Phytase activity as a novel metabolic feature in Bifidobacterium

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Abstract
Phytase activity has been detected for the first time in Bifidobacterium spp. These bacteria were able to dephosphorylate phytic acid (myo-inositol hexaphosphate, IP 6) and generate several myo-inositol phosphate intermediates (IP 3–IP 5). B. globosum and B. pseudocatenulatum were optimally active at neutral-alkaline pH and B. adolescentis, B. angulatum and B. longum at acid pH. B. pseudocatenulatum showed the highest levels of phytase activity. This species produced maximum activity in the exponential phase of growth and when fructo-oligosaccharides were used as carbon source in the culture medium. The potential role of phytase activity from Bifidobacterium spp. in the reduction of the antinutritional properties of IP 6 is discussed.

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1. Introduction
The human gut is populated by at least 500 different species, which coexist in dynamic equilibrium with the host. The beneficial microbial groups develop an array of metabolic, trophic and protective functions, which have profound repercussions on human health [1]. Among them, nutrient metabolism represents an important biochemical activity of the human body and results in salvage of energy, generation of absorbable compounds and production of vitamins and other essential nutrients [2,3]. As a consequence, dietary strategies that favour the prevalence of health-promoting intestinal bacteria have been developed. These include the use of prebiotic oligosaccharides and selected bacterium strains (probiotics) as food supplements [4]. Fructooligosaccharides (FOS) are examples of prebiotics that stimulate the growth and metabolism of bifidobacteria [5]. Bifidobacteria selectively colonize the intestinal tract of breast-fed infants and are also relevant colonic bacteria in adults [6,7]. This group is considered to be one of the major microbial stimuli for newborns and one of the most important sources of probiotics. However, the information about the metabolic activities of bifidobacteria that could serve nutritional functions is still limited and mostly focused on polysaccharide degradation [8]. The metabolism of these compounds leads to the generation of small free fatty acids (butyrate, propionate, and acetate), which are utilized as energy sources and are thought to play additional roles, for instance in the solubilization and absorption of minerals [9,10].

Phytic acid (myo-inositol hexaphosphate, IP 6) or phytate is the primary storage form of phosphorus in plant seeds and is associated with fibre in many foods,
such as soy- and cereal-based products. It consists of an inositol, which is a hexahydroxycyclohexane in chair conformation, with six phosphate ester bonds. The phosphate groups confer on it a high negative charge and therefore a strong chelating ability, which reduce the dietary bioavailability of amino acids and minerals such as Ca$^{2+}$, Zn$^{2+}$ and Fe$^{2+}$ [11]. However, the antinutritional properties of phytates can be reduced through their enzymatic dephosphorylation into lower phosphorylated products [12]. Furthermore, the generation of these intermediate products (IP$_{1-6}$) and myo-inositol could also have relevant health implications, as they are involved in the regulation of vital cellular functions [13].

Phosphatases constitute a diverse group of enzymes that catalyze the hydrolysis of phosphomonoester bonds of a wide variety of phosphate esters. Phytases are a subgroup of phosphatases with general preference for phytate, which is hydrolysed in a stepwise manner generating phosphoric acid and myo-inositol phosphates [14]. Non-specific acid phosphatases constitute another subgroup of phosphatases, which show high hydrolysis rates with monophosphorylated compounds but low level of activity against phytate. However, this group of enzymes could also contribute to the hydrolysis of myo-inositol with lower number of phosphate groups [15–17]. Phytases are particularly important in human nutrition for their possible role in the degradation of phytate during both food processing and gastrointestinal transit [18]. Previous studies have demonstrated that the degradation of phytate in the stomach and intestine is mainly due to dietary phytases and, probably, to the metabolic activity of the colonic microflora [18,19]. So far, the only phytic acid degrading bacteria identified in human faeces are members of the genera Bacteroides and Clostridium and the Gram-negative bacteria Escherichia coli and Klebsiella pneumoniae [20,21]. This biochemical property has not been attributed to intestinal isolates of the genera Lactobacillus and Bifidobacterium, which are important integrants of the gut microflora and the preferred source of probiotics. This activity has only been screened in Lactobacillus strains isolated from food fermentations. These isolates rarely produce phytase activity although they normally possess non-specific acid phosphatase activity [15,17,22].

