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Selected Lactic Acid Bacteria Synthesize Antioxidant Peptides during Sourdough Fermentation of Cereal Flours

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A pool of selected lactic acid bacteria was used for the sourdough fermentation of various cereal flours with the aim of synthesizing antioxidant peptides. The radical-scavenging activity of water/salt-soluble extracts (WSE) from sourdoughs was significantly ($P < 0.05$) higher than that of chemically acidified doughs. The highest activity was found for whole wheat, spelt, rye, and kamut sourdoughs. Almost the same results were found for the inhibition of linoleic acid autoxidation. WSE were subjected to reverse-phase fast protein liquid chromatography. Thirty-seven fractions were collected and assayed *in vitro*. The most active fractions were resistant to further hydrolysis by digestive enzymes. Twenty-five peptides of 8 to 57 amino acid residues were identified by nano-liquid chromatography-electrospray ionization-tandem mass spectrometry. Almost all of the sequences shared compositional features which are typical of antioxidant peptides. All of the purified fractions showed *ex vivo* antioxidant activity on mouse fibroblasts artificially subjected to oxidative stress. This study demonstrates the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through the proteolysis of native cereal proteins.

Interest in health-promoting functional foods, dietary supplements, and pharmaceutical preparations containing peptides deriving from food proteins is markedly increasing (24). Bioactive peptides are defined as specific protein fragments that have positive effects on body functions or conditions and that may influence human health (23). Usually, bioactive peptides correspond to cryptic sequences from native proteins, which are released mainly through hydrolysis by digestive, microbial, and plant proteolytic enzymes, and their levels generally increase during food fermentation (24). *In vitro* and *in vivo* studies show a large spectrum of biological functions attributed to bioactive peptides, such as opioid-like (19), mineral binding (9), immunomodulatory (15), antimicrobial (25), antioxidative (27), antithrombotic (46), hypocholesterolemic (53), and antihypertensive (21) activities. The release of various bioactive peptides (e.g., angiotensin I-converting enzyme [ACE]-inhibitory peptides) from milk proteins through proteolysis by lactic acid bacteria is the best documented (24). Recently, interest in antioxidant peptides derived from food proteins has increased, and evidence that bioactive peptides prevent oxidative stresses associated with numerous degenerative aging diseases (e.g., cancer and arteriosclerosis) is accumulating (2).

Overall, antioxidants have many applications in food industries. The delay of food discoloration and deterioration, which occur as a consequence of oxidative processes, markedly improves food preservation. The radical-mediated oxidation of fats and oils is one of the major causes of spoilage for lipid-containing foods during processing and storage (36). Antioxidants are extensively tested for the absence of carcinogenicity and other toxic effects in themselves, in their oxidized forms, and in the products of their reactions with food constituents; for their effectiveness at low concentrations; and for the absence of the ability to impart an unpleasant flavor to the food in which they are used (28). The use of antioxidants in food products is governed by regulatory laws of individual countries or by internal standards (28). Even though many synthetic compounds have antioxidant properties, only a few of them have been accepted as GRAS (generally recognized as safe) for use in food products by international bodies such as the Joint FAO/WHO Expert Committee on Food Additives and the European Community's Scientific Committee for Food. Toxicological studies are crucial in determining the safety of an antioxidant and also in determining the acceptable daily intake (ADI) levels (28). ADIs for widely used antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and gallates, have changed over the years mainly because of their toxicological effects in various species (20, 28, 33, 50), and new toxicological data for some of the synthetic antioxidants cautioned against their use (28).

Natural antioxidants recently attracted the attention of many food manufacturers as a result of the desire to produce healthy foods (28). Biologically active peptides with potential antioxidant activity have been derived from many animal and plant protein sources (35, 43, 49). They were already isolated from peanut kernels, rice bran, sunflower protein, alfalfa leaf protein, corn gluten meal, frog skin, yam, egg yolk protein, milk kefir, soymilk kefir, medicinal mushrooms, mackerel, curry leaves, cotton leafworm, casein, algal protein waste, wheat gluten, and buckwheat protein (43). It was argued that antioxidant peptides act as inhibitors of lipid peroxidation, as direct scavengers of free radicals, and/or as agents to chelate transition metal ions that catalyze the generation of radical species (43). Antioxidant peptides usually are constituted by 2 to 20 amino acidic residues and have molecular masses of less than 6.0 kDa (26, 48). The antioxidant activity seems to be strongly correlated with amino acid composition, conformation, and hydrophobicity (5).

Cereals are staple foods in the human diet. They are considered one of the most important sources of dietary carbohydrates, proteins, vitamins, minerals, and fibers for people all over the world.

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TABLE 1 Characteristics of the flours used for sourdough fermentation

Flour[b]	Result by parameter[a]					
	Moisture (%)	Carbohydrate (% of d.m.)	Protein (% of d.m.)	Lipid (% of d.m.)	Fiber (% of d.m.)	Ash (% of d.m.)
Whole wheat	12.3 ± 0.3	67.1 ± 1.1	11.0 ± 0.7	1.7 ± 0.1	9.6 ± 0.1	1.2 ± 0.1
Durum wheat	12.5 ± 0.5	63.2 ± 1.0	12.9 ± 0.8	2.8 ± 0.2	8.8 ± 0.5	1.5 ± 0.1
Rye	9.8 ± 0.3	77.5 ± 1.5	9.4 ± 0.6	1.7 ± 0.1	14.6 ± 0.6	1.5 ± 0.1
Spelt	10.4 ± 0.1	67.1 ± 1.2	15.1 ± 0.3	2.5 ± 0.1	6.8 ± 0.3	2.5 ± 0.2
Oat	12.0 ± 0.3	62 ± 0.8	12.6 ± 0.8	12.3 ± 0.6	11.4 ± 0.6	1.9 ± 0.1
Rice	11.9 ± 0.3	80.1 ± 1.2	5.9 ± 0.9	1.4 ± 0.1	2.4 ± 0.2	0.6 ± 0.2
Kamut	11.7 ± 0.2	69.2 ± 0.5	13.5 ± 0.3	2.1 ± 0.2	8.3 ± 0.1	0.9 ± 0.3
Barley	12.1 ± 0.1	70.5 ± 0.4	10.5 ± 0.1	1.6 ± 0.2	9.1 ± 0.2	0.9 ± 0.1
Maize	12.5 ± 0.4	76.8 ± 0.8	8.7 ± 0.1	2.7 ± 0.2	2.1 ± 0.3	0.6 ± 0.3

[a] d.m., dry matter. Mean values ± standard deviations are reported.
[b] For each flour, three samples were analyzed twice.

