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Review:

Phytate: impact on environment and human nutrition. A challenge for molecular breeding*

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Abstract: Phytic acid (PA) is the primary storage compound of phosphorus in seeds accounting for up to 80% of the total seed phosphorus and contributing as much as 1.5% to the seed dry weight. The negatively charged phosphate in PA strongly binds to metallic cations of Ca, Fe, K, Mg, Mn and Zn making them insoluble and thus unavailable as nutritional factors. Phytate mainly accumulates in protein storage vacuoles as globoids, predominantly located in the aleurone layer (wheat, barley and rice) or in the embryo (maize). During germination, phytate is hydrolysed by endogenous phytase(s) and other phosphatases to release phosphate, inositol and micronutrients to support the emerging seedling. PA and its derivatives are also implicated in RNA export, DNA repair, signalling, endocytosis and cell vesicular trafficking. Our recent studies on purification of phytate globoids, their mineral composition and dephytinization by wheat phytase will be discussed. Biochemical data for purified and characterized phytases isolated from more than 23 plant species are presented, the dephosphorylation pathways of phytic acid by different classes of phytases are compared, and the application of phytase in food and feed is discussed.

Key words: Phytase, Phytic acid, Iron bioavailability, Antinutritional factor, Purple acid phosphatase, Cereal
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INTRODUCTION

A complete understanding of the biosynthesis of phytic acid (PA) in plants based on a single model species cannot be achieved and comparative studies are needed. Even if the same core set of inositol phosphate kinases (Ipks) should exist in the plant kingdom, the coordination of biosynthesis, translocation, site of accumulation and storage vary among species. This is exemplified by the fact that in wheat, barley and rice the majority of phytate accumulates in the aleurone cells and only minor amounts in the embryo. The distribution of phytate is just opposite in the maize seeds, which means that within relatively closely related grasses different control points exist. PA biosynthesis initiates shortly after flowering and it accumulates during development until seed maturation and desiccation. During this period of plant development changes in growth conditions occurs: rain, drought, high temperature and pathogens. Individual inositolphosphate kinases accept a broad range of substrates and it is also evident that rice and barley Ipks have phosphatase and isomerase activity. These multiple activities provide degrees of freedom for controlling and fine tuning the PA biosynthesis and accumulation, but it also poses a challenge for molecular breeding strategies, because mutations in one kinase gene can be compensated for by other activities.

During the past years, attention has been focused on PA as an antinutritional factor in the diet of humans because of their inability to utilize phytate. The low bioavailability of the minerals bound in the PA can lead to deficiencies in human populations where

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staples like wheat, rice and maize are the major or the only source of nutrition. In the case of livestock production, excretion of PA can lead to accumulation of P in soil and water, and subsequently to eutrophication of fresh water streams and near coastal seawaters. Low-PA mutant seeds can potentially reduce these problems. Many low PA mutants have been produced in most of the important crop. However, many examples indicate that random mutations seem to affect PA biosynthesis throughout the plant life cycle and not just in the seeds. At present, little is known about the impact of these mutations on agronomical important quality parameters such as stress response or disease susceptibility of plants. Therefore methods acting exclusively on the biosynthesis of PA are needed to produce sustainable low PA seeds.

NUTRITIONAL ASPECTS

Micronutrient malnutrition and distribution

The main micronutrient deficiencies in the world involve Fe, Zn and Vitamin A (WHO, 2002). The three factors are closely related and, in the correct combination, the uptake of one will enhance the uptake of another, and especially fats or proteins from meat will enhance uptake of all of them (Gibson *et al*., 2006; Lonnerdal, 2000; Storcksdieck *et al*., 2007). In developing countries plant foods are the major staples of the diet. Particularly in diets based on unrefined cereals or legumes the bioavailability of several micronutrients, such as Ca, Fe, Zn, I and some vitamins can be quite low, causing metabolic disorders related to these nutritional factors. Consequently, improving the nutritional value of this type of foods would improve the nutritional status of the entire population. The transgenic rice line "Golden Rice" is a good example of how the effect of extra β-carotene can decrease Vitamin A deficiency (Zimmermann and Hurrell, 2002). Unfortunately, similar work has not yet been pursued in wheat, the staple cereal of developed countries.

The mineral bioavailability of wheat depends on the cultivar, environment and harvest year. Positive correlations between Zn and Fe have been found in both spring and winter wheat, but these elements were negatively correlated with Mn and P. Furthermore, genotype seems to influence Fe concentrations (Morgounov *et al*., 2007), while its content is positively correlated with grain size rather than phytate content, suggesting that ferritin deposits are involved in storage of Fe (Grusak *et al*., 1999). Zn uptake depends more on geographical location (Morgounov *et al*., 2007) but positive associations between phytate and zinc have been reported as well as evidence that the position on the spike also influences the composition of the minerals in grain (Liu *et al*., 2006).

Mineral, phosphorous and phytate content is much higher in the bran than in the whole grain (Guttieri *et al*., 2003; Iskander and Morad, 1986; Steiner *et al*., 2007), but also within the bran fractions differences occur. Pearling of wheat has shown that the outer 0~4% layer of wheat has the highest Zn content, whereas the next outer 4%~8% layer has the highest phytase activity, phytate and iron contents. However, the differences are small in the outer 0~12% layer, proving that storage of all of these compounds takes place in the bran (Liu Z.H. *et al*., 2007).

Factors involved in micronutrient malnutrition outside the grain are, for example, the chemical form of the nutrient in the food matrix, interactions with other compounds or treatment of the food prior to ingestion. Iron bioavailability will be used to exemplify it.

Iron uptake and bioavailability

There are two types of iron in the human diet; both of them are mostly absorbed in the proximal part of the duodenum. Heme iron origins from meat products and consists of iron complexed with the porphyrin ring from either haemoglobin or myoglobin. It only accounts for approximately 10%~20% of dietary iron, but for up to 50% of the iron actually entering the body (Carpenter and Mahoney, 1992). Specific receptors for heme iron on the microvilli of the enterocytes have been identified (Krishnamurthy *et al*., 2007; Worthington *et al*., 2001) and the iron is easily absorbed and split from the complex by heme oxygenase inside the cell (Raffin *et al*., 1974).

