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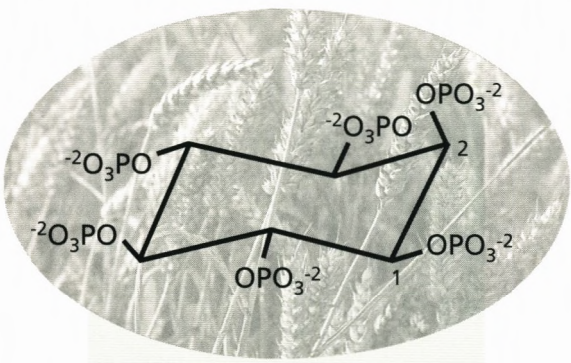

Henrik Brinch-Pedersen

Phytate and Phytase in Plants
(Review)

Ministry of Food, Agriculture and Fisheries
Danish Institute of Agricultural Sciences

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(Review)

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Preface

Being the major storage compound of phosphorus in plants, phytic acid (phytate, *myo*-inositol 1,2,3,4,5,6- hexa*kis*phosphate) is of special importance in plant biology. Plant tissues with storage function such as seeds, tubers, pollen and roots contain large amounts of this acid.

The enzymes responsible for the initial steps in the degradation of phytic acid are referred to as phytases. They comprise a special class of phosphatases that catalyse a sequential hydrolysis of phytic acid to lower inositol phosphates and, in some cases, *myo*-inositol.

The present review describes the present state of knowledge within the field of phytic acid biosynthesis and deposition, phytase enzymes from different organisms, the degradation of phytic acid mediated by phytase, the role of phytate and phytase in nutrition, and the available evidence on the performance and biological significance of phytase as an additive to animal feed or as a heterologous enzyme in transgenic plants.

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Research Centre Flakkebjerg
February 2000

Contents

The Storage of Phosphorus in Plants

Phytic acid (phytate, *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate) (Figure 1A) is the main storage form of phosphorus in plants. It is deposited as phytin (Figure 1B), a mixed salt with counterions including Fe^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+} (Lott, 1984). In leaves phytin is a temporary phosphate reserve and the deposits in the grains ensure that the developing seedlings can be provided with a continuous supply of phosphate and minerals that are liberated from the molecule during germination. Removal of the first phosphate groups from phytin requires a specific enzyme, phytase, while the remaining phosphate groups may be cleaved off by phosphatases with broad substrate specificities. Phytin may also serve as an energy resource and the lower *myo*-inositol phosphates appear to be essential in signal transduction pathways (Raboy and Gerbasi, 1996). The content of phytin in seeds may account for 0.4% to 5% of the seed dry weight in legumes and oilseeds and 0.5% to 1.9% in cereals (Reddy *et al.*, 1982). Up to 87% of the total phosphorus in the seed may be present as phytin bound phosphate e.g. in soybean and wheat 53% and 72% of the total phosphorus is present in phytin (Lolas *et al.*, 1976).

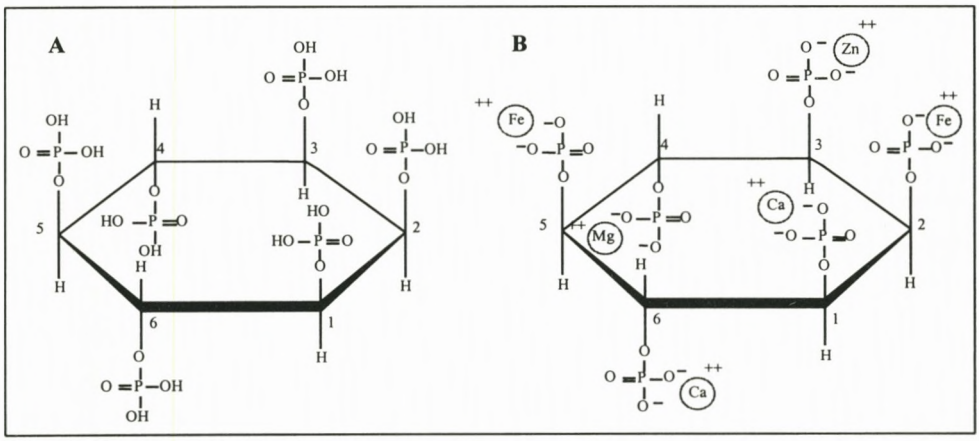

Figure 1. The structure of **(A)** phytic acid and **(B)** the mixed salt phytin.

Deposition and accumulation of phytin in the seed

Phytin in seeds are deposited in single-membrane storage microbodies (protein bodies), mainly as discrete inclusions (globoids) (Lott, 1984). Globoid crystals consist predominantly

of phytin salt complexes of primarily potassium and magnesium, although minor amounts of other metals such as zinc, iron, manganese, copper and calcium have been reported (O´Dell *et al.*, 1972). Additionally, some protein-phytin-mineral complexes have also been reported to be present in protein bodies in barley, rye, wheat and soybean (Lott, 1984).

Phytin accumulates rapidly during seed development, and is in monocots predominantly deposited in the outer layers (aleurone and pericarp) and the germ, whereas legumes and oilseeds hold their phytin in the endosperm and cotyledons (O´Dell *et al.*, 1972; Pernollet, 1978). In rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) 80% and 87% of the phytin is localised in the outer layers, while 7.6% and 12.9% is found in the germ (scutellum and embryo) (O´Dell *et al.*, 1972). Maize is an exception to the typically localisation pattern seen in the other cereals since 88% of the phytin is confined to the germ (O´Dell *et al.*, 1972).

In dicot species, phytin is deposited in the endosperm, the embryo, and the cotyledons. In some cases the phytin is localised to specific tissues. In the yellow lupine (*Lupinus luteus* L.) phytin inclusions are thus found in the five to ten subepidermal cell-layers of the cotyledon (Sobolev *et al.*, 1976), whereas phytin deposits in soybean (*Glycine max* L.) are distributed evenly throughout the cotyledon tissues (Lott, 1984). The castor bean endosperm contains 97% to 98% of the phytin while the remaining 3% primarily are confined to the cotyledons (Greenwood *et al.*, 1984).

Phytin is also present in the pollen of several plant species and the concentration has been suggested to relate to the style length (Jackson *et al.*, 1982; Helsper *et al.*, 1984). An analysis of 25 angiosperm species revealed that species with a mean style length longer than 5 mm also had a significant amount of phytin in the pollen (0.05% to 2.1% by dry weight). Pollen of *Triticum aestivum* L, *Hordeum bulbosum* L. and *Secale cereale* L., all having a mean style length of about 2 mm, lacked phytin. In contrast, pollen of *Zea mays* L., with a mean style length of 250 mm, contained 0.26% phytin by dry weight. This correlation did, however, not hold for three gymnosperm species that contained 0.1 % to 5.9% phytin by dry weight in the pollen irrespective of that these species only form short pollen tubes. Thus the function of phytin may not only be restricted to pollen tube development but may in gymnosperms also be important for pollen development.

Biosynthesis of Phytic Acid

Myo-inositol

Free *myo*-inositol is generated from D-glucose by three enzymatic steps: **A)** hexokinase (EC 2.7.1.1); **B)** 1L-*myo*-inositol 1-phosphate synthase (EC 5. 5. 1. 4) and **C)** *myo*-inositol 1-phosphate phosphatase (EC 3.1.3.25), (Figure 2).

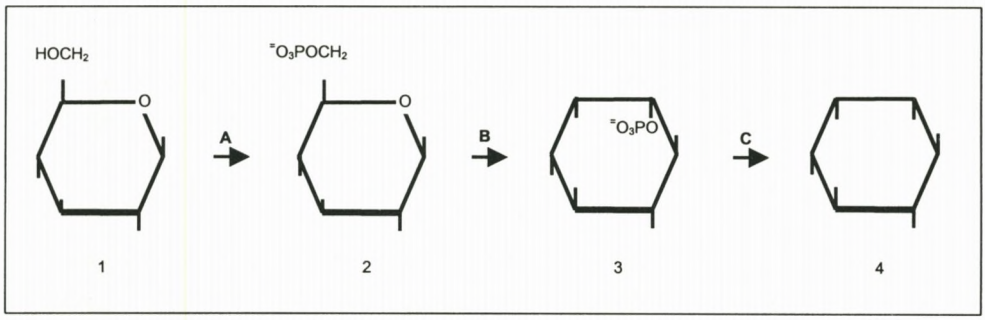

Figure 2. The conversion of **(1)** D-glucose to **(4)** free *myo*-inositol via **(2)** D-glucose 6-phosphate and **(3)** 1L-*myo*-inositol 1-phosphate catalysed by **(A)** hexokinase, **(B)** 1L-*myo*-inositol 1-phosphate synthase, and **(C)** *myo*-inositol monophosphatase (Loewus, 1990)

After phosphorylation of D-glucose to D-glucose 6-phosphate by hexokinase, the next step involves *myo*-inositol 1-phosphate synthase. In the reaction described in figure 3 the bond that links phosphate to carbon remains undisturbed. The intermediates (within brackets) are tightly bound to the enzyme as revealed by studies using isotopically labelled substrates as well as partial reaction sequences (Loewus, *et al.*, 1984). The enzyme exhibits an absolute requirement for NAD^+, since the first product 1L-*myo*-inositol 1-phosphate, is generated by an NAD^+ catalysed oxidation of D-glucose 6-phosphate at carbon 5 to 5-keto-D-glucose 6-phosphate (Maeda and Eisenberg, 1980; Mauck *et al.*, 1980). After the oxidation, the removed hydrogen is transferred as a hydride ion to the B position of carbon 4 on the nicotinamide moiety of NAD^+ (Bryun and Jenness, 1981; You, 1985).