In this work, phytase and general phosphatase activities have been screened in different Bifidobacterium spp., representative of common intestinal isolates and probiotic species. The ability of certain species to reduce the levels of phytate and generate lower phosphorylated derivatives was detected for the first time. The optimal conditions for phytase activities as well as the study of the environmental factors that regulate their synthesis are reported, providing relevant information about their potential as novel technology or probiotic traits.

### 2. Materials and methods

#### 2.1. Bacterial strains and culture conditions

The Bifidobacterium strains used in this work are listed in Table 1. The bacterial strains were routinely cultured in modified Garche broth [23]. For enzyme activity assays and determinations of myo-inositol phosphates,
the same culture medium was used but inorganic phosphate \( (K_2HPO_4/NaH_2PO_4) \) was replaced by 0.65 g L\(^{-1}\) sodium phytate and 0.1 M 3-[N-Morpholino] propane-sulfonic acid (MOPS) buffer and the contents of yeast extract and peptone were reduced to 1 and 10 g L\(^{-1}\) respectively, in order to achieve a low-phosphate growth condition that could promote the synthesis of the enzymes responsible for phosphatase and phytase activities. This medium was inoculated at 5% (v/v) with 18-h old cultures previously propagated in the same conditions (Gas Pak Linegal Chemicals GmbH, Poland). For end-point determinations, cultures were incubated at 37 °C and in anaerobic conditions (Gas Pak Linegal Chemicals GmbH, Poland). For end-point determinations, cultures were incubated until the stationary phase of growth was reached (14–24 h). Cells were harvested by centrifugation (6000 g, 10 min, 4 °C) and washed with 50 mM Tris–HCl, (pH 6.5). The culture supernatant was filtered through a 22 µm Millipore filter (Milllex-GV, Bedford, MA, USA) and used for determinations of the content of myo-inositol phosphates. The cell pellets were suspended in 50 mM sodium acetate-acetic acid (pH 5.5) and used for enzyme activity assays. To analyse the time course of phytase activity and the content of myo-inositol phosphates during different phases of growth of the selected species (B. pseudocatenulatum), aliquots of the culture were withdrawn at different times (0, 3, 6, 9, 12, 15, 18, 24, 30, 36 and 48 h) and treated as described above. Bacterial growth was monitored by measuring the optical density at 600 nm and by plate count in modified Garche medium containing 1.8% (w/v) agar.

The effects of the composition of the culture medium on phytase production and the content of myo-inositol phosphates were determined in the described standard Garche medium containing different concentrations of lactose (5, 10 and 20 g L\(^{-1}\)), replacing lactose (10 g L\(^{-1}\)) by equivalent concentrations of either glucose or fructo-oligosaccharides (FOS, Wako Pure, Japan, grade of polymerisation: 2–4), and including inorganic phosphate alone or together with FOS (2 g L\(^{-1}\) K\(_2\)HPO\(_4\)).

2.2. Phytase and phosphatase activity assays

Acid phosphatase activity was determined by monitoring the rate of hydrolysis of \( p \)-nitrophenyl phosphate [15]. The reaction mixture consisted of 250 µl 0.1 M sodium acetate-acetic acid (pH 5.5) containing 5 mM \( p \)-nitrophenyl phosphate and 250 µl enzyme sample. After incubation at 50 °C for 15 min, the reaction was stopped by adding 500 µl 1.0 N NaOH. The \( p \)-nitrophenol released was determined by measuring the absorbance at 405 nm. One unit of phosphatase activity (U) was defined as the amount of enzyme that produces 1 µmol of \( p \)-nitrophenol per hour at 50 °C.