The second type of iron is non-heme iron from plants. Unlike heme-iron, its uptake depends on the composition of the meal and other factors in the degradation pathway. Absorption by the enterocytes involves reduction from ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron (Mckie *et al*., 2001) before co-transport with a proton across the membrane by divalent metal

transporter 1 (DMT1) (Andrews, 1999; Gunshin *et al*., 1997). Iron is then released into the bloodstream by ferrous ion transporter (FPN) and absorbed by cells through the "transferrin cycle": iron-transferrin is bound by transferrin receptors and internalised by endocytosis. Proton pumps create an acidic environment inside the endosome and iron is released from the transferrin. Iron is now available to the cell either for biologically active compounds or for storage, and the transferrin and transferrin receptors are recycled back to the membrane and the cycle can be repeated (Benito and Miller, 1998).

Beside the dietary factors described in the next section, a very important factor, the constitution of the body, also regulates iron absorption. Humans have no physiologically regulated pathway for iron excretion (Andrews and Schmidt, 2007) but have the primary physiological factors involved in non-heme iron absorption, which are summarised in Table 1.

Dietary factors involved in iron absorption

As previously mentioned, absorption of non-heme iron depends on its surroundings. Most of it is found as ferric iron, which precipitates as iron hydroxide at pH>3 if it is not kept in solution by chelators such as ascorbic acid, peptides or certain sugars and amino acids. In the following, the effects and mechanisms of some of the most influential factors on iron bioavailability will be described.

Vitamins: Ascorbic acid (the active form of Vitamin C) keeps iron available for absorption through several mechanisms. First, it promotes acidic conditions in the stomach and intestines, thereby providing optimal conditions for iron absorption; second, it chelates ferric iron and maintains it in a stable and soluble complex, even at higher pH. Finally it reduces ferric iron to its ferrous form, thereby preventing it from precipitating as ferric hydroxide (Teucher *et al*., 2004). Vitamin A or β-carotene also enhances iron absorption through formation of soluble iron complexes and to a certain extent it can reverse the effect of several inhibitors such as phytates and polyphenols (Layrisse *et al*., 2000).

Meat is an important enhancer of the bioavailability of non-heme iron. The "meat factor" is still largely unexplored, but recent findings suggest that it is due to peptides of myosin, generated by pepsin degradation in the gut, which binds and keeps iron in solution (Storcksdieck *et al*., 2007). Other suggestions to explain the "meat factor" involve sulphydryl groups of e.g., cysteine, to reduce ferric iron to ferrous iron (Mulvihill and Morrissey, 1998), and the induction of gastric juice production by proteins (Carpenter and Mahoney, 1992).

Weaker chelators, such as EDTA, organic acids or amino acids also contribute to iron solubility and these iron salts (and also ferrous sulphate) are some of the most commonly used iron fortificants (Hertrampf and Olivares, 2004; Hurrell *et al*., 2004; Salovaara *et al*., 2003b).

Table 1 Physiological factors that influence absorption of non-heme iron

Factor	Comment
Iron status	This is probably the most important factor in regulation of iron absorption. Iron uptake is negatively regulated by the hormone hepcidin, which is produced by the liver and regulated by iron levels, blood oxygen concentration and inflammation (Atanasiu *et al*., 2007; Nicolas *et al*., 2002)
Gastric acidity	Keeping the iron soluble through low pH is essential for iron absorption. Gastric juice with pH>2 can barely solubilise iron in bread, for example, as Fe^{2+} is far more soluble at intestinal pH than Fe^{3+} (Salovaara *et al*., 2003b). Furthermore, the acidity denatures proteins and gives optimal conditions for pepsin, thereby releasing iron from protein complexes and at the same time providing weak chelators to keep the iron soluble. Finally, as iron is co-transported with a proton, a positive pH gradient enhances iron uptake (Bezwoda *et al*., 1978; Kim *et al*., 1993; Salovaara *et al*., 2003b)
Transit time	Gastric emptying slows down in some iron-deficient rats, thereby increasing absorption time in duodenum. This factor is not well proven yet, as other experiments in iron-deficient rats showed no change in transit time (Huebers *et al*., 1990; Salovaara *et al*., 2003a)
Mucus secretion	Iron ions must cross a layer of secreted mucus before they reach the microvilli of the mucosal cells. Mucins bind cations in general and can inhibit or enhance iron absorption depending on the composition of minerals in the intestine (Conrad and Umbreit, 2002)
Health	As iron homeostasis is regulated through iron uptake more than excretion, diseases that cause abnormalities in the intestine and stomach can of course affect iron status. Mutations in iron transporters or regulators of their expression also influence iron status in general, but most often the phenotype of these genetic defects will be iron overload of tissues more than anaemia (Anderson *et al*., 2005; Annibale *et al*., 2000; Atanasiu *et al*., 2007)

Contradictory to this, iron uptake is inhibited by strong chelators such as PA and some polyphenols that have a chatechol group in their structure, able to form very stable chelates (Brune *et al*., 1989; Tuntawiroon *et al*., 1991). In this group of substances, tannic acid is a particularly strong inhibitor of iron absorption; in vitro experiments have shown that a molar ratio of 1:10 of tannic acid:iron results in 92% inhibition of iron absorption; in comparison, to reach the same result a molar ratio of 10:1 of phytic acid:iron is required (Glahn *et al*., 2002). However, the type of iron involved in these experiments is an important factor, as $FeSO_4$ has higher solubility than $FeCl_3$ in complexes with PA (Engle-Stone *et al*., 2005). Ascorbic acid and meat can to some extent reverse the inhibition of iron absorption by PA, but not tannic acid. Furthermore, the combination of tannic acid and fish is even able to decrease iron solubility further.

