because of the specific activities toward cereal proteins, the use of the above pool of lactobacilli for synthesizing ACE-inhibitory peptides during sourdough fermentation may deserve an interest. Several reports (*9, 16, 17*) also showed the presence of GAD activity in lactic acid bacteria. Recently, strains of lactic acid bacteria isolated from cheeses, but also populating sourdoughs, were shown to possess the *gad* gene and to synthesize the relevant concentration of GABA (*9*). Currently, no literature data are available on the synthesis of GABA during sourdough fermentation by selected lactic acid bacteria.

This article aimed at investigating the synthesis of ACE-inhibitory peptides and GABA during sourdough fermentation of white wheat, wholemeal wheat, and rye flours by selected lactic acid bacteria.

MATERIALS AND METHODS

Microorganisms and Enzymes. *Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A, and *Lactobacillus hilgardii* 51B were previously selected on the basis of their capacity to hydrolyze gliadins (*15*). *Lb. sanfranciscensis* LS3, LS10, LS19, LS23, LS38, and LS47 were selected on the basis of their peptidase systems, with particular reference to activities toward Pro-rich peptides (*18*).The above 10 lactobacilli (pool 1) were used in the mixture for sourdough fermentation. *Lactobacillus plantarum* C48 and *Lactococcus lactis* subsp. *lactis* PU1 were isolated from cheeses and selected for their capacity for synthesizing GABA (*9*). *Lactobacillus* strains were propagated for 24 h at 30 °C in MRS broth (Oxoid, Basingstoke, Hampshire, England) with the addition of fresh yeast extract (5%, v/v) and 28 mM maltose at a final pH of 5.6. *Lc. lactis* subsp. *lactis* PU1 was propagated for 24 h at 30 °C in M17 broth (Oxoid). When used for sourdough fermentations, *Lactobacillus* and *Lactococcus* cells were cultivated until the late exponential phase of growth was reached (ca. 10 h), washed twice in 50 mM phosphate buffer, pH 7.0, and resuspended in the tap water used for making the dough. Proteases of *Aspergillus oryzae* (500,000 Hemoglobin Unit on the Tyrosine basis, HUT/g) (enzyme 1, E1) and *Aspergillus niger* (3,000 Spectrophotometric Acid Protease Unit, SAPU/g) (enzyme 2, E2), routinely used for bakery applications, were supplied by BIO-CAT (BIO-CAT Inc., Troy, Virginia, USA).

The number of lactic acid bacteria was estimated by plating serial dilution of sourdoughs on MRS (lactobacilli) or M17 (lactococci) agar media (Oxoid) at 30 °C for 48 h. Total mesophilic bacteria were enumerated on PCA agar medium (Oxoid) at 30 °C for 48 h.

Sourdough Fermentation. The characteristics of the flours used in this study were as follows: (i) white wheat flour (*Triticum aestivum*, Appulo cv): moisture, 14.2%; protein (N × 5.70), 11.5%, of dry matter (d.m.); fat, 1.6% of d.m.; ash, 0.6% of d.m.; and total soluble carbohydrates, 1.5% of d.m. (ii) Wholemeal wheat flour (*Triticum aestivum*, Appulo cv): moisture, 13.4%; protein, 11.9%, of d.m.; fat, 1.8% of d.m.; ash, 0.6% of d.m.; and total soluble carbohydrates, 1.9% of d.m. (iii) Rye flour (*Secale cereale*): moisture, 10%; protein, 10% of d.m.; fat, 2.5% of d.m.; ash, 1.5% of d.m; and total soluble carbohydrates, 1.8% of d.m.

For each flour, the following sourdoughs were prepared: A, 120 g of flour and 280 g of tap water (dough yield of 330, semiliquid dough), containing pool 1 at the initial cell density of 5×10^8 CFU/g of dough; B, as sourdough A with the addition of 200 ppm of both E1 and E2; C, 120 g of flour and 280 g of tap water, containing *Lb. plantarum* C48 at the initial cell density of 5×10^8 CFU/g of dough; D, 120 g of flour and 280 g of tap water, containing *Lc. lactis* subsp. *lactis* PU15 at the initial cell density of 5×10^8 CFU/g of dough; E, as sourdough A with the addition of *Lb. plantarum* C48 (5×10^8 CFU/g); F, as sourdough A with the addition of *Lc. lactis* subsp. *lactis* PU15 (5×10^8 CFU/g); and G, 250 g of flour and 150 g of tap water (dough yield of 160), containing pool 1 and *Lc. lactis* subsp. *lactis* PU15 at the cell density of 5×10^8 CFU/g of dough. For each flour, two chemically acidified doughs, having a dough yield of 330 or 160 (CT1 and CT2, respectively), without bacterial inoculum and addition of commercial proteases, were acidified to pH 3.5 by a mixture of lactic and acetic acids (molar ratio 4:1) and used as the controls. Sourdoughs and chemically acidified doughs were incubated for 24 h at 37 °C. Incubation of semiliquid doughs was under stirring conditions (ca. 200 rpm). For each condition, four independent fermentations were carried out.

