Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours

Running title: antioxidant peptides in sourdough

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Abstract

A pool of selected lactic acid bacteria was used for sourdough fermentation of various cereal flours with the aim to synthesize 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 the linoleic acid autoxidation. WSE were subjected to Reverse Phase Fast Protein Liquid Chromatography. Thirty-seven fractions were collected and *in vitro* assayed. The most active fractions were resistant to further hydrolysis by digestive enzymes. Twenty-five peptides, having the size from 8 to 57 amino acid residues were identified by nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra. Almost all the sequences shared compositional features which are typical of antioxidant peptides. All 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 proteolysis of native cereal proteins.
Introduction

The interest for 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 a positive impact on the body function or condition, and may, ultimately, influence the human health (23). Usually, bioactive peptides correspond to cryptic sequences from native proteins, which are mainly released through hydrolysis by digestive enzymes, microbial and plant proteolytic enzymes and, more in general, increase during food fermentation (24). In vitro and some in vivo studies show a large spectrum of biological functions attributed to bioactive peptides: opioid like (19), mineral-binding (9), immunomodulatory (15), antimicrobial (25), antioxidative (27), antitrombohotic (46), hypocoolesterolemic (53) and antihypertensive (21). 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, the interest for antioxidant peptides, deriving from food proteins, has increased, and the evidences that bioactive peptides prevent oxidative stresses associated with numerous degenerative aging diseases (e.g., cancer and arteriosclerosis) are accumulating (2).

Overall, antioxidants have a large application also in food industries. The delay of food discoloration and deterioration, which occur as the consequence of oxidative processes, markedly improves food preservation. 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 carcinogenity and other toxic effects in themselves, in their oxidised forms, and in their reaction products with food constituents, for their effectiveness at low concentrations, and for the absence of the ability to impart an unpleasant flavour to the food in which they are used (28). The use of antioxidants in food products is governed by regulatory laws of the individual country or by internal standards (28). Even though many synthetic compounds have antioxidant properties, only a few of them have
been accepted as GRAS (generally recognised 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 BHA, BHT and gallates have changed over the years 
mainly because of their toxicological effects in various species (20, 33, 50, 28) and new 
toxicological data on some of the synthetic antioxidants cautioned against their use (28).

In the recent past, natural antioxidants attracted the attention of many food manufacturers as a 
result of the necessity to produce healthy foods (28). Biologically active peptides with potential 
antioxidant activity were derived from many animal and plant protein sources (35, 49, 43). 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 and soymilk kefir, medicinal 
mushroom, mackerel, curry leaves, cotton leafworm, casein, algae protein waste, wheat gluten 
and buckwheat protein (43). It was argued that antioxidant peptides act as inhibitors of lipid 
peroxidation, direct scavengers of free radicals and/or as agents to chelate transition metal ions 
that catalyze the generation of radical species (43). Usually, antioxidant peptides are constituted 
by 2-20 amino acidic residues, and have molecular masses less than 6.0 kDa (26, 48). The 
antioxidant activity seems to be strongly correlated to the amino acid composition, conformation 
and hydrophobicity (5).

Cereals are staple foods in the human diet. They are considered one of the most important source 
of dietary carbohydrates, proteins, vitamins, minerals and fibers for people all over the world. A 
large proportion of cereals is processed into foods and beverages by fermentation (e.g., 
sourdough) prior to consumption. Although, several biological activities such as ACE inhibition, 
antimicrobial and anticancer were reported for cereal peptides (38, 8, 40, 7, 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 the structure relationships with antioxidant properties.

Materials and methods

Microorganisms

Lactobacillus alimentarius 15M, 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 towards Pro-rich peptides (12). The above 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%, v/v) and 28 mM maltose at the final pH of 5.6 (modified MRS, mMRS). When used for sourdough fermentations, lactic acid bacteria 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 re-suspended in tap water (total bacteria count <100 cfu/ml) used for making the dough. Enumeration of lactic acid bacteria was carried out by plating serial dilution 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 x 100/flour weight) of 330. Fermentation with the pool of selected lactic acid bacteria (initial cell density of 5 x 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.