Large proportions of cereals are processed into foods and beverages by fermentation (e.g., sourdough) prior to consumption. Although biological activities such as ACE inhibition and antimicrobial and anticancer activities were reported for cereal peptides (7, 8, 38, 40, 41), to the best of our knowledge only a few studies (43) have investigated the potential release of antioxidant peptides during cereal fermentation.

This study aimed at investigating the *in vitro* and *ex vivo* antioxidant potential of different cereal flours, which were subjected to sourdough fermentation by selected lactic acid bacteria. Bioactive peptides were purified and identified to determine structural relationships with antioxidant properties.

MATERIALS AND METHODS

Microorganisms. *Lactobacillus alimentarius* 15 M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A, and *Lactobacillus hilgardii* 51B were previously selected based on their capacity to hydrolyze gliadins (14). *L. sanfranciscensis* LS3, LS10, LS19, LS23, LS38, and LS47 were selected based on their peptidase systems, with particular reference to activities toward Pro-rich peptides (12). The strains were used in mixture for sourdough fermentation. Lactobacilli were propagated for 24 h at 30°C in MRS broth (Oxoid, Basingstoke, Hampshire, England), with the addition of fresh yeast extract (5%, vol/vol) and 28 mM maltose, at a final pH of 5.6 (modified MRS [mMRS]). When used for sourdough fermentations, lactic acid bacterial 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 tap water (total bacteria count, <100 CFU/ml), which was used for making the dough. The enumeration of lactic acid bacteria was carried out by plating serial dilutions of sourdoughs on mMRS agar medium (Oxoid) at 30°C for 48 h.

Sourdough fermentation. As determined by AACC official methods (1), the characteristics of the flours used in this study are reported in Table 1. Each flour was used to prepare a sourdough containing 120 g flour and 280 g of tap water, with a dough yield (DY; dough weight × 100/flour weight) of 330 (DY 330). Fermentation with the pool of selected lactic acid bacteria (initial cell density of 5×10^7 CFU/g of dough) was carried out at 37°C for 24 h under stirring conditions (ca. 200 rpm). Control doughs (DY 330) without bacterial inoculum were chemically acidified to pH 3.5 by a mixture of lactic and acetic acids (molar ratio, 4:1) and incubated under the same conditions.

WSE. Water/salt-soluble extracts (WSE) were prepared from each dough according to the method originally described by Osborne (32) and further modified by Weiss et al. (51). 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, vortexed 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 *in vitro* assays of the antioxidant activity. The concentrations of peptides in the WSE and purified fractions were determined by the *o*-phtaldialdehyde (OPA) method (6). 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.

DPPH radical-scavenging activity. The scavenging effect of WSE and purified fractions on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was measured according to the method of Shimada et al. (45), with some modifications (52). Freeze-dried samples were dissolved in 0.1 M phosphate buffer (pH 7.0) at a peptide concentration of 1 mg/ml, and 2 ml of each solution was added to 2 ml of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was read at 517 nm. The absorbance measured after 10 min was used for the calculation of the DPPH radicals scavenged by WSE or purified peptide fractions (39). A lower absorbance represents a higher DPPH radical-scavenging activity. The scavenging effect was expressed as shown in the following equation: DPPH radical-scavenging activity (%) = [(blank absorbance − sample absorbance)/blank absorbance] × 100. Butylated hydroxytoluene (BHT) and α-tocopherol (1 mg/ml) also were assayed as antioxidant references.

Inhibition of linoleic acid autoxidation. The antioxidant activity of WSE and purified fractions also was measured according to the method of Osawa and Namiki (31), with some modifications. After freeze-drying, 1.0 mg of each sample was suspended in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) and added to 1 ml of linoleic acid (50 mM), which was previously dissolved in ethanol (99.5%). Incubation in a glass test tube, tightly sealed with a silicon rubber cap, was allowed at 60°C in the dark for 8 days. The degree of oxidation was determined by measuring the values of ferric thiocyanate according to the method described by Mitsuta et al. (30). One hundred microliters of the sample was mixed with 4.7 ml of 75% (vol/vol) ethanol, 0.1 ml of 30% (wt/vol) ammonium thiocyanate, and 0.1 ml of 0.02 M ferrous chloride, which had been dissolved in 1 M HCl. After 3 min, the degree of color development, which represents the oxidation of linoleic acid, was measured spectrophotometrically at 500 nm. BHT and α-tocopherol (1 mg/ml) also were assayed as antioxidant references. A negative control (without antioxidants) also was considered.

Purification of antioxidant peptides. WSE were fractionated by ultrafiltration (Ultrafree-MC centrifugal filter units; Millipore) by using three different membrane sizes with 50-, 30-, and 10-kDa cutoffs (fractions A, B, and C, respectively). Aliquots of 400 μl of WSE were centrifuged at 10,000 × *g* for 60 min. After ultrafiltration, fractions were used for the DPPH radical-scavenging activity assay. The 10-kDa partially purified fractions (C) were further fractionated (37 fractions) by reverse-phase fast performance liquid chromatography (RP-FPLC) using a Resource RPC column and ÄKTA FPLC equipment with the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Aliquots containing 1 mg/ml of peptides were added to 0.05% (vol/vol)

trifluoroacetic acid (TFA) and centrifuged at 10,000 × *g* for 10 min. The supernatant was filtered with a 0.22-μm-pore-size filter 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 acetonitrile (CH_3CN) containing 0.05% TFA. The concentration of CH_3CN was increased linearly from 5 to 46% between 16 and 62 min and from 46 to 100% between 62 and 72 min. Solvents were removed from collected fractions by freeze-drying. The fractions were redissolved in sterile water and subjected to *in vitro* assays for antioxidant activity.

Proteolysis and heat stability of purified fractions. The purified fractions from WSE, which showed the highest antioxidant activities, were subjected to sequential protein hydrolysis by digestive enzymes according to the method described by Pasini et al. (34). Briefly, freeze-dried aliquots corresponding to 10 mg of peptides were suspended in 400 μl of 0.2 N HCl (pH 2.0) containing 0.05 mg/ml of pepsin (EC 3.4.23.1) (Sigma-Aldrich CO., St. Louis, MO) and homogenized in a Sterilmixer Lab (PBI International). After 30 min of incubation at 37°C under stirring conditions (150 rpm), 115 μl of a solution of 1 M boric acid and 0.5 N NaOH, adjusted to pH 6.8 with 5 N HCl and containing 0.25 mg/ml of pancreatin (Sigma) and 0.0087 mg/ml of trypsin (EC 3.4.21.4) (Sigma), was added. The resulting pH was 7.6. Pancreatic digestion lasted 150 min. Digested samples were heated for 5 min at 100°C and centrifuged at 12,000 × *g* for 20 min to recover the supernatants. After treatments, samples were subjected to *in vitro* assays for antioxidant activity.

