Analysis of *myo*-inositol hexakisphosphate hydrolysis by *Bacillus* phytase: indication of a novel reaction mechanism

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Phytic acid (*myo*-inositol hexakisphosphate, InsP_6) hydrolysis by *Bacillus* phytase (PhyC) was studied. The enzyme hydrolyses only three phosphates from phytic acid. Moreover, the enzyme seems to prefer the hydrolysis of every second phosphate over that of adjacent ones. Furthermore, it is very likely that the enzyme has two alternative pathways for the hydrolysis of phytic acid, resulting in two different *myo*-inositol trisphosphate end products: Ins(2,4,6)P_3 and Ins(1,3,5)P_3. These results, together with inhibition studies with fluoride, vanadate, substrate and a substrate analogue, indicate a reaction mechanism different from that of other phytases. By combining the data presented in this study with (1) structural information obtained from the crystal structure of *Bacillus amyloliquefaciens* phytase [Ha, Oh, Shin, Kim, Oh, Kim, Choi and Oh (2000) Nat. Struct. Biol. **7**, 147–153], and (2) computer-modelling analyses of enzyme–substrate complexes, a novel mode of phytic acid hydrolysis is proposed.

Key words: HPLC, metal-dye detection, phytic acid.

INTRODUCTION

The hydrolysis of phosphomonoesters in biological systems is an important process. It is linked to energy metabolism, metabolic regulation and a wide variety of cellular signal transduction pathways [1]. The biological hydrolysis of a wide variety of phosphomonoesters is catalysed by a diverse group of enzymes: the phosphatases. These enzymes can be classified as alkaline phosphatases, purple acid phosphatases, high-molecular-mass acid phosphatases, low-molecular-mass acid phosphatases and protein phosphatases; the classes differ in their pH optima, substrate specificities, metal-ion requirements and even reaction mechanisms [1]. Perhaps the most obvious difference is the presence or absence of metal-ion cofactors. Alkaline phosphatases contain two Zn^{2+} ions and one Mg^{2+} ion per enzyme subunit. The two Zn^{2+} ions form a binuclear centre bridged by the product phosphate, whereas the Mg^{2+} ion is not directly in contact with the phosphate. A base-labile, acid-stable phosphoserine intermediate has a critical role in alkaline phosphatases. For a plausible reaction mechanism of alkaline phosphatases see the review by Coleman [2]. Purple acid phosphatases also use metal-ion cofactors to catalyse the hydrolysis of phosphomonoesters. The mammalian purple acid phosphatases harbour a binuclear iron centre, whereas plant enzymes have an iron–zinc centre. A plausible, although not exclusive, role of these centres is to provide the hydroxide nucleophile for displacing phosphate from the phosphoryl intermediate [1]. Acid phosphatases lack the metal-ion cofactors. In general, acid phosphatases hydrolyse monophosphate esters by a two-step mechanism. This includes a covalent phosphoryl enzyme (E-PO_3^-) and a non-covalent $E \cdot P_i$ complex. There is substantial evidence for base-stable, acid-labile phosphohistidine as the phosphorylated intermediate [3,4].

Despite the abundance and divergence of phosphatases, these enzymes are virtually unable to hydrolyse phosphomonoesters in phytic acid (*myo*-inositol hexakisphosphate, InsP_6). The salt form, phytate, is the most abundant source of phosphate in plants, accounting for up to 80% of total phosphorus in cereals and legumes [5]. Because the hydrolysis of phytic acid is of great importance in biological systems, a specific class of enzymes hydrolysing phytic acid has evolved: the phytases. These enzymes hydrolyse phytic acid to less phosphorylated *myo*-inositol phosphates (in some cases to free *myo*-inositols), releasing P_i. Phytase is widespread in Nature, occurring in micro-organisms, plants and some animal tissues [6]. The role of phytase in each of these organisms is different. In plants, phytase is induced during germination to provide the growing seedling with P_i and the plant with concomitant free *myo*-inositol, which is an important growth factor [5]. The hydrolysis of phytate also releases cations, such as K^+, Mg^{2+}, Zn^{2+} and Ca^{2+} bound to phytic acid. The role of *myo*-inositol phosphate intermediates in the transport of materials into the cell has also been established. In particular, *myo*-inositol trisphosphates are important in transport as secondary messengers and in signal transduction in plants [7,8]. In micro-organisms, phytase is most frequently induced in response to phosphate starvation so as to release phosphate from surrounding plant-derived phytic acid for the use of the organism. The role of phytases in animal cells is more obscure. However, a phytase-like enzyme, multiple inositol polyphosphate phosphatase (MIPP), has the capacity to regulate the cellular activities of phytic acid and Ins(1,3,4,5,6)P_5 [9]. Furthermore, MIPP-generated downstream metabolites, e.g. Ins(1,4,5)P_3, are themselves physiologically active as a Ca^{2+}-mobilizing signal [10]. The evolutionary conservation of MIPP within the inositol phosphate pathway suggests a significant role for MIPP throughout higher eukaryotes [11].

