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Expression of bifidobacterial phytases in *Lactobacillus casei* and their application in a food model of whole-grain sourdough bread

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ABSTRACT

Phytases are enzymes capable of sequentially dephosphorylating phytic acid to products of lower chelating capacity and higher solubility, abolishing its inhibitory effect on intestinal mineral absorption. Genetic constructions were made for expressing two phytases from bifidobacteria in *Lactobacillus casei* under the control of a nisin-inducible promoter. *L. casei* was able of producing, exporting and anchoring to the cell wall the phytase of *Bifidobacterium pseudocatenulatum*. The phytase from *Bifidobacterium longum* spp. *infantis* was also produced, although at low levels. *L. casei* expressing any of these phytases completely degraded phytic acid (2 mM) to lower *myo*-inositol phosphates when grown in MRS medium. Owing to the general absence of phytase activity in lactobacilli and to the high phytate content of whole grains, the constructed *L. casei* strains were applied as starter in a bread making process using whole-grain flour. *L. casei* developed in sourdoughs by fermenting the existing carbohydrates giving place to an acidification. In this food model system the contribution of *L. casei* strains expressing phytases to phytate hydrolysis was low, and the phytate degradation was mainly produced by activation of the cereal endogenous phytase as a consequence of the drop in pH. This work shows the capacity of lactobacilli to be modified in order to produce enzymes with relevance in food technology processes. The ability of these strains in reducing the phytate content in fermented food products must be evaluated in further models.

1. Introduction

The impact of diet on health has led to an increasing demand for functional foods, where high fiber products, as whole grain meal, play an important physiological role in the maintenance of general well-being and health. Epidemiological findings indicate a protective role of whole grain foods against several diseases such as diabetes, certain cancers, cardiovascular disease and obesity, including an improved regulation of blood glucose levels (McIntosh et al., 2003). Refined grains are characterized by a limited nutritional value, whereas whole grains are a better source of fiber, vitamins, minerals and other biologically active compounds as phenolic compounds, lignans, phytosterols and phytic acid. Processing may modify the amount and bioavailability of some of them (Slavin, 2004). Sourdough fermentation is a traditional process employed since ancient times in baking (Katina et al., 2005). Generally, the microbiota involved in sourdough fermentation is composed of yeasts and lactic acid bacteria (LAB), which represent the majority of the sourdough's microbiota, with counts ranging from 10^8 to 10^9 CFU per g of sourdough. The strains of LAB most frequently found in sourdough belong to the species *Lactobacillus sanfranciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum* (De Vuyst and Neysens, 2005). During sourdough fermentation LAB produce a number of metabolites which have been shown to have a positive effect on the texture and staling of bread, e.g. organic acids, exopolysaccharides (EPS) and/or enzymes (Arendt and Dal Bello, 2007). This results in an enhancement of the nutritional and sensory quality of bread (Katina et al., 2005). The sourdough could also increase the bioavailability of minerals. As was mentioned above, whole grain cereals contain significant amounts of phytic acid (*myo*-inositol (1,2,3,4,5,6)-hexakisphosphate, InsP_6) or its salts (phytates). The phytic acid is an organic acid common in plants which functions in the storage of phosphorus and cations for growth and it is a well-known inhibitor of mineral, proteins and trace element bioavailability (Sandberg et al., 1999). Notwithstanding, some works have reported positive effects of phytate as antioxidant, anti-diabetes or anti-cancer agent (Kumar et al. 2010).

The phytate hydrolysis decreases the negative effects on mineral absorption and generates lower *myo*-inositol phosphates with potential specific biological activity that may positively affect human health (Shi et al., 2006). Phytases are the enzymes capable of sequentially dephosphorylating phytic acid to products of lower chelating capacity and higher solubility, unlocking the inhibitory effect on mineral absorption (Haros et al., 2009). Cereals have their own endogenous phytase activity that could be enhanced by the low pH resulting from the use of sourdough in the breadmaking process, but this activity is not sufficient to efficiently degrade phytate (Greiner and Konietzny, 2006; Sanz-Penella