Other divalent cations, such as Ca^{2+} (Hallberg *et al*., 1992; Perales *et al*., 2006), Zn^{2+} (Camara *et al*., 2007), Co^{2+} and Mn^{2+} (Yeung *et al*., 2005) competitively inhibit iron absorption, probably because they use the same transporters (the DMT1) to enter the enterocytes, or because they co-precipitate with iron in phytic acid salts. The mode of Ca^{2+}-inhibition is still being debated (Perales *et al*., 2006; Roughead *et al*., 2005), as Ca^{2+} seems to have larger impact on heme iron absorption than on non-heme iron absorption, which could be due to mucosal uptake inhibition.

The interactions between inhibitors and enhancers decide the final absorption level of the element in the gut. The sums of the effects are, in general terms, predictable, and several algorithms to predict the percentage of non-heme iron absorption from food are available in literature (Conway *et al*., 2007).

Despite these cofactors involved in iron uptake, the main inhibitor of Fe absorption is phytic acid, and its chemical properties and functions will be described in the following section.

CHEMISTRY OF PHYTIC ACID

Myo-inositol (1,2,3,4,5,6) hexa*kis*phosphate

Inositol phosphates consist of an inositol ring and at least one phosphate group. Breaking the name into its separate parts describes the exact structure and appearance: the prefix "*myo*" refers to the conformation of the hydroxyl groups on the inositol ring. The nine possible configurations of the inositol ring have been annotated in a number of ways, but the adopted nomenclature is according to the set of rules suggested by Posternak (1965).

The conformation *myo*-inositol thus has one plane of symmetry, going directly from the most left to the most right atom (Fig.1). The D/L-prefixes specify the numbering direction of carbons in the inositol ring, where the D annotates counterclockwise and L clockwise counting, respectively. In general chemistry, numbering of the atoms should always follow the lowest possible route. Confusions regarding *myo*-inositols and enzymes related to them have led the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB, 1989) to recommend that the atoms in the *myo*-inositol ring should always be numbered according to the D configuration. The numbering should be initiated at the atom that is esterified in inositol phospholipids according to Agranoff (1978), using Agranoff's turtle as a reminder. The four limbs and tail of the turtle are coplanar and represent the five equatorial hydroxyl

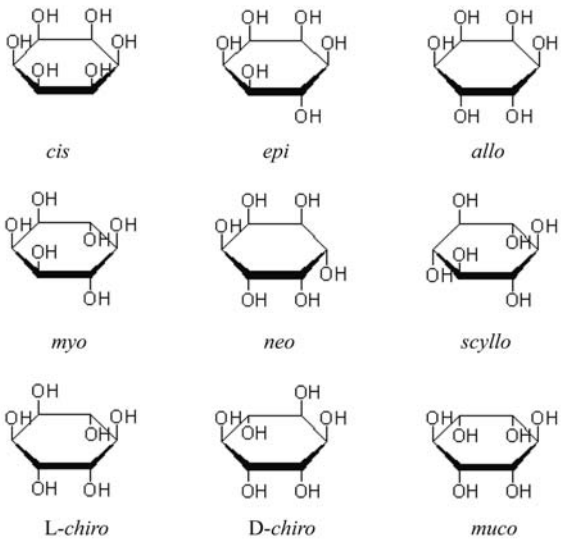

Fig.1 The nomenclature of the nine stereoisomers of inositol (Haworth projections). Seven have a mirror axis in the molecule and numbering of the carbon atoms can be performed either counterclockwise (D) or clockwise (L). Only the *chiro* form has specific D and L conformations (software: ChemDraw 10.0, Cambridgesoft.com)

groups. The turtle's head is erect and represents the axial hydroxyl group. Looking down at the turtle from above, the numbering of the turtle will begin at the right paw and continue past the head to the other limbs, thus numbering the inositol in the counter-clockwise (D) direction (Shears, 2004). This way, the plane of symmetry in the "*myo*" conformation will always go through C2 and C5, and the L (clockwise) configuration will start at the turtle's left paw and the D configuration will start at the turtle's right paw.

The consequence is a simplification of the naming of the enzymes related to the inositol phosphates: any phytase does not automatically produce D/L-*myo*-inositol (1,2,3,4,5) penta*kis*phosphate, thereby obeying the rules of numbering according to the lowest possible route. The phytase more specifically is named according to the D-*myo*-inositol penta*kis*phosphate that indicates initiation point of hydrolysis. According to this set of rules, a 3-phytase will initiate dephosphorylation at the C3 atom and have D-*myo*-inositol (1,2,4,5,6) penta*kis*phosphate as the product. As the enzyme hydrolyses additional phosphate groups from the inositol ring, the numbering of the groups will remain the same regardless of which phosphate is hydrolysed and the configurational counting will not change between the intermediates. Confusions surrounding the enzymes and their products are however not yet entirely resolved. The same enzyme some authors annotate as a 4-phytase, others refer to as a 6-phytase. Here, the IUPAC-IUB recommendations are used and readers should note that a D-6-phytase and an L-4-phytase produce the same product.

Myo-inositol is the major nutritionally relevant form of inositol, and although some of the other stereoisomers also are found in nature, they will not be addressed in this paper.

Myo-inositol (1,2,3,4,5,6) hexa*kis*phosphate has 6 groups of phosphates attached to the inositol ring. Using the prefix "hexa*kis*" instead of "hexa" indicates that the phosphates are not internally connected (Johnson and Tate, 1969) and the compound is consequently a polydentate ligand, which is a chelator that can bind to more than one coordination site of the metal atom. Each of the phosphate groups is esterified to the inositol ring and together they can bind up to 12 protons in total. The acidity of the protons varies from very strong acids to very weak (pK_a up to 9.4) although ionic strength of the solution and temperature influence these values (Brown *et al*., 1961; Costello *et al*., 1976; Torres *et al*., 2005).

Trivial names for D-*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate are IP_6, $InsP_6$ or phytic acid (PA). The last two will be used here depending on context and $InsP_1$~$InsP_5$ will also be used regarding lower *myo*-inositol phosphates counted in D configuration unless the prefix L is explicitly added.

