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BACTERIAL PHYTASE: POTENTIAL APPLICATION, *IN VIVO* FUNCTION AND REGULATION OF ITS SYNTHESIS

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REVIEW

ABSTRACT

The stepwise release of phosphate from phytate, the major storage form of phosphate in plant seeds and pollen, is initiated by a class of enzymes that have been collectively called phytases. The classification is solely due to the *in vitro* capability of these enzymes to accept phytate as a substrate. Phytases have been studied intensively in recent years because of the great interest in such enzymes for reducing phytate content in animal feed and food for human consumption. They have a wide distribution in plants, microorganisms, and in some animal tissues. Due to several biological characteristics, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases have considerable potential in commercial applications. In bacteria, phytase is an inducible enzyme and its expression is subjected to a complex regulation, but phytase formation is not controlled uniformly among different bacteria. It was suggested that phytase is not required for balanced growth of bacterial cells, but may be synthesised in response to a nutrient or energy limitation.

Key words: bacterial phytase, biotechnological application, dephosphorylation, occurrence, phytase formation, phytate

INTRODUCTION

During the last 20 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology. These enzymes belong to a special class of phosphomonoesterases [*myo*-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8 and *myo*-inositol hexakisphosphate 6-phosphorylase, EC 3.1.3.26] and are capable of initiating the stepwise release of phosphate from phytate [*myo*-inositol (1,2,3,4,5,6)hexakisphosphate], the major storage form of phosphate in plant seeds and pollen (40).

Phytases were originally proposed as an animal feed additive to enhance the nutritional quality of plant material in feed for simple-stomached animals by liberating phosphate (53). More recently, addition of phytase has been seen as a way to reduce the level of phosphate pollution in areas of intensive animal production. Numerous studies have shown the effectiveness of supplemental microbial phytases in improving utilization of phosphate from phytate (8,29,43,77,87). Therefore, inorganic phosphate supplementation in the diets for simple-stomached animals can be obviated by including adequate amounts of phytase and as a result, the faecal phosphate excretion of these animals may be reduced by up to 50%.

In addition, a biotechnological application of phytase in the food area was taken into consideration (41,96). Because phytate acts as an anti-nutrient by binding to proteins and by chelating minerals (5,63), addition of phytase can improve the nutritional value of plant-based foods by enhancing protein digestibility

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and mineral availability through phytate hydrolysis during digestion in the stomach or during food processing (63,69). Since certain *myo*-inositol phosphates have been proposed to have novel metabolic effects (57,61,71,86), phytases may also find application in food processing to produce functional foods (41).

OCCURENCE

Phytases have a wide distribution in plants, microorganisms, and in some animal tissues (40,84). Recent research has shown that microbial phytases are most promising for a biotechnological application (58,84). Although phytases from several species of bacteria, yeast and fungi have been characterized (40,58,84), commercial production currently focuses on the soil fungus *Aspergillus*. However, due to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases are a real alternative to the fungal enzymes. Phytases have been detected in various bacteria, such as *Pseudomonas* sp. (32,64), *Bacillus* sp. (6,35,38,62,73), *Raoultella* sp. (17,68,70,79), *Escherichia coli* (18), *Citrobacter braakii* (37), *Enterobacter* (93) and anaerobic rumen bacteria, particularly in *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella* sp., *Mitsuokella multiacidus* (92), and *Mitsuokella jalaludinii* (42). With lactic acid bacteria the results were inconsistent; a few strains seem to have a quite low phytase activity, but with the majority of strains the detection of significant phytase activity failed (19,75,78,95). Only very recently it was shown that lactic acid bacteria isolated from sourdoughs exhibit a considerable phytate degrading capacity (9). Among the different lactic acid bacteria strains isolated from sourdoughs, *Lactobacillus sanfranciscensis*, which is considered as a key sourdough lactic acid bacterium, was identified as the best phytase producer.

Generally, the phytases produced by fungi are extracellular, whereas the enzymes from bacteria are mostly cell associated. The only bacteria showing extracellular phytase activity are those of the genera *Bacillus* (6,35,38,62,73) and *Enterobacter* (93). The phytases of *Escherichia coli* have been reported to be periplasmatic enzymes (18) and phytase activity in *Selenomonas ruminantium* and *Mitsuokella multiacidus* was found to be associated with the outer membrane (10).