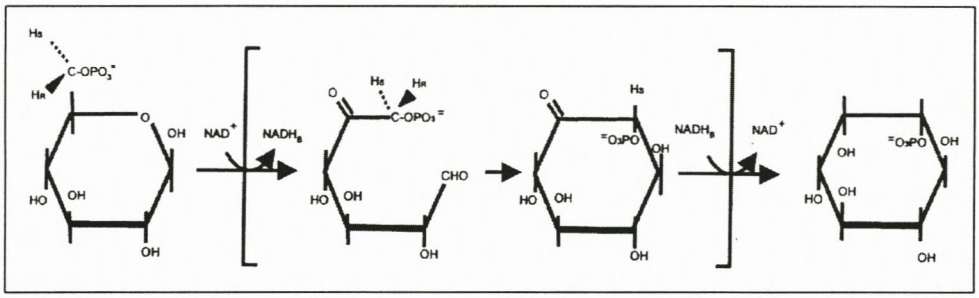

Figure 3. The mechanism for conversion of D-glucose 6-phosphate to 1L-*myo*-inositol 1-phosphate by 1L-*myo*-inositol 1-phosphate synthase (Loewus *et al.*, 1984).

Cyclisation of D-glucose 6-phosphate is required for the formation of 1L-myo-inositol 1-phosphate. During this a specific hydrogen is lost from position 6 of 5-keto-D-glucose 6-phosphate. The enzyme removes the 6-R hydrogen whereas the 6-S hydrogen is retained in the product (Loewus *et al.*, 1980). The reductive partial reaction by enzyme-bound NADH of the second putative intermediate *myo*-inosose-2 1-phosphate (D-2,4,6/3,5-pentahydroxy-cyclohexanone 2-phosphate) transfers a hydride from the B position of carbon 4 on the nicotinamide ring of NADH to the carbonyl group to generate 1L-*myo*-inositol 1-phosphate (Bryun and Jenness, 1981; Loewus *et al.*, 1982).

The synthesis of 1L-*myo*-inositol 1-phosphate by *myo*-inositol 1-phosphate synthase is a critical point as it is the sole biosynthetic route to *myo*-inositol. The enzyme exists in a cytoplasmatic form in a wide range of plants, animals and fungi. It has also been observed in several bacteria and a chloroplast form is present in alga and higher plants (Majumder *et al.*, 1997). Northern analysis and *in situ* hybridisation of a recently isolated rice cDNA with high homology to the *myo*-inositol 1-phosphate synthase of yeast and other plants showed that transcripts accumulated to high levels in embryos but was undetectable in shoots, roots, and flowers. Strong signals were detected in the scutellum and aleurone at 4 days and increased until 7 days after anthesis whereafter it gradually decreased. Phytin containing globoids appeared 4 days after anthesis in the scutellum and aleurone, coinciding with the presence of the *myo*-inositol 1-phosphate synthase transcripts. The temporal and partial patterns of accumulation of the transcripts and globoids suggest that this gene directs phytin biosynthesis in rice seeds (Yoshida *et al.*, 1999).

Conversion of 1L-*myo*-inositol 1-phosphate to free *myo*-inositol is catalysed by *myo*-inositol monophosphatase (Figure 2, enzyme C). The enzyme is a Mg^{2+} dependent alkaline phosphatase with high affinity for both 1D- and 1L-*myo*-inositol 1-phosphate. Its has a low affinity for *myo*-inositol 2-phosphate and little or no affinity for hexose phosphates or the model phosphatase substrate *p*-nitrophenyl phosphate (Eisenberg and Parthasarathy, 1987). In tomato *myo*-inositol monophosphatase appears to be encoded by a small gene family and three different cDNA's have been characterised encoding distinct but highly conserved enzymes (Gillaspy *et al.*, 1995). In particular, cells associated with the vasculature expressed high levels of the protein, suggesting a co-ordinated regulation between phloem transport and synthesis of inositol.

Phosphorylation of myo-inositol

Phytic acid appears to be formed by a series of sequential phosphorylations of *myo*-inositol by a *myo*-inositol kinase. Early studies showed that free *myo*-inositol in germinating mung beans is phosphorylated by a Mg^{2+} ATP dependent kinase (Majumder *et al.*, 1972). The product, *myo*-inositol-1-phosphate, is identical to that produced by *myo*-inositol 1-phosphate synthase, however the kinase phosphorylates *myo*-inositol more efficient than glucose. Likewise, a stepwise addition of phosphate residues was reported in germinating mung beans using the same Mg^{2+} ATP dependent kinase generating *myo*-inositol 1,3,4,5,6 penta*kis*phosphate (Chakrabarti and Majumder, 1978). The stereospecificity of the positional *myo*-inositol polyphosphates of the intermediates was not determined. Additional data from mung bean described a phosphorylation of *myo*-inositol 1,3,4,5,6-penta*kis*phosphate to *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate by a *myo*-inositol hexa*kis*phosphate-adenosine diphosphate phosphotransferase (Biswas *et al.*, 1978). From immature soybean seeds *myo*-inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase has been purified (Phillippy *et al.*, 1994). The kinase specifically phosphorylated the 2-position on the *myo*-inositol 1,3,4,5,6-penta*kis*phosphate inositol ring, but could also utilise *myo*-inositol 1,4,5,6-tetra*kis*posphate as a substrate.

More comprehensive data from the slime mould *Dictyostelium* illustrate that the concept of a sequential phosphorylation to pentakisphosphate by a single kinase described for mung bean is probably misleading. Instead, the sequential phosphorylation is mediated by a series of kinases with different preferences and specificities (Stephens and Irvine, 1990; Kaay *et al.*,

1995). The sequential phosphorylation of inositol appears to occur via *myo*-inositol 3-phosphate, whereafter *myo*-inositol 3,6-diphosphate, *myo*-inositol 3,4,6-triphosphate, *myo*-inositol 1,3,4,6- tetra*kis*phosphate, and *myo*-inositol 1,3,4,5,6-penta*kis*phosphate are generated (Figure 4). The phosphates in position 3 and 5 of *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate additionally takes part in two futile cycles in which *myo*-inositol 1,2,4,5,6-penta*kis*phosphate and *myo*-inositol 1,2,3,4,6-penta*kis*phosphate are rapidly formed by dephosphorylation of *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate, only to be rephosphorylated to yield their precursor.

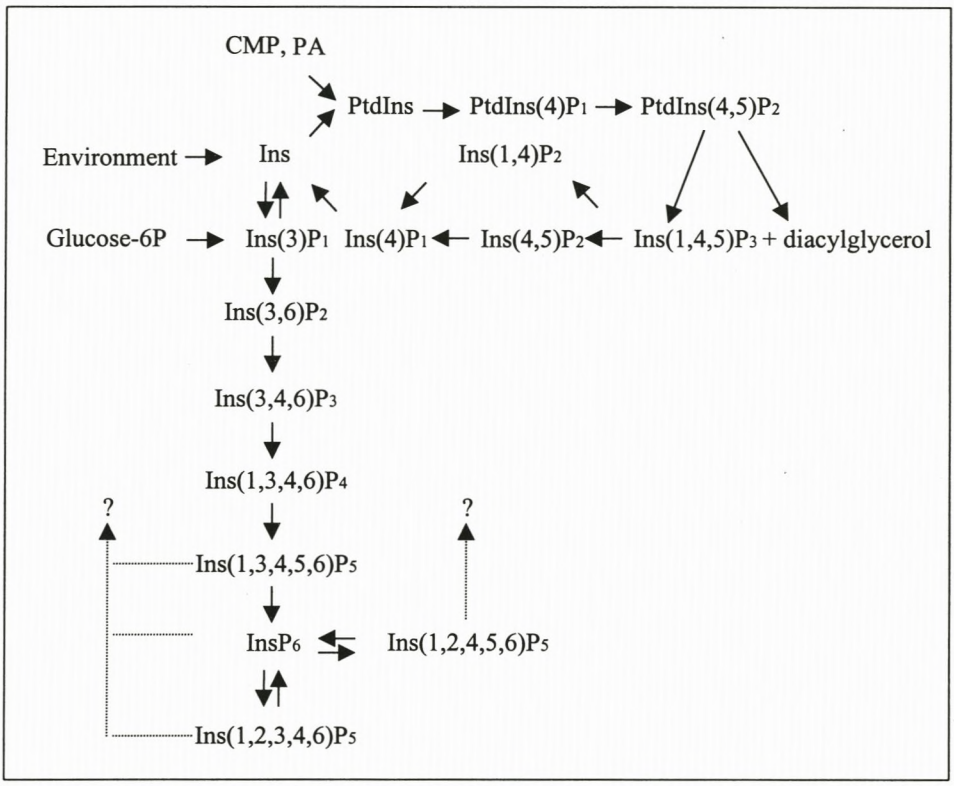

Figure 4. The inositol metabolism in *Dictyostelium*. Abbreviations: Ptdlns, phosphatidylinositol; CMP, PA, cystidine monophosphate phosphatidic acid, (Stephens and Irvine, 1990).

Also in plants, the involvement of multiple kinases in *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate synthesis is suggested. Three enzyme fractions isolated from *Lemna gibba*,

could together convert *myo*-inositol to *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate. One fraction converted *myo*-inositol to *myo*-inositol monophosphate, a second to triphosphate, and a third to *myo*-inositol hexa*kis*phosphate (Bollman *et al.*, 1980). Moreover, Murthy (1996) proposed two pathways for phytic acid synthesis (Figure 5). Pathway 1 shared *myo*-inositol 3-phosphate and *myo*-inositol 1,3,4,5,6-penta*kis*phosphate with the *Dictyostelium* pathway, however the pathway included *myo*-inositol 1,2,3,5,6-penta*kis*phosphate and different di-, tri- and tetrakisphosphates. The second pathway started from *myo*-inositol 2-phosphate and ended with *myo*-inositol 2,3,4,5,6-penta*kis*phosphate involving intermediates all being different from pathway 1 and the *Dictyostelium* pathway.