Water/Salt-Soluble Extracts. Water/salt-soluble extracts were prepared from each dough following the method originally described by Osborne (*19*) and modified by Weiss et al. (*20*). An aliquot of each dough (containing 7.5 g of flour) was diluted with 30 mL of 50 mM Tris-HCl (pH 8.8), held at 4 °C for 1 h vortexing at 15-min intervals, and centrifuged at 20,000*g* for 20 min. The supernatants, containing the water/salt-soluble nitrogen fraction, were used for the ACE inhibition assay and to determine the concentration of GABA. The concentration of peptides in the water/salt-soluble extracts was determined by the *o*-phtaldialdehyde (OPA) method (*21*). A standard curve prepared using tryptone (0.25 to 1.5 mg/mL) was used as the reference. The use of peptone gave a similar standard curve.

ACE Inhibition Assay. The determination of the ACE-inhibitory activity was carried out by reversed-phase fast performance liquid chromatography (RP-FPLC) analysis, modified in agreement with the spectrophotometric method described by Cushman and Cheung (*22*), and Wu and Ding (*23*). This method does not contain the step of ethyl acetate extraction, and it is particularly suitable for extremely complex mixture of peptides such as protein hydrolyzates (*24*). Two hundreds microliters of hippuryl-L-histidyl-L-leucine (HHL) solution (5 mM in Na-borate buffer, pH 8.3, containing 300 mM NaCl) was mixed with 60 μL of the sample or water (control) and 40 μL of ACE (100 mU/mL 10 mM in phosphate buffer, pH 7.0, containing 500 mM NaCl). The mixture was incubated for 60 min at 37 °C; then, the reaction was stopped by adding 250 μL of 1 M HCl. The mixture was injected directly onto a Resource RPC C_{18} column (6.4 × 100 mm, particle size 15 μM; GE Healthcare Bio-Sciences AB) to separate the product and hippuric acid (HA) from HHL. The column was eluted with a mobile phase composed of water and acetonitrile (CH_3CN) containing 0.05% trifluoroacetic acid (TFA), at a flow rate of 1 mL/min, and the detector was monitored at 228 nm. The CH_3CN content was increased linearly from 5 to 46% between 16 and 62 min. The inhibition activity was calculated using the following equation: inhibition activity (%) = [(Pc − Ps)/(Pc − Pb)]100, where Pc is the HA-peak area of the control, Ps is the HA-peak area of the reaction mixture (sample), and Pb is the HA-peak area of the reaction mixture without ACE. The concentration of an ACE-inhibitor (crude fraction) needed to inhibit 50% of ACE activity was defined as IC_{50} and determined by regression analysis of ACE inhibition (%) versus peptide concentration. Because of some limitations of the OPA method for determining peptide concentration, IC_{50} values are considered as IC_{50} apparent values. Values of the percentage of ACE inhibition are the average of three separated assays, and the coefficient of variation was always lower than 2%.

Concentration of GABA. The water/salt-soluble extracts were filtered through a Millex-HA 0.22-μM pore size filter (Millipore Co., Bedford, MA). Total and individual free amino acids (FAAs) contained in the water-soluble extracts were analyzed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) with a Na-cation-exchange column (20 by 0.46 cm internal diameter). A mixture of amino acids at a known concentration (Sigma Chemical Co., Milan, Italy) was added to cysteic acid, methionine sulfoxide, methionine sulfone, tryptophan, ornithine, glutamic acid, and GABA and used as standard. Proteins and peptides in the samples were precipitated by the addition of 5% (vol/vol) cold solid sulfosalicylic acid, holding at 4 °C for 1 h and centrifuging at 15,000*g* for 15 min. The supernatant was filtered through a 0.22 μM pore size filter and diluted, when necessary, with sodium citrate (0.2 M, pH 2.2) loading buffer. Amino acids were postcolumn derivatized with ninhydrin reagent and detected by absorbance at 440 (proline and hydroxyproline) or 570 nm (all the other amino acids).

Isolation of Peptides from Water/Salt-Soluble Extracts. Peptides were separated from water/salt-soluble extracts by RP-FPLC, using a

Resource RPC column and ÄKTA FPLC equipment with a UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). This method of separation is largely used for the isolation of biologically active peptides from complex food matrices (*25*). A volume of water-soluble extract containing ca. 30 mg of peptides was separated. Aliquots (ca. 2 mL) of the water-soluble extracts were added to 0.05 (vol/vol) TFA and centrifuged at 10,000*g* for 10 min. The supernatant was filtered through a Millex-HA 0.22 μM pore size filter (Millipore Co.) and loaded onto the column. Gradient elution was performed at a flow rate of 1 mL/min using a mobile phase composed of water and CH_3CN, containing 0.05% TFA. The CH_3CN content was increased linearly from 5 to 46% between 16 and 62 min. Solvents were removed from the collected 2-mL fractions by freeze-drying. The fractions were redissolved in 600 μL of water and assayed for ACE inhibition activity.