Water/salt-soluble extracts
Water/salt-soluble extracts (WSE) were prepared from each dough according to the method
originally described by Osborne (32) and further modified by Weiss, Vogelmeier, and Gorg (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, vortexing at 15-min intervals, and centrifuged at 20,000 x g for 20
min. The supernatants, containing the water/salt-soluble nitrogen fraction, were used to in vitro
assay the antioxidant activity. The concentration of peptides in the WSE and purified fractions
was 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 radical was measured according to the method of Shimada, Fujikawa, Yahara, and
Nakamura, (45) with some modifications. Freeze-dried samples were dissolved in 0.1 M
phosphate buffer at pH 7.0, at the final concentration of 1 mg/ml of peptides, and 2 ml of each
solution were 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 resulting solution was
read at 517 nm. The absorbance measured after 10 min was used for the calculation of the DPPH
scavenged by WSE or purified peptide fractions (39). A lower absorbance represents a higher
dPPH scavenging activity. The scavenging effect was expressed as shown in the following
equation: DPPH scavenging activity (%) = [(blank absorbance – Sample absorbance) / Blank
Inhibition of linoleic acid autoxidation

The antioxidant activity of WSE and purified fractions was also 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), previously dissolved on ethanol (99.5%). Incubation in a glass test tube, tightly sealed with 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, Yasumoto, and Iwami (30). One hundred microliters of the above sample were mixed with 4.7 ml of 75% (v/v) ethanol, 0.1 ml of 30% (w/v) ammonium thiocyanate, and 0.1 ml of 0.02 M ferrous chloride, dissolved in 1 M HCl. After 3 min, the degree of color development that represents the oxidation of linoleic acid was measured spectrophotometrically at 500 nm. Butylatedhydroxytoluene (BHT) and α-tocopherol (1 mg/ml) were also assayed as antioxidant references. A negative control (without antioxidants) was also considered.

Purification of antioxidant peptides

WSE were fractionated by ultra-filtration (Ultrafree-MC centrifugal filter units, Millipore) by using three different membrane sizes: 50, 30 and 10 kDa cut-off (fractions A, B and C, respectively). Aliquots of 400 µl of WSE were centrifuged at 10,000 x g for 60 min. After ultra-filtration, fractions were used for DPPH radical scavenging activity assay. The 10 kDa partially purified fractions (C) were further fractionated (37 fractions) by Reversed-Phase Fast Performance Liquid Chromatography (RP-FPLC), using a Resource RPC column and an 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% (v/v) absorbance x 100. Butylatedhydroxytoluene (BHT) and α-tocopherol (1 mg/ml) were also assayed as antioxidant references.
trifluoroacetic acid (TFA) and centrifuged at 10,000 x 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 (CH₃CN), containing 0.05% TFA. The concentration of CH₃CN 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 re-dissolved 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, Simonato, Giannattasio, Peruffo, and Curioni (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, containing 0.25 mg/ml of pancreatin (Sigma) and 0.0087 mg/ml of trypsin (EC 3.4.21.4) (Sigma), were added. The resulting pH was 7.6. Pancreatic digestion was lasting 150 min. Digested samples were heated for 5 min at 100°C and centrifuged at 12,000 x 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 activity were subjected to a second step of purification through RP-HPLC, under the conditions described previously, and using an ÄKTA Purifier apparatus (GE HealthcareBio-Sciences Corp., Piscataway, New
Jersey, USA). The centers of the peaks were collected, freeze dried and used for mass spectrometry analysis.

Identification of peptides was carried out by nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS), using a Finningan LCQ Deco XP Max ion trap mass spectrometer (ThermoElectron) through the nano-ESI interface. According to manufacturer’s instrument settings for nano-LC-ESI-MSMS analyses, MS spectra were automatically taken by Xcalibur software (ThermoElectron), in positive ion mode. MS/MS spectra were processed using the software BioWorks 3.2 (ThermoElectron) generating peaklists suitable for database searches. Peptides were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, England) and NCBI nr protein database (National Centre for Biotechnology Information, Bethesda, USA). For 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, Know, Kim, & Zhao (5), and the validated peptide sequences explained all the major peaks in the MS/MS spectrum.

Effect of purified peptides on viability of oxidation induced cells

Mouse fibroblasts (Balb 3T3, clone A31, ATCC CCL-163TM) were cultured under humidified atmosphere (5% CO2, 37°C) using Dulbecco’s Modified Eagle Medium (DMEM) which was supplemented with 10% (w/v) fetal bovine serum (FBS), 1 mM glutamine and 100 µg/ml penicillin-streptomycin. The culture medium was renewed every two days and the cultures after four passages were used to determine the viability. Cell viability was measured using the MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenytetrazolium 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 MTT assay, cells were seeded in the
96-well plate at the density of 5x 10^4 cells/well and incubated for 16 h. Subsequently, cells were treated with the antioxidant purified fractions and incubated for further 16 h. The final concentration of peptides in the reaction mixture was 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, 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 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, were added and incubation (37°C) in the dark was allowed for 1 h. Finally, 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 by an Aytok EL808microplate reader (Winooski, VT, USA). 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 the control culture before oxidative stress.