Identification of antioxidant peptides. The fractions of WSE with the highest radical-scavenging activities were subjected to a second step of purification through RP-HPLC under the conditions described previously and using an ÄKTA purifier apparatus (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The centers of the peaks were collected, freeze-dried, and used for mass spectrometry (MS) analysis.

The identification of peptides was carried out by nano-liquid chromatography-electrospray ionization-tandem MS (nano-LC-ESI-MS/MS) using a Finningan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron) through the nano-ESI interface. According to the manufacturer's instrument settings for nano-LC-ESI-MS/MS analyses, MS/MS spectra were automatically taken by Xcalibur software (ThermoElectron) in the positive ion mode. Spectra were processed using the software BioWorks 3.2 (ThermoElectron), generating peak lists suitable for database searches. Peptides were identified using an MS/MS ion search with the Mascot search engine (Matrix Science, London, England) and of the NCBInr protein database (National Center for Biotechnology Information, Bethesda, MD). For the identification of peptides, the following parameters were considered: enzyme, none; instrument type, ESI-trap; peptide mass tolerance, ± 0.1%; and fragment mass tolerance, ± 0.5 Da. Results from peptide identification were subjected to a manual evaluation as described by Chen et al. (5), and the validated peptide sequences explained all of the major peaks in the MS/MS spectra.

Effect of purified peptides on viability of oxidation-induced cells. Mouse fibroblasts (Balb 3T3; clone A31; ATCC CCL-163TM) were cultured under a humidified atmosphere (5% CO_2, 37°C) using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (wt/vol) fetal bovine serum (FBS), 1 mM glutamine, and 100 μg/ml penicillin-streptomycin. The culture medium was renewed every 2 days, and after four passages the cultures were used to determine cell viability. Cell viability was measured using the MTT [3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide] method (18), and the capacity of succinate dehydrogenase to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into visible formazan crystals was assessed. For the MTT assay, cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated for 16 h. Cells subsequently were treated with the antioxidant-purified fractions and incubated for a further 16 h. The final concentrations of peptides in the reaction mixture were 1.20, 0.81, 2.33, 0.41, 1.79, 2.88, 1.69, and 1.67 mg/ml for WSE from fraction 3 of whole wheat, fractions 2 and 3 of spelt, fractions 5 and 36 of rye, and fractions 2, 3, and 37 of kamut, respectively. A negative control, without the addition of peptide fractions, was used. α-Tocopherol (250 and 500 μM) was used as the positive control. Following the removal of FBS, cells were exposed to 150 μM hydroxide peroxide for 2 h. For each well, 250 μl of MTT (0.5 mg/ml final concentration) dissolved in DMEM was added, and incubation (37°C) in the dark was allowed for 1 h. Finally, dimethylsulfoxide (DMSO) (250 μl) was added to solubilize the formazan which was formed. The level of formazan was determined by measuring the optical density at 570 nm with a Biotek EL808 microplate reader (Winooski, VT). The relative cell viability was determined as the level of MTT converted into formazan salt. Data were expressed as the mean percentage of viable cells compared to that of the control culture before oxidative stress.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA); the pair comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$ using Statistica for Windows.

RESULTS

Sourdough fermentation. After 24 h of fermentation at 37°C, lactic acid bacteria reached cell densities ranging from 1.2 ± 0.4 to $6.2 \pm 0.5 \times 10^9$ CFU/g. The lowest values were found during the sourdough fermentation of oat, rice, and maize flours. No significant ($P > 0.05$) differences were found among cell densities of sourdoughs made with whole wheat, durum wheat, rye, spelt, kamut, and barley flours. Before fermentation, the pHs were 5.20 ± 0.05, 5.27 ± 0.06, 5.82 ± 0.04, 5.45 ± 0.08, 4.56 ± 0.02, 5.15 ± 0.02, 5.37 ± 0.03, 5.23 ± 0.03, and 4.45 ± 0.02 for whole wheat, durum wheat, rye, spelt, oat, rice, kamut, barley, and maize doughs, respectively. After fermentation, the pHs ranged from 3.40 ± 0.03 to 3.88 ± 0.05. The lowest pHs were found for rice (3.26 ± 0.02) and whole wheat (3.40 ± 0.03) sourdoughs. The highest value was found for oat sourdough (3.88 ± 0.05).

***In vitro* antioxidant activity of WSE.** WSE from sourdoughs and control doughs had pHs ranging from 6.5 ± 0.2 to 7.0 ± 0.2 as a consequence of extraction with the Tris-HCl buffer. As determined by the OPA method, the concentrations of peptides of WSE were 0.68 ± 0.04, 0.41 ± 0.02, 1.12 ± 0.05, 0.68 ± 0.05, 0.31 ± 0.01, 0.18 ± 0.02, 0.61 ± 0.03, 0.71 ± 0.03, and 0.91 ± 0.04 mg/ml for whole wheat, durum wheat, rye, spelt, oat, rice, kamut, barley, and maize sourdoughs, respectively. The concentrations of peptides of WSE from the chemically acidified doughs was ca. 2 to 3 times lower than those of the respective sourdoughs.

During the radical-scavenging assay, the colored stable DPPH radical is reduced to DPPH-H (nonradical form) in the presence of an antioxidant or a hydrogen donor. The DPPH radical without antioxidants was stable over time. Under the assay conditions, 100% of activity corresponds to the complete scavenging of DPPH radicals (50 μM final concentration) after 10 min of incubation with the antioxidant compounds. According to previous studies (38, 50), the color intensity of the DPPH radical showed a logarithmic decline in the presence of BHT. The radical-scavenging activity toward the stable radical DPPH was in the range of 3.0% ± 0.2% to 5.5% ± 0.2% for WSE chemically acidified doughs (Fig. 1). No significant ($P > 0.05$) differences were found between samples. BHT (1 mg/ml) showed an activity of ca. 65% (10 min). Almost all WSE from sourdoughs showed a marked increase of the radical-scavenging activity compared to that of the chemically acidified doughs. Durum wheat, maize, and barley sourdoughs did not show appreciable increases. The highest scavenging activity was found for whole wheat (48.0% ± 0.4%), kamut (39.0% ± 0.2%), spelt (37.3% ± 0.4%) and rye (35.4% ± 0.5%) sourdoughs. Based on these results, WSE from chemically acidified doughs were not further characterized.