Phytase activity was determined by measuring the amount of liberated inorganic phosphate from sodium phytate. The reaction mixture consisted of 400 µl 0.1 M sodium acetate-acetic acid (pH 5.5), containing 1.2 mM sodium phytate and 200 µl enzyme sample. After incubation at 50 °C for 30 min, the reaction was stopped by adding 100µl 20% trichloroacetic acid solution [24]. An aliquot was analysed to determine the liberated inorganic phosphate with the ammonium molybdate method by measuring the absorbance at 405 nm [25]. One unit of phytase activity (U) was defined as the amount of enzyme that produces 1 µmol of inorganic phosphorous per hour at 50 °C.

In every case, data of enzyme activities are the mean of two replicates twice analysed.

2.3. Protein quantification

For quantification of the protein content in cell suspensions, samples were diluted in 1 N NaOH and incubated at 100 °C for 5 min. The protein content was quantified in the supernatant obtained after centrifugation (6000 g, 5 min, 4 °C) by the micromethod of Biuret [26]. Bovine-serum albumin was used as a standard.

2.4. Characterization of optimal conditions for phytase activity

To determine the effect of pH on phytase activities, enzyme assays were conducted at 50 °C, in a pH range from 3.0 to 8.5 using 50 mM sodium citrate/citric acid (pH 3.0–3.5), 50 mM sodium acetate/acetic acid (pH 4.0–5.5), 50 mM MES-NaOH (pH 6.0–6.5) and 50 mM Tris–HCl (pH 7.0–8.5) as buffers. To determine the effect of temperature, phytase activities were measured at optimum pH, over a temperature range from 37 to 70 °C. Activity assays were carried out according to the standard procedure described above.

2.5. HPLC analysis of inositol phosphates generated from phytic acid

The ability of different strains to hydrolyse phytic acid and generate myo-inositol penta-, tetra- and tris-phosphate (IP\(_5\), IP\(_4\) and IP\(_3\)) during growth was tested by HPLC according to the method of Sandberg and Ahderinne [27]. Briefly, 10 ml of culture supernatant was lyophilised, suspended in 15 ml 0.025 M HCl and transferred to mini-columns filled with Dowex AG 1-X8 resin 200–400 Mesh (Bio-Rad, Hercules, CA, USA). myo-Inositol phosphates were eluted with 2 N HCl (5 × 4 ml) and air-dried at 40 °C. The dried residue was dissolved in the mobile phase used for HPLC separations. HPLC analyses were carried out in a Shimadzu chromatograph (pump LC-6A, system controller SCL-6B, Shimadzu Japan), equipped with a refractive index detector (RID-6A, Shimadzu, Japan). Samples (20 µl) were loaded onto a Kromasil C\(_{18}\) column (5 µm, 4.6 by 150 mm, Barcelona, Spain). Elution was carried out
at 0.4 ml min\(^{-1}\) flow rate (35 °C) in the mobile phase, which consisted of methanol–0.05 M formic acid (51:49) with 1.5% (v/v) tetrabutylammonium hydroxide, adjusted at pH 4.3 with 9 M sulphuric acid. A mixture of standards obtained during hydrolysis of phytic acid sodium salt was used for identification of the different inositol phosphates [28]. Quantitative analysis was conducted using an external standard of sodium phytate (Sigma, St. Louis, MO, USA).