Statistical Analysis

Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey’s procedure at P<0.05, using the statistical software, Statistica for Windows (Statistica7.0 per Windows).

Results

Sourdough fermentation

After 24 h of fermentation at 37°C, lactic acid bacteria reached values of cell density ranging from 1.2 ± 0.4 to 6.2 ± 0.5 x 10^9 cfu/g. The lowest values were found during sourdough fermentation of oat, rice, and maize flours. No significant (P>0.05) differences were found
between cell densities of sourdoughs made with the whole wheat, durum wheat, rye, spelt, kamut and barley flours. Before fermentation, the values of pH 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 values of pH values ranged from 3.40 ± 0.03 to 3.88 ± 0.05. The lowest values of pH 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 water/salt-soluble extracts**

Water/salt-soluble extracts (WSE) from sourdoughs and control doughs had values of pH ranging from 6.5 ± 0.2 to 7.0 ± 0.2, as the consequence of the extraction with the Tris-HCl buffer. As determined by the OPA method, the concentration of peptides of WSE was 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 concentration of peptides of WSE from the chemically acidified doughs was ca. 2-3 times lower compared to the respective sourdoughs.

During radical scavenging assay, the colored stable DPPH radical is reduced to non-radical DPPH-H, when in the presence of an antioxidant or a hydrogen donor. DPPH radical without antioxidants was stable over the time. Under the assay conditions, the 100% of activity corresponds to the complete scavenging of DPPH radical (50µM final concentration) after 10 min of incubation by the antioxidant compounds. According to previous studies (38, 50), the color intensity of DPPH showed a logarithmic decline when in the presence of BHT. The radical scavenging activity towards the stable radical DPPH was in the range 3.0 ± 0.2 - 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
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 the above results, WSE from chemically acidified doughs were not further characterized.

Lipid peroxidation is thought to proceed via radical mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (35). The absorbance at 500 nm of WSE from sourdoughs was higher than the positive controls, thus showing a lower inhibition of linoleic acid autoxidation (Fig. 2). In agreement with the previous findings on 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 ultra-filtration (cut-off 50, 30 and 10 kDa) and further assayed for radical scavenging activity. All fractions from ultra-filtration showed activity towards DPPH radical, suggesting that the molecular mass of antioxidant compounds was lower 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 containing 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 inhibition of linoleic acid autoxidation. Based on DPPH radical scavenging assay, eight fractions with the highest activity were selected (Fig. 3). In details, they were fraction 3 (activity ca. 27%, concentration of peptides 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 the fractions showed an inhibition of the linoleic
peroxidation similar to that 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 decrease of the antioxidant
activity lower than the 10% when compared to undigested fractions (35, 42, and 35% of activity,
respectively). No significant (P>0.05) decrease was found for the other fractions (antioxidant
activity was 27% for fraction 3 from whole wheat; 89 and 76% for fractions 2 and 3 from spelt;
49 and 49% for fractions 2 and 3 from kamut). 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, having the size from 8 to 57 amino acid residues, and molecular masses
varying from 769.8 to 5338.5 Da were identified by the nano-LC-ESI-MS/MS analysis (Table
2). All the peptides were found in the NCBI nr database as 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) and total net charge 0, were identified from fraction 3 of
whole wheat sourdough. Four and three peptides having from 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 positive or neutral total net charge. Four and five different
peptides were identified from fractions 5 and 36 of rye sourdough, respectively. The major part of the sequences contained 26 - 53 amino acid residues. The sequences n.11, n.13, n.14 and n.15 were shorter (10-16 amino acid residues). Except for the sequences n.12, n.17 and n.18, the hydrophobic ratio was higher than 48%. The total net charge of sequences n.18 and n.20 was negative, while it was positive or neutral for all 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 (less than 21 amino acid residues). Except for the sequence n.22, the hydrophobic ratio was higher than 35%. All the peptides identified from fractions 2 and 3 had positive or neutral total net charge, while sequences n.24 and n.25 from fraction 37 had negative total net charge.