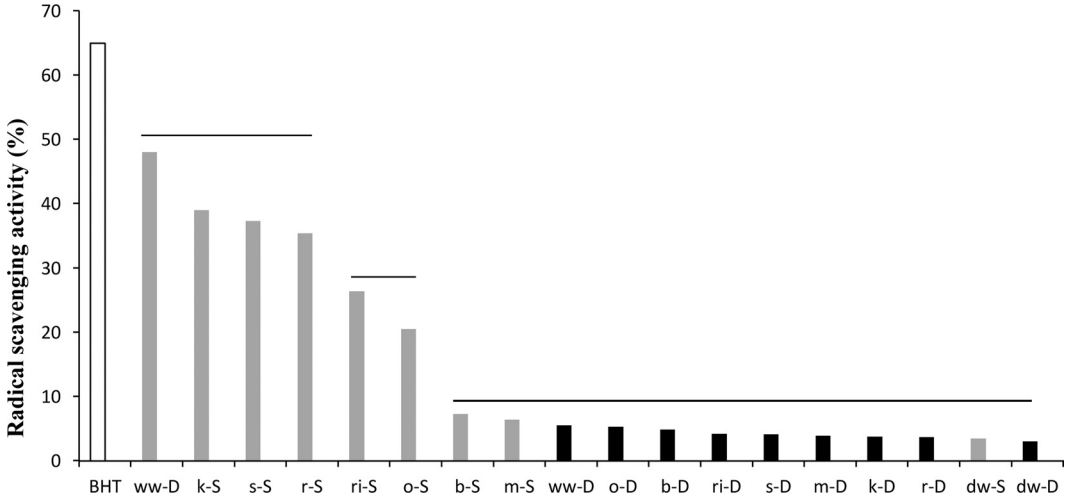

FIG 1 DPPH radical-scavenging activity of the WSE from chemically acidified doughs (black bars; -D) and sourdoughs (grey bars; -S) fermented with the pool of selected lactic acid bacteria. BHT (white bar) (1 mg/ml) was used as the positive control. ww, whole wheat; dw, durum wheat; r, rye; s, spelt; o, oat; ri, rice; k, kamut; b, barley; m, maize. Data are the means from three independent experiments. Means connected by the same horizontal line are similar by the Tukey's comparison test ($P > 0.05$).

Lipid peroxidation is thought to proceed via the radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (35). The absorbance of WSE at 500 nm from sourdoughs was higher than that of the positive controls, thus showing a lower inhibition of linoleic acid autoxidation (Fig. 2). In agreement with the previous findings for radical-scavenging activities, the oxidation of linoleic acid was markedly inhibited by the addition of WSE from whole wheat, spelt, rye, and kamut sourdoughs. WSE from durum wheat, rice, oat, barley, and maize sourdoughs showed weak activities.

Aiming at the subsequent identification of antioxidant peptides, WSE of whole wheat, spelt, rye, and kamut sourdoughs were subjected to ultrafiltration (cutoffs of 10, 30, and 50 kDa) and further assayed for radical-scavenging activity. All fractions from ultrafiltration showed activity toward the DPPH radical, suggesting that the molecular mass of antioxidant compounds was less than 10 kDa (data not shown).

Purification and characterization of antioxidant peptide fractions. Thirty-seven fractions were collected by the RP-FPLC separation of each WSE (aliquots contained 10 mg of peptides, as determined by the OPA method). The peptide profiles of the different WSE were similar (Fig. 3).

Collected fractions were freeze-dried, dissolved in ca. 600 μl of distilled water, and assayed for DPPH radical-scavenging activity and the inhibition of linoleic acid autoxidation. Based on the DPPH radical-scavenging assay, eight fractions with the highest

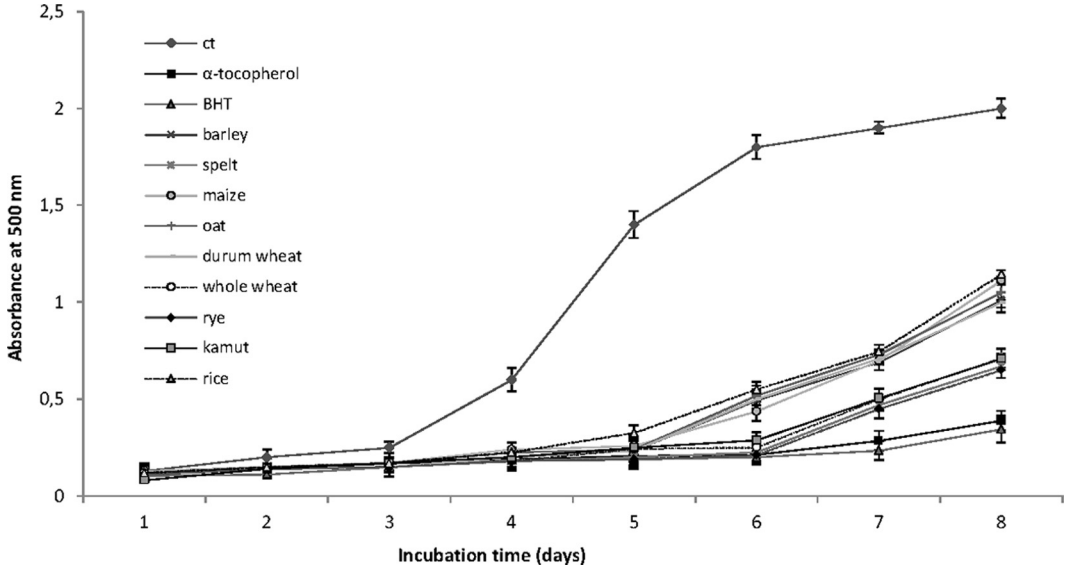

FIG 2 Lipid peroxidation inhibitory activity of the WSE from sourdoughs fermented with the pool of selected lactic acid bacteria. The activity was measured under a linoleic acid oxidation system for 8 days. BHT and α-tocopherol (1 mg/ml) were used as the positive controls. A negative control, without antioxidants, also was considered (ct). Data are the means from three independent experiments. Bars represent standard deviations.