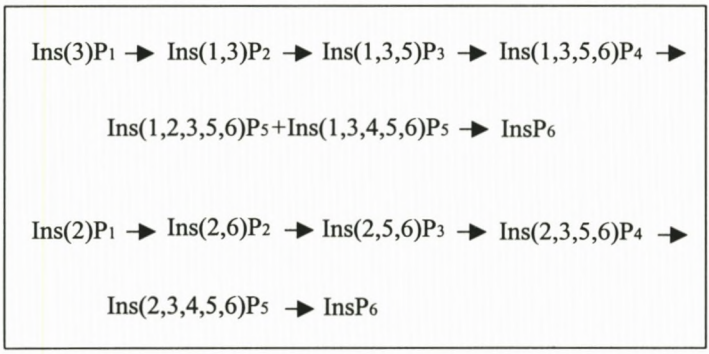

Figure 5. The plant biosynthesis of *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate ($InsP_6$) from *myo*-inositol 3-phosphate ($Ins(3)P_1$ and *myo*-inositol 2-phosphate ($Ins(2)P_1$) (Murthy, 1996).

So far only one gene encoding a kinase involved in *myo*-inositol phosphorylation has been isolated from plants. A partial cDNA from Arabidopsis (Wilson and Majerus, 1997) encoding a putative *myo*-inositol 1,3,4-triphosphate 5/6 kinase was expressed in *E. coli* and shown to produce inositol 1,3,4,6-tetrakisphosphate and inositol 1,3,4,5-tetrakisphosphate in a ratio of 1:3 when offered inositol 1,3,4-triphosphate as a substrate.

The mutagenesis approach currently undertaken in maize and barley might prove to be essential for the identification of the individual genes involved in phytic acid biosynthesis. In both maize and barley two loci have been identified that when mutated result in a drastic decrease in the concentration of phytic acid and a corresponding increase in free phosphate (Raboy and Gerbasi, 1996; Ertl *et al.*, 1998; Larson *et al.*, 1998; Raboy, 1998). Mutants homozygous for the mutated *lpa*1 locus give rise to an allele specific decrease in phytic acid

amounting to 50-95% with no accumulation of the lower inositol phosphates. In maize, the *lpa*1 mutation maps to the position of the locus for *myo*-inositol-1-phosphate synthase. In barley, *lpa*1 is known to reside on chromosome 2H but it remains to be proven if this locus codes for *myo*-inositol-1-phosphate synthase. The other, and less frequent mutation, is referred to as *lpa*2. In contrast to the situation for the *lpa*1 mutations, the reduction in phytic acid is in maize accompanied by the accumulation of lower *myo*-inositol phosphates, in particular *myo*-inositol 1,2,4,5,6-penta*kis*phosphate and *myo*-inositol 2,3,4,5,6-penta*kis*phosphate while in barley the prominent form is *myo*-inositol 1,2,3,4,6-penta*kis*phosphate. At Risø a similar mutagenesis program has also lead to the identification of two mutants in the phytic acid biosynthesis of barley and it remains to be seen if the two mutations are in the same loci, *lpa*1 and *lpa*2 as described for maize and barley.

Compartmentalisation of phytic acid synthesis

It has been suggested on the basis of studies in developing seeds that phytic acid synthesis takes place in the cytoplasm prior to its deposition within protein bodies (Greenwood and Bewley, 1984). The course of events begins with phytin synthesis in cisternal endoplasmic reticulum, followed by migration of phytin bearing vesicles towards the protein body. After discharge of the vesicular contents within the protein body, phytin rich particles are accumulated as a globoid. Thus in *Dictyostelium* lysates, a cytosolic route exhibiting formation of *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate by sequential phosphorylation of inositol (Figure 4) has been reported (Stephens and Irvine, 1990).

However nucleus associated phosphorylation could take place in plants. Studies in *Dictyostelium* indicate that the stepwise phosphorylation of inositol to phytic acid, can occur via nucleus associated phosphorylation (Kaay *et al.*, 1995). HPLC analysis indicated that *myo*-inositol 1,4,5-phosphate in the nucleus is converted into phytic acid via sequential phosphorylation at the 3-, 6- and 2-positions.

Phytase

Classification of phytases

The enzymes responsible for the initial steps in the degradation of phytic acid are referred to as phytases. They comprise a special class of phosphatases that catalyse a sequential hydrolysis of phytic acid to lower inositol phosphates and, in some cases, to inositol. A rich diversity of phytases occurs in a variety of organisms including plants and micro-organisms (Irving, 1980a; Wodzinski and Ullah, 1996). Differences in pH optima, substrate specificity and specificity of hydrolysis have been identified.

Phytase are often described according to their pH optimum as acid phytase or alkaline phytase, also referred to as the pH 5 and the pH 8 phytase respectively. Based on the specificity of the initial hydrolysis, the International Union of Biochemistry (1979) recognises two classes of acid phytase, the 6-phytases (EC 3.1.3.26), and the 3-phytases (EC 3.1.3.8). The 6-phytase hydrolyses the phosphate ester at the L-6 (or D-4) position of phytic acid, and the 3-phytase hydrolyses the phosphate ester at the D-3 position. Later results have shown that this definition may be too rigorous as phytases may diverge from the 6-, 3-phytase terminology in their action on phytic acid.

Active site determination

Phytases belong to the family of histidine phosphatases, a subclass of phosphate metabolising enzymes, all utilising a phosphohistidine intermediate in their phosphoryl transfer reaction (Etten, 1982). Analyses for conserved motifs in the sequences of diverse phosphate liberating enzymes have led to the identification of the active site comprising a tripeptidic region in Arg-His-Gly, the N-terminal segment of the protein. The Arg-His-Gly motif is highly conserved in phosphatases from organisms such as *Escherichia coli*, *Aspergillus niger*, *Saccharomyces cerevisia*, *Schizosaccharomyces pombe*, *Homo sapiens*, rat and mouse. The histidine class phosphatases uses the positive charge of the guanido group of arginine for the recognition and anchoring of the negatively charged phosphate group. The phosphate group is

transiently transferred to the histidine group to form an unstable phosphoenzyme complex before hydrolytic cleavage to form orthophosphate (Etten, 1982).

Plant phytase

Acidic phytase shows a broad affinity for various phosphorylated substrates. Thus the wheat bran pH 5 phytase catalyses the hydrolysis of several intermediate forms of phosphorylated *myo*-inositol, spanning from *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate to *myo*-inositol 2-phosphate (Figure 6) (Lim and Tate, 1971;1973). Mung bean cotyledon phytase, in spite of a pH optimum of 7.5, exhibits the enzymatic properties of the group of acidic phytases (Maiti and Biswas, 1979). Unlike the pH 5 phytases, the pH 8 phytases are highly specific for phytic acid, and none of the *myo*-inositol phosphates containing three or fewer ester groups can act as substrate (Baldi *et al.,* 1988; Barrientos *et al.*, 1994). Alkaline phytase has been identified in lily pollen and in a variety of legumes (Scott and Loewus, 1986b; Baldi *et al.*, 1988; Scott, 1991; Barrientos *et al.*, 1994).

Although plant phytase activity has been described in numerous cases, only phytases from a few sources have been purified to homogeneity. The most notable are from mung bean cotyledon (Maiti and Biswas, 1979), soybean cotyledon (Gibson and Ullah, 1988), maize seedlings and roots (Laboure *et al.*, 1993; Hübel and Beck, 1996), scallion leaves (Phillippy, 1998), and rye (Greiner *et al.*, 1998). However, so far it has only been possible to isolate a cDNA for maize phytases (Maugenest *et al.,* 1997; Maugenest *et al.*, 1999).

Microbial phytase

The number of micro-organisms screened for phytase production is enormous. Of all the organisms surveyed the *Aspergilli* fungi produces the most active extracellular phytase (Wodzinski and Ullah, 1996). Consequently, a number of phytases from this organism have been purified to homogeneity and the genes isolated. Thus, a phytase with a pH optimum of 5.0 and 2.5 was isolated from a culture filtrate of *Aspergillus niger*, biochemically characterised, partially sequenced and the *phy*A gene for the enzyme cloned (Ullah and Dischinger, 1992; Piddington *et al.,* 1993; Hartingsveldt *et al.*, 1993). A second enzyme from

Aspergillus niger encoded by the *phy*B gene with pH optimum 2.5 was initially considered to be an acid phosphatase (Erlich *et al.*, 1993; Piddington *et al.*, 1993). However, additional studies have shown that it is able to hydrolyse phytic acid at pH 2.5 but not at pH 5 (Ullah and Phillippy, 1994). Also the *Aspergillus niger phy*B gene has been cloned (Ehrlich *et al.*, 1993). From *Aspergillus fumigatus* the *phy*A gene has been cloned and found to encode an enzyme with a pH range of 2.5 to 7.5 (Pasamontes *et al.*, 1997). A *phy*A gene from *Aspergillus terreus* has been isolated and the resulting phytase enzyme with a pH optimum around 5.5 described (Mitchell *et al.*, 1997).