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et al., 2012a). Bacteria of the genus *Lactobacillus* are the main players in sourdough fermentation and LAB:yeast ratio is generally 100:1 (De Vuyst and Neysens, 2005). Yeasts usually show low phytase activity and for high yeast phytase activity to take place, conditions must favor the expression of the phytase genes (Andlid et al., 2004). As far as we know, no real phytases from lactobacilli have been described in the literature. Some reports exist describing the partial degradation of phytate by particular *Lactobacillus* strains (Anastasio et al., 2010; De Angelis et al., 2003; López et al., 2000; Zamudio et al., 2001), but this activity is due to the expression of unspecific phosphatases that act on phytate, although with very low efficiency (Haros et al., 2009; Sandberg and Andlid, 2002; Zamudio et al., 2001). On the contrary, phytase activity has been described for strains of the genus *Bifidobacterium* and the corresponding genes and enzymes have been characterized, showing that they belong to the Histidine-Acid Phosphatase family (Tamayo-Ramos et al., 2012). The purified bifidobacterial phytases have been applied in several food processes (García-Mantrana et al., 2014; Iglesias-Puig et al., 2014; Sanz-Penella et al., 2012b) and the strain *Bifidobacterium pseudocatenulatum* ATCC27919 has been used in both direct and indirect breadmaking processes (Sanz-Penella et al., 2009, 2012a), showing its potential in the reduction of phytates in breads and in the increase of mineral bioavailability. However, the condition of strict anaerobic and fastidious microorganisms of bifidobacteria limits their use in food fermentations. The purpose of this work was to construct *Lactobacillus casei* strains expressing the phytases from *B. pseudocatenulatum* and *Bifidobacterium longum* spp. *infantis* and their application as starters in the breadmaking process of whole-grain bread.

2. Materials and methods

2.1. Materials

Commercial Spanish whole wheat flour was purchased from the local market. The characteristics of the flour in dry basis were: moisture 14.04 ± 0.08%, protein (N × 5.7) 11.64 ± 0.08%, lipid content 1.67 ± 0.03% and ash 1.36 ± 0.01. Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as starter for the breadmaking process and *Lactobacillus casei* strains genetically modified to produce phytases from bifidobacteria were used as starter in sourdough fermentation. In order to construct these strains *L. casei* BL23 (wild type) and *L. casei* BL23 [*nisRK*] (Hazebrouck et al., 2007), a BL23 derivative in which the *nisRK* two-component system from *Lactococcus lactis* has been integrated in its genome, were used. *L. lactis* MG1363 was used as a host for cloning.

2.2. Methods

2.2.1. Culture media and growth conditions

L. casei strains were grown in MRS medium (Oxoid) at 37 °C under static conditions and *L. lactis* was grown in M17 medium (Oxoid) supplemented with 0.5% glucose at 30 °C. Antibiotics for plasmid selection (erythromycin and chloramphenicol) were used at 5 μg/ml when added individually and at 2.5 μg/ml when they were used together.

2.2.2. Construction of genetically modified Lactobacillus casei expressing phytase genes from bifidobacteria

The phytase genes from *B. pseudocatenulatum* ATCC27919 (BIFPSEUDO_03792) and *B. longum* spp. *infantis* ATCC15697 (BLON_0263) were amplified by PCR with the following primer pairs: (5´-GAACCATGGGGATAATGGCGAAAAAC/5′-CACAAGCTTTCAC GTCACGTTTGAACCGGTTTTG) and.