In the pH range 0.5~10.5 phytic acid keeps the sterically stable conformation with one axial and five equatorial groups (Fig.2). At higher pH the phytic acid will flip to the reverse conformation with five axial and one equatorial group. A similar phenomenon is detected within the $InsP_5$-group, especially the $InsP_5$s with phosphorylation intact on all three carbon atoms C1, C3 and C5 as these groups are able to form a "chelation cage" that will use a cation to stabilise the otherwise more unstable conformation (Volkmann *et al*., 2002). For the same reason, crystallisation of PA also favors this conformation (He *et al*., 2006; Rodrigues-Filho *et al*., 2005). Lower inositol phosphates keep the stabile 5 eq/1 ax conformation over the entire pH range (Barrientos and Murthy, 1996).

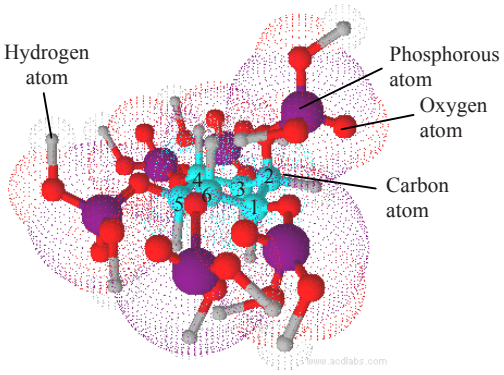

Fig.2 *Myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate in boat formation showing the 5 equatorial and the 1 axial group. Red oxygen, purple phosphorous and grey hydrogen atoms form phosphate groups on each of the blue carbon atoms that have been numbered according to the IUPAC-IUB recommendation. The special relationships between the atoms have been indicated with dots (software: ChemSketch 10.0, ACDlabs.com)

Phytate

The chelating effect of the phosphate groups causes PA to bind readily to mineral cations, especially to Cu^{2+} and Zn^{2+} which appear to have a high affinity for inositol phosphates. The order of the

ability of the mineral cations to form complexes in vitro with inositol phosphates has been found to be $Cu^{2+}>Zn^{2+}>Cd^{2+}$ for all $InsP_3$~$InsP_6$ at pH 3~7, but binding strength is weaker for the lower inositol phosphates (Persson *et al*., 1998). Similar binding assays using only phytic acid and none of the lower inositol phosphates show the order $Cu^{2+}>Zn^{2+}>Ni^{2+}>Co^{2+}>Mn^{2+}>Fe^{3+}>Ca^{2+}$ in one study (Vohra *et al*., 1965) and the order $Zn^{2+}>Cu^{2+}>Co^{2+}>Mn^{2+}>Ca^{2+}$ in another (Maddaiah *et al*., 1964). Despite of these results, the compositions of minerals in PA stores do not necessarily follow these orders of affinity. Recent finding shows that PA is stored in vivo in complexes not only with these minerals, but to a much larger extents with Mg, Ca and K (Bohn *et al*., 2007).

In presence of excess phytic acid, formation of soluble complexes between PA and a metal ion displaying 1:1 stoichiometries predominates. However, when metal ions are in excess, an insoluble solid called phytate is formed (Torres *et al*., 2005). The stoichiometry itself influences the solubility of phytate, as a very low or a very high metal ion:phytic acid ratio increases the solubility of the salt (Bullock *et al*., 1995; Cheryan *et al*., 1983; Nolan *et al*., 1987). However, heterologues precipitation with a combination of more than one type of metal ions seems to be increasing phytate formation. When especially Ca^{2+} is present simultaneously with another metal ion, it increases the proportion of the phytic acid-metal aggregate (Wise and Gilburt, 1983; Simpson and Wise, 1990). In many cases (Mn, Co, Ni, Cu, Sn and Zn), pure phytic acid-metal salt precipitates when the stoichiometry between PA and the ion is between 1:2 and 1:5, but addition of chloride ions to the solution can deprotonise the phytic acid further and form complexes with up to 6 ions per PA molecule (Vasca *et al*., 2002; Vohra *et al*., 1965).

The pH is another factor influencing the solubility of phytic acid (Cheryan, 1980). Its Ca, Cd, Zn and Cu salts tend to be soluble at pH lower than 4~5, whereas Mg-phytate is 100% soluble at acid pH up to pH 7.5 (Brown *et al*., 1961; Nolan *et al*., 1987). Solubility studies of bran phytate prove that at gastric pH (approximately pH 2), Ca is actually not bound to PA and this component does not contribute to the solubility of the Ca ion (Siener *et al*., 2001).

Because the resulting phytate precipitate is amorphous and full of co-crystallised water (up to 22 H_2O per PA molecule (Veiga *et al*., 2006)), the structure of the phytate salts cannot be obtained by crystallisation techniques. Attempts to resolve the structure of phytic acid-Fe^{3+} salt have revealed quite a high binding capacity with almost covalent-strong bonds between P-O-Fe. Precipitation initiates when at least four out of the six phosphate groups, are able to bind with the Fe^{3+}, leading to iron phytate with 2~4 iron atoms per molecule of PA (Mali *et al*., 2006). In the most idealised version of the salt, the ferric ions will be organised in a grid where each phosphate group is bound to two iron atoms, and each iron atom is bound to three phosphate groups and shared between two PA molecules (Thompson and Erdman, 1982). This is, however, a very constraint structure and in most cases phytic acid will not be completely saturated with ferric irons.

Functions of the *myo*-inositol phosphates

The primary functions of PA in seeds are storage of phosphates as energy source and antioxidant for the germinating seed (Raboy, 2003). Phytate as the mineral bound salt of PA is also an important mineral reserve in seeds, and it is stored in protein storage vacuoles in the aleurone cell layer or the embryo of the seed. Lower inositol phosphates are also involved in stress responses, membrane biogenesis and intracellular signalling (Storcksdieck *et al*., 2007; Loewus and Murthy, 2000).