Identification of Peptides. Fractions of the water/salt-soluble extract from wholemeal wheat flour dough were subjected to a second step of purification through RP-FPLC under the conditions described previously. The centers of the peaks were collected, freeze-dried, and used for mass spectrometry analysis.

The identification of peptides was performed by the Proteome Factory (Proteome Factory AG, Berlin, Germany) using nano-liquid chromatography coupled to electrospray ionization tandem mass spectrometry (nanoLC-ESI-MS/MS). The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Boeblingen, Germany), PicoTip emitter (New Objective, Woburn, USA), and an Esquire 3000 plus ion trap MS (Bruker, Bremen, Germany). After trapping and desalting the peptides on an enrichment column (Zorbax SB C18, 0.3 × 5 mm, Agilent) using 1% acetonitrile/0.5% formic acid solution for five minutes, peptides were separated on a Zorbax 300 SB C18, 75 μM × 150 mm column (Agilent) using an acetonitrile/0.1% formic acid gradient from 5 to 40% acetonitrile within 40 min. MS spectra were automatically taken by Esquire 3000 plus according to manufacturer instrument settings for nanoLC-ESI-MSMS analyses. Peptides were identified using a MS/MS ion search of the Mascot search engine (Matrix Science, London, England) and NCBInr protein database (National Center for Biotechnology Information, Bethesda, USA). Ion charge in search parameters for ions from ESI-MS/MS data acquisition were set to "1+, 2+ or 3+", according to the instrument and method common charge state distribution.

Hydrolysis of the Peptide Fractions by Tripsin and Chymotrypsin. Aliquots (750 μL) of crude peptide fractions at the ACE-inhibitory concentrations were incubated with 10 μL of trypsin or chymotripsin (2 and 4 mg/mL, respectively), and 40 μL of 0.25 M Tris-HCl, pH 8.0, at 37 °C for 50 min. The reaction was stopped with 100 μL of 0.1% TFA, and samples were analyzed by RP-FPLC. Insulin chain A (240 μg/mL) was used as the control, and the concentrations of trypsin and chymotripsin were standardized to give ca. 80% hydrolysis of insulin chain A.

RESULTS

ACE-Inhibitory Activity of Water/Salt-Soluble Extracts. After fermentation, all sourdoughs (A-G) had values of pH in the range of 3.5−3.9 and contained cell densities of lactic acid bacteria from 1×10^9 to 5×10^9 CFU/g of dough. Chemically acidified doughs (CT1 and CT2) contained total mesophilic bacteria at a cell density lower than 3.0 CFU/g.

First, ACE-inhibitory activity was assayed under semiliquid conditions (sourdoughs A−F). Apart from the flour used, the ACE-inhibitory activity of the water/salt-soluble extract from chemically acidified dough (CT1) ranged from 10 to 14% (**Table 1**). Very few peptide peaks were found in the water/salt-soluble extract of CT1 (**Figure 1A**, **B**, and **C**). The ACE-inhibitory activities of sourdough A fermented with pool 1 were 82, 95, and 89% for white wheat, wholemeal wheat, and rye flours, respectively. When proteases E1 and E2 were added together with pool 1 (sourdough B), the ACE-inhibitory activity significantly ($P < 0.05$) decreased to 47, 48, and 45% for white wheat, wholemeal wheat, and rye flours, respectively. As shown by the RP-FPLC chromatograms of the water/salt-soluble extracts, the use of proteases (**Figure 1D**, **E**, and **F**) caused an almost complete degradation of the peptide peaks found in the central zone of the acetonitrile gradient of sourdough A (**Figure 1G**, **H**, and **I**). When GABA-producing strains (*Lb. plantarum* C48 or *Lc. lactis* subsp. *lactis* PU1) were used alone (sourdoughs C and D, respectively), the ACE-inhibitory activity of the water/salt-soluble extracts did not significantly ($P < 0.05$) vary with respect to that found in the control CT1. When GABA-producing strains were used together with pool 1, the ACE-inhibitory activity did not significantly ($P < 0.05$) differ from those found for sourdough A. When dough yield was decreased to 160, the ACE-inhibitory activity of the sourdough G was found to be 39, 63, and 56% for white wheat, wholemeal wheat, and rye flours, respectively. These values were markedly lower than those found for sourdough F started with the same lactic acid bacteria (pool 1 and *Lc. lactis* subsp. *lactis* PU1) but under semiliquid conditions (dough yield of 330). The ACE-inhibitory activity of CT2 did not significantly ($P < 0.05$) vary with respect to control CT1.

