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Improving the antioxidant properties of quinoa flour through fermentation with selected autochthonous lactic acid bacteria

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ABSTRACT

Lactic acid bacteria strains, previously isolated from the same matrix, were used to ferment quinoa flour aiming at exploiting the antioxidant potential. As *in vitro* determined on DPPH and ABTS radicals, the scavenging activity of water/salt-soluble extracts (WSE) from fermented doughs was significantly ($P < 0.05$) higher than that of non-inoculated doughs. The highest inhibition of linoleic acid autoxidation was found for the quinoa dough fermented with *Lactobacillus plantarum* T0A10. The corresponding WSE was subjected to Reverse Phase Fast Protein Liquid Chromatography, and 32 fractions were collected and subjected to *in vitro* assays. The most active fraction was resistant to further hydrolysis by digestive enzymes. Five peptides, having sizes from 5 to 9 amino acid residues, were identified by nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra. The sequences shared compositional features which are typical of antioxidant peptides. As shown by determining cell viability and radical scavenging activity (MTT and DCFH-DA assays, respectively), the purified fraction showed antioxidant activity on human keratinocytes NCTC 2544 artificially subjected to oxidative stress.
This study demonstrated the capacity of autochthonous lactic acid bacteria to release peptides with antioxidant activity through proteolysis of native quinoa proteins. Fermentation of the quinoa flour with a selected starter might be considered suitable for novel applications as functional food ingredient, dietary supplement or pharmaceutical preparations.

1. Introduction

The interest for health-promoting functional foods, dietary supplements and pharmaceutical preparations, which contain peptides deriving from food proteins, is markedly increasing (Shadidi and Li, 2015). 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 (Kits and Weiler, 2003). Among the various bioactivities described, antioxidant activity, together with antihypertensive, immunomodulatory, antimicrobial, and antitumoral activities, gained a growing interest from the scientific community, food industry, and consumers (Korhonen and Pihlanto, 2007; Sarmadi and Ismail, 2010; Shadidi and Li, 2015).

Overall, the interest for antioxidant peptides has increased thanks to the abundant evidences of the *in vivo* prevention of oxidative stresses, which are mainly associated to degenerative aging diseases (*e.g.*, cancer and arteriosclerosis) (Adebiyi et al., 2009). Antioxidants have a large potential for food industries. Delay of food discoloration and deterioration, which occur because of the oxidation, undoubtedly enhances 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 (Rajapakse et al., 2005).

Biologically active peptides with potential antioxidant activity were derived from many animal and plant protein sources (Korhonen and Pihlanto, 2007; Shadidi and Li, 2015). Production and/or isolation from peanut kernels, rice bran, sunflower protein, alfalfa leaf protein, corn gluten meal, frog skin, yam, egg-yolk protein, milk-kefir and soymilk kefir, mushroom, mackerel, curry leaves, cotton leaf worm, casein, algae protein waste, wheat gluten and buckwheat protein were already reported (Sarmadi and Ismail, 2010). Recently, antioxidant peptides were isolated from various cereal flours fermented with sourdoughs (Coda et al., 2012). In particular, a pool of lactic acid bacteria, selected based on proteolytic activities, had the capacity to release antioxidant peptides (8 to 57 amino acid residues) during sourdough

Abbreviations: ABTS, 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]; ADI, acceptable daily intake; BHT, butylated hydroxytoluene; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DY, dough yield; FBS, fetal bovine serum; GRAS, generally recognized as safe; MTT, (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide); nano-LC-ESI-MS/MS, nano-Liquid Chromatography-Electrospray Ionisation-Mass Spectra/Mass Spectra; OPA, *o*-phtaldialdehyde; PUFA, polyunsaturated fatty acids; QF, quinoa flour; ROS, reactive oxygen species; RP-FPLC, Reversed-Phase Fast Performance Liquid Chromatography; RSA, radical scavenging activity; TFA, trifluoroacetic acid; TFAA, total free amino acids; TTA, total titratable acidity; WSE, water/salt-soluble extracts.

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fermentation of whole wheat, spelt, rye, and kamut doughs. The antioxidant activity of these peptides on mouse fibroblasts, which were artificially subjected to oxidative stress, was also described (Coda et al., 2012).

Quinoa (*Chenopodium quinoa* Willd.) is a seed crop, which is traditionally cultivated in the Andean region since thousands of years. Commonly, quinoa grains and flour are used for human consumption and animal feeding (Rizzello et al., 2015a). Quinoa has the capacity of adapting to a range of agro-ecological conditions, showing tolerance to frost, salinity and drought, and having the potential to grow on marginal soils. These features, together with an undoubtedly high nutritional value, determine the worldwide interest for this crop (Stikic et al., 2012). During the last years, the production of quinoa markedly increased, thus emphasizing the opportunity to cultivate this crop in various regions (Stikic et al., 2012). FAO selected the quinoa as one of the crops that are destined to offer food security in the 21st century (Jacobsen et al., 2003). The high nutritional value of quinoa seeds is mainly due to the high concentrations of proteins, minerals, and vitamins (Fleming and Galwey, 1995). Quinoa proteins are rich in amino acids like lysine, threonine and methionine, which are deficient in cereals.

Recently (Rizzello et al., 2015a), autochthonous lactic acid bacteria were isolated from quinoa flour and spontaneously fermented doughs (Rizzello et al., 2015a). Strains, selected for pro-technological features, were used as starters to get quinoa sourdough. Free amino acids, soluble fibers, total phenols, phytase and antioxidant activities, and the *in vitro* protein digestibility markedly increased during fermentation. These results encouraged the use of quinoa and selected starters for the manufacture of novel and healthy leavened baked goods (Rizzello et al., 2015a).