Phytic acid accumulates during seed development until the seeds reach maturity and accounts for 60%~90% of total phosphorous content in cereals, legumes, nuts and oil seeds (Lott *et al*., 2000; 2001). It is however found in most eukaryotic tissues, where it is kept adherent to the cell walls through phosphoinositides, or in complexes with proteins or ions (Torres *et al*., 2005; Veiga *et al*., 2006). Phytic acid is found in ten-fold higher concentrations in the brains of rats as in the kidney, indicating that it has great potential outside the plant kingdom (Grases *et al*., 2001a).

In eukaryotes in general, three main features of PA keep it involved in a number of metabolic processes: its chelating properties and its ability to function as a phosphate donor/acceptor makes it ubiquitous/abundant in numerous cell systems. Moreover, the lower inositol phosphates are involved in a number of cell signalling pathways and finally PA may

consequently act as a precursor of compounds with this function.

One of the best known properties of PA is its antioxidative ability by binding and thereby inactivate Fe ions in solution. This prevents the ferric irons from participating in the Fenton reaction (the formation of the hydroxyl radical ·OH as a consequence of oxidation of Fe^{2+} to Fe^{3+} during reaction of Fe^{2+} with H_2O_2 or peroxides). PA is even better than the in vitro commonly used EDTA (Graf *et al*., 1984; Wong and Kitts, 2001). These radicals are highly reactive molecules that rapidly and non-specifically react with proteins, lipids or DNA, thereby causing cell injury or cell death. Alzheimer's and Parkinson's diseases, cirrhosis, arthritis and cancer have all been linked with radicals (Benzie, 2003) and PA may therefore be a potential inhibitor of all of these illnesses.

The chelating ability of PA is also used in preservation, where historic ink, made from gallic acid from the tannins and Fe ions, has been used on paper: phytic acid prevents iron catalysed oxidation of the cellulose in the paper, thereby doubling the half-life of the documents (Neevel, 1995). $InsP_5$s with various different combinations of conformations of the phosphate groups are also capable of inhibiting radical formation, as long as the 1,2,3-(equatorial-axial-equatorial) phosphate conformation is kept intact (Hawkins *et al*., 1993), which has also proven useful in paper preservation (Sala *et al*., 2006). Quite recently, the ability of PA to inhibit Fe-dependent reactions has also been tested with some success in storage of meat (Stodolak *et al*., 2007).

In mammalian organisms, PA has been implicated in starch digestibility and blood glucose response (Lee *et al*., 2006), in the prevention of dystropic calcifications in soft tissues (Grases *et al*., 2004) and kidney stone formation (Grases *et al*., 1998; Selvam, 2002), and in the lowering of cholesterol and triglycerides (Jariwalla, 1999; Onomi *et al*., 2004). PA has also been suggested to be part of a structure that could inhibit transcription of the viral genome from HIV-1 (Filikov and James, 1998), and apparently it has been tested in toothpaste as a tool for preventing plaque formation (Vasca *et al*., 2002).

At the cellular level, PA or inositol phosphate intermediates are involved in gene regulation, efficient export of mRNA, RNA-editing and DNA repair (York *et al*., 1999; York, 2006). The lower inositol phosphates such as $Ins(1,4,5)P_3$ take part in cell signalling cascades (Berridge and Irvine, 1989) and pathways leading to versatile functions within Ca^{2+} mobilisation and signalling (Efanov *et al*., 1997; Larsson *et al*., 1997). They also contribute to protein folding (Macbeth *et al*., 2005) and trafficking (Shears, 2004), endo- and exocytosis (Efanov *et al*., 1997; Saiardi *et al*., 2002), oocyte maturation (Angel *et al*., 2002), and cell division and differentiation (Berridge and Irvine, 1989).

The involvement of phytic acid in cancer therapy is also widely discussed (Vucenik and Shamsuddin, 2006) and the potential of PA in cancer treatment are shown in Table 2. As with the previously mentioned functions, most of the effects of phytic acid are related to its chelating abilities, to the lower inositol's involvement in signalling pathways or to their phosphate donor/acceptor capabilities. After intake, phytic acid is dephosphorylated to lower inositol phosphates and these can act as an antioxidant by inhibiting iron mediated oxidative reactions, enhancing immunity by increasing Natural Killer cell function and activity, or stimulate bacterial killing by neutrophils. Furthermore, the compounds can normalise abnormal cell proliferation, induce cell differentiation and apoptosis and inhibit angiogenesis. In addition, inositol phosphates modify Phases I and II metabolising enzymes by causing G0/G1 arrest in cancer cells and modulate oncogene expression and prevent tumor metastasis formation.

It should be noted that although many of these functions of lower inositol phosphates have been shown in vitro, the human small intestines show very little phytase activity (Iqbal *et al*., 1994). Even though

Table 2 Cancer prevention and treatment functions of phytic acid according to Vucenik and Shamsuddin (2006)

Enhancement	Other effects	Inhibition
Cell differentiation	Antioxidant	Cell proliferation
Apoptosis	Oncogene regulation	Tumor metastasis
Immune function	Regulation of Phases I and II enzymes	Angiogenesis
		Inflammation

mammalian cells are able to synthesize the inositol phosphates themselves (York, 2006), endogenous synthesis of phytic acid is minor (Grases *et al*., 2001a; 2001b). Using phytic acid in cancer therapy would therefore be dependent on daily intake of phytic acid. This treatment is influenced by the fact that absorption rate of PA itself is low (Grases *et al*., 2006) and it is suggested that there is a maximum to the uptake in the gut, which again is regulated by PA plasma levels (Grases *et al*., 2001c; Kemme *et al*., 2006). The effect of phytic acid in cancer therapy is therefore still under investigation and so far the results have mainly been in connection with colon cancer, where uptake is not a prerequisite.