Several phytases have been cloned and characterized, including fungal phytases from *Aspergillus niger* [12], bacterial phytase from *Escherichia coli* [13] and a mammalian phytase [9]. These enzymes do not show any apparent sequence similarity to each other. However, they share a highly conserved sequence motif, RHGXRXP (single-letter amino acid codes), which is found at the active sites of acid phosphatases [14]. Furthermore, they contain a remote C-terminal His-Asp motif (HD motif) that is likely to take part in the catalysis. Phytases are therefore said to form the phytase subfamily of histidine acid phosphatases [15].

Abbreviations used: MDD, metal-dye detection; MIHS, *myo*-inositol hexasulphate; MIPP, multiple inositol polyphosphate phosphatase; PhyC, *Bacillus* phytase.

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The reaction mechanism of *E. coli* phytase has been revealed [4,16]. On the basis of these results the following reaction mechanism for phytase is suggested: (1) the positive charge of the guanido group of the arginine residue in the tripeptide RHG interacts directly with the phosphate group in the substrate making it more susceptible to nucleophilic attack, (2) the histidine residue serves as a nucleophile in the formation of a covalent phosphohistidine intermediate, and (3) the aspartic residue from the C-terminal HD motif protonates the substrates leaving group.

Highly thermostable phytases have been isolated and cloned from *Bacillus* species independently by two groups [17–19]. These two enzymes are virtually identical: both contain 383 amino acid residues with over 90% sequence identity. They do not align with any other phytases or phosphatases in the sequence databank, nor do they contain the conserved active-site motif RHGXRXP. Unlike other phytases, these enzymes are dependent on Ca^{2+} ions for stability and activity [17,18]. These divergences imply a different mode of hydrolysis of phytic acid.

To expand our knowledge of the catalytic mechanism of *Bacillus* phytase (PhyC), we studied the hydrolytic pathway, reaction intermediates and end product of the enzyme. On the basis of the results presented here, on the three-dimensional structure of *Bacillus amyloliquefaciens* phytase [20] and on the analyses of computer models of enzyme–substrate complexes, we suggest a novel mode of hydrolysis of phytic acid.

EXPERIMENTAL

Source of PhyC

The phytase gene (*phyC*) [17] from *B. subtilis* strain VTT E-68013 (Culture Collection of Technical Research Center of Finland) was expressed in *B. subtilis* strain BD170 (*Bacillus* Genetic Stock Center; Dr D. R. Zeigler, Department of Biochemistry, Ohio State University, Columbus, OH, U.S.A.) (J. Kerovuo, unpublished work). The PhyC was purified to homogeneity by precipitation with $(NH_4)_2SO_4$ [17].

Assays of phytic acid hydrolysis

The spectrum of inositol phosphates generated from phytic acid and the total P_i released from phytic acid were determined. In inhibition studies, various concentrations of inhibitor were added to the enzyme reaction. Samples were withdrawn at different time points and reactions were stopped by adding equal volumes of trichloroacetic acid (5%, w/v). For a time-course study of phosphate release, and for inhibition studies, the enzymic activity was measured as described by Shimizu [21]. For metal-dye detection (MDD)-HPLC analysis the samples stopped with trichloroacetic acid were centrifuged and supernatants were treated with active charcoal to remove remaining phytase enzyme. Samples corresponding to 8 nmol of phytic acid at zero time were applied to the HPLC column. All assays were run in duplicate.