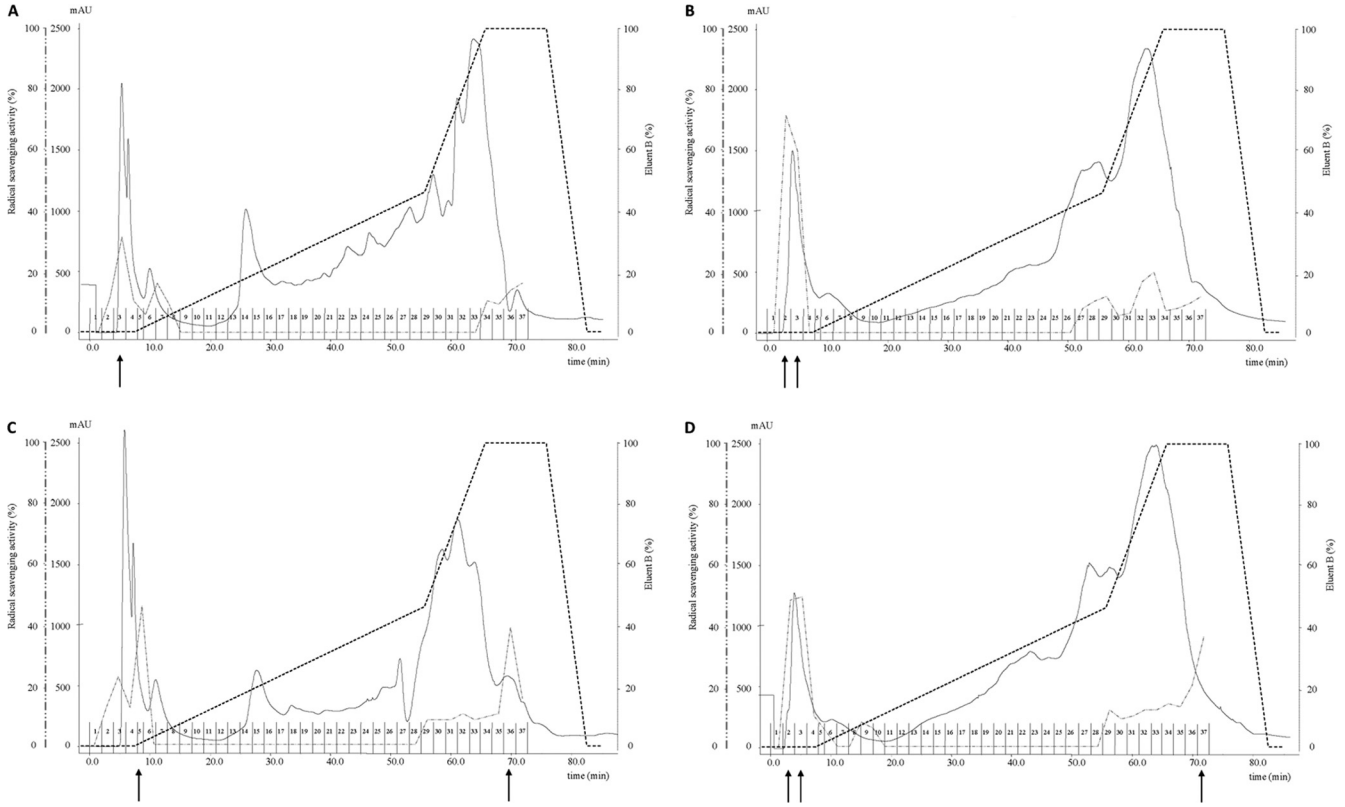

FIG 3 RP-FPLC chromatograms of WSE of whole wheat (A), spelt (B), rye (C), and kamut (D) sourdoughs fermented with the pool of selected lactic acid bacteria. The dashed lines refer to the percentages of radical scavenging activity (line of dashes and dots) and to the gradient of eluent B (line of dashes only). Arrows indicate peptide fractions with the highest antioxidant activity. ct, control.

levels of activity were selected (Fig. 3). They were fraction 3 (activity of ca. 27%, peptide concentration of 1.20 ± 0.04 mg/ml) from whole wheat; fractions 2 (ca. 90%, 0.81 ± 0.03 mg/ml) and 3 (ca. 75%, 2.33 ± 0.04 mg/ml) from spelt; fractions 5 (ca. 45%, 0.41 ± 0.02 mg/ml) and 36 (ca. 38%, 1.79 ± 0.05 mg/ml) from rye; and fractions 2 (ca. 48%, 2.88 ± 0.03 mg/ml), 3 (ca. 49%, 1.69 ± 0.05 mg/ml), and 37 (ca. 36%, 1.67 ± 0.04 mg/ml) from kamut. All of the fractions showed inhibitions of linoleic peroxidation similar to those of α-tocopherol (80.6%) and BHT (82.3%) (Fig. 4). In particular, fraction 3 from spelt and fraction 36 from rye showed the highest activity (83.9%). No statistical correlation was found between the concentration of peptides and the antioxidant activities.

The purified fractions were subjected to sequential hydrolysis by pepsin, trypsin, and pancreatin, which mimicked the digestive process. As determined by the free radical-scavenging assay, fraction 37 from kamut and fractions 5 and 36 from rye showed a less than 10% decrease in antioxidant activity compared to that of undigested fractions (35, 42, and 35% of activity, respectively). No significant ($P > 0.05$) decrease was found for the other fractions (the antioxidant activity was 27% for fraction 3 from whole wheat, 89 and 76% for fractions 2 and 3 from spelt, respectively, and 49 and 49% for fractions 2 and 3 from kamut, respectively). The antioxidant activity of the digested peptide fractions was not affected by heating for 5 min at 100°C.

Isolation and identification of antioxidant peptides. Twenty-five peptides of 8 to 57 amino acid residues and molecular masses varying from 769.8 to 5,338.5 Da were identified by nano-LC-ESI-MS/MS analysis (Table 2). All of the peptides were found in the NCBInr database as being encrypted in different cereal proteins (accession numbers are reported in Table 2).

Two peptides of 14 and 8 amino acid residues, with hydrophobic ratios of 64 and 50% (sequences n.1 and n.2, respectively), respectively, and a total net charge of 0 were identified from fraction 3 of whole wheat sourdough. Four and three peptides having 8 to 21 amino acid residues were identified from fractions 2 and 3 of spelt sourdough, respectively. Except for the sequences n.4 and n.9 (Table 2), the hydrophobic ratio was higher than 50%. Except for the sequence n.6, all peptides have net positive or neutral total charges. Four and five different peptides were identified from fractions 5 and 36 of rye sourdough, respectively. The major part of the sequences contained 26 to 53 amino acid residues. The sequences n.11, n.13, n.14, and n.15 were shorter (10 to 16 amino acid residues). Except for those of sequences n.12, n.17, and n.18, the hydrophobic ratio was greater than 48%. The total net charge of sequences n.18 and n.20 was negative, while it was positive or neutral for all of the other sequences. Two peptides from fractions 2 and 37 and three peptides from fraction 3 of kamut sourdough were identified. Sequences n.24 and n.25 of fraction 37 had the highest number of amino acid residues (57 and 52, respectively). The other sequences were shorter (fewer than 21 amino acid residues). Except for the sequence n.22, their hydrophobic ratios were higher than 35%. All of the peptides identified from fractions