Effect of purified peptides on viability of oxidation induced cells

To investigate the capacity of the purified peptides to act as radical scavenger, cultured mouse fibroblasts were grown in the presence of eight purified fractions from whole wheat, spelt, rye, and kamut sourdoughs. Afterwards, cells 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 the cell survival compared to the negative control (55.4 ± 0.2% of cell viability after oxidative stress) (Fig. 5). In particular, the purified fraction 3 of spelt sourdough showed a significantly ($P < 0.05$) higher activity compared to 500 µM α-tocopherol (93.6 ± 0.2 vs. 87.2 ± 0.1%). Other three purified fractions (2 of kamut, and 5 and 36 of rye sourdoughs) induced a cell viability higher than that found for 250 µM α-tocopherol (77.6 ± 0.1, 73.9 ± 0.4, 78.6 ± 0.4% vs. 70.8 ± 0.3%).

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 level of various bioactive compounds, retards starch bioavailability, thus decreasing the glycaemic index, and increases mineral bioavailability (42; 10; 13). To our knowledge, this is the first study reporting the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through proteolysis of native cereal proteins. In the areas of human nutrition and biochemistry, natural antioxidants from food sources are largely studied since their potential health benefits with no or little 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 non hazardous immune-reaction (43). It is presumable that bioactive peptides deriving from cereal proteins are not immunogenic/allergenic toward healthy people, but only for subjects suffering of 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 long time and with semi-liquid conditions, which are indispensable to fully exploit microbial proteolysis (38, 16). 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 ten strains, previously selected based on proteinase and peptidase activities towards wheat proteins (37). Proteinase activity and, especially, a large portfolio of peptidases are the pre-requisites to release bioactive peptides during sourdough fermentation (17, 11). 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 may possess 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 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. Water/salt-soluble extracts (WSE) from several sourdoughs, which had almost the same level of acidity of the controls, showed an elevated \textit{in vitro} radical scavenging activity and 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 \( \alpha \)-tocopherol.

Despite many cereals can be closely related species, 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 (concentration of peptides 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 at the end of the acetonitrile gradient. The \textit{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 the positive controls. As previously reported (38, 29), purified fractions can show higher bioactivity with respect to the raw extract, as the consequence of the higher concentration of the active compound compared to the other constituents of the matrix.

To be active, antioxidant peptides should have the capacity to overcome hydrolysis and modifications at the intestine level, and to reach their targets (43). Overall, the antioxidant activity of the purified fractions seemed to be not affected by sequential \textit{in vitro} treatments with digestive enzymes. A slight decrease of the antioxidant activity was only found for fraction 37 of kamut sourdough, and fractions 5 and 36 of rye sourdough, which contained peptides having the
highest size. After the \textit{in vitro} assays, the \textit{ex vivo} antioxidant activity of the purified fractions was determined towards mouse fibroblasts, which were artificially subjected to oxidative stress. As shown by MTT assays, all the purified fractions exhibited a marked protective effect. In particular, fractions 3 of spelt sourdough and 36 of rye sourdough, which also showing the highest \textit{in vitro} inhibition of linoleic acid autoxidation, had an effect on the survival of mouse fibroblasts which was comparable to that of \( \alpha \)-tocopherol. However, specific \textit{in vivo} test should be carried out in order 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 as antioxidant (43). A mixtures of peptides was identified in all the active fractions. Overall, it was hypothesized that the strongest antioxidant activity would be ascribed to the synergic effect between peptides rather than to the individual activity of the single peptide (5). Almost all the sequences showed typical features of well known antioxidant peptides such as the 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 GTIFFSQGDGPTSVTGVSGLPKPLHGDFHVHALGDTNCGMSTGPHFNPTGK, respectively found in fractions 3 of whole wheat sourdough and 36 of rye sourdough, all the other sequences are constituted totally (e.g., HPVPPKKK from fraction 3 of spelt sourdough) or for the major part by the above amino acids. Hydrophobic amino acids enhance the solubility of peptides in lipids, thus facilitating the access to hydrophobic radical species and to hydrophobic PUFAs (Polyunsaturated Fatty Acids) (43). Indeed, 14 of the 25 sequences had hydrophobic ratios higher than 50%. The highest levels of hydrophobicity were found for the sequences MAPAAAVAAEAGSK (64%), EALEAMFL (75%), LCPVHRAADL (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