Table 1. Concentration of Peptides (mg/mL) and of γ-Aminobutyric Acid (GABA) (mg/kg), and ACE-Inhibitory Activity (%) of the Water/Salt Soluble Extracts of White Wheat, Wholemeal Wheat, and Rye Flour Sourdough[a]

sourdough	flour	peptide concentration[b] (mg/mL)	GABA concentration (mg/kg of dough)	ACE-inhibitory activity (%)
CT1	white wheat	0.28 a	0.80 a	14 a
	wholemeal wheat	0.53 a	2.87 a	12 a
	rye	0.77 a	3.78 a	10 a
A	white wheat	2.90 b	13.79 ab	82 b
	wholemeal wheat	3.26 b	10.97 ab	95 b
	rye	6.43 c	11.68 ab	89 b
B	white wheat	4.57 bc	nd[c]	47 c
	wholemeal wheat	6.73 c	nd	48 c
	rye	13.13 d	nd	45 c
C	white wheat	1.2 a	12. 65 b	14 a
	wholemeal wheat	1.7 a	100.71 cd	15 a
	rye	2.9 b	44.61 c	15 a
D	white wheat	1.25 a	16.68 b	15 a
	wholemeal wheat	1.69 a	96.71 cd	16 a
	rye	3.4 b	58.87 c	15 a
E	white wheat	2.88 b	42.67 c	83 b
	wholemeal wheat	3.19 bc	125.86 cd	95 b
	rye	6.40 c	70.20 cd	88 b
F	white wheat	2.92 b	55.57 c	82 b
	wholemeal wheat	3.30 b	126.09 cd	96 b
	rye	6.48 c	77.61 cd	89 b
CT2	white wheat	0.51 a	22.36 bc	11 a
	wholemeal wheat	1.03 a	55.24 c	12 a
	rye	1.55 a	38.63 c	10 a
G	white wheat	5.32 bc	100.84 cd	39 c
	wholemeal wheat	5.91 bc	258.71 d	63 c
	rye	11.54 d	167.76 d	56 c

[a] For details of the sourdough formulas, see Materials and Methods. [b] The peptide concentration of the water/salt-soluble extracts was determined by the OPA method. Data are the mean of three independent fermentations, and the values in the same column with different letters (a−d) differ significantly ($P < 0.05$). [c] nd, not determined.

Concentration of GABA. First, the concentration of GABA was determined under semiliquid conditions (sourdoughs A−F). The concentration of GABA in the control CT1 was very low