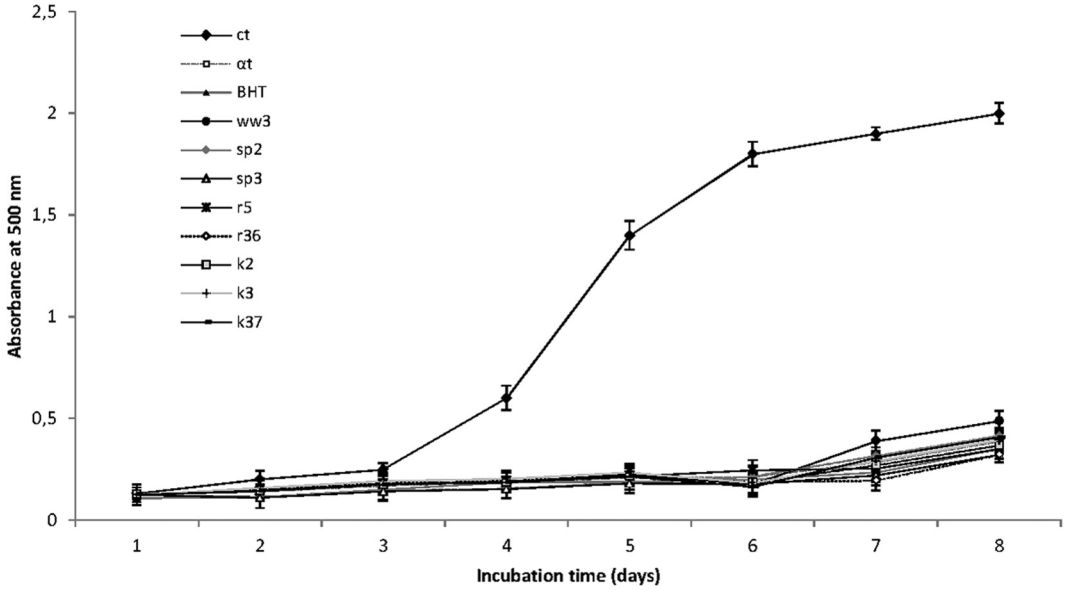

FIG 4 Lipid peroxidation inhibitory activity of the purified peptide fractions of the WSE from sourdoughs fermented with the pool of selected lactic acid bacteria. The activity was measured in a linoleic acid oxidation system in the dark for 8 days. BHT and α-tocopherol (1 mg/ml) were used as the positive controls. ct, negative control (without antioxidants); αt, α-tocopherol; ww3, fraction 3 of whole wheat; sp2 and sp3, fractions 2 and 3 of spelt, respectively; r5 and r36, fractions 5 and 36 of rye, respectively; and k2, k3, and k36, fractions 2, 3, and 37 of kamut, respectively. Data are the means from three independent experiments. Bars represent standard deviations.

2 and 3 had net positive or neutral charges, while sequences n.24 and n.25 from fraction 37 had net negative charges.

Effect of purified peptides on viability of oxidation-induced cells. To investigate the capacity of the purified peptides to act as radical scavengers, cultured mouse fibroblasts were grown in the presence of eight purified fractions from whole wheat, spelt, rye, and kamut sourdoughs. Cells then were treated with hydroxide peroxide. Under the experimental conditions, cell viability was evaluated by assaying the capacity of functional mitochondria to catalyze the reduction of MTT to formazan salt via mitochondrial dehydrogenases. α-Tocopherol and all purified fractions increased cell survival compared to that of the negative control (55.4% $\pm$ 0.2% of cell viability after oxidative stress) (Fig. 5). In particular, purified fraction 3 of spelt sourdough showed a significantly ($P < 0.05$) higher activity than that induced by 500 μM α-tocopherol (93.6% $\pm$ 0.2% versus 87.2% $\pm$ 0.1%). Three other purified fractions (2 of kamut and 5 and 36 of rye sourdoughs) induced cell viability higher than that induced by 250 μM α-tocopherol (77.6% $\pm$ 0.1%, 73.9% $\pm$ 0.4%, 78.6% $\pm$ 0.4%, and 70.8% $\pm$ 0.3%, respectively).

DISCUSSION

Sourdough fermentation has a well-known role in improving the nutritional properties of wheat, rye, and oat baked goods (22). It stabilizes or increases the levels of various bioactive compounds, retards starch bioavailability (thus decreasing the glycemic index), and increases mineral bioavailability (10, 13, 42). To our knowledge, this is the first study reporting the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through the proteolysis of native cereal proteins. In the areas of human nutrition and biochemistry, natural antioxidants from food sources are studied largely because of their potential health benefits with few or no side effects. Low molecular mass, low cost, high activity, and easy absorption are the main features of antioxidant peptides. Although synthetic antioxidants are more effective, natural antioxidants have a simpler structure, higher stability, and nonhazardous immune reactions (43). It is presumed that bioactive peptides deriving from cereal proteins are not immunogenic/allergenic toward healthy people but are immunogenic/allergenic for subjects suffering from specific allergies, intolerance, and/or sensitivity to them.

Whole wheat, durum wheat, rye, spelt, oat, rice, kamut, barley, and maize are the most common cereal flours used for making fermented baked goods. Sourdough fermentation by selected lactic acid bacteria was allowed for a long time and under semiliquid conditions, which are indispensable to fully exploit microbial proteolysis (16, 38). Under these processing parameters, a moderate but not too extensive degradation of the flour proteins occurred (38). The pool of lactic acid bacteria used for sourdough fermentation included 10 strains, which were previously selected based on proteinase and peptidase activities toward wheat proteins (37). Proteinase activity and, especially, a large portfolio of peptidases are the prerequisites to release bioactive peptides during sourdough fermentation (11, 17). The hydrolyzing activities responsible for the degradation of cereal proteins are not widespread in sourdough lactic acid bacteria, and in general it is very rare that a unique microbial strain possesses all the necessary enzymes (13). Previously, the same pool of lactic acid bacteria was successfully used to degrade epitopes responsible for celiac disease (37) and to synthesize ACE-inhibitory peptides during the sourdough fermentation of wheat and rye flours (38).