REGULATION OF PHYTASE FORMATION

In bacteria, phytase is an inducible enzyme and its expression is subjected to a complex regulation, but phytase formation is not controlled uniformly among different bacteria (49). Until now, phytase production was studied in some detail only in *Escherichia coli* (18,80) and *Raoultella terrigena* (17,94). In non-limiting media the formation of both the *Escherichia coli* and the *Raoultella terrigena* phytase was turned off in exponentially growing bacteria and started as soon as the cultures entered the stationary phase. Because the synthesis of the enzymes started as soon as the growth rate began to fall, it was suggested that either a nutrient or an energy limitation, known to occur in the stationary phase, could be at the origin of its induction. Among the nutrient limitations tested, only carbon starvation was able to provoke an immediate synthesis of the *Raoultella terrigena* phytase (17), whereas in *Escherichia coli*, phytase synthesis was triggered, when bacteria were starved for inorganic phosphate, while carbon, nitrogen, and sulfur limitation were ineffective (80). A tight regulatory inhibition of phytase formation by inorganic phosphate levels was generally observed in all microbial phytase producers, including moulds, yeast and bacteria, with the exception of *Raoultella terrigena* and the rumen bacteria (17,92). The repression of phytase synthesis by inorganic phosphate seems to be less significant with higher medium composition complexities. It is not known, however, what components in the complex media account for the reduced repression.

In *Escherichia coli*, the primary response to the limitation of a specific nutrient was shown to be an activation of a certain set of genes that allow a better uptake of the nutrient present in low concentration or the utilisation of other substances that belong to the same class of nutrients. These nutrient-specific systems include the cyclic AMP(cAMP) and its receptor the catabolite activator protein (CAP) for the use of alternative carbon sources, the NtrB/NtrC/σ^{54} regulon that is induced under nitrogen limitation, and the PhoB/PhoR regulon that is induced under phosphorus limitation (26). However, if the environment is totally exhausted for an essential nutrient, the cells enter into the stationary phase. The formation of several dozen proteins is stimulated during transition into stationary phase and a core set of proteins is induced regardless of the class of nutrient for which the cells are starved. As mentioned above, phytase formation in *Escherichia coli* is induced in non-limiting media upon entry into the stationary phase and under anaerobic conditions (18). The expression of the phytase-encoding gene *appA* was shown to be strongly dependent on the *rpoS*-encoded sigma factor σ^S (1), which has been identified as a central regulator for many stationary-phase-responsive genes. Very often σ^S-dependent genes are regulated by several promotors and only one of them is controlled by σ^S. Thus, not all genes identified as σ^S-controlled are entirely dependent on σ^S for expression. In minimal medium, starvation for phosphate, but not for glucose or ammonia, resulted in a strong stimulation of *rpoS* expression followed by an increase in phytase activity.

In addition, phytase expression depends on the nature of the carbon source used for growth. Glucose, which is known to cause catabolite repression, has been widely used to improve phytase production. That cAMP-CAP, rather than the carbon source itself, are directly involved in this regulation was shown in *Escherichia coli* (49). Synthesis of the phytases in both

Escherichia coli and *Raoultella terrigena* have been reported to be negatively regulated by cAMP (80,94), which is suggested to be involved in the amphibolic metabolism of glucose and galactose as well as directly or indirectly in controlling the expression of an important stationary growth regulator. For example, *rpoS* transcription in *Escherichia coli* was described to be negatively controlled by the cAMP-CAP complex (26).

For several *Raoultella* sp. it was reported that phytate is needed to induce phytase production (17,70,79). Substrate induction was also found in *Mitsuokella jalaludinii* (42), whereas phytate had no effect on the formation of phytase in *Escherichia coli* (18). Phytase formation in *Pseudomonas* sp. (32) and *Raoultella aerogenes* (79) was reported to be significantly induced in the presence of *myo*-inositol as the sole carbon source. In the other *Raoultella* sp. studied *myo*-inositol was ineffective (17,70).