3. Results and discussion

3.1. Detection of phytase and phosphatase activities in Bifidobacterium strains

Phytase and phosphatase activities were screened in nine strains belonging to different species of the genus Bifidobacterium, representative of common intestinal isolates and probiotic species (Table 1). These activities were assayed in cell suspensions of every tested strain since the bacterial phytases characterized so far have been demonstrated to be either periplasmic or cell associated enzymes, with the exception of those found in Bacillus subtilis, Lactobacillus amylovorus and Enterobacter sp. 4 that seemed to be extracellular [14]. The assayed strains hydrolysed phytic acid at higher rates than \(p\)-nitrophenylnphosphate suggesting that they produce phytases, a subgroup of phosphatases with preference for the former substrate (Table 1). The only exception was B. breve, which appeared to exhibit a general phosphatase activity but not real phytase. In addition, the ratios between phytase and phosphatase activities (R) at 50 °C were determined as a preliminary indication of the type of enzymes (general phosphatase or phytase) responsible for phytate degradation (Table 1). The strain of the species B. breve showed a R value below 1, whereas the rest of the strains showed values higher than 1. The activity ratio (R) was especially high for B. angulatum, B. longum and B. pseudocatenulatum, showing values of 5.50, 4.33 and 6.60, respectively. The specific activities of bifidobacteria (except for B. breve) against phytic acid varied from 0.10 to 0.61 U mg protein\(^{-1}\) when measured at 50 °C. The specific activities against phytic acid retained at physiologically relevant temperature (37 °C) varied from 0.04 to 2.01 U mg protein\(^{-1}\). The highest activities at both temperatures (50 and 37 °C) were found in strains of the species B. animalis, B. globosum, B. longum and B. pseudocatenulatum (Table 1).

3.2. Degradation of phytic acid during growth by different Bifidobacterium strains

The ability of the different strains to hydrolyse IP\(_6\) and generate myo-inositols with lower numbers of phosphate groups (IP\(_3\)–IP\(_3\)) during growth was tested by HPLC. The myo-inositol contents were determined in the culture supernatants of every strain grown until the stationary phase (Table 1). The relative IP\(_6\) hydrolysis was in the range from 0.56% to 12.81% except for B. pseudocatenulatum, which degraded 100% of initial IP\(_6\) (Table 1). myo-Inositol intermediates were also analysed in the culture medium. IP\(_5\), which is the first product generated in the stepwise degradation of IP\(_6\), was not detected in most of the analysed samples. The accumulation of IP\(_4\) and IP\(_3\) in the growth media greatly varied amongst strains regardless of their ability to dephosphorylate the initial substrate (IP\(_6\)). Whereas the relative hydrolysis of IP\(_6\) catalysed by B. angulatum and B. globosum strains were similar, the amounts of IP\(_4\) and IP\(_3\) generated by B. angulatum were about 9- and 3-fold higher, respectively, than those generated by B. globosum. These results reflected differences in the catalytic properties of phytase activities that could be of interest for the controlled generation of desirable inositol phosphates. In fact, the studies carried out by Wyss et al. [16] have revealed that fungal and E. coli phytases have substantial differences in their biochemical properties and specificities. The strains of the species B. adolescentis, B. angulatum, B. globosum, B. longum and B. pseudocatenulatum showed the highest relative hydrolysis of IP\(_6\), which is the more reliable indication of their ability to metabolise phytate. In addition, these strains showed high phytase activities and phytase/phosphatase activity ratios, which overall support their selection for further analysis.

3.3. Characterization of optimal conditions for phytase activity

The effects of pH and temperature on phytase activities of the selected bifidobacteria strains were determined in order to elucidate their putative roles during food processing and in the gastrointestinal tract. The optimal pH for phytase activities in cell suspensions were 6.0–6.5, 5.0–5.5, 7.0–7.5, 5.0–5.5 and 6.5–7.0 for B. adolescentis, B. angulatum, B. globosum, B. longum and B. pseudocatenulatum, respectively (Fig. 1). Accordingly, two types of phytase activities could be defined, neutral or slightly alkaline in B. globosum and B. pseudocatenulatum and acid in B. adolescentis, B. angulatum and B. longum, although the last two species also retained high levels of activity (80–40%) at neutral pH. The phytase activity profiles of B. longum and B. globosum and B. pseudocatenulatum as function of pH also revealed other peaks of maximum activity that could be the result of the effects of the different buffers used to cover the whole pH range or the existence of more than one type of enzyme contributing to phytate degradation. The widest active pH range was exhibited by the phytase activity of B. angulatum, retaining considerable levels of activity from pH 3.5 to 8.5. In contrast, the remaining
optimal activity at different pHs could also increase their effectiveness in the gastrointestinal tract. In vitro experiments have already demonstrated that combinations of alkaline and acid phytases induce a more efficient hydrolysis of phytate in the gastrointestinal tract of animals [30].