This study aimed at investigating the antioxidant potential of quinoa flour, which was subjected to fermentation with autochthonous and selected lactic acid bacteria. Bioactive peptides were purified, identified and characterized for the antioxidant properties *in vitro*, also using human keratinocytes NCTC 2544.

2. Materials and methods

2.1. Microorganisms

Lactic acid bacteria strains, previously (Rizzello et al., 2015a) isolated from quinoa, were used in this study. *Lactobacillus plantarum* T0B3, T0A10, T0A6, T0A2, T0C2, T0C3 and T0C1, and *Lactobacillus rossiae* T0A16 were from quinoa flour (T0); *Lactobacillus plantarum* T1B6, T1B16, T1A14, T1A12, T1A11 and T1C17, and *Pediococcus pentosaceus* T1B11, T1A13 and T1C1 were from quinoa flour dough (DY, dough weight × 100 / flour weight, of 160) subjected to spontaneous fermentation at 30 °C for 16 h (T1); and *L. plantarum* T6B4, T6B14, T6B10, T6A14, T6A10, T6A4, T6C16, T6C20 and T6C5 were from quinoa type I sourdough (T6), which was made and propagated through the traditional protocol commonly used for wheat flour fermentation, without using starter cultures or baker's yeast (Pontonio et al., 2015). All the strains were previously identified genotypically through sequencing of the 16S rDNA gene (Pontonio et al., 2015).

Lactic acid bacteria were cultivated on different culture broth, depending on the isolation medium (Rizzello et al., 2015a): MRS (Oxoid, Basingstoke, Hampshire, United Kingdom) (strains labeled with letter A); modified MRS (mMRS), containing 1% [wt/vol] maltose, and 5% [vol/vol] fresh yeast extract, pH 5.6, (Oxoid) (strains labeled with letter B), and SDB (sourdough bacteria broth) (strains labeled with letter C).

2.2. Fermentation

Organic quinoa (*Chenopodium quinoa*) dehulled seeds, imported from Argentina (Fundacion Nuevagestion, San Ignacio de Loyola, Jujuy), were used in this study. Quinoa flour (QF) was obtained from seeds through the laboratory mill Ika-Werke M20 (GMBH, and Co. KG, Staufen, Germany). Protein (total nitrogen × 5.7), lipids, ash and moisture contents were determined according to the AACC approved methods 46-11A, 30-10.01, 08-01, and 44-15A, respectively (AACC, 2010). The determination of insoluble and soluble dietary fibers was carried out by AOAC approved methods 991.42 and 993.19, respectively (Horwitz and Latimer, 2006).

Total carbohydrates (%) were calculated as the difference: 100 − (moisture + proteins + lipids + ash). The characteristics of the flour used in this study are reported in Table 1.

The 26 autochthonous lactic acid bacteria strains were cultivated into their respective media at 30 °C for 24 h. Cells were harvested singly by centrifugation (10,000 ×*g*, 10 min, 4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0) and re-suspended in tap water at the cell density of *ca.* 8.0 log cfu/mL.

Quinoa flour (30 g) and 70 mL of tap water, containing the above cellular suspension of each lactic acid bacterium (cell density in the dough of *ca.* log 7.0 cfu/g), were used to prepare 100 g of dough (DY of 330). Mixing was done for 5 min. Doughs were fermented at 37 °C for 24 h, under stirring conditions (*ca.* 200 rpm), according to the conditions previously set-up by Coda et al. (2011b) to maximize the release of antioxidant peptides. Two not inoculated quinoa doughs (DY 330) were produced. Incubation was lasting 0 (Ct0) and 24 h (Ct24) at 37 °C and doughs were used as controls.

The pH value of doughs was determined by a pH meter (Model 507, Crison, Milan, Italy) with a food penetration probe. Total titratable acidity (TTA) was determined after homogenization of 10 g of dough with 90 mL of distilled water, and expressed as the amount (mL) of 0.1 M NaOH needed to reach the value of pH of 8.3.

Lactic acid bacteria were enumerated by plating serial dilutions of doughs into MRS, mMRS, or SDB agar media, supplemented with cycloheximide (0.1 g L). Plates were incubated at 30 °C for 48 h, under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid).

2.3. Water/salt-soluble extracts

Water/salt-soluble extracts (WSE) were prepared from each dough, according to the method originally described by Osborne (1907) and further modified by Weiss et al. (1993), at the end of incubation. An aliquot of each dough (containing 3.75 g of flour) was diluted with 15 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 stored at −20 °C before the *in vitro* assay for determining the antioxidant activity. The peptide concentration of WSE was determined by the *o*-phtaldialdehyde (OPA) method (Church et al., 1983). A standard curve was prepared using tryptone (0.25 to 1.5 mg/mL) and used as the reference. The use of peptone gave a similar standard curve. The concentration of total free amino acids (TFAA) of WSE was 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), as described by Rizzello et al. (2010).

Table 1
Characteristics of the quinoa flour used for lactic acid bacteria fermentation.

	Proximal composition
Moisture (%)	11.4 ± 0.6
Proteins (%)	12.5 ± 0.8
Lipids (%)	5.3 ± 0.4
Carbohydrates (%)	69.3 ± 3.5
Soluble fibers (%)	1.2 ± 0.4
Insoluble fibers (%)	7.9 ± 2.0
Ash (%)	1.9 ± 0.2

Three samples were twice analyzed. Mean values ± standard deviations were reported.