IN VIVO FUNCTION OF BACTERIAL PHYTASES

The complex mode of regulation does not shed much light on the role of bacterial phytases. The stationary phase induction suggests that phytase is not required for balanced growth, and that this enzyme may be synthesised in response to a nutrient or energy limitation. This suggestion could also explain why there is, with the exception of sourdough bacteria, no clear evidence for lactic acid bacteria with phytase-producing ability. Lactic acid bacteria are adapted to environments rich in nutrients and energy and therefore, it never may have been an evolutionary selection for lactic acid bacteria with the capability to produce a phytase.

The efficient induction or depression of phytase formation by phosphate starvation in most bacteria (18,40) raises the question of a possible role in providing the cell with phosphate hydrolysed from molecules such as phytate. This hypothesis is, for example, supported by the identification of a phytase in the stalk of *Caulobacter crescentus*, a gram-negative alpha-purple proteobacterium, which is an oligotroph that lives in aquatic environments dilute in nutrients (31). Phosphate is the limiting nutrient in the environments in which *Caulobacte*r is found and one of the hypothesized functions of the stalk is phosphate uptake. Stalks elongate when phosphate is limiting and increasing the surface area available for phosphate uptake as well as the presence of a phytase would allow the uptake of the organically phosphate by the stalk.

In addition, ruminants seem to digest phytate through the action of phytase produced by microbial flora in the rumen (92). In contrast to most other bacteria, anaerobic rumen bacteria are capable of tolerating a high level of phosphate without any negative impact on phytase production. This unique ability leads to a more efficient phytate hydrolysis in the rumen, even under the high phosphate levels in the rumen fluid of ruminants fed concentrated feed. The phosphate generated by splitting of phytate is utilized by both the microbial flora and ruminant host.

Because phytase formation was observed when bacterial cells had to adapt to environmental fluctuations imposed before the onset of growth or when actively growing cells are stressed, it was suggests that phytase could be involved in a signal transduction mechanism of metabolic regulation (94). Several *myo*-inositol phosphates have recently been identified in gram-negative bacteria and are probably involved in signal transduction. For instance, *Salmonella dublin* excretes an *myo*-inositol polyphosphate 4-phosphatase, which contributes to its virulence by subverting cellular *myo*-inositol phosphate signalling reactions (56). Furthermore, two proteins with *myo*-inositol monophosphate phosphatase activity implicated in the control of gene expression have also been reported in *Escherichia coli* (30) and *Rhizobium leguminosarum* (33).

In higher plants phytases occur predominantly in grains, seeds and pollen (40,63). These enzymes were reported to be responsible for phytate degradation during germination to make phosphate, minerals and *myo*-inositol available for plant growth and development (63). A low phytase activity is also found in the plant root (27,47). Root phytase has been suggested as one of the mechanisms of plants to improve utilisation of soil phosphate. Organic forms of phosphate generally account for at least 50% of total soil phosphate and it is known that a major component occurs in from of inositol penta- and hexakisphosphates (65,66). Due to the low phytase activity in roots and the inability of phytase secretion into the rhizosphere, however, phytate appears to be only poorly utilised by plants (25). Thus it was suggested, that soil microorganisms colonising the plant rhizosphere and producing extracellular phytase activity, such as *Bacillus* and *Enterobacter* ssp., could act as plant growth promoting rhizobacteria (PGPR) by making phytate phosphate available to the plant (28). Recently, the importance of phosphate availability from soil phytate for plant nutrition under phosphate limitation was demonstrated by enabling the plants to utilise phytate phosphate through expression of extracellular secreted *Aspergillus* phytase in the plant root (65), and by adding of purified phytase as well as soil microorganisms expressing extracellular phytase activity to the rooting medium (28,66).

BIOTECHNOLOGICAL APPLICATION

Phytases are of great interest for biotechnological applications, in particular for the reduction of phytate content in feed and food (44,84). Depending on the application, a phytase in which there is commercial interest should fulfil a series of quality criteria. Enzymes used as feed additives should be effective in releasing phytate phosphate in the digestive tract, stable to resist inactivation by heat from feed processing and storage, and cheap to produce. Thermostability is a particularly important issue since

feed pelleting is commonly performed at temperatures between 65 and 95ºC. Although phytase inclusion using an after-spray apparatus for pelleted diets and/or chemical coating of phytase may help bypass or overcome the heat destruction of the enzyme, thermostable phytases will no doubt be better candidates for feed supplements. Naturally-occurring phytases having the required level of thermostability for application in animal feeding have not been found in nature thus far (44). Thermostability of phytases is therefore still a major concern for animal feed applications. Therefore, it comes as no surprise that isolation and characterization, and engineering of thermostable enzymes, as well as the search for the determinants of thermostability, are hot spots of current research (38,45,59). Likewise, an enzyme that can tolerate long-term storage or transport at ambient temperatures is undisputedly attractive.