Separation of inositol phosphates by HPLC

The isomer identity of inositol phosphates generated from phytic acid hydrolysis was investigated with post-column MDD-HPLC [22]. MDD-HPLC was performed as follows: acidic elution was performed on a chemically inert HPLC system (10Avp-series; Shimadzu, Kyoto, Japan) equipped with Resource Q (1 ml) Mono Q HR 5/20 column configuration (Pharmacia, Uppsala, Sweden). Samples were applied automatically and an LC-10Ai pump delivered solvent A (0.2 mM HCl/15 μM YCl_3) and solvent B (0.5 M HCl/22.5 μM YCl_3) at 1 ml/min. Inositol phosphates were separated on a concave gradient: 0 min, 10% B; 10 min, 14.5% B; 23 min, 28% B; 31 min, 44% B; 35 min, 60% B; 40 min, 90% B; 43 min, 100% B; 62 min, 100% B. Detection of inositol phosphates was performed by mixing PAR reagent [300 μM 2-(4-pyridylazo)resorcinol/1.6 M triethanolamine, made to pH 9.0 with HCl] at 0.55 ml/min into the eluent. Compound detection at 546 nm was performed with a photodiode-array detector (SPD-M10Avp).

The assignment of inositol phosphate isomers was performed by comparing peak retention times in chromatographic profiles of unknown samples with the corresponding peaks in a profile of a chemical hydrolysate of phytic acid. The elution order of peaks in chemical hydrolysates of phytate was established by Mayr [22].

RESULTS

Phytic acid degradation: kinetics and intermediates

As a first step towards understanding the reaction mechanism of PhyC, a time-course analysis of P_i release from phytic acid was performed (Figure 1). The reaction, which was essentially complete after 60 min, yielded a ratio of product (P_i) to substrate (phytic acid) very close to 3, indicating that three phosphate groups per molecule of phytate were released. This strongly suggested that phytate degradation by the PhyC generated an inositol trisphosphate as an end product.

For further analysis of the degradation mechanism, an additional time-course study was performed to identify phytic acid degradation products by isomer-specific MDD-HPLC analysis (Figure 2). With this system, the assignment of isomer identities can be performed with a chemical hydrolysate of phytic acid as a reference. The chromatographic profile of the zero-time control indicated the substrate peak (phytic acid, peak I) and a trace amount of D,L-Ins(1,2,4,5,6)P_5 (peak H). After 10 min of incubation, D,L-Ins(1,2,4,5,6)P_5 (peak H) appeared as the major degradation product, accompanied by small amounts of D,L-Ins(1,2,3,4,5)P_5 (peak G), Ins(1,2,3,4,6)P_5 (peak F), Ins(2,4,5,6)P_4 (peak E) and Ins(1,2,3,5)P_4 (peak C). After 30 min of incubation, the quantities of phytic acid and D,L-Ins(1,2,4,5,6)P_5 had decreased and the levels of Ins(2,4,5,6)P_4 (peak E) and Ins(1,2,3,5)P_4 (peak C) had increased. Finally, after 90 min of incubation, only one major inositol trisphosphate peak remained (peak A), indicating Ins(1,3,5)P_3 and/or Ins(2,4,6)P_3 as the end product(s) of phytic acid degradation by PhyC. A small shoulder of peak A (peak B) suggested the presence of minor amounts of D,L-Ins(1,4,5)P_3.

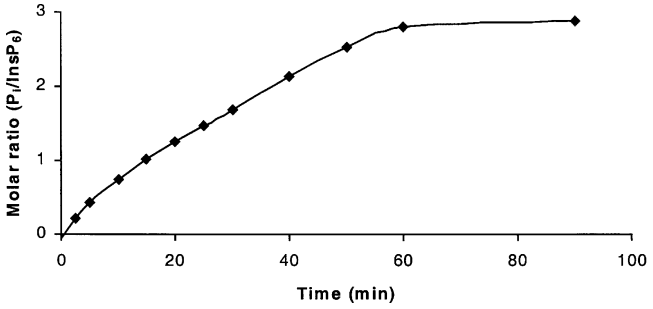


Figure 1 Time course of release of P_i from phytic acid by PhyC

The molar amount of P_i released/mol of phytic acid is shown. After 60 min, approx. 3 mol of P_i is released/mol of phytic acid. Prolonged incubation does not release more phosphate, indicating that PhyC hydrolyses three of six phosphate groups in phytic acid.