Regarding the negative functions of phytic acid, the outcome of a homogenous and high PA diet has long been known to be mineral deficiency and malnutrition (McCance and Widdowson, 1949; Cheryan, 1980). The negative effects of PA are consequences of the same chemical features as the potential positive effects. The capability of binding minerals makes phytic acid an antinutritional factor, because the solubility of the phytic acid:metal-complexes are low at the pH of the major part of the intestines. Regarding lower inositol phosphatese, $InsP_5$s are also able to complex cations, whereas inositol phosphates with only three or four phosphate groups attached to the inositol ring do not inhibit mineral absorption independently. They are however able to interact with PA and thereby contribute to the negative mineral absorption (Sandberg *et al*., 1999). Furthermore, phosphorous in the form of phytic acid is largely unavailable as a nutritional factor to monogastric animals because insufficient degradation capabilities in the gastrointestinal tract prevent the phosphorous from being biologically available. Feeds for pigs and poultry are therefore traditionally supplemented with inorganic phosphate to meet the nutritional requirements for optimal growth of the animals. The excess of phosphorous bond in phytic acid is then excreted through the faeces and spread as manure into the soil. The potential eutrophication of fresh water streams, lakes and near costal areas can then cause cyanobacterial blooms, hypoxia and death of aquatic animals and production of nitrous oxide, a potential greenhouse gas (Vats *et al*., 2005). In the laboratory, phytic acid in faeces also inhibits polymerase chain reactions (PCR), thereby preventing PCR-based diagnostic tests for detecting microorganisms in livestock (Thornton and Passen, 2004).

Storage of phytic acid

Phytic acid and its co-precipitated cations are stored in electron dense spherical particles named globoids (Pfeffer, 1872). The globoids are localised predominantly in the aleurone layer (wheat and barley) or in the embryo (maize) (Odell *et al*., 1972). They are compartmentalised inside protein storage vacuoles in the seeds. Protein storage vacuoles in general contain three morphologically distinct regions: a matrix that contains most of the soluble storage proteins, crystalloids composed of proteins yet to be investigated but organised in lattice structure, and globoids of PA or oxalate crystals (Lott, 1980). How many of these regions are present in the seed is species dependent. Protein storage vacuoles of wheat are composed only of matrix proteins and phytate globoids (Morrison *et al*., 1975) which make the cells highly susceptible to extraction of phytate. Consequently, even a minor rupture of the aleurone cells means release of phytate into the surrounding environment (Antoine *et al*., 2004). Morrison *et al*.(1975) also reported the detection of a layer of lipid droplets around the wheat "aleurone grain". Membranes surrounding the globoids have been reported in other species such as *Brassicaceae* (Gillespie *et al*., 2005) and *Solanaceae* (tomato) (Jiang *et al*., 2001). Certain reports of the phenomenon in wheat has however not been published to date, but the findings of Morrison *et al*.(1975) could be parts of an inner membrane in the protein storage vacuole, which have been broken during staining and cutting of the samples.

The size of the phytate globoids depends on the amount of phytate in the grain. In wildtype (WT) wheat, globoids up to 4 μm in diameter have been detected (Antoine *et al*., 2004), whereas a low phytic acid (lpa) wheat mutant (Js-12-LPA) with the same amount of P in the grains but lowered phytate concentration has smaller globoids, organised in clusters (Joyce *et al*., 2005). This phenomenon has also been observed in WT wheat grown under low P conditions (Batten and Lott, 1986) and in lpa mutations in other cereals, such as rice (Liu *et al*., 2004), maize (Lin *et al*., 2005) and barley (Ockenden *et al*., 2004).

The compositions of the globoids seem to be the same for globoids from WT and lpa wheat. Using

scanning transmission electron microscopy-energy-dispersive X-ray analyses on section of wheat grains, P, K, Mg and Ca are found in high concentration in globoids (Joyce *et al*., 2005), whereas especially Cu and Zn are lowered in lpa compared to WT (Guttieri *et al*., 2003). Minerals were recently quantified by inductively coupled plasma-mass spectrometry (ICP-MS) of phytate globoids purified from wheat bran (Bohn *et al*., 2007). Although Cu has high affinity to phytic acid, there is no indication that Cu-phytate globoids are the primary storage facility for this element and K>Mg>Ca>Fe (in concentration order) were found as the main minerals (Bohn *et al*., 2007). Fe is also found in both lpa and WT wheat, but its distribution is restricted because globoids near the embryo were shown to contain the relatively highest amounts of this element in WT wheat (Lott and Spitzer, 1980).

Dephytinisation and nutrition

The chelating properties of PA not only result in the binding of cations in seeds. When released during food or feed processing or in the gut, PA also binds minerals and makes them unavailable as nutritional factors. Iron and zinc uptake have both been shown to be inhibited when the phytic acid:metal ratio increases above 10:1 (Gharib *et al*., 2006; Glahn *et al*., 2002). In human studies, phytic acid has been reported to inhibit absorption of iron, zinc, calcium, magnesium and manganese but surprisingly not copper (Bohn *et al*., 2004; Davidsson *et al*., 1995; Egli *et al*., 2004; Hallberg *et al*., 1989; Lonnerdal, 1997; Phillippy, 2006; Reddy *et al*., 1996). Removal or degradation of PA would therefore increase the bioavailability of many cations and the nutritional value of the meal, and several strategies to reduce it are therefore considered.

Milling of cereals removes the phytic acid, but this treatment also removes the major parts of the minerals and dietary fibres and cannot therefore be a nutritional solution to the problem. Similarly, soaking or extracting in aqueous solutions can remove up to two thirds of the PA by the action of endogenous phytase activity, but loss of minerals, water-extractable proteins and vitamins also occurs (Hurrell, 2004). Heat treatments have minor effects (Pontoppidan *et al*., 2007) usually due to leaching of minerals into the boiling water. Different processing and cooking methodologies for reduction of PA have been compared between wheat variety, and the results have been that if one method is efficient in reduction of PA in one wheat variety, this may not apply for another. Furthermore, the method with the highest phytic acid reduction (germination for 48 h) still only reduced its content by up to 40% (Masud *et al*., 2007). Avoiding PA formation in the first place or catalysing its degradation by the use of PA hydrolysing enzymes would therefore be more beneficial approaches to dephytinisation.