Detailed characterisations have also been performed on microbial phytases such as *Bacillus subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992; Kerovuo *et al.*, 1998), *Escherichia coli* (Greiner *et al.*, 1993), *Myceliophthora thermophila* (Mitchell *et al.*, 1997), *Klebsiella terrigena* (Greiner *et al.*, 1997), *Thermomyces lanuginosus* (International patent application number: PCT/US97/04559; Berka *et al.*, 1998), *Emericella nidulans* and *Talaromyces thermophillus* (Wyss *et al.*, 1999).

Regulation of phytase synthesis and activity

Although a small amount of endogenous phytase activity has been detected in ungerminated and dry seeds, e.g. peas (Gibson and Ullah, 1990), a large increase in phytase activity and a decrease in phytic acid content can be observed when seeds are germinating or even shortly after water imbibition. Thus, a 2-fold increase in phytase activity was observed in maize during germination and several varieties of peas exhibited an 8- to 30-fold increase in phytase activity from day 1 to day 5 of germination (Gibson and Ullah, 1990). In soybean, an approximately 10-fold increase in phytase activity was observed, with a maximum level of activity at days 8 to 10 after germination (Gibson and Ullah, 1988). A 24 hrs incubation of wheat, rye, and hulled and dehulled barley seeds with water, resulted in a 46-77% reduction of phytate content, indicating a high phytase activity (Fredlund *et al.*, 1997).

The increase in phytase activity during germination can be a result of either an activation of a pre-existing phytase or a *de novo* synthesis. In addition to the low phytase activity present in dormant seeds of peas, two additional forms are present exclusively in germinating seeds indicating a *de novo* synthesis (Gibson and Ullah, 1990). In soybeans, phytase activity is

observed in ungerminated seeds, however in both soybean and maize, an increase in immunospecific staining on western blot appears during germination, indicating a *de novo* synthesis of the enzyme (Gibson and Ullah, 1988; Laboure *et al.*, 1993). In maize, no transcript was present in dry seeds, however mRNA accumulated during the first day of germination, to reach a maximum after 2 days, and then decreased in young seedlings (Maugenest *et al.*, 1997).

Phytase activity is regulated by the level of inorganic phosphate in seeds of *Phaseolus vulgaris* (Chang and Schwimmer, 1977; Lolas and Markakis, 1977) in wheat bran (Lim and Tate, 1971;1973), in soybean cotyledons (Gibson and Ullah, 1988), in *Lilium longiflorum* pollen (Scott and Loewus, 1986a; Lin *et al.*, 1987), and in *Petunia hybrida* pollen (Jackson and Linskens, 1982). Also, the *E. coli* phytase activity is inhibited by inorganic phosphate (Greiner *et al.*, 1993). In contrast, low phosphate levels appear to induce phytase gene expression. Thus, in cell suspension cultures of soybean, phytase and acid phosphatase excretion was induced after 3 to 4 days of growth under phosphate deficient conditions (Gibson and Christen, 1987). Likewise, in *Aspergillus niger*, the phytase encoding *phy*A gene is transcriptionally upregulated under low phosphate conditions (Hartingsveldt *et al.*, 1993).

In summary, phytase gene expression may be upregulated at the transcriptional level by low inorganic phosphate levels while the phytase enzyme appears to be inhibited by high levels of inorganic phosphate, i.e., a feed back inhibition.

The action of plant phytase

The sequence of hydrolysis of phosphate groups has been investigated in wheat (Lim and Tate, 1971; 1973), mung bean (Maiti and Biswas, 1979), and lily pollen (Baldi *et al.*, 1988; Barrientos *et al.*, 1994). Additionally, the *myo*-inositol composition in barley aleurone cells has been characterised *in vitro* as being stereochemically very much similar to the degration products of phytic acid after the action of wheat bran phytase (Brearley and Hanke, 1996).

Acid plant phytase mediated degradation of phytic acid to *myo*-inositol appears to be rather complex and has been studied in most detail for the wheat bran phytase (Figure 6). Thus, Tomlinson and Ballou reported the substrate specificity of a crude wheat bran phytase

preparation already in 1961 and 1962. Further characterisation was performed and subsequently the wheat bran phytase was resolved into two fractions named F1 and F2 (Johnson and Tate, 1969; Lim and Tate , 1971; 1973). The crude extract characterised by Tomlinson and Ballou and the later F1 fraction yielded the same products from phytic acid, perhaps indicating that the F2 fraction was a relatively minor component.

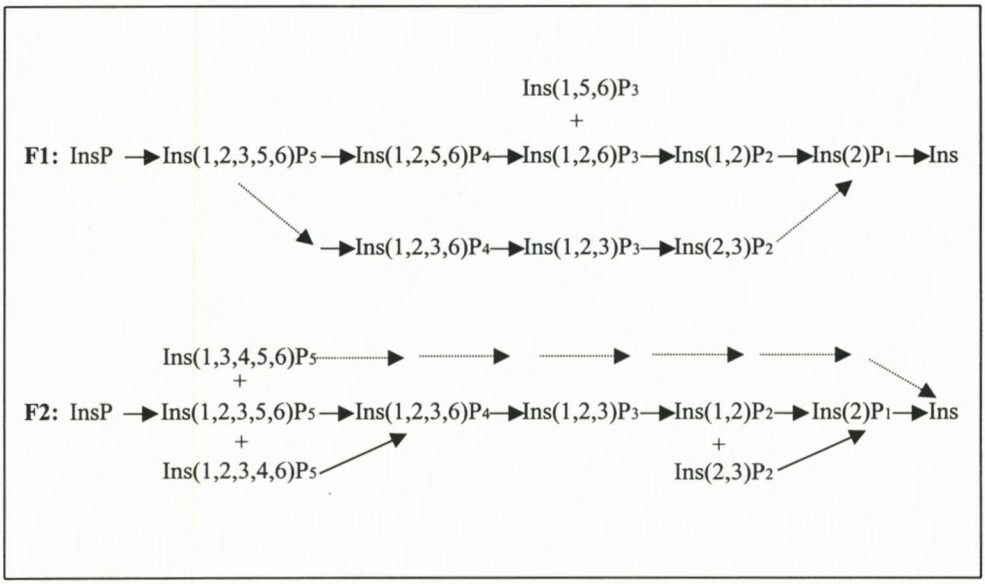

Figure 6. The hydrolysis of phytic acid ($InsP_6$) to *myo*-inositol (Ins), catalysed by wheat bran phytase fraction 1 (**F1**) and fraction 2 (**F2**) respectively.

As illustrated in Figure 6, the phytase activity of F1 initially removes the D-4 phosphate, yielding *myo*-inositol 1,2,3,5,6-penta*kis*phosphate. During the subsequent F1 mediated step, two different tetra*kis*phosphates are generated, namely a minor component, *myo*-inositol 1,2,3,6-tetra*kis*phosphate and a major product, *myo*-inositol 1,2,5,6-tetra*kis*phosphate. Further dephosphorylation yields *myo*-inositol 1,2,3-triphosphate and *myo*-inositol 1,2,6-triphosphate (Tomlinson and Ballou, 1962). *Myo*-inositol 1,2-diphosphate and /or *myo*-inositol 2,3-diphosphate were identified when treating *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate with the crude phytase, and it has been shown that crude wheat bran phytase yields only a single *myo*-inositol monophosphate, *myo*-inositol 2-phosphate (Irving, 1980b). In a study using a commercial available preparation of wheat phytase, much of the above conclusions was

confirmed by NMR, but additionally the formation of *myo*-inositol 1,5,6-triphosphate from *myo*-inositol 1,2,5,6-tetra*kis*phosphate was reported (Phillippy, 1989). Only two *myo*-inositol tetrakisphosphate products were characterised, since *myo*-inositol 1,2,6-triphosphate and *myo*-inositol 1,2,3-triphosphate were identified as products following the action of the crude phytase on *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate as well (Tomlinson and Ballou, 1962).

In contrast to the results obtained with the F1 fraction, the F2 fraction of wheat bran phytase generates three chromatographically distinct peaks of *myo*-inositol penta*kis*phosphates (Lim and Tate, 1971; 1973), i.e., *myo*-inositol 1,2,3,5,6-penta*kis*phosphate as the major product and smaller amounts of *myo*-inositol 1,3,4,5,6-penta*kis*phosphate and *myo*-inositol 1,2,3,4,6-penta*kis*phosphate. Further catabolism of the 1,2,3,5,6 and 1,2,3,4,6 isomers yielded the same product, *myo*-inositol 1,2,3,6-tetra*kis*phosphate, which is the same isomer as the minor *myo*-inositol tetra*kis*phosphate component produced by crude wheat bran phytase. A single triphosphate was identified as *myo*-inositol 1,2,3-triphosphate. Further breakdown yielded a mixture of *myo*-inositol 2,3-diphosphate and *myo*-inositol 1,2-diphosphate, with the latter dominating, and finally *myo*-inositol 2-monophosphate and *myo*-inositol.

The broad substrate affinity of the acid phytases is confirmed when comparing the wheat bran/barley aleurone intermediates with the intermediates identified in other plant species. No clear consensus for the pattern of the penta*kis*phosphates is present in wheat, barley, mung bean and soybean. In soybean, *myo*-inositol 1,3,4,5,6-penta*kis*phosphate is represented as in wheat and barley, but along with *myo*-inositol 1,2,3,4,6-penta*kis*phosphate, it is as a minor component compared with *myo*-inositol 1,2,4,5,6-penta*kis*phosphate (Phillippy and Bland, 1988). In mung bean seedlings, 1,2,4,5,6; 1,2,3,4,5; 1,3,4,5,6 and *myo*-inositol 1,2,3,4,6-penta*kis*phosphate were all identified, but the latter two represented less than 4% total *myo*-inositol penta*kis*phosphate in [^{3}H] *myo*-inositol or [^{32}P]P_i- labelled preparations (Stephens, 1990; Stephens *et al.*, 1991). In barley myo-inositol 1,2,3,4,5- and -1,2,3,4,6 penta*kis*phosphate are the dominating penta*kis*phosphate while *myo*-inositol 1,2,3,4,6 penta*kis*phosphate is only present in small amounts in soybean and mung bean seedlings compared to *myo*-inositol 1,2,4,5,6 penta*kis*phosphate and *myo*-inositol 1,2,3,4,5 penta*kis*phosphate.