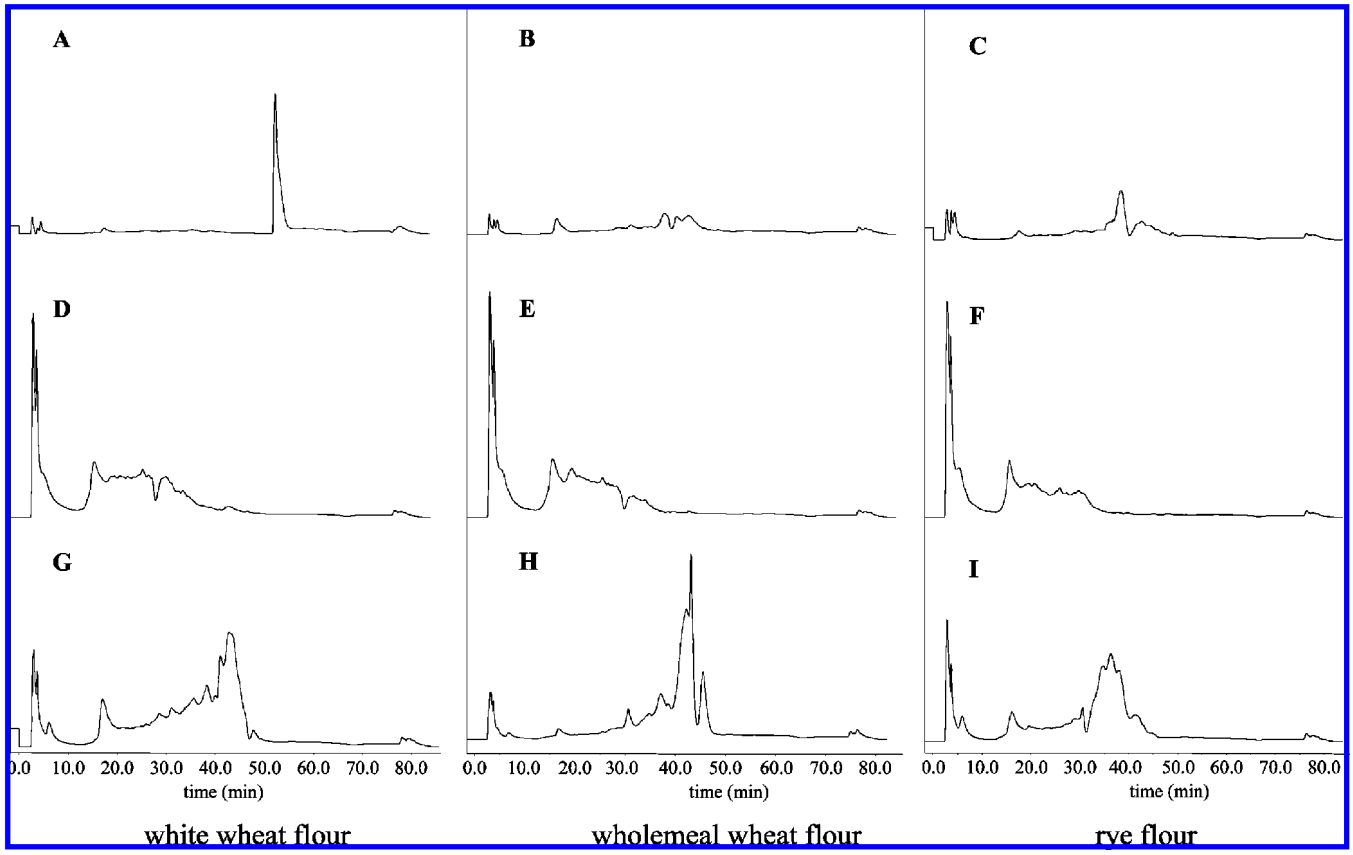

Figure 1. RP-FPLC chromatograms of the water/salt-soluble extracts of white wheat, wholemeal wheat, and rye flour sourdoughs: chemically acidified (**A**, **B**, and **C**); started with pool 1 of selected lactic acid bacteria pool and proteases (**D**, **E**, and **F**); started with pool 1 of selected lactic acid bacteria pool (**G**, **H**, and **I**). For details of the sourdough formulas see Materials and Methods.

and did not significantly ($P < 0.05$) vary between the flours used (**Table 1**). The concentration of GABA in sourdough A fermented with pool 1 was 13.79, 10.97, and 11.68 mg/kg for white wheat, wholemeal wheat, and rye flours, respectively. When GABA-producing strains were used alone, the concentration of GABA markedly increased depending on the flour. Sourdough C started with *Lb. plantarum* C48 had concentrations of GABA of 12.65, 100.71, and 44.61 mg/kg for white wheat, wholemeal wheat, and rye flours, respectively. Sourdough D started with *Lc. lactis* subsp. *lactis* PU1 also gave the highest synthesis of GABA when wholemeal wheat was used (96.71 mg/kg). The concentration of GABA further increased when GABA-producing strains were individually added to pool 1. In particular, the highest synthesis of GABA was found in sourdoughs E and F with wholemeal wheat used as substrate (125.86 and 126.09 mg/kg, respectively). The combination of both GABA-producing strains with pool 1 did not further increase the synthesis of GABA (data not shown). When dough yield was decreased to 160, the concentration of GABA in sourdough G started with the pool 1 and *Lc. lactis* subsp. *lactis* PU1 increased to 100.84, 258.71, and 167.76 mg/kg for white wheat, wholemeal wheat, and rye flours, respectively. Almost similar results were found when *Lb. plantarum* C48 was used (data not shown). The concentration of GABA in CT2 was higher than that in control CT1. The concentration of GABA increased to 55.24 mg/kg, especially when wholemeal flour was used.

Isolation and Identification of ACE-Inhibitory Peptides. The water/salt-soluble extract of sourdough F made with wholemeal wheat flour (dough yield of 330) and fermented with pool 1 and *Lc. lactis* subsp. *lactis* PU1 was used for isolation and identification of potential ACE-inhibitory peptides. This water/salt-soluble extract had the highest ACE-inhibitory activity together with the highest concentration of GABA under semiliquid conditions. Thirty-four fractions of a volume of the water/salt-soluble extract, containing 30 mg of peptides, were collected by RP-FPLC. Before the ACE-inhibitory assay, the fractions were freeze-dried and dissolved in ca. 600 μL of distilled water. Except for fractions eluting in the early zone of the acetonitrile gradient, all the other fractions had a concentration of peptides lower than 1 mg/mL. Only 3 fractions distributed in the central zone of the acetonitrile gradient showed ACE-inhibitory activity higher than 80% (**Figure 2**). They were fraction 16, 82.30%; 17, 85.77%; and 22, 81.85%. All these fractions were subjected to a second step of purification through RP-FPLC. Peptides from these fractions were subjected to mass spectra analyses by nanoLC-ESI-MS/MS. Identification of peptides was carried out through MS/MS ion search of the Mascot search engine (Matrix Science, London, England) and NCBInr protein database (National Center for Biotechnology Information, Bethesda, USA). Before database searching, the following parameters were specified: species, *Triticum aestivum* and other cereal species belonging to *Oryza*, *Hordeum*, and *Secale* genus; enzyme, none; type of instrument, ESI-trap; peptide mass tolerance, $\pm$ 0.1%; and fragment mass tolerance, $\pm$ 0.5 Da. Results from peptide identification were subjected to a manual evaluation as described by Chen et al. (*26*), and the validated peptide sequences explained all the major peaks found in the MS/MS spectrum. All fractions contained a mixture of peptides, and the respective sequences are listed in **Table 2**. Fraction 16 contained 4 peptides: DPVAPLQRSGPEI, PVAPQLSRGLL, ELEIVMASPP, and QILLPRPGQAA. Fraction 17 contained 4 peptides: PVAPLQRSGPE, PRSGNVGESGL, VAPSRPTPR, and DIIIPD. Fraction 22 contained 3 peptides that differed