The ability of a phytase to hydrolyse phytate in the digestive tract is determined by its enzymatic properties. As the stomach is the main functional site of supplemental phytase, an enzyme with a acidic pH optimum and high resistance to pepsin is certainly desirable. Up to now, two main types of phytases have been identified; acid phytases with an pH optimum around pH 5.0 and alkaline phytases with an pH optimum around pH 8.0 (40). Most of the so far described microbial phytases belong to the acidic ones and their pH optima range from 4.0 to 5.5. They are encoded by genes evolved from acid phosphatases and share the highly conserved sequence motif RHGxRxP considered to be the phosphate acceptor site near the N-terminus (83). In addition, they contain near the C-terminus a conserved HD-motif where the aspartate is proposed to be the proton donor for the substrate leaving group. All these phytases belong to the subfamily of histidine acid phosphatases and do not need any co-factor for optimal activity.

Some bacterial phytases, especially those of the genera *Bacillus* and *Enterobacter*, exhibit a pH optimum in the range from 6.0 to 8.0 (6,35,38,62,73,93). Therefore, they would be more beneficial as feed additives for poultry as their pH optimum is close to the physiological pH of the poultry crop. The phytase-encoding genes isolated from the genera *Bacillus* do not have homology to the sequences of any other phosphatase listed in the databases (16,35,39). Even the putative active site motifs RHGxRxP and HD found in histidine acid phosphatases are absent. They seem to be evolved from an alkaline *Bacillus* phosphatase ancestor. These enzymes have a six-bladed-propeller folding architecture with six calcium-binding sites in each protein molecule (74). Binding of three calcium ions to high-affinity calcium binding sites results in a dramatic increase in thermostability by joining loop segments remote in the amino acid sequence. Binding of three additional calcium ions to low-affinity calcium binding sites at the top of the molecule turns on the catalytic activity of the enzyme by converting the highly negatively charged cleft into a favourable environment for the binding of phytate.

It was shown that the phytases of *Escherichia coli* and *Citrobacter braakii* are more resistant to pepsin and pancreatin than the commercially available *Aspergillus niger* phytase (37,67). In addition, the phytase of *Citrobacter braakii* was resistant to trypsin (67). Compared to the phytase from *Escherichia coli*, the corresponding enzymes from *Bacillus* exhibit a similar sensitivity to pancreatin, but a much higher susceptibility to pepsin (76). The phytases of *Escherichia coli* and *Citrobacter braakii* display further favourable characteristics for its use as a feed additives. They exhibit k_{cat}/K_M values, which validates the kinetic efficiency of an enzyme for a given substrate, of 1.34×10^7 M^{-1} s^{-1} for the *Escherichia coli* (13) and 1.03×10^7 M^{-1} s^{-1} for the *Citrobacter braakii* enzyme (37), which are the highest values reported for phytases. The *Escherichia coli* phytase has furthermore an exceptional high pH stability under acidic conditions. Even exposure at pH 2.0 for several hours did not result in a significant loss of activity (18).

The effectiveness and limitations of phytase supplementation may also depend on substrate specificity. *In vitro* feed experiments suggest that phytases with broad substrate specificity are better suited for animal nutrition purposes than phytases with narrow substrate specificity (88). Phytases with a broad substrate specificity readily degrade phytate to *myo*-inositol monophosphate with no major accumulation of intermediates, whereas phytases with narrow substrate specificity resulted in *myo*-inositol tris- and bisphosphate accumulation during phytate degradation coupled with a progressive rate of phosphate release. So far, only a few phytases have been described as highly specific for phytate such as the alkaline phytases from *Bacillus subtilis* (62,73) and *Bacillus amyloliquefaciens* (38). Furthermore, the acid phytases from *Escherichia coli* (18), *Raoultella terrigena* (17), *Aspergillus niger* and *Aspergillus terreus* (88) have been reported to be rather specific. Unfortunately, broad substrate specificity currently must be paid for dearly in that it is coupled with low specific activity. In the future it will be a challenge to combine high specific phytase activity with broad substrate specificity.