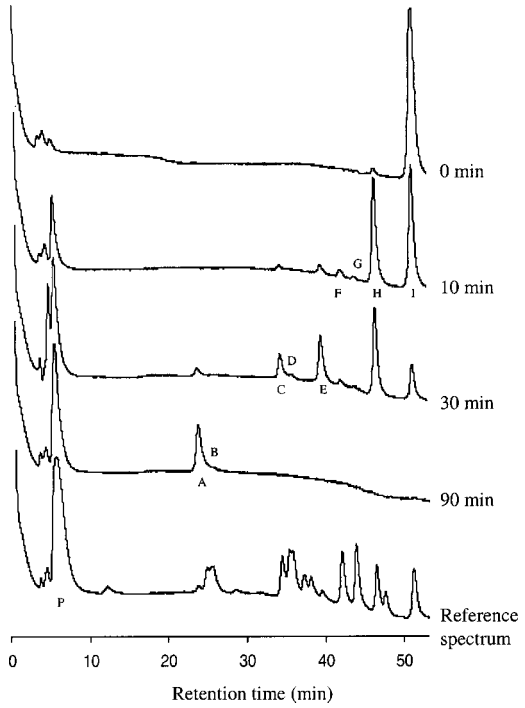


Figure 2 Isomer assignment of the inositol phosphates generated by PhyC

Superposed chromatograms of the reaction products obtained from phytic acid after 0, 10, 30 and 90 min of incubation are shown. The reference spectrum of the assigned peaks [22] is at the bottom. The peaks were identified as follows: A, Ins(1,3,5)P_3/Ins(2,4,6)P_3; B, D,L-Ins(1,4,5)P_3; C, Ins(1,2,3,5)P_4; D, D,L-Ins(1,2,4,5)P_4; E, Ins(2,4,5,6)P_4; F, Ins(1,2,3,4,6)P_5; G, D,L-Ins(1,2,3,4,5)P_5; H, D,L-Ins(1,2,4,5,6)P_5; I, phytic acid; P, P_i. The D,L prefix is used to indicate that the HPLC system could not discriminate between the stereoisomers. See the text for details.

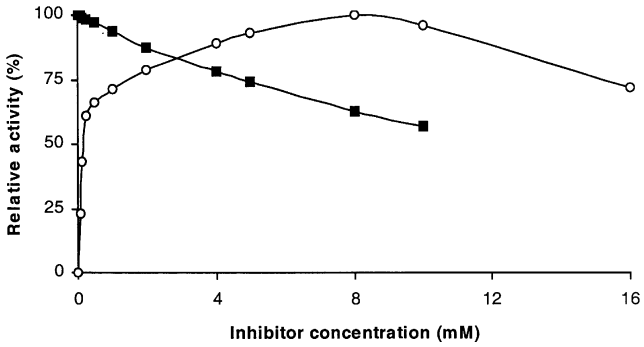


Figure 3 Effects of vanadate (■) and phytic acid (○) concentrations on PhyC activity

Fluoride and MIHS had no effect on PhyC in the concentration ranges tested (0–10 mM and 0–2 mM respectively).

Inhibition studies

Apart from degradation kinetics and intermediates we also studied the inhibitory effect of 'classic' phytase/phosphatase activity inhibitors such as fluoride, vanadate and phytic acid (Figure 3). Vanadate was found to be a relatively weak inhibitor of PhyC activity, whereas inhibition by fluoride was not observed at all in the concentration range 0–10 mM. *myo*-Inositol hexasulphate (MIHS), a structural analogue of the substrate, was not an inhibitor of PhyC; no effect on enzyme activity was detected in the concentration range 0–2 mM. The PhyC is relatively insensitive to otherwise very potent phytase/phosphatase activity inhibitors and is not prone to competitive product inhibition of phytic acid hydrolysis caused by P_i.

Computer modelling studies

To obtain a view of substrate binding, the phytic acid molecule was manually docked to the active site by using the crystal structure of the PhyC phytase determined at 2.0 Å resolution (T. Parkkinen, J. Kerovuo and J. Rouvinen, unpublished work). Figure 4 shows the position of the substrate. Phytic acid seems to bind to the enzyme sideways on. One phosphate group (in the 3-or the 1-position) is oriented inside the active site and its neighbouring phosphate groups would also bind to the enzyme. In the right-hand panel of Figure 4 the amino acid residues that might take part in substrate binding or catalysis are marked.