(5'AATCCATGGCAACACGAGTGATG/5'GACAAGCTTTCAGACCGAACT TCCGGTACGTGCC), respectively (underlined sequences correspond to the NcoI and HindIII sites introduced for cloning). The PCR reaction was carried out in a mixture containing Expand High Fidelity 1× buffer, 100 ng of genomic DNA of each strain, 200 μM of dNTPs, 10 pmol of each primer, and 1 μl of Expand High Fidelity Polymerase (Roche), in a volume of 50 μl. The PCR thermal conditions were as follows: 1 cycle at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2.5 min and a last cycle of 72 °C for 7 min. The amplified genes were examined by agarose gel electrophoresis, purified with the Illustra GFX PCR and gel band DNA purification kit (GE Healthcare) and digested with NcoI y HindIII for cloning into the pNG8048e vector (Steen et al., 2007) digested with the same endonucleases. Vector and phytase genes were ligated with T4 ligase (Invitrogene) and the ligation mixtures were used to transform *L. lactis* MG1363 electrocompetent cells (Holo and Nes, 1995) using a GenePulser apparatus (Biorad) and 0.2 cm electroporation cuvettes. After electroporation, cells were resuspended in 5 ml of M17 medium containing 0.5 M saccharose, 0.5% glucose, 2 mM $CaCl_2$ and 10 mM $MgCl_2$ and incubated for 1 h at 30 °C. After this period transformants were plated on M17 plates containing 0.5 M saccharose, 0.5% glucose and 5 μg/ml chloramphenicol and incubated overnight at 30 °C. The recombinant plasmids were purified from the lactococcal transformants with the Illustra Plasmid Isolation Kit (GE Healthcare) and examined by sequencing analysis. The obtained plasmids were electrotransformed to competent cells of *L. casei* BL23 [*nisRK*] as previously described (Posno et al., 1991). Transformants were recovered on MRS plates containing erythromycin and chloramphenicol after incubation at 37 °C.

2.2.3. Phytase induction experiments

L. casei BL23 [*nisRK* pNG80548e], *L. casei* BL23 [*nisRK* pNGPHY*pseudo*] and *L. casei* BL23 [*nisRK* pNGPHY*longum*] were grown in 20 ml of MRS medium overnight. These cultures were diluted into 50 ml of fresh MRS medium with antibiotics to an OD_{550} of 0.4, and incubated at 37 °C for 1.5 h. At the end of the incubation each culture was divided in two parts and nisin (Sigma-Aldrich) was added at a concentration of 20 ng/ml to one of them. Then, cultures were further incubated for 3 h at 37 °C. The cells were centrifuged at 9000 × *g* for 15 min (Hermle Z383K centrifuge), the pellet was washed twice with PBS and frozen at −20 °C until use. Phytase expression was analyzed by SDS-PAGE. To this end, to 10 μl of bacterial cells resuspended in PBS, 10 μl of 2× Laemmli buffer were added. After boiling for 5 min, samples were centrifuged at 12,000 × *g* for 5 min and loaded onto 10% SDS-PAGE gels that were stained with Coomassie blue.

2.2.4. Preparation of crude extracts for phytase determination

L. casei strains harboring different plasmids were induced for phytase production and the phytase activity was determined in different cellular fractions. Induced cells were washed twice with Tris–HCl 50 mM pH 7.5 and resuspended in the same buffer. Eight hundred microliters of this suspension were mixed with 1 g of glass beads (0.1 mm diameter) and broken in a BeadBeater apparatus (Biospec Products) for 4 cycles of 40 s at maximal speed with 1 min intervals in which the tubes were kept on ice. Unbroken cells were removed after centrifugation for 5 min at 14,000 × *g* at 4 °C. Protein concentration in the crude extracts was determined with the BioRad Dye-binding Protein Assay Kit using BSA as a standard. Cell wall proteins were obtained by enzymatic digestion. The reaction was carried out in 100 μl of Tris–HCl 50 mM pH 7.5 containing bacterial cells to an OD_{550} of 1 and 0.5 M saccharose, 5 mg/ml lysozyme and 5 U/ml mutanolysin. The suspension was incubated at 37 °C for 30 min and bacteria were removed by centrifugation 5 min at 14,000 × *g* and 4 °C. The supernatant was collected and used as crude cell wall extract. The enzymatic extract of sourdoughs were prepared following the method reported by Haros et al. (2001).

2.2.5. Determination of phytase activity

The phytase activity was determined using 500 μl of 0.1 M sodium acetate pH 5.5, containing 1.2 mM phytic acid dipotassium salt (Sigma-Aldrich) and 100 μl of each fraction (whole cells, crude extracts, cell wall extracts or sourdough extracts) (Haros et al., 2001, 2005). After 15 min of incubation at 50 °C, the reaction was stopped with 100 μl of