Reducing phytate content through lpa mutants have been attempted through knock-out of genes involved in PA biosynthesis. With the only exception of one barley mutant accumulating Fe, the mineral composition does not dramatically change in lpa mutants, indicating that there is no direct link between mineral distribution and phytic acid biosynthesis (Joyce *et al*., 2005; Liu J.C. *et al*., 2004; Liu K. *et al*., 2007; Ockenden *et al*., 2004). Chicks fed lpa barley or corn, respectively excrete 33% less phosphate and they show enhanced growth and bone structure (Jang *et al*., 2003) as compared to chicks on a normal diet. Similar results have been reported for growing pigs (Veum *et al*., 2002) and rats (Poulsen *et al*., 2001) on lpa barley diet. Still, almost the same results on growth performance can be achieved by fortifying with inorganic phosphorous instead of using lpa mutants; the main difference between lpa diets and phosphorous fortified diets is therefore the higher phosphorous excretion from the latter. In human studies, Fe, Ca and Zn uptake increased when meals were based on lpa maize (Adams *et al*., 2002; Hambidge *et al*., 2005; Mendoza *et al*., 1998) and all in all this would be a reasonable approach to increase the bioavailability of micronutrients.

Due to the involvement of the lower inositol phosphates in plant cell metabolism, production of the perfect lpa mutant has however turned out to be quite a challenge. Since many kinases and tranferases are involved in the synthesis of phytic acid (Josefsen *et al*., 2007), the mutants often find ways to circumvent the knocked out pathway. Furthermore, the yield or germination ability is affected if PA content is reduced more than 50% (Raboy, 2007), thereby making this approach unattractive from an economic perspective. The best result so far is the maize lpa1 mutant, which is mutated in an embryo-specific ATP-

binding cassette (ABC)-tranporter and it is able to hold up to 90% reduction of PA without compromising seed viability. The effect on mineral distribution in this mutant is yet to be evaluated (Shi *et al*., 2007).

Instead of blocking its biosynthesis, an attempt to reduce PA in wheat products has been performed by introducing the *Aspergillus niger* phytase gene *phy*A into a wheat variety by particle bombardments of immature wheat embryos (Holm *et al*., 2002). The concerted action of wheat phytase and *Aspergillus* phytase has proven to be an efficient way of increasing the rate of PA degradation in transgenic wheat flour, although experiments in food or feed applications have not been reported yet.

The most successful dephytinisations so far involve endogenous enzymatic activity during germination, but this is a species dependent phenomenon where some plants are more sensitive to the treatment than others. Wheat, barley and rye all have high phytase activity in the grain, whereas maize, millet and sorghum have low initial phytase activity that increase rapidly after germination (Egli *et al*., 2002). Adding exogenous phytase to food and feed is therefore under investigation in many forms and the next section will present the general classes of phytases and some of the applications.

PHYTASES

Definition

The term phytase (*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate phosphohydrolase) is defined as a class of phosphatases with the in vitro capability to release at least one phosphate from PA, thereby releasing phosphate and lowering inositol phosphates and potentially chelated minerals.

The earliest reports of a phytase activity are from the blood of calves (McCollum and Hart, 1908), and rice bran (Suzuki *et al*., 1907), indicating from its discovery, that this enzyme is found in diverse organisms. Later discoveries have also found phytases in bacteria, yeast and fungi. As previously mentioned, most monogastric animals, including humans, lack the enzyme in their digestive track, making PA hydrolysis in the gut dependent on mucosal or bacterial enzymes or on non-enzymatic hydrolysis by gastrointestinal acidity. The number of enzymes described as phytases has increased rapidly over the years and details regarding the enzymatic properties can be found in several reviews (Haefner *et al*., 2005; Oh *et al*., 2004; Vats and Banerjee, 2004).

IUPAC-IUBMB (the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology) currently acknowledges three classes of phytase enzymes, which initiate the dephosphorylation of PA at different positions on the inositol ring, and produce different isomers of the lower inositol phosphates. Within each class of phytase structural differences can be found, and not all enzymes within a certain class hydrolyze phosphate from PA through the same mechanism. Similarly, the enzymes can be grouped as acidic, neutral or alkaline phosphatases depending on the optimum pH of activity. All phytases however have pronounced stereo specificity and a strong preference for equatorial phosphate groups over axial groups (Lei and Porres, 2003).

EC 3.1.3.8: the 3-phytases

The largest group of phytases to date contains the 3-phytases (EC 3.1.3.8), which in general are found in fungi and bacteria. Structurally, most of the 3-phytases show homology to β-propeller phosphatase (BPP), or histidine acid phosphatases (HAP).

BPPs are tightly bound to three Ca ions and need two adjacent phosphate groups to bind to the "cleavage site" and to the "affinity site" before hydrolysis can occur. The end product has been suggested to be inositol-triphosphate—either Ins(1,3,5)P_3 or Ins(2,4,6)P_3 (Kerovuo *et al*., 2000; Shin *et al*., 2001), but most recent data give evidence that Ins(2,4,6)P_3 is sole the end product (Oh *et al*., 2006), confirming the equatorial preference of most phytases.

Most bacterial, fungal and plant phytases belong to the HAPs. Within this structural classification, there are two phytase subgroups: Some show broad substrate specificity but low specific activity for PA, whereas others have narrow substrate specificities but high specific activity for PA. All members of the HAP class share two conserved active site motifs, RHGXRXP and HD (van Etten *et al*., 1991), and hydrolyse metal-free phytate in the acidic pH-range. However, they do not necessarily share any additional regions of sequence similarity (Maugenest *et al*.,

1997). HAPs can initiate hydrolysis of phytic acid on either the C3 or the C6 position of the inositol ring and produce *myo*-inositol monophosphate (in particular Ins(2)P due to its axial position) as the final product (Greiner and Carlsson, 2006; Mullaney and Ullah, 2003; Oh *et al*., 2006).