DISCUSSION

Inhibition characteristics indicate a robust enzyme

Fluoride and vanadate are known inhibitors of different phytases and phosphatases [23–27]. It is suggested that vanadate, as a transition-metal oxoanion, inhibits phosphomonoesterases by forming complexes resembling the trigonal bipyramidal geometry of the transition state [28]. Neither fluoride nor vanadate had a significant effect on the activity of PhyC. MIHS is a potent competitive inhibitor of both the phyA and phyB enzymes of *Aspergillus ficuum* (K_i values of 4.6 and 0.2 μM respectively) [29]. The finding that MIHS does not inhibit the PhyC activity indicates a reaction mechanism different from that of other phytases.

Substrate inhibition at relatively low concentrations is common to phytases [30–33]. This is probably due to competitive inhibition by product, as recognized for bacterial, fungal and oat spelt phytase [13,34,35]. PhyC was inhibited by less than 30% at the highest substrate concentration tested (16 mM). Because the enzyme cleaves three phosphates from phytic acid, the P_i concentration at the end of the reaction at 16 mM substrate would be close to 48 mM. PhyC is therefore not prone to competitive inhibition by product.

P_i also inhibits phosphatases that use a non-covalent $E \cdot P_i$ complex as an intermediate [1]. The active site in these enzymes is highly constrained and is barely large enough to accommodate the phosphate moiety [36]. This leaves the remainder of the substrate exposed to solvent and explains the poor substrate specificity. Fungal phytases exhibit poor substrate specificity. However, PhyC is highly specific for phytic acid [17,18]; this fact and the results obtained from the inhibition studies indicate that the active site is not arranged as in other phytases (or phosphatases). It is likely that the enzyme's active site is formed to accommodate most of the substrate, not just the phosphate moiety. The computer modelling studies supported this presumption and are discussed below.

Inositol trisphosphate(s) as the end products of a dual degradation pathway

On the basis of the data in Figure 2, the following hydrolysis pathway is suggested (pathway I): the enzyme removes phosphates stepwise from the 3-position and the 1-position. Whether the hydrolysis sequence is 3/1 or 1/3 cannot be distinguished from the data. However, the outcome is the same: the formation