Together, these data indicate limited substrate specificity for the phytases during the initial hydrolytic step. This may also be the case for the subsequent steps. Although *myo*-inositol

1,2,3,4 tetra*kis*phosphate and *myo*-inositol 1,2,5,6 tetra*kis*phosphate are the predominant forms in barley and mung bean, other types of tetra*kis*phosphates are generated in soybean seedlings and wheat. If focusing on wheat and barley, *myo*-inositol 1,2,3 triphosphate and *myo*-inositol 1,2,6 triphosphate are hydrolysed to the diphosphates *myo*-inositol 1,2 diphosphate and *myo*-inositol 2,3 diphosphate and finally to *myo*-inositol 2 phosphate (wheat and barley) or *myo*-inositol 3 phosphate (barley).

An alkaline pH 8.0 phytase activity has been identified and extensively studied in pollen of *Lilium longiflorum* (Baldi *et al.*, 1988; Barrientos, 1994). The initial hydrolysis of the phosphate ester by alkaline phytase occurs at the D-5 position of phytic acid to yield *myo*-inositol 1,2,3,4,6-penta*kis*phosphate (Barrientos *et al.*, 1994). The two subsequent dephosphorylations occur adjacent to the D-5 hydroxyl group to yield *myo*-inositol 1,2,3-triphosphate as the final product. The physiological significance of alkaline phytases in relation to acidic phytases remains to be elucidated. Likewise, it is not known if *myo*-inositol 1,2,3-triphosphate has a biological role.

Role of Phytin and Phytase in Nutrition

The nutritional aspect of feeding non-ruminants with high phytin diets

In the dry seed and in the digestive tract of non-ruminants animals there is only a limited phytase activity (Gibson and Ullah, 1990; Lantzsch *et al.*, 1992; Usayran and Balnave, 1995). Ruminants readily utilise the phytate phosphorus of seeds because of the phytase produced by rumen micro-organisms. In contrast, digestible phosphate compounds have to be added to seed based feed for pigs and poultry to compensate for the low digestibility of phytin. Large amounts of phytin phosphorus pass through the gastrointestinal tract and only 30% of the phosphorus eaten by pigs are deposited during growth. Of the remaining phosphorus, 50% is excreted with the manure and 20% with the urine, reflecting unutilised phytate-P and supplemental inorganic-P respectively (Poulsen, 1996). The undigested phytin causes environmental phosphorus pollution when the manure is used as fertiliser to croplands.

The strong association between phytate and important dietary cations reduces the absorption of these elements in the digestive tract of non-ruminants (Hurrell *et al.*, 1992). This is a major problem in the developing world in populations feeding primarily on cereals where iron, zinc and phosphate deficiencies cause serious health problems, including anaemia's, rickets, osteoporosis and bone deformities (Lott, 1984). Several studies have documented that an enhancement of phytin phosphorus utilisation also increases the absorption of i.e. zinc, magnesium and iron (Robenson and Edwards, 1994; Murry, 1995; Sandberg *et al.*, 1996). Strategies for improving the bioavailability of phosphorus for animal feed are accordingly also very relevant for the generation of mineral dense food for human consumption.

Phytate has further been shown to inhibit peptic digestion of proteins and to interfere with the activity of proteolytic enzymes, probably as a result of the formation of protein/phytin complexes (Lott, 1984).

Application of phytase to feed

It has been known for several years that a supplementation of cereal diets with phytase of *Aspergillus niger* NRRL 3135 enhances the release of phosphate from phytin and consequently reduces the phosphate excretion from non-ruminants (Nelson *et al.*, 1968; 1971). Later studies of broilers have further demonstrated that the addition of enzyme even improves the performance compared to that attained by adding supplemental inorganic phosphate (Simmons *et al.*, 1990; Jongbloed *et al.*, 1992).

The environmental load imposed by the excess phosphate in animal manure has been one of the driving forces in the development of phytase as a feed additive. European countries such as Denmark, The Netherlands and Germany, that all have large scale animal husbandry, are now via legislative measures trying to balance the nitrogen and phosphate input with that required by the crops. For the last 50 years more phosphate has been added to the cultivated soils in Denmark than has been removed by the crops. Formerly, this was considered a minor problem since phosphate is firmly bound in the soil in an organic or inorganic form. It is apparent, however, that water and wind drainage and erosion as well as drainage in poor soils results in a significant transfer of phosphate to sweet water as well as coastal areas, causing massive alga growth and anaerobic conditions.

Application of phytase to animal feed can drastically reduce the requirements for a supplementation with dietary phosphate and reduce the phosphate excretion. It has been estimated (Table 1) that if phytase was used as a feed ingredient in the diet of all the momogastric animals in the United States, the amount of excreted phosphorus could be reduced with a total of $8.23*10^7$ kg per year (Wodzinski and Ullah, 1996). The total value of the phytin phosphorus in the animal feed has been estimated to amount $1.68*10^8$ $ per year (data for 1993, Wodzinski and Ullah, 1996).

Table 1. Effect of use of phytase on abatement of phytate pollution (Wodzinski and Ullah, 1996).

Animal	No. in US in 1992	Average live wt. (kg)	kg of feed/ animal	g P excreted/ animal if supplemented with P	g P excreted/ animal if supplemented with phytase	kg P/year not excreted
Broilers	$6.14*10^9$	2.01	3.8	14.5	8.4	$3.75*10^7$
Layers	$3.64*10^8$		36.4/year	139/year	80.5/year	$2.20*10^7$
Ducks	$1.8*10^7$	2.95	7.08	27 (estimate)	15.6/year (estimate)	$2.81*10^5$
Turkeys	$2.89*10^8$	9.91	26.4	101 (estimate)	58.5 (estimate)	$1.69*10^7$
Pigs	$5.78*10^7$	80.4	265	271 (estimate)	177	$5.62*10^6$
Total	-	-	-	-	-	$8.23*10^7$

Increasing the Phytase Activity of Plants by Transformation

Transformation of plants with phytase genes for improved growth performance of non-ruminants

The success of using phytase as a feed additive has stimulated research to generate transgenic plants with a higher phytase potential in the mature seed. A transformation strategy addressed to the overexpression of a homologous or heterologous phytase gene in plant seeds could lead to an increase in the bioavailability of the phosphorus and essential minerals of the seed.

Results obtained in tobacco and wheat indicates that transformation with the *Aspergillus niger* phytase encoding gene *phy*A, driven by a constitutive promoter can increase the total phytase activity in the seeds (Pen *et al.*, 1993; Brinch-Pedersen *et al.*, 2000a). Seeds of transgenic plants exhibited normal phenotype even when the phytase enzyme accumulated in amount up to 1% of the soluble protein in the seed. The enzyme showed to be stable since storage of transgenic seeds, intact or milled, for up to 12 months at 4^0C or room temperature caused no significant decrease in phytase activity level. In a broiler feeding trial, the phytase accumulated in tobacco seeds proved to be effective for increasing the bioavailability of phosphate (Pen *et al.*, 1993). The addition of milled transgenic seeds, *Aspergillus niger* phytase or inorganic phosphate had comparable effects on the growth rate (Table 2). In transgenic wheat grains, *in vitro* studies have shown that the heterologous phytase is capable of reducing the amounts of phytic acid and lower *myo*-inositol phosphates. Consequently the amount of inorganic phosphate is increased significantly (Brinch-Pedersen *et al.*, 2000b).

Recent studies have further documented that a fully functional *Aspergillus niger* phytase can be accumulated in transgenic alfalfa seeds and soybeans (Beudeker and Pen, 1994; Denbow *et al.*, 1998). In addition, *Aspergillus niger* phytase has been synthesised and stored in an active form in tobacco and canola leaves and in soybean cell suspension cultures (Verwoerd *et al.*, 1995; Koegel *et al.*, 1997; Li *et al.*, 1997).

Table 2. Effect of phytase-transgenic seeds on the growth rate of broilers over a 4-week period (Pen *et al.*, 1993).

Dietary Supplementation[1]	Phosphate[2] (g/kg diet)	Phytase Activity.[3] (FTU/kg diet)	Growth (g) Age (weeks)		
			0-2	2-4	0-4
None	5.0	-	203[c]	492[bc]	695[c]
Inorganic P	6.5	-	314[a]	729[a]	1043[a]
A. niger phytase	5.0	202	276[b]	665[a]	941[b]
A. niger phytase	5.0	404	293[ab]	692[a]	985[ab]
Control seeds[4]	5.0	-	193[c]	515[b]	708[c]
Transgenic seeds[4,5]	5.0	295	299[ab]	693[a]	992[ab]
Stat. significance of difference: P<			0.001	0.001	0.001
SED[6]			13.2	34.8	44.7

[1]Differences in the amount of diet, caused by the supplementation was not compensated. The highest dietary supplementation's were 33 g/kg feed (diets 5 and 6), while maximally 8 g/kg was added in the other diets.

[2]The basal diet contains 3.3 g/kg plant phytate and 1.7 g/kg of available phosphorus.