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NANGELCPNNMCCSQWGYCGLGSEFCGNGCQSGACCPEK, 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 is at the N-terminal was found for the sequences HPVPPKKK and HKEMQAIFDVYIMFIN from fractions 3 of spelt sourdough and 23 of kamut sourdough, and at the C-terminal was found for the sequences KVALMSAGSMH and GVSNAAVVAGGH from fractions 15 of rye sourdough and 2 of kamut sourdough. His at the N-terminal acts mainly as metal ion chelator, while at C-terminal His is an effective scavenger against various radicals (4). Four sequences have Ala or Leu at the N- or C-terminal, which was already shown as 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 sourdough fermentation of various cereal flours. The fermentation conditions here employed are applicable at industrial level for the making of bakery products with high nutritional value and additive-free. The purified peptides exhibited bioactive properties compatible with various antioxidant mechanisms, thus indicating a 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.
Acknowledgements

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References


**Legend to figures**

**Figure 1.** Radical scavenging activity towards DPPH of the water/salt-soluble extracts (WSE) from chemically acidified doughs (■, -D) and sourdoughs (■, -S) fermented with the pool of selected lactic acid bacteria. Butylatedhydroxytoluene (■, BHT) (1mg/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 of three independent experiments. Means connected by the same horizontal line are similar by the Tukey’s comparisons test (P>0.05).

**Figure 2.** Lipid peroxidation inhibitory activity of the water/salt-soluble extracts (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. Butylatedhydroxytoluene (BHT) and α-tocopherol (1mg/ml) were used as the positive controls. A negative control, without antioxidants, was also considered (ct). Data are the means of three independent experiments. Bars of standard deviations are represented.

**Figure 3.** Reverse Phase-Fast Protein Liquid Chromatography (RP-FPLC) chromatograms of water/salt-soluble extracts (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 percentage of radical scavenging activity (….) and to the gradient of eluent B (---). Arrows indicate peptide fractions with the highest antioxidant activity.

**Figure 4.** Lipid peroxidation inhibitory activity of the purified peptide fractions of the water/salt-soluble extracts (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. Butylatedhydroxytoluene (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; r5 and r36, fractions 5 and 36 of rye; and k2, k3 and k36,
fractions 2, 3 and 37 of kamut. Data are the means of three independent experiments. Bars of standard deviations are represented.

**Figure 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 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 measure through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ct, control without antioxidants; αt1 and αt2, α-tocopherol 500 and 250 µg/l, 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 of three independent experiments twice analyzed. Means connected by the same horizontal line are similar by the Tukey’s comparisons test (P>0.05).
Table 1. Characteristics of the flours used for sourdough fermentation.

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<thead>
<tr>
<th></th>
<th>Whole wheat</th>
<th>Durum wheat</th>
<th>Rye</th>
<th>Spelt</th>
<th>Oat</th>
<th>Rice</th>
<th>Kamut</th>
<th>Barley</th>
<th>Maize</th>
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<td>Moisture</td>
<td>12.3±0.3</td>
<td>12.5±0.5</td>
<td>9.8±0.3</td>
<td>10.4±0.1</td>
<td>12.0±0.3</td>
<td>11.9±0.3</td>
<td>11.7±0.2</td>
<td>12.1±0.1</td>
<td>12.5±0.4</td>
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<td>Carbohydrates (% of d.m.)</td>
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<td>63.2±1.0</td>
<td>77.5±1.5</td>
<td>67.1±1.2</td>
<td>62±0.8</td>
<td>80.1±1.2</td>
<td>69.2±0.5</td>
<td>70.5±0.4</td>
<td>76.8±0.8</td>
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<tr>
<td>Proteins (% of d.m.)</td>
<td>11.0±0.7</td>
<td>12.9±0.8</td>
<td>9.4±0.6</td>
<td>15.1±0.3</td>
<td>12.6±0.8</td>
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<td>10.5±0.1</td>
<td>8.7±0.1</td>
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<td>Lipids (% of d.m.)</td>
<td>1.7±0.1</td>
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<td>2.5±0.1</td>
<td>12.3±0.6</td>
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<td>Fibres (% of d.m.)</td>
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<tr>
<td>Ash (% of d.m.)</td>
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For each flour, three samples were twice analyzed. Mean values ± standard deviations were reported.
Table 2. Sequences of peptides contained in the purified fractions of the water/salt-soluble extracts (WSE) of whole wheat, spelt, rye and kamut sourdoughs fermented with the pool of selected lactic acid bacteria.

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<th>Fractions</th>
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<th>Calculated mass</th>
<th>Expected mass</th>
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aSequences were obtained by mass spectrometry and confirmed by database search using NCBI accession numbers.
| Gene ID | Description | MW1 | MW2 | p-value | Matched Gene
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* The single-letter amino acid code is used.
Figure 2.