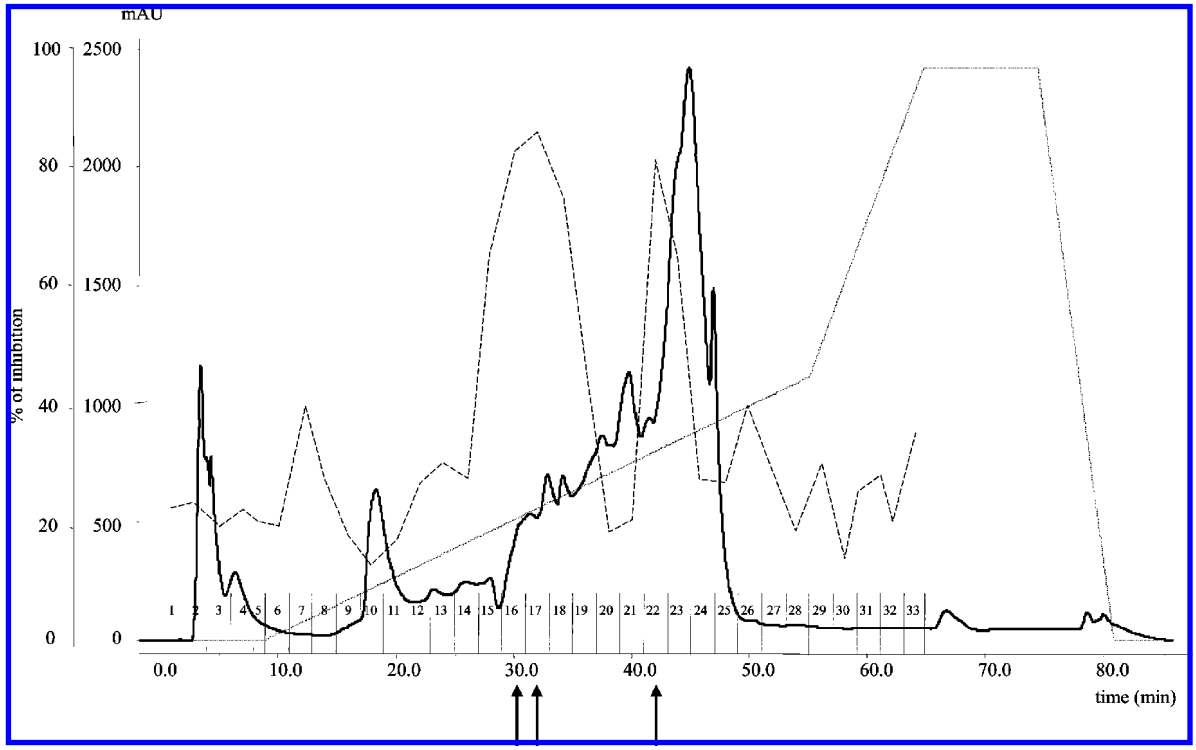

Figure 2. RP-FPLC chromatogram of the water/salt-soluble extract of wholemeal wheat sourdough (dough yield of 330) started with pool 1 of selected lactic acid bacteria. The dashed line refers to the percentage of ACE inhibition. Arrows indicated peptide fraction with the highest ACE-inhibitory activity. For microbial composition of pool 1 see Materials and Methods.

Table 2. Sequences of Peptides Contained in Crude Fractions of the Water/Salt-Soluble Extract of Wholemeal Wheat Sourdough (Dough Yield of 330) Started with Pool 1[a] of Selected Lactic Acid Bacteria

fraction	sequence[b]	IC_{50}[c]	score	charge	calculated mass	expected mass	delta	source protein NCBI accession number
n.16	DPVAPLQRSGPEI	0.19 mg/mL	78	2	1149.6142	1150.2854	0.67	**gil231540**, β-amylase (1,4-α-D-glucan maltohydrolase)
	PVAPQLSRGLL		43	2	1149.6870	1150.2854	0.60	**gil125564773**, hypothetical protein Osl_031385
	ELEIVMASPP		27	2	1084.5474	1084.0654	0.48	**gil32488664**, OSJNBa0087O24.14
	QILLPRPGQAA		22	2	1162.6822	1162.7254	0.04	**gil55733859**, unknown protein
n.17	PVAPLQRSGPE	0.54 mg/mL	72	2	1149.6142	1150.2854	0.67	**gil231540**, β-amylase (1,4-α-D-glucan maltohydrolase)
	PRSGNVGESGL		36	2	1149.6870	1072.1854	0.62	**gil123957**, α-amylase/trypsin inhibitor CM3 precursor
	VAPSRPTPR		30	1	1084.5474	980.5327	0.98	**gil18542913**, putative hsr201 hypersensitivity-related protein
	DIIIPD		26	1	1162.6822	683.9327	0.44	**gil19551090**, putative polyprotein
n.22	PRSGNVGESGLID	0.45 mg/mL	60	2	1299.6419	1300.2654	0.62	**gil123957**, α-amylase/trypsin inhibitor CM3 precursor
	DPVAPLQRSGPEI		50	2	1149.6142	1150.2854	0.67	**gil231540**, β-amylase (1,4-α-D-glucan maltohydrolase)
	DPVAPLQRSGPEIP		44	2	1262.6983	1263.2654	0.57	**gil231540**, β-amylase (1,4-α-D-glucan maltohydrolase)
	PVAPLPRKGS		31	2	1020.6080	1021.2454	0.64	**gil115439091**, Os01g0670800
	DPVAPLQRSGPE		28	2	1020.5716	1021.2454	0.63	**gil231540**, β-amylase (1,4-α-D-glucan maltohydrolase)
	SFTAGARTFNFDENPCDYFQGGKIKAT		23	3	2984.3763	2984.4082	0.03	**gil14275754**, P-type ATPase