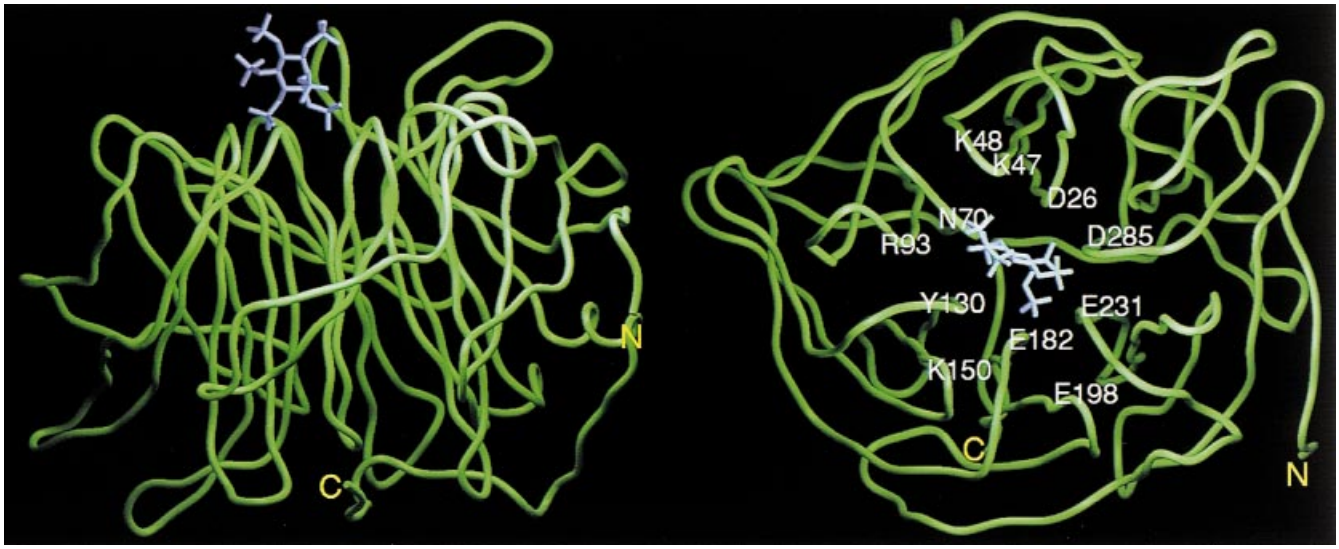


Figure 4 Computer model of binding of phytic acid to the active site of PhyC

The model is shown in two orientations. The phytic acid molecule is in purple and the main chain of the enzyme is in green. The Ca^{2+} ions are not shown. The putative residues for the substrate binding or catalysis are marked on the right panel. The residues are numbered in accordance with the mature protein. The docking was performed manually with the program XtalView [40] and the pictures were created with the program SETOR [41].

of Ins(2,4,5,6)P_4. It is likely that after the hydrolysis of the first phosphate the substrate is released from and rebound to the active site, with the concomitant hydrolysis of the second phosphate, to yield Ins(2,4,5,6)P_4. Removal of the phosphate at the 5-position from Ins(2,4,5,6)P_4 clearly results in the formation of the end product Ins(2,4,6)P_3. However, the formation of an Ins(1,2,3,5)P_4 intermediate [and an Ins(1,3,5)P_3 end product] cannot be explained by a stepwise hydrolysis of the 3-phosphate and the 1-phosphate. A plausible explanation might be an alternative pathway (pathway II): Ins(1,2,3,5)P_4 is formed by the stepwise hydrolysis of phosphates in the 6-position and the 4-position. Again, the hydrolysis sequence (6/4 or 4/6) is not discerned. However, in contrast with Ins(2,4,5,6)P_4 formation, no preceding InsP_5 intermediate accumulates. Only small amounts of D,L-Ins(1,2,3,5,6)P_5 and/or D,L-Ins(1,2,3,4,5)P_5 are detected. This suggests that substrate remains tightly bound to the enzyme until the phosphates in the 6-position and the 4-position have been removed. After the dephosphorylation of these two phosphates, the Ins(1,2,3,5)P_4 reaction intermediate is released. This reaction intermediate binds to the enzyme, again with concomitant hydrolysis of the phosphate in the 2-position, resulting in the formation of the Ins(1,3,5)P_3 end product.