[3]Phytase activity was determined in the supplement prior to addition to the diet.

[4]Seeds were milled and added as such.

[5]Seeds of this line had an expression level of 0.4% of soluble protein.

[6]SED, standard error of differences between two means.

[a, b, c]Values within a column having identical superscript letter are not significantly different.

Effect of transgenic phytase seeds on phosphorus excretion from non-ruminants

The effect on phosphorus excretion was assessed in a feed experiment where broilers were fed transgenic soybeans containing phytase (Denbow *et al.*, 1998). Thus, 1200U phytase activity per kilo of transgenic soybeans caused about 50% reduction in the phosphorus excretion, when compared to a diet supplemented with an intermediate level (0.16%) of dietary nonphytate phosphorus (Table 3). In addition, the excretion of phosphorus was in the experiments with the transgenic soybeans reduced on average 11% when compared to experiments where feed based on wild type soybean was added a commercial microbial derived phytase (Natuphos). The reduced excretion reflects an approximately 10% increased

phosphorus digestibility of the feed with the endogenous phytase compared with feed supplemented with phytase. The results illustrate that diets low in non phytate phosphorus but with endogenous phytase activity ensure growth rates similar to that of diets supplemented with inorganic phosphorus. Moreover the bioavailability of phosphate in the former type of feed was higher than when using the same feed supplemented with exogenous commercially available phytase from *Aspergillus niger* phytase (Natuphos).

Table 3. Effect of feeding phytase transformed soybean (TSB) or Natuphos phytase on growth, phosphorus digestibility and excretion (Adapted from: Denbow *et al.*, 1998).

Diets	BW[a] gain week 2 to 3	Feed intake week 2 to 3	Gain: feed week 2 to 3	Phosphorus Digestibility	Phosphorus excretion
	(g)	(g)	(g/kg)	(% of intake)	(g/kg DM[b] intake)
Basal	407	618	658	47.2	2.56
B+0.08P	436	669	650	42.8	3.01
B+0.16P	487	739	659	44.0	3.56
B+0.24P	488	749	649	40.0	3.90
B+400U phytase TSB	435	643	678	55.8	2.15
B+800U phytase TSB	451	707	638	60.4	1.98
B+1200U phytase TSB	474	720	658	61.6	1.82
B+400U phytase Natuphos	416	616	677	50.6	2.34
B+800U phytase Natuphos	440	686	641	53.3	2.24
B+1200U phytase Natuphos	454	710	639	55.1	2.10

[a]BW, body weight

[b]DM, dry matter

Heat stability

Currently available industrial phytases all originate from *Aspergillus niger*. The temperature profile of the enzyme is asymmetric. It is sluggish from 20^{0}C to 30^{0}C and displays its highest activity at 58^{0}C. The activity rapidly declines at 65^{0}C and at 68^{0}C only 2% of its activity is retained (Gibson and Ullah, 1990; Engelen *et al.*, 1994). The thermal inactivation appears however, to be partly transient since a subjection to 68^{0}C for 10 min and subsequent transfer to 58^{0}C resulted in regaining of 40% of the original activity (Gibson and Ullah, 1990). The limited thermostability is a drawback of the phytases originating from *Aspergillus niger* since modern feed-pelleting processes utilise a heat treatment (Gibson, 1995).

However, *in planta* synthesised *Aspergillus niger* phytase shows altered thermal stability when compared to the isolated fungal derived enzyme (Li *et al.*, 1997). After preincubation at 63^{0}C for 10 min, 90% of the activity remained for the recombinant phytase, whereas only 75% remained for the fungal phytase. Above 63^{0}C, activity declined rapidly for the fungal and the recombinant enzyme. At 70^{0}C, the recombinant enzyme still retained about 20% of the activity while the fungal enzyme was completely inactivated. Prolonged incubation (63^{0}C for 1 hr), resulted in a 60% loss of activity for the fungal enzyme compared to 20% loss of activity for recombinant phytase. It was suggested that the changes in thermal stability of the recombinant enzyme might be attributed to the addition of two amino acids at the translational fusion site or differences in glycosylation between phytases synthesised *in planta* and in fungi (Li *et al.*, 1997). Further studies may reveal the mechanisms determining the improved thermostability of the plant derived phytase.

Phytases with superior heat stability has recently been described from *Aspergillus fumigatus* and *Thermomyces lanuginosus*. The *A. fumigatus* enzyme was able to withstand temperatures up to 100^{0}C for 20 min while loosing only 10% of the initial enzymatic activity (Pasamontes *et al.*, 1997). In addition, the enzyme showed high activity at a pH range of 2.5 to 7.5. The high thermostability of this enzyme is remarkable and similar phytases or acid phosphatases have so far not been described. Thus, *Thermomyces* phytase retained activity at assay temperatures only up to 75^{0}C (Berka *et al.*, 1998).

References

Baldi, B.G. Scott, J.J., Everand, J.D. and Loewus, F.A. (1988). Localization of constitutive phytases in lily pollen and properties of the pH 8 form. Plant Sci., **56**, 137-147.

Barrientos, L., Scott, J.J, and Murthy, P.P.N. (1994). Specificity of phytic acid by alkaline phytase from lily pollen. Plant Physiol., **106**, 1489-1495.

Berka, R. M., Rey, M. W., Brown, K. M., Byun, T. and Klotz, A. V. (1998). Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl. Environ. Microbiol., **64**, 4423-4427.

Beudeker, R. F. and Pen, J. (1994). Development of plant seeds expressing the Aspergillus niger phytase gene for use in monogastric animal feeds. In: The Proceedings of the 3rd International Symposium on the Biosafety Results of Field Tests of Genetically modified Plants and Microorganisms, Jones, D. D. (ed), University of California, 299-304.

Biswas, S., Maity, I.B., Chakrabarti, S. and Biswas, B.B. (1978). Purification and characterisation of *myo*-inositol hexaphosphate-adenosine diphosphate from *Phaseolus aureus*. Arch Biochem Biophys, 185, 557-566.

Bollman, O., Strother, S. and Hoffman-Ostenhof, O. (1980). The enzymes involved in the synthesis of phytic acid in *Lemna gibba* (studies on the biosynthesis of cyclitols, XL). Mol. Cell Biochem. Biophys., **30**, 171-175.

Brearley, C.A. and Hanke, D.E. (1996). Inositol phosphates in barley (*Hordeum vulgare* L.) aleurone tissue are stereochemically similar to the products of breakdown of InsP_6 *in vitro* by wheat-bran phytase. Biochem. J., 318, 279-286.

Brinch-Pedersen, H., Olesen, A., Rasmussen, SK:, Holm, PB. (2000a). Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. Mol Breeding, in press.

Brinch-Pedersen, H., Hatzack, F., Holm, PB. (2000b). The effect of *in planta* production of non-secreted and secreted microbial phytase on the *myo*-inositol phosphate profile in transgenic wheat grains. Manuscript in preparation.

Bryun, M. S. and Jenness, R. (1981). Stereo specificity of 1L-*myo*-inositol 1-phosphate synthase for nicotinamide adenine dinucleotide. Biochemistry, **20**, 5174-5177.

Chakrabarti, S. and Majunder, A. L. (1978). Phosphoinositol kinase from plant and avian sources. In: Wells, W.W., Eisenberg, F.Jr (eds). Cyclitols and Phosphoinositide. Academic Press, New York, 69-82.

Chang, R. and Schwimmer, S. (1977). Characterisation of phytase of beans (*Phaseolus* vulgaris). J. Food Biochem., **1**, 45-56.

Denbow, D. M., Graubau, E. A., Lacy, G. H., Kornegay, E. T., Russell, D. R. and Umbeck, P. F. (1998). Soybeans transformed with a fungal phytase gene improve phosphorus availability for broilers. Poul. Sci., **77**, 878-881.

Ehrlich, K.C., Montalbano, B.G., Mullaney, E.J., Dischinger, H.C.Jr. and Ullah, A.H.J. (1993). Identification and cloning of a second phytase gene (*phy*B) from *Aspergillus niger* (*ficum*). Biochem. Biophys. Res. Com., **195**, 53-57.

Eisenberg, F. and Parthasarathy, R. (1987), Measurement of biosynthesis of *myo*-inositol from glucose 6-phosphate. Methods Enzymol, **141**, 127-143.

Engelen, A. J., Heeft, F. C. ven der, Randsdorp, P. H. G. and Smit, E. L. C. (1994). Simple and rapid determination of phytase activity. J. of AOAC Internat., **77**, 760-764.

Etten van-, R. L. (1982). Human prostatic acid phosphatase: A histidine phosphatase. Ann. NY Acad. Sci., **390**, 27-51.

Ertl, D. S., Young, K. A. and Raboy, V. (1998). Plant genetic approaches to phosphorus management in agriculture production. J. Environm. Qual., **27**, 299-304.

Fredlund, K., Asp, N.-G., Larsson, M., Marklinder, I., and Sandberg, A.-S. (1997). Phytate reduction in whole grains of wheat, rye, barley and oats after hydrothermal treatment. J. Cereal Sci., **25**, 83-91.

Gibson, D.M. and Christen, A.A. (1987). Is expression of phytase activity regulated by phosphate concentration? Plant Physiol., (Suppl), **83**, 770.

Gibson, D.M. and Ullah, A.H.J., (1988). Purification and characterisation of phytase from cotyledons of germinating soybean seeds. Arch Biochem Biophys, **260**, 503-513.

Gibson, D. M. and Ullah, A. B. J. (1990). Phytases and their action on phytic acid. In: Morré, D.J., Boss, W.F. and Loewus F.A., (eds), Inositol Metabolism in Plants, Wiley-Liss Inc, New York, 77-92.