Finally, a phytase will not be competitive if it cannot be produced in high yield and purity by a relatively inexpensive system. Recently, economically competitive expression and/or secretion systems for microorganisms have been developed (50,52,90). Reduction of phytate levels or increase in phytase activity in the plant seed itself are alternative strategies for improving nutrient management in animal production. Increased phytase activity in the plant seed by heterologous expression of a fungal phytase was already achieved and it was shown that only limited amounts of transgenic seeds are required in compound feeds to ensure proper degradation of phytate (4,46,60,81). A different strategy to overcome the problems using phytases as a feed additive such as cost, inactivation at the high temperatures required for pelleting feed, and loss of activity

during storage, might be to add those enzymes to the repertoire of digestive enzymes produced endogenously by swine and poultry. In the meantime, swine were generated with a gene from *Escherichia coli* for the production of a phytase in the salvia (11,12). It was shown that provision of salivary phytase activity enables essentially complete digestion of dietary phytate, largely relieving the requirement for phosphate supplementation, and reduces faecal phosphate output by up to 75%. This reduction even exceeds the 40% reduction reported for pigs fed expensive phytase supplements.

Since it is evident that the inability of plants to utilize phosphate from soil phytate is associated with a lack of extracellular phytase activity, an opportunity exists for using gene technology to improve the ability of plants to utilise phytate phosphate. Extracellular secretion of a phytase from *Aspergillus niger* by plant roots was shown to enable the plants to obtain phosphate from soil phytate (65). A more effective utilization of phosphate from soil and fertiliser sources would be particularly beneficial to agriculture throughout the world.

The food industry may also be interested in using phytases; on the one hand to improve mineral bio-availability by reducing phytate content of a given food, on the other hand to produce functional foods. Certain *myo*-inositol phosphates have been suggested to have beneficial health effects, such as reducing the risk of heart disease (34,61), renal stone formation (54,57), and certain type of cancers (3,14,71,72,82,85,86,91). The number and position of the phosphate groups on the *myo*-inositol ring is thereby of great significance for their physiological functions. Independent of their bacterial, fungal or plant origin, the acid phytases studied so far in respect to phytate degradation release five of the six phosphate groups of phytate, and the final degradation product was identified as *myo*-inositol (2) monophosphate (7,15,20,21,22,24,48,51,55,89). This indicates that all those phytases have a strong preference for equatorial phosphate groups, whereas they are virtually unable to cleave the axial one. Only in rare cases traces of free *myo*-inositol were detected. On the other hand, the alkaline phytases are not capable of accepting a *myo*-inositol phosphate with three or fewer phosphate residues as a substrate (2,23,36). Thus, a *myo*-inositol trisphosphate isomer is the end product of phytate degradation by these alkaline phytases. Depending on the phytase used, different phosphate residues of phytate may be released at different rates and in different order. Therefore, every phytase might be useful in the production of foods with a regulated content and composition of *myo*-inositol phosphate with beneficial health effects.

FINAL REMARKS

Due to several biological characteristics, bacterial phytases have considerable potential in commercial and environmental applications. The *Escherichia coli* phytase for example displays several favourable characteristic for its use as an feed supplement, an acidic pH optimum close to the physiological pH of the stomach of pigs and chicken, high pH stability under acidic conditions, high catalytic efficiency, greater resistance to pepsin than the commercially available *Aspergillus niger* phytase. It was already shown in *in vivo* experiments, that the *Escherichia coli* phytase enhanced the availability of phosphate from phytate at the same range or even slightly better than the commercially available *Aspergillus* phytase (29). For plant transformation, however, the highly specific *Bacillus* phytases seem to be the best candidates, because a phytase with a broad substrate specificity introduced into plants might disturb several metabolic pathways of plant cells. Thus, it is important to realise that any single phytase may never be able to meet the diverse needs for all commercial and environmental applications. Therefore, there is ongoing interest in screening microorganisms, including bacteria for novel and efficient phytases.

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