Notwithstanding an activation of cereal-endogenous enzymes due to acidification (16), the antioxidant activity of chemically acidified doughs (controls) was very low. WSE from several sourdoughs, which had almost the same level of acidity as that of the controls, showed an elevated *in vitro* radical-scavenging activity

TABLE 2 Sequences of peptides contained in the purified fractions of WSE of whole wheat, spelt, rye, and kamut sourdoughs fermented with the pool of selected lactic acid bacteria

Fraction (fraction no.) and peptide no.	Sequence[a]	Score	Charge	Mass			Source protein NCBI accession no. and description
				Calculated	Expected	Change	
Whole wheat (3)							
1	MAPAAVAAAEAGSK	17	2	1,243.6230	1,243.0196	−0.6034	GH32_ORYSJ; probable indole-3-acetic acid-amidosynthetase; P0C0M2
2	DNIPIVIR	14	2	938.5549	937.5898	−0.9651	AKH2_MAIZE; bifunctional aspartokinase/homoserine dehydrogenase 2; P49080
Spelt (2)							
3	AIAGAGVLSGYDQLQILFFGK	13	3	2,167.1677	2,166.0752	−1.0925	ADT1_MAIZE; ADP, ATP carrier protein 1, mitochondrial; P04709
4	GNQEKVLELVQR	18	2	1,411.7783	1,412.4122	0.6339	FH16_ORYSJ; formin-like protein 16
5	PAGSAAGAAP	14	2	769.8311	769.4197	−0.4114	C3H31_ORYSJ; zinc finger CCH domain-containing protein 31; Q7XPK1
6	EALEAMFL	12	3	924.1021	923.5322	−0.5699	IAA31_ORYSJ; auxin-responsive protein IAA31; P0C133
Spelt (3)							
7	AAGAAAAARSAGQCGR	16	3	1,387.6738	1,388.3942	0.7204	SAP17_ORYSJ; zinc finger AN1 domain-containing stress-associated protein 17; Q6H595
8	ITFAAYRR	12	2	998.1621	997.6323	−0.5298	NIP41_ORYSJ; aquaporin NIP4-1; Q9ASI1
9	HPVPPKKK	14	3	912.2177	912.5011	0.2834	CCF11_ORYSJ; putative cyclin-F1-1; Q6K1Z1
Rye (5)							
10	VFVDEGLEVLGWRPVPFNV SVVGRNAK	18	2	2,982.6080	2,983.0406	0.4326	GLTB_MAIZE; ferredoxin-dependent glutamate synthase, chloroplastic; P23225
11	RLSLPAGAPVTVAVSP	14	2	1,535.8101	1,534.9327	−0.8774	CSLD4_ORYSJ; cellulose synthase-like protein d4 *Oryza sativa* subsp. *japonica*; Q2QNS6
12	NANGELCPNNMCCSQWG YCGLGSEFCGNGCQSG ACCPEK	13	3	4,033.4843	4,032.5905	−0.8938	AGI_ORYSJ; lectin; Q0JF21
13	LCPVHRAADL	14	2	1,095.3231	1,094.6324	−0.6907	CSLD4_ORYSJ; cellulose synthase-like protein d4; Q2QNS6
Rye (36)							
14	PAEMVAAALDR	12	2	1,484.7511	1,483.8343	−0.9168	SLY1_OTYSJ; SEC1 family transport protein SLY1; Q851W1
15	KVALMSAGSMH	14	3	1,131.2679	1,131.3802	0.1123	MLOH1_HORVU; MLO protein homolog 1; O49873
16	DLADIPQQQRLMAGLALVV ATVIFLK	13	3	2,822.6092	2,822.9826	0.3734	CP51_SORBI; obtusifoliol 14-alpha demethylase; P93846
17	KNGSIFNSPSATAATIIHGHN YSGLAYLDFVTSK	13	2	3,580.7950	3,581.6329	0.8379	KSL6_ORYSJ; Ent-isokaur-15-ene synthase; A4KAG8
18	GTIFFSQEGDGPTSVTGSVSG LKPGLHGFHVHALGDT TNGCMSTGPHFNPTGK	12	2	5,338.5201	5,338.2306	−0.2895	SODC2_ORYSJ; superoxide dismutase (Cu-Zn); P28757
Kamut (2)							
19	YEWEPTVPNFDVAKDVTDM	13	2	2,255.0093	2,254.3380	−0.6713	KRP3_ORYSJ; cyclin-dependent kinase inhibitor 3; Q2R185
20	GVSNAAVVAGGH	12	3	1,037.5254	1,038.6974	1.1720	SUT5_ORYSI; sucrose transport protein SUT5; A2X6E6

Continued on following page

TABLE 2 (Continued)

Fraction (fraction no.) and peptide no.	Sequence[a]	Score	Charge	Mass			Source protein NCBI accession no. and description
				Calculated	Expected	Change	
Kamut (3)							
21	DAQEFKR	13	2	892.4403	891.7372	−0.7031	HFN40_MAIZE; suppressor protein HFN40; P82865
22	PPGPGPGPPPPPGAAGRG GGG	14	3	1,704.8721	1,703.9566	−0.9155	FH18_ORYSJ; formin-like protein 18; Q6MWG9
23	HKEMQAIFDVYIMFIN	14	1	2,000.3734	1,999.0342	−1.3392	ZRP4_MAIZE; *O*-methyltransferase ZRP4; P47917
Kamut (37)							
24	TGGGSTSSSSSSSSLGGGASR GSVVEAAPPATQGAAAAN APAVPVVVVDTQEAGIR	15	3	5,124.5196	5,124.3480	−0.1715	SLR1_ORYSJ; DELLA protein SLR1; Q7G7J6
25	DTAAGYVAPPDPAVSTGDY GLAGAEAPHPHESAVMS GAAAAAVAPGGEAYTR	15	3	4,921.2889	4,920.3312	−0.9577	DHR25_ORYSJ; dehydrin Rab25; P30287

[a] Single-letter amino acid codes are used.

and the inhibition of linoleic acid autoxidation. Sourdoughs from whole wheat, rye, spelt, and kamut had the highest activities, which, however, at the concentration assayed were lower than those of BHT and α-tocopherol.

Many cereals are closely related species, but many factors, such as the high level of polymorphism, the ratio between different protein fractions (solubility classes), the amino acid composition and sequence, and the molecular mass of the individual polypeptides, differentiate the technological, structural, nutritional, and functional properties of flours (44). Thus, differences of the functional properties of peptides deriving from different cereals were expected. Aiming at purifying antioxidant peptides, eight fractions (the concentration of peptides was lower than 3 mg/ml) from whole wheat, spelt, rye, and kamut sourdoughs were selected after RP-FPLC separation. Six fractions eluted in the early zone of the acetonitrile gradient. The other two were eluted at the end of the acetonitrile gradient. The *in vitro* antioxidant activity of some of these fractions (3 and 36 from spelt and rye, respectively) was higher than that of the synthetic antioxidants used as positive controls. As previously reported (29, 38), purified fractions can show higher bioactivity than the raw extract as the consequence of the higher concentration of the active compound compared to that of the other constituents of the matrix.