The effects of the temperature on phytase activities of the different selected strains were also determined (data not shown). Enzyme activity was optimal at 60 °C in all the analysed strains, except for \( B. \) adolescentis that showed a maximum at 50 °C. The optimal temperatures for phytase activities associated with bifidobacteria cells are in the range of the values (45–70 °C) found for the enzymes of bacterial origin that have been characterized to date [14]. The activities retained by the selected strains at 37 °C, which is the normal corporal temperature, were from 21% to 70%. These results suggest that phytases from bifidobacteria could be partly active in the human gut. In addition, these activities could play a role during food fermentations since temperatures around 37 °C are commonly applied (e.g. sourdough fermentations) [22]. The only exception was \( B. \) globosum that only retained 7% of its optimal activity at 37 °C.

3.4. Effect of the composition of the growth medium on phytase and phosphatase activities

The phosphorous and carbohydrate sources used in the growth medium are some of the known environmental factors that regulate the synthesis of microbial phytases. When \( B. \) bifidobacterium strains were grown in the presence of inorganic phosphate the activity against phytic acid was markedly reduced (45–87%). The synthesis of phytases is generally induced when limiting concentrations of phosphorous are present in the growth medium in yeast, moulds and bacteria [14,31,32]. The concentration of the carbon source (lactose) used in the growth medium used for \( B. \) bifidobacterium strains also had important effects on phytase production (Fig. 2). Most of the studied strains showed the highest specific phytase activity when using high concentrations of lactose (10–20 g L\(^{-1}\)). In contrast, \( B. \) pseudocatenulatum showed maximal specific phytase activity when using the lowest concentration of lactose (5 g L\(^{-1}\)) although it also limited its growth yield (data not shown). The incorporation of different types of carbon source (either lactose or glucose or FOS) to the growth medium also seemed to modulate the synthesis of the enzymes responsible for these activities in \( B. \) bifidobacterium (Fig. 2). \( B. \) angulatum, \( B. \) globosum and \( B. \) longum showed the highest specific activities and growth yields (data not shown), in the presence of 10 g L\(^{-1}\) lactose and \( B. \) adolescentis in the presence of 20 g L\(^{-1}\) lactose. In contrast, \( B. \) pseudocatenulatum showed maximal specific phytase activity in the presence of FOS, which is a prebiotic that also promotes its growth. In addition,
the presence of FOS in the growth medium partly alleviated (55%) the repression caused by the presence of inorganic phosphate on phytase activity in *B. pseudocatenulatum*. However, this effect was not detected for the other selected bifidobacterial strains. Thus, the regulation pattern for these activities varies depending on the considered species. In *B. pseudocatenulatum* limiting concentrations of the carbon source as well as the use of complex oligosaccharides, such as FOS, appeared to induce the synthesis of the activity. Similarly, the carbon source and its concentration are critical factors for phytase production in other bacteria, with glucose at 1–2% (w/v) being normally the preferred substrate [14,31]. Here, carbon sources alternative to glucose have been proved to be more suitable in terms of both growth yields and specific phytase activities for *Bifidobacterium*, probably due to the particular physiological characteristics of this genus.