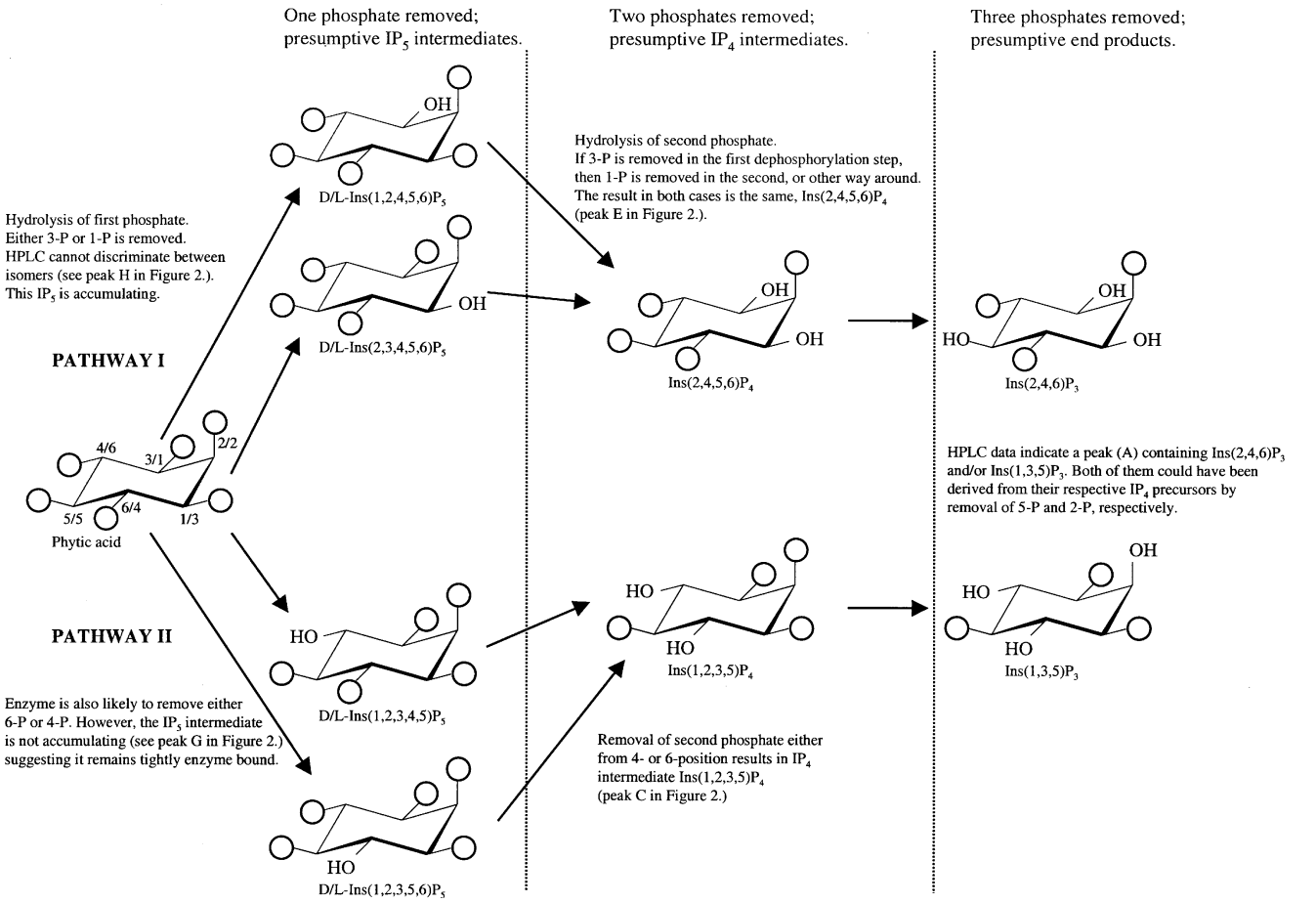
Characteristic for the formation of both InsP_4 isomers and the concomitant InsP_3 end products is the hydrolysis of every second phosphate instead of adjacent ones. The presumptive hydrolysis pathways of phytic acid by PhyC are presented in Scheme 1.

The other phytases yielding *myo*-inositol trisphosphate as the end product are the alkaline phytase from lily pollen [Ins(1,2,3)P_3] [26], the phytase from *Typha latifolia* pollen (stereoisomer not determined) [37], and the rat hepatic MIPP [Ins(1,4,5)P_3] [9]. Most fungal phytases hitherto characterized hydrolyse phytic acid to *myo*-inositol monophosphate [38]. The studies on *B. subtilis* phytase indicated that *myo*-inositol monophosphate is the end product of phytic acid hydrolysis [39]. However, it could be that the enzyme preparation used in that study contained some non-specific phosphatase that hydrolysed the formed *myo*-inositol trisphosphates to *myo*-inositol monophosphate during prolonged incubation.

Indications of a novel mode of phytic acid hydrolysis

PhyC is significantly different from other phytases [17–19]. The results presented in this study further suggest a novel mode of hydrolysis. Strong evidence for this suggestion was provided recently when the crystal structure of the *B. amyloliquefaciens* phytase was published [20]. The structure reveals a new folding architecture of a six-bladed propeller for phosphatase activity. There are six Ca^{2+} ions in the enzyme: three have high-affinity binding sites and are needed for enzyme stability; the three low-affinity ions regulate the catalytic activity of the enzyme. These Ca^{2+} ions turn the predominantly negatively charged shallow cleft into a favourable electrostatic environment for the binding of phytic acid. Ha et al. [20] suggest that the low-affinity Ca^{2+} sites comprise the active site and are responsible for the Ca^{2+}-dependent catalytic activity of PhyC. They further suggested that three Ca^{2+} ions in the active site participate directly in the catalysis by binding the phosphate group(s) of the substrate and stabilizing the quinquivalent transition-state intermediate.