[a] For microbial composition of pool 1, see Materials and Methods. [b] Single-letter amino acid code is used. [c] IC_{50} is the concentration of ACE-inhibitor needed to inhibit 50% of ACE-activity.

just for one or two C-terminal amino acids (DPVAPLQRSGPE, DPVAPLQRSGPEI, and DPAVAPLQRSGPEIP) and 3 other peptides such as PVAPLPRKGS, PRSGNVGESGLID, and SFTAGARTFNFDENPCDYFQGGKIKAT. All the peptides contained in fractions 16, 17, and 22 showed a prevalence of hydrophobic and neutral residues (> of the 67% of the total amino acids). Besides, fractions 16, 17, and 22 showed a marked overlapping for some of the identified sequences. The peptide DPVAPLQRSPGEI was contained in both fractions 16 and 22. The above sequence differed in fraction 17 for the N- and C-terminal residues only. The peptide PRSGNVGESGL found in fraction 17 was also identified in fraction 22 as a part of the

longer peptide PRSGNVGESGLID. The encrypted sequence VAP was included in 2, 2, and 4, peptides of those identified in fractions 16, 17, and 22, respectively.

The apparent IC_{50} of the crude peptide fractions are shown in **Table 2**. The values of the apparent IC_{50} for crude peptide fractions may be overestimated due to the presence of free amino acids in the preparation, which interfered with the calculation of the peptide concentration, and more generally, the over estimation is due to the possible breakdown of the large peptides as the result of ACE activity (*27*). All fractions showed IC_{50} values lower than 0.6 mg/mL. In particular, the crude peptide fraction 16 had an IC_{50} of 0.19 mg/mL.

Hydrolysis of Crude Fractions by Trypsin and Chymotrypsin. Peptide fractions were treated with trypsin and chymotrypsin. Under assay conditions, which caused ca. 80% hydrolysis of insulin chain A, all peptide preparations were resistant to hydrolysis (data not shown).

DISCUSSION

ACE (dipeptidyl carboxypeptidase, EC3.4.15.1) plays an important role in regulating blood pressure. In the rennin−angiotensin system, ACE cleaves the dipeptide at the C-terminal portion of angiotensin I and produces the potent vasopressor angiotensin II. This induces the release of aldosterone that causes the retention of sodium ions by the kidney and elevates blood volume, thus increasing blood pressure. ACE also inactivates the vasodilatator bradykinin (*2*). Consequently, ACE inhibitors contained in functional foods may deserve an interest, especially as coadjutants in medical therapy.

Currently, milk proteins, through enzyme hydrolysis and/or milk fermentation by selected lactic acid bacteria, are the main sources for ACE-inhibitory peptides in functional foods (*2*). A number of ACE-inhibitory peptides were effective in lowering the blood pressure of moderately hypertensive humans or SHR (*8, 28*). Very often, this inhibition was related to common amino acid sequences, which seemed to be the prerequisite for biological function, apart from the protein source and enzyme processing (*2*). Recently, some reports have shown that ACE-inhibitory peptides are also released from cereal proteins (*3–6*). None of these studies have considered the synthesis of ACE-inhibitory peptides through sourdough fermentation.