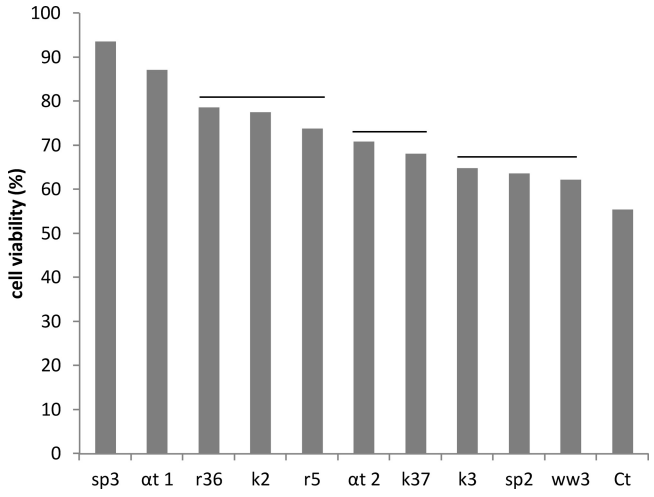

FIG 5 Effect of purified peptide fractions on cell viability of mouse fibroblasts. Mouse fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) without fetal bovine serum and were incubated with purified fractions for 16 h. Oxidative stress was artificially induced by incubating cultured cells with 150 μM hydroxide peroxide for 2 h. The percentage of viable cells with respect to untreated cultures was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ct, control without antioxidants; αt1 and αt2, α-tocopherol at 500 and 250 μg/liter, respectively; ww3, fraction 3 of whole wheat; sp2 and sp3, fractions 2 and 3 of spelt, respectively; r5 and r36, fractions 5 and 36 of rye, respectively; and k2, k3, and k36, fractions 2, 3, and 37 of kamut, respectively. Data are the means from three independent experiments analyzed twice. Means connected by the same horizontal line are similar by the Tukey's comparison test ($P > 0.05$).

To be active, antioxidant peptides should have the capacity to overcome hydrolysis and modifications at the intestinal level and to reach their targets (43). Overall, the antioxidant activity of the purified fractions seemed not to be affected by sequential *in vitro* treatments with digestive enzymes. A slight decrease of the antioxidant activity was found only for fraction 37 of kamut sourdough and fractions 5 and 36 of rye sourdough, which contained peptides having the largest size. After the *in vitro* assays, the *ex vivo* antioxidant activity of the purified fractions was determined for mouse fibroblasts, which were artificially subjected to oxidative stress. As shown by MTT assays, all of the purified fractions exhibited a marked protective effect. In particular, fractions 3 of spelt sourdough and 36 of rye sourdough, which also showed the highest *in vitro* inhibition of linoleic acid autoxidation, had an effect on the survival of mouse fibroblasts comparable to that of α-tocopherol. However, a specific *in vivo* test should be carried out to evaluate the effect on human health.

Twenty-five peptides were found in the purified fractions by nano-LC-ESI-MS/MS analysis. None of these peptides was previously reported to be an antioxidant (43). A mixture of peptides was identified in all of the active fractions. Overall, it was hypothesized that the strongest antioxidant activity was ascribed to the synergic effect between peptides rather than to the individual ac-

tivity of the single peptides (5). Almost all of the sequences showed features typical of well-known antioxidant peptides, such as low molecular mass (43). The presence of amino acids, such as Tyr (Y), Trp (W), Met (M), Lys (K), Pro (P), Cys(C), His (H), Val (V), Leu (L), and Ala (A), would be ascribed, for different reasons, to the antioxidant activity of peptides (42). Except for the sequences DNIPIVIR and GTIFFSQEGDGPTSVTGSVSGLKPGLHGFHVH ALGDTTNGCMSTGPHFNPTGK, found in fractions 3 of whole wheat sourdough and 36 of rye sourdough, respectively, all of the other sequences are constituted totally (e.g., HPVPPKKK from fraction 3 of spelt sourdough) or mostly by the amino acids described above. Hydrophobic amino acids enhance the solubility of peptides in lipids, thus facilitating access to hydrophobic radical species and to hydrophobic PUFAs (polyunsaturated fatty acids) (43). Indeed, 14 of the 25 sequences had hydrophobic ratios above 50%. The highest levels of hydrophobicity were found for the sequences MAPAAVAAAEAGSK (64%), EALEAMFL (75%), LC PVHRAADL (60%), and PAEMVAAALDR (70%). The SH group of Cys (C) has a crucial antioxidant activity due to its direct interaction with radicals (34). The sequence NANGELCPNNMCCSQ WGYCGLGSEFCGNGCQSGACCPEK, which was identified in fraction 5 of rye sourdough, contains 8 cysteine residues. It was hypothesized that the antioxidant activity of His-containing peptides is related to the hydrogen-donating, lipid peroxyl radical-trapping, and/or the metal ion-chelating ability of the imidazole group (3, 36). Eight sequences contained His residues. In particular, His at the N terminus was found for the sequences HPVP PKKK and HKEMQAIFDVYIMFIN from fractions 3 of spelt sourdough and 23 of kamut sourdough, and His at the C terminus was found for the sequences KVALMSAGSMH and GVSNAAVV AGGH from fractions 15 of rye sourdough and 2 of kamut sourdough. His at the N terminus acts mainly as a metal ion chelator, while at the C terminus His is an effective scavenger against various radicals (4). Four sequences have Ala or Leu at the N or C terminus, which was already shown to be a typical feature for antioxidant peptides (43, 47). Amino acids with aromatic residues may donate protons to electron-deficient radicals. This property improves the radical-scavenging activity of peptides (43). Sixteen sequences contained one or more aromatic amino acids.

This study shows that selected lactic acid bacteria have the capacity to synthesize antioxidant peptides during the sourdough fermentation of various cereal flours. The fermentation conditions employed here are applicable at the industrial level for making additive-free bakery products with high nutritional value.

The purified peptides exhibited bioactive properties compatible with various antioxidant mechanisms, thus indicating presumptive protection against free radicals. These features could lead to the production of innovative functional foods and the design of new synthetic peptides for food/pharmaceutical applications.

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