The catalytic mechanism of hydrolysis by HAPs is proposed to be as follows: the histidine residue in the conserved motif is used to make a nucleophilic attack on the carbon of interest, and an aspartic acid in the C-terminal of the enzyme stabilises the leaving group by acting as a proton donor (Ostanin and van Etten, 1993; Ostanin *et al*., 1992). A water molecule is consumed in the hyrolysis of the intermediate, but the phospho-histidine interaction is stable enough for crystallisation, as it has been done e.g., with a heat stable HAP phytase from *Aspergillus fumigatus* (Xiang *et al*., 2004).

A well studied phytase from the class of 3-phytases is isolated from baker's yeast, *Saccharomyces cerevisiae*. This phytase is extracellular and its expression of it can be induced when grown in a medium containing $InsP_6$ as the sole phosphorous source (Andlid *et al*., 2004). The extracellular yeast 3-phytase has been studied intensively as its potential during baking is obvious (Turk *et al*., 1996). Unfortunately, the endogenous phytase of yeast is repressed by the concentrations of phosphate in the dough during leavening (Andlid *et al*., 2004). Constitutive expression of yeast phytase is possible by deletion of genes expressing negative regulators (Veide and Andlid, 2006), but recent investigations have to a large extent advanced to focusing on the use of baker's yeast as host for heterologues expression of microbial phytases due to their higher thermostability (Kaur *et al*., 2007).

The sequential dephosphorylation of phytic acid by an *S. cerevisiae* phytase is depicted in Fig.3. Not all subproducts have been confirmed yet, but the major pathway is from phytic acid over Ins(1,2,4,5,6)P_5 through Ins(1,2,5,6)P_4 and Ins(1,2,6)P_3 to probably Ins(1,2)P_2. End product is Ins(2)P (Andlid *et al*., 2004; Greiner and Alminger, 2001).

EC 3.1.3.72: the 5-phytase

Only a single 5-phytase (EC 3.1.3.72) has been detected so far. This alkaline phytase from lily pollen is interesting, because it is the only one in the family of phytases that initiates hydrolysis with an attack of a phosphate group in the plane of symmetry (the "turtle's tail"), thereby creating yet another symmetrical compound as shown in Fig.4 (Barrientos *et al*., 1994).

The pollen phytase shows highest activity at pH 8.0 and temperature 55 °C (Jog *et al*., 2005). It has the conformation of an HAP phytase, but with the exception of the active site, the amino acid sequence homology is higher towards multiple inositol polyphosphate phosphatase (MINPP) from humans or rats (Mehta *et al*., 2006).

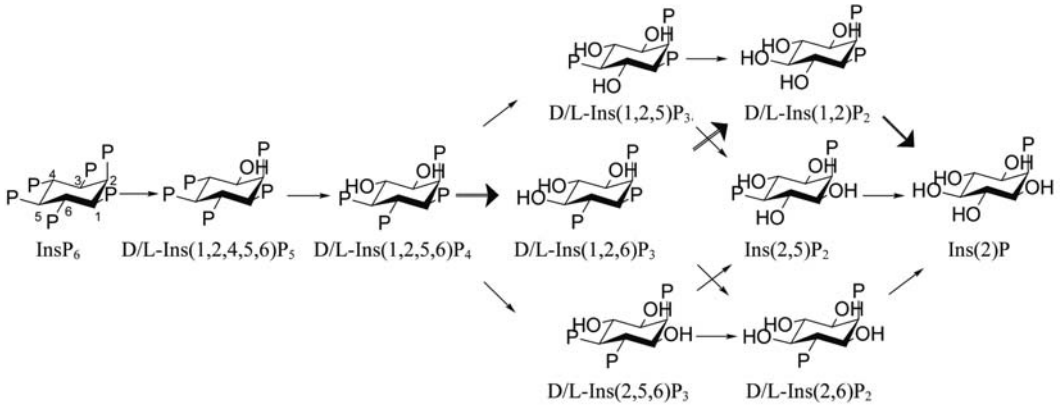

Fig.3 Dephosphorylation of phytic acid by the 3-phytase from *Saccharomyces cerevisiae*. Hydrolysis of phytic acid initiates on C3 of the inositol ring and the adjacent phosphates are removed consecutively. Fat arrows indicate major pathway of dephosphorylation, narrow arrows indicate possible pathways that have not been excluded (modified from Greiner *et al*., 2001a). End product is Ins(2)P. In this figure, P represents inorganic phosphate and the rest of the molecules have been omitted for clarity (Software: ChemSketch 10.0)

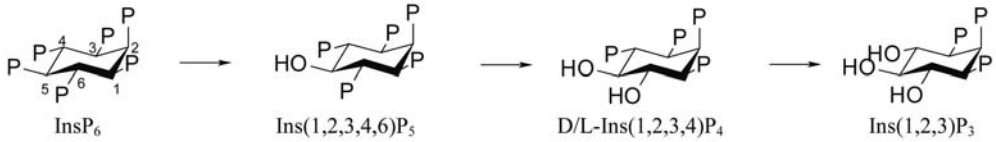

Fig.4 Dephosphorylation of phytic acid by the 5-phytase from lily pollen. Hydrolysis of phytic acid initiates on C5 of the inositol ring and the adjacent phosphates are removed consecutively. End product is Ins(1,2,3)P_3 (modified from Barrientos *et al*., 1994). In this figure, P represents inorganic phosphate and the rest of the molecules have been omitted for clarity (software: ChemDraw Ultra 10.0)

EC 3.1.3.26: the 4/6-phytases

The 4/6-phytases (EC 3.1.3.26) act on the carbon atom next to C5 of the inositol ring. The official name should be 4-phytase, but traditionally it has been called a 6-phytase. Several structurally different phytases are found in this group: The purple acid phosphatase (PAP), the ADP phosphoglycerate phosphatase (