This study used white wheat, wholemeal wheat, or rye flours for producing 7 different sourdoughs. White wheat and rye flours are the most largely used flours for making baked goods. Wholemeal wheat flour generally harbors higher concentrations of bioactive substances/precursors and functional enzymes with respect to white wheat flour (*1*). Fermentation by selected lactic acid bacteria was allowed for 24 h at 37 °C which is compatible with the most common sourdough fermentations (*29*). The highest ACE-inhibitory activity was found by fermenting wholemeal wheat flour with pool 1 under semiliquid conditions. Pool 1 contained 10 strains of lactobacilli, which have been selected previously for proteinase and peptidase activities toward wheat proteins (*29*). Notwithstanding the activation of wheat and rye flour endogenous enzymes due to lactic acidification, the ACE-inhibitory activity of the chemically acidified doughs was very low. Since fungal proteases were successfully used together with pool 1 for degrading epitopes responsible for celiac disease (*29*), the same formula was used for generating ACE-inhibitory peptides. Nevertheless, fungal proteases caused highly extensive proteolysis and decreased ACE-inhibitory activity. An intense proteolysis of the protein matrix also caused the breakdown of biological peptides in milk products (*30*). As expected, the supplementation of pool 1 with GABA-producing strains did not modify ACE-inhibitory activity. When dough yield decreased from 330 to 160, the ACE-inhibitory activity also decreased. As shown for the hydrolysis of gliadin epitopes (*31*), semiliquid sourdough fermentation seemed to be indispensable to favor interactions between enzymes and substrates and to fully exploit the potential of the proteolytic enzymes of pool 1. Fourteen peptides were identified from the wholemeal wheat sourdough. These whole peptide sequences were not reported previously as ACE-inhibitory peptides (*2, 28*). As usual for ACE-inhibitory peptides (*2, 28*), the molecular masses were low (<1,600 Da), except for the peptide SFTAGARTFNFDEN-PCDYFQGGKIKAT. The major part of the identified peptides contained common motifs: PVAPLQRSGPE (β-amylase [1,4-α-D-glucan maltohydrolase] as protein source), PRSGNVGES-GL (α-amylase/trypsin inhibitor CM3 precursor as protein source), and VAP (contained in 8 of 14 peptides). Previously, the epitope VAP was found either as anthipertensive peptide (corresponding to one of the $á_{s1}$-casokinins) or as the encrypted sequence of well-known ACE-inhibitory peptides from milk proteins (*2*). Several reports showed that ACE-inhibitory peptides containing the motif VAP were markedly anthipertensive toward humans and SRH (*2, 28*). The three crude fractions isolated from wholemeal wheat sourdough had apparent values of IC_{50} in the range 0.19−2.34 mg/mL, which is comparable with the most potent ACE-inhibitory hydrolyzates of casein (*27*), soy (*32*), and rice proteins (*3*).

Apart from the type of fermentation, the use of wholemeal wheat flour also allowed the highest concentration of GABA. This is probably because the endogenous concentration of GABA and especially because the levels of glutamic acid and glutamate decarboxylase (GAD) activity are higher in wheat bran and germ, and in whole grain than in white wheat flour (*33*). Under semiliquid conditions, fermentation of wholemeal wheat by pool 1 gave a concentration of GABA markedly lower than that found with *Lb. plantarum* C48 or *Lc. lactis* subsp. *lactis* PU1. The capacity for producing GABA seemed to be strain specific. A core fragment of the *gad* gene was identified from both the above strains that had the highest activity among 440 isolates from the cheese matrix (*9*). Overall, GABA confers resistance to bacterial cells under acidic conditions, and the GAD decarboxylation process has also been coupled with the synthesis of energy in *Lactobacillus* sp. strain E1 (*9*). The concentration of GABA further increased (258.71 mg/kg) in wholemeal wheat sourdough started with pool 1 and *Lc. lactis* subsp. *lactis* PU1 when the dough yield decreased to 160. Contrary to proteolysis for generating ACE-inhibitory peptides, the increased amount of flour seemed to be related to the increased concentration of the precursor glutamic acid for GAD endogenous and/or microbial activity. The daily intake of fermented milk (10 mg of GABA) for 12 weeks decreased blood pressure by 17.4 Hg in hypertensive human patients (*7*). Foods enriched with GABA are defined as FOSHU (Foods for Specified Health Use) by the Japanese government (*34*). Besides treatment for sleeplessness, depression, and autonomic disorders, chronic alcohol-related symptoms, stimulation of immune cells, and prevention of diabetic conditions have been related to the administration of GABA (*9*).

Overall, epidemiological findings have indicated a protective role of whole grain foods against several Western diseases (*35, 36*). Wholemeal flour is rich in fiber, minerals, vitamins, and many phytochemicals (e.g., bioactive compounds) such as phenolic compounds, sterols, tocopherols and tocotrienols, lignans, and phytic acid (*1, 11*). Besides the positive effect on texture and flavor, sourdough fermentation may increase the functional value

of whole grains. This study shows that selected lactic acid bacteria used during sourdough fermentation of especially wholemeal flour may synthesize ACE-inhibitory peptides and GABA with a potential antihypertensive effect.

ABBREVIATIONS USED

DF, dietary fibers; ACE, angiotensin I-converting enzyme; SHR, spontaneously hypertensive rats; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; PLP, pyridoxal 5′-phosphate; HUT, hemoglobin unit on the tyrosine basis; SAPU, spectrophotometric acid protease unit; OPA, *o*-phtaldialdehyde; RP-FPLC, reversed-phase fast performance liquid chromatography; HHL, hippuryl-L-histidyl-L-leucine; HA, hippuric acid; FAAs, free amino acids; TFA, trifluoroacetic acid; nanoLC-ESI-MS/MS, nano-liquid chromatography coupled to electrospray ionization tandem mass spectrometry; NCBI, National Center for Biotechnology Information; FOSHU, foods for specified health use.

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