On the basis of computer-modelled substrate docking, and by combining the enzyme–substrate model with the phytic acid hydrolysis results, we suggest the following mode of action: the substrate binds to the shallow cleft sideways by using the phosphate groups in the 2- and 4-positions, for example. These phosphates are likely to bind to Ca^{2+} ions and to positively charged amino acid residues present in the cleft. The phosphate moiety in the middle (in this case the one in the 3-position) would then face the active site, as shown in Figure 4, and could consequently be cleaved. The Ins(1,2,4,5,6)P_5 intermediate thus formed would then be released from the enzyme. The Ins(1,2,4,5,6)P_5 intermediate could then bind to the cleft again, now using the phosphate groups in the 2- and 6-positions for binding. The phosphate in 1-position would then face the active



Scheme 1 Scheme of the hydrolysis pathway(s) of PhyC

Carbon atoms in the inositol ring are numbered for the D,L-configuration (D/L respectively). Presumptive reaction intermediates and end products are referred to the corresponding assigned peaks in Figure 2.

site and could consequently be cleaved, resulting in the formation of Ins(2,4,5,6)P_4. Analogously, the phosphate in the 5-position would be cleaved, resulting in the formation of Ins(2,4,6)P_3. The Ins(2,4,6)P_3 could still bind to the enzyme but there would be no more phosphate groups available to face the active site, so Ins(2,4,6)P_3 would be the end product of hydrolysis by phytic acid. The reaction intermediates might compete for the active site with substrate, as they might bind in such a way that no phosphate groups would face the active site. Therefore, the intermediates (and the end products) would slow down the enzyme reaction. This was actually observed: a slower conversion of these compounds to lower *myo*-inositol phosphates occurred than with phytic acid (results not shown).

A question remaining is the formation of two different InsP_4 reaction intermediates and the resulting two different end products, Ins(2,4,6)P_3 and Ins(1,3,5)P_3. Phytases are generally divided into 3- and 6-phytases (the position of the phosphate cleaved first). Furthermore, the cleavage of an axial phosphate in the 2-position is very uncommon in phytases. The formation of the Ins(2,4,6)P_3 and Ins(1,3,5)P_3 end products would require hybrid 3-/6-phytase activity, including the cleavage of an axial phosphate in the 2-position in the latter case. A plausible explanation for the formation of two different end products and for the cleavage of an axial phosphate is that the enzyme could twist the inositol ring of the bound substrate into a more planar conformation. Thus the concept of axial and equatorial phosphate would be diminished and substrate could bind in two ways: either with phosphates in the 2-, 4- and 6-positions or phosphates in the 1-, 3- and 5-positions to dock to the substrate-binding pocket. The first binding and hydrolysis step would determine the end product of hydrolysis. The phosphate groups in the substrate used in docking to the substrate-binding pocket are those remaining in the InsP_3 end product: Ins(2,4,6)P_3 and Ins(1,3,5)P_3 respectively. Thus it could be said that the enzyme has both 3- and 6-phytase activities. However, the 3- and 6-phosphates are not cleaved from the same molecule. The difference between these two pathways is that in pathway II the InsP_5 intermediate is not released from the enzyme, because it does not accumulate. The non-accumulation of InsP_5 intermediate is probably not due to a rapid Ins$P_5 \rightarrow$ InsP_4 conversion because the InsP_4 intermediates [Ins(2,4,5,6)P_4 and Ins(1,2,3,5)P_4] evolve and diminish at the same rate (although not in the same amounts; results not shown). Therefore in pathway II the InsP_5 intermediate probably remains bound to the enzyme until the second phosphate has been cleaved. The cleavage of Ins(1,2,3,5)P_4 to Ins(1,3,5)P_3 further indicates the preference for alternate phosphate groups, even over an equatorial phosphate.

An important feature of the structure of PhyC seems to be the tunnel passing through the enzyme. The tunnel leads exactly to the cleavable phosphate moiety (see Figure 4) in the active site. There are water molecules present in the tunnel in the crystal structure [20]. The cleaved phosphate could be transferred away from the enzyme through that tunnel.

To confirm the mode of action suggested here, work is continuing on complex crystals of PhyC with the end products, and also by means of mutagenesis studies and kinetic studies with supplemented reaction intermediates and end products.

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