Gibson, K. (1995). The pelleting stability of animal feed enzymes. In: Hartingsveldt, W, van, Hessing, M., Lugt, J. P. van, and Somers, W. A. C. (Eds.), Proceedings of the second european symposium on feed enzymes. TNO Nutrition and Food Research Institute, Zeist, The Netherlands. 157-162.

Gillaspy, G. E., Keddie, J. S. Oda, K. and Gruissem, W, (1995). Plant inositol monophosphatase is a lithium-sensitive enzyme encoded by a multigene family. Plant Cell, **7**, 2175-2185.

Greenwood, J.S. and Bewley, J.D. (1984). Subcellular distribution of phytin in the endosperm of developing castor bean: A possibility for its synthesis in the cytoplasm prior to deposition within protein bodies. Planta, 160, 113-120.

Greenwood, J.S., Gifford, D.J. and Bewley, J.D. (1984). Seed development in *Ricinus communis* cv. Hala (castor bean). Accumulation of phytic acid in the developing endosperm and embryo in relation to the deposition of lipid, protein and phosphorus. Can. J. Bot., **62**, 255-261.

Greiner, R., Konietzny, U. and Jany, Kl.-D. (1993). Purification and characterisation of two phytases from *Escherichia coli*. Arch. Biochem. Biophys., **303**, 107-113.

Greiner, R., Haller, E., Konietzny, U. and Jany, K. D. (1997). Purification and characterisation of a phytase from *Klebsiella terrigena*. Arch. Biochem. Biophys., **341**, 201-206.

Greiner, R., Konietzny, U. and Jany, K.D. (1998). Purification and properties of phytase from rye. J. Food Biochem., **22**, 143-161.

Hartingsveldt, W. van, Zeijl, C.M.J. van, Harteveld, G.M., Gouka, R.J., Suykerbuyk, M.E.G., Luiten, R.G.M., Paridon P.A. van, Selten, G.C.M., Veenstra, A.E., Gorcom, R.F.M. van, and Hondel, C.A.M.J.J. van den. (1993). Cloning, characterisation and overexpression of the phytase-encoding gene (*phy*A) of *Aspergillus niger*. Gene, **127**, 87-94.

Helsper, J. P. F. G., Linskens, J. F. and Jackson, J. F. (1984). Phytate metabolism in *Petunia* pollen. Phytochemistry, **23**, 1841-1845.

Hübel, F. and Beck, E. (1996). Maize root phytase. Plant Physiol., **112**, 1429-1436.

Hurrell, R. F., Juillerat, M. A., Reddy, M. B. Lynch, S. R., Dassenko, S. A. and Cook, J. D. (1992). Soy protein, phytate, and iron absorption in humans. Am. J. Clin. Nutr., **56**, 573-578.

International patent application number: PCT/US97/04559: Polypeptides having phytase activity and nucleic acids encoding same.

International Union of Biochemistry (1979). Enzyme nomenclature: Recommendations (1978) of the nomenclature Committee of the International Union of Biochemistry. Academic Press, New York, 242-247.

Irving, G. C. J. (1980a). Phytase. in: Inositol Phosphates: Their Chemistry, Biochemistry and Phsiology. New York. Elsevier Scientific Publishing Company. 85-138.

Irving, G. C. J. (1980b). Intermediates in the dephosphorylation of inositol hexakisphosphates by phytase enzymes. In: Inositol Phosphates: Their Chemistry, Biochemistry and Phsiology. New York. Elsevier Scientific Publishing Company. 99-117.

Jackson, J.F. and Linskens, H.F. (1982). Phytic acid in *Petunia hybrida* pollen is hydrolyzed during germination by a phytase. Acta. Bot. Neerl., **31**, 441-447.

Jackson, J. F., Jones, G. and Linskens, H. F. (1982). Phytic acid in pollen. Phytochemistry, **21**, 1255-1258.

Johnson, L.F. and Tate, M.E. (1969). The structure of *myo*-inositol pentaphosphates. Ann. N.Y. Acad. Sci., **165**, 526-532.

Jongbloed A. W., Mroz, Z. and Kemme, P. A. (1992). The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. J. Anim. Sci., **70**, 1159-1168.

Kaay, J. van der, Wesseling, J. and Haastert, J.M. van (1995). Nucleus-associated phosphorylation of Ins(1,4,5)P_3 to InsP_6 in *Dictyostelium*. Biochem J., **312**, 911-917.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N. and Apajalahti, J. (1998). Isolation, Characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol., **64**, 2079-2085.

Koegel, R. G. Austin-Phillips, S. Bingham, E. T. Straub, R. J. and Cook, M. E. (1997). Phytase from transgenic alfalfa. In: Research Summaries, US Dairy Forage Research Center, 21-22.

Laboure, A-M., Gagnon, J. and Lescure, A-M. (1993). Purification and characterisation of a phytase (*myo*-inositol-hexakisphosphate phosphohydrolase) accumulated in maize (*Zea mays*) seedlings during germination. Biochem. J., **295**, 413-419.

Lantzsch, H.,J., Hillenbrand, S., Scheuermann, S.E. and Menke, K.H. (1992). Comparative study of phosphorus utilization from wheat, barley and corn diets by young rats and pigs. J. Anim. Physiol. Anim. Nutr., **67**, 123-132.

Larson, L. R., Young, K. E., Cook, A., Blake, T. K. and Raboy, V. (1998). Linkage mapping two mutations that reduce phytic acid content of barlley grain. Theor. Appl. Genet., **97**, 141-146.

Li, J., Hegeman, C.E., Hanlon, R.W., Lacy, G.H., Densow, D.M. and Grabau, E.A. (1997). Secretion of active recombinant phytase from soybean cell-suspension cultures, *Plant Physiol.* **114**, 1103-1111.

Lim, P.E. and Tate, M.E. (1971). The phytases. I. Lysolecithin-activated phytase from wheat bran. Biochim. Biophys. Acta, **250**, 155-164.

Lim, P.E. and Tate, M.E. (1973). The phytases. II. Properties of phytase fractions F1 and F2 from wheat bran and the *myo*-inositol phosphates produced by fraction F2. Biochim Biophys Acta, **302**, 316-328.

Lin, J-J., Dickinson, D.B. and Ho, T-HD, (1987). Phytic acid metabolism in lily (*Lilium longiflorum* Thunb). Plant Physiol, **83**, 408-413.

Loewus, M.W., Loewus, F.A., Brillinger, G-U., Otsuka, H. and Floss, H.G. (1980). Stereochemistry of the 1L-myo-inositol 1 phosphate synthase reaction. J Biol Chem, **255**, 11710-11712.

Loewus, M.W., Sasaki, K., Leavitt, A.L., Munsell, L., Sherman, W.R. and Loewus, F.A. (1982). The enantiomeric form of *myo*-inositol 1-phosphate produced by *myo*-inositol kinase in higher plants. Plant Physiol, **70**, 1661-1663.

Loewus, M. W., Bedgar, D. L. and Loewus, F. A. (1984). 1L-myo-inositol 1-phosphate synthase from pollen of *Lilium* longiflorum. J Biol Chem, **259**, 7644-7647.

Loewus, F.A. (1990). Inositol biosynthesis, In: Morré, D.J., Boss, W.F. and Loewus F.A., (eds), Inositol Metabolism in Plants, Wiley-Liss Inc, New York, 13-19.

Lolas, G. M., Palamidis, N. and Markakis, P. (1976). The phytic acid-total phosphorus relationship in barley, oats, soybeans and wheat. Cereal Chem., **53**, 867-871.

Lolas, G.M. and Markakis, P. (1977). The phytase of navy beans (*Phaseolus vulgaris*). J. Food Sci., **42**, 1094-1106.

Lott, J. N. A. (1984). Accumulation of seed reserves of phosphorus and other minerals, In: Murray, D.R. (Ed), Seed Physiology, Academic Press, New York, 139-166.

Maeda, T. and Eisenberg, F. (1980). Purification, structure and catalytic properties of 1L-myo-inositol 1-phosphate synthase from rat testis. J Biol Chem, **255**, 8458-8464.

Maiti, I.B. and Biswas, B.B. (1979). Further characterisation of phytase from *Phaseolus aureus*. Phytochem, **18**, 316-318.

Majerus, P.W., Connolly, T.M., Bansal, V.S., Inhorn, R.C., Ross, T.S. and Lips, D.L. (1988). Inositol phosphates: synthesis and degradation. J. Biol. Chem., **263**, 3051-3054.

Majumder, A.N.L., Mandal, N. C. and Biswas, B. B. (1972). Phosphoinositol kinase from germinating mung bean seeds. Phytochemistry, **11**, 503-508.

Majumder, A.N.L., Johnson, M. D. and Henry, S. A. (1997). 1L-*Myo*-inositol-1-phosphate synthase. Biochim. Biophys. Acta, **1348**, 245-256.

Mauck, L. A., Wong, Y-H. and Sherman, W. R. (1980). 1L-*myo*-inositol 1-phosphate synthase from bovine testis: Purification to homogeniety and partial characterusation. Biochemistry, **19**, 3623-3629.

Maugenest, S., Martinez, I. and Lescure, A-M. (1997). Cloning and characterisation of a cDNA encoding a maize seedling phytase. Bichem. J., **322**, 511-517.

Maugenest, S., Martinez, I., Godin, B., Perez, P, and Lescure, A-M. (1999). Structure of two maize phytase genes and their spatio-temporal expression during seedling development. Plant Mol. Biol., **39**, 503-514.