### 3.5. Evolution of phytase activities and myo-inositol phosphate contents in different culture media and during different growth phases of *B. pseudocatenulatum*

*B. pseudocatenulatum* was selected on the basis of its higher phytase activity for more detailed studies. The evolution of phytase activities as well as the transformation of IP₆ into intermediate degradation products with lower numbers of phosphate groups (IP₃–IP₅) was determined at different growth phases and in media of different composition (Figs. 3 and 4). The production of phytase activity was growth-phase dependent showing a maximum in the exponential phase of growth (9–12 h of incubation) in the three different tested media: (i) the standard modified Garche broth (ii) the standard medium containing inorganic phosphate (Pi) and (iii) the standard medium containing FOS instead of lactose (Fig. 3). The activity levels sharply decreased beyond the optimum, reaching almost constant values after 18–20 h of incubation (Fig. 3(a)). The presence of inorganic phosphate dramatically reduced the production of phytase activity while the addition of FOS, which promotes bacterial growth, increased phytase activity during the incubation period in comparison with the activities found in cells grown in the standard medium (Fig. 3(a)).

The degradation of IP₆ as well as the generation of IP₃–IP₅ was simultaneously monitored (Fig. 4). The growth curves obtained in various media are shown in Fig. 3(b). The lag phase and the time at which maximum growth yields were achieved were longer in cells grown in the medium containing Pi (Fig. 3(b)). This also caused a delay in the time at which the degradation of IP₆ was initiated, although finally the initial IP₆ was completely dephosphorylated in the three tested media (Fig. 4). The first degradation products (IP₂ and IP₄), resulting from the removal of one and two phosphate groups from IP₆, respectively, appeared after 6 h of incubation in the presence of FOS (Fig. 4(c)), after 9 h in the standard medium (Fig. 4(a)) and after 12 h in the presence of Pi (Fig. 4(b)), when a significant reduction in IP₆ levels was also detected (Fig. 4). In FOS containing medium, the degradation of IP₆, IP₃ and IP₄ was completed after 9 h of incubation (Fig. 4(c)) when
maximum phytase activity was also detected in cell suspensions (Fig. 3(a)). By this time, a peak corresponding to IP₃ appeared and was progressively degraded during the incubation time (Fig. 4(c)). The complete degradation of IP₄ and IP₅ was delayed in the standard medium and it was not achieved in that containing Pi, where high levels of IP₃ remained after 25 h of incubation. As anticipated, the presence of Pi drastically reduced the production of phytase activity resulting in a limited degradation of the myo-inositol derivatives with lower numbers of phosphate groups Therefore, the composition of the growth medium is critical to achieve higher and more stable levels of phytase activity allowing the complete degradation of IP₆ to, at least, inositol phosphates with less than three phosphate groups.

In general, the dephosphorylation degree achieved by the produced phytases in a concrete ecosystem is of great importance for mineral bioavailability. Studies carried out in humans and animals indicated that IP₆ and IP₅ are direct inhibitors of iron and zinc absorption and that IP₄ and IP₃ contribute to the negative effect on iron absorption by interacting with higher phosphorylated inositol phosphates. According to these data the complete degradation of IP₆ to at least IP₃ is favourable for improvement of iron absorption from cereal- and soy-containing products [33].

In summary, Bifidobacterium spp. have been demonstrated to possess phytase activities with different catalytic and regulation characteristics, which could contribute to phytic acid degradation during food processing and along the gastrointestinal transit. B. pseudocatenulatum is a especially promising intestinal phytic acid degrading bacteria whose activity is promoted in the presence of prebiotics (FOS). This biochemical property constitutes a novel metabolic trait that, together with the small free fatty acids generated from the metabolism of FOS, could contribute to the improvement of mineral absorption in the intestine. However, recent studies using a phytate-enriched chemostat inoculated with human faecal bacteria did not detect any phytate metabolising activity from Bifidobacterium spp. [21]. Therefore, further studies on the biochemical and

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**Fig. 3.** Evolution of specific phytase activity in *B. pseudocatenulatum* during different phases of growth and medium composition. (a) Specific phytase activities expressed as a percentage of the maximum value obtained, which was rated as 100%; (b) growth curves. Symbols: standard Garche medium (○); standard medium containing Pi (■); standard medium replacing lactose by FOS (▲).
genetic characterization of *B. pseudocatenulatum* enzyme as well as on the application of the producer strain in model systems would be required to progress the understanding of its potential nutritional role.

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