Mitchell, D. B. Vogel, K. Weimann, B. J. Pasamontes, L. and van loon, A. P. G. M. (1997). The phytase subfamily of histidine acidphosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology, **143**, 245-252.

Murry, A.C. Jr. (1995). Effect of microbial phytase on calcium and phosphorus digestibility and serum mineral concentrations in growing and finishing pigs. In: Annual Report, Department of Animal and Dairy Science, The University of Georgia, pp 2. Available on www as: http://www.ads.uga.edu/annrpt/1995/s301murr.htm.

Murthy, P. P. N. (1996). Inositol phosphates and their metabolism in plants. In: Biswas, B. B. and Biswas, S., (eds), Subcellular Biochemistry, vol 26, Plenum Press, New York, 227-255.

Nelson, T. S., Shieh, T. R., Wodzinski, R.J. and Ware, J.H. (1968). The availability of phytate phosphorus in soya bean meal before and after treatment with a mold phytase. Poultry Sci., **47**, 1842-1848.

Nelson, T. S., Shieh, T. R., Wodzinski, R.J. and Ware, J.H. (1971). Effects of supplemental phytase on the utilization phytate phosphorus by chicks. J. Nutr., **101**, 1289-1294.

O'Dell, B. L., de Boland, A. R. and Koirtyohann, S. R. (1972). Distribution of phytate and nutritionally important elemants among the morphological components of cereal grains. J Agric Food Chem, **20**, 718-721.

Pasamontes, L., Haiker, M., Wyss, M., Tessier, M. and van Loon, A.P.G.M. (1997). Gene cloning, purification, and characterisation of a heat-stable phytase from the fungus *Aspergillus fumigatus*. Appl. Environ. Microbiol., **63**, 1696-1700.

Pen, J., Verwoerd, T.C., Paridon, P.A. van, Beudeker, R.F., Elzen, P.J.M. van den, Geerse, K., Klis, J.D. van der, Versteegh. H.A.J., Ooyen A.J.J van and Hoekema, A. (1993). Phytase-containing transgenic seeds as a novel feed addetive for improved phosphorus utilisation. Bio/Tech., **11**, 811-814.

Pernollet, J-C. (1978). Protein bodies of seeds: Ultrastructure, biochemistry, biosynthesis and degradation. Phytochemistry, **17**, 1473-1480.

Phillippy, Q. (1989). Identification by two-dimensional NMR of *myo*-inositol tris- and tetrakis(phosphates) formed from phytic acid by wheat phytase. J. Agric. Food Chem., **37**, 1261-1265.

Phillippy, B.Q. and Bland, J.M. (1988). Gradient ion chromatography of inositol phosphates. Anal. Biochem., **175**, 162-166.

Phillippy, B. Q., Ullah, A. H. and Ehrlich, F. C. (1994). Purification and some properties of inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase from immature soybean seeds. J. Biol. Chem., **269**, 28393-28399.

Phillippy, B. Q. (1998). Purification and catalytic properties of a phytase from scallion (*Allium fistulosum* L.) leaves. J. Agric. Food Chem., **46**, 3491-3496.

Piddington, C. S., Huston, C. S., Paloheimo, M., Cantrell, M., Miettinen-Oinonen, A., Nevalainen, H. and Rambosek, J. (1993). The cloning and sequencing of the genes encoding phytase (*phy*) and pH 2.5-optimum acid phosphatse (*adh*) from *Aspergillus* var *awamori*. Gene, **133**, 55-62.

Poulsen, H.D. (1996). Fosfor i svineproduktion. Jord og Viden, **19**, 8-10.

Powar, W.K. and Jagannathan, V. (1982). purification and properties of phytate-specific phosphatase from *Bacillus* subtilis. J. Bacteriol., **151**, 1102-1108.

Raboy, V. (1998). The genetics of seed storage phosphorus pathways. In: Lynch, J.P. and Deikman, J., (eds), Phosphorus in plant biology: Regulatory roles in molecular, cellular,

organismic, and ecosystem processes. Current Topics in Plant Physiology, vol. 19, Rockville, Maryland U.S.A. 192-203.

Raboy, V. and Gerbasi, P. (1996). Genetics of *myo*-inositol phosphate synthesis and accumulation, in: Biswas, B. B. and Biswas, S., (eds), Subcellular Biochemistry, *myo*-inositol phosphates, phosphoinositdes and signal transduction, **26**, Plenum Press, New York.

Reddy, N.R., Sathe, S.K. and Salunkhe, D.K. (1982). Phytates in legumes and cereals. Adv, Food Res., 28, 1-92.

Robenson, K. D. and Edwards, H. M. Jr. (1994). Effect of 1,25-dihydroxychlolecalciferol and phytase on zinc utilisation in broiler chicks. Poult. Sci., **73**, 1312-1326.

Sandberg, A. S., Hulthen, L. R. and Turk, M. (1996). Dietary *Aspergillus niger* phytase increases iron absorption in humans. J. Nutr., **126**, 476-480.

Scott, J.J. and Leowus, F.A. (1986a). Phytate metabolism in plants. In: Graf, E. (Ed), Phytic Acid: Chemistry and Applications, Pilatus Press, Minneapolis, MN, 23-42.

Scott, J.J. and Leowus, F.A. (1986b). Calcium-activated phytase from pollen of *Lilium longiflorum*. Plant Physiol, **82**, 333-335.

Scott, J. J. (1991). Alkaline phytase activity in nonionic detergents of legume seeds. Plant Physiol., **95**, 1298-1301.

Shimizu, M. (1992). Purification and characterisation of phytase from bacillus subtilis. Biosci. Biotech. Biochem., **56**, 1266-1269.

Simons, P.C.M., Versteeg, H.A.J., Jongbloed, A.W., Kemme, P.A., Slump, P., Bos, K.D. Wolters, M.G.E., Beudeker, R.F. and Verschnoor, G.J. (1990). Improved phosphorus availability by microbial phytase in broilers and pigs. Brit. J. Nutr., **64**, 525-540.

Sobolev, A.M., Buzulukova, N.P., Dmitrieva, M.I. and Barbashova, A.K. (1976). Structural-bio-chemical organisation of aleurone grains inseeds of yellow lupine. Soviet Plant Physiol., **23**, 739-746.

Stephens, L.R. (1990). Preparation and separation of inositol tetra*kis*phosphates and inositol penta*kis*phosphates and the establishment of enantiomeric configurations. In: Irvine, R.F. (ed.), Methods in Inositide Research. Raven Press, New York, 9-29.

Stephens, L.R. and Irvine, R.F. (1990). Stepwise phosphorylation of *myo*-inositol leading to *myo*-inositol hexa*kis*phosphate in *Dictyostellium*. Nature, **346**, 580-583.

Stephens, L.R., Hawkins, P.T., Stanley, A.F., Moore, T., Poyner, D.R., Morris, P.J., Hanley, M.R., Kay, R.R. and Irvine, R.F. (1991). *myo*-Inositol penta*kis*phosphates: Structure, biologocal occurrence and phosphorylation to *myo*-inositol hexa*kis*phosphate. Biochem. J., **275**, 485-499.

Tomlinson, R.V. and Ballou, C.E. (1961). Complete characterisation of the *myo*-inositol polyphosphates from beef brain phosphoinositide. J. Biol. Chem., **236**, 1902-1906.

Tomlinson, R.V. and Ballou, C.E. (1962). *myo*-Inositol polyphosphate intermediates in the dephosphorylation of phytic acid by phytase. Biochem., **1**, 166-171.

Ullah, A. H. J. and Dischinger, H. C. Jr, (1992). Identification of residues involved in active-site formation in *Aspergillus ficum* phytase. Ann. N. Y. Acad. Sci. , **672**, 45-51.

Ullah, A.H.J. and Phillippy, B.Q. (1994). Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphatase using *myo*-inositol phosphates. J. Agric. Food Chem., **42**, 423-425.

Usayran, N. and Balnave, D. (1995). Phosphorus requirements of laying hens fed on wheat-based diets. Br. Poultry Sci., **36**, 285-301.

Verwoerd, T. C., Paridon, P. A. van, Ooyen, A. J. J. van, Lent, J. W. M. van, Hoekema, A. and Pen, J. (1995). Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. Plant Physiol., **109**, 1199-1205.

Wilson, M. P. and Majerus, P. W. (1997). Charecterization of a cDNA encoding *Arabidopsis thaliana* inositol 1,3,4-triphosphate 5/6-kinase. Biochem. Biophys. Res. Commun., **232**, 678-681.

Wodzinski, R. J. and Ullah, A. H. (1996). Phytase. Adv. Appl. Microbiol., **42**, 263-302.

Wyss, M., Pasamontes, L., Friedlein, A., Remy, R., Tessier, M., Kronenberger, A., Middendorf, A., Lehmann, M., Schnoebelen, L., Rothlisberger, U., Kusznir E., Wahl, G., Muller, F., Lahm, H. W., Vogel, K. and van Loon A. P. G. M. (1999). Biophysical characterization of fungal phytases (*myo*-inositol hexa*kis*phosphate phosphohydrolases): Molecular size, glycosylation pattern, and engineering of proteolytic resistance. Appl. Environ. Microbiol., **65**, 359-366.

Yoshida, K. T., Wada, T., Koyama, H., Mizobuchi-Fukuoka, R. and Naito, S. (1999). Temporal and spatial patterns of accumulation of the transcript of *myo*-inositol-1-phosphate synthase and phytin-containing particles during seed development in rice. Plant Physiol., **119**, 65-72.

You, K-S. (1985). Stereospecificity for nicotinamide nucleotides in enzymatic and chemical hydride transfer reactions. CRC Crit. Rev. Biochem., **17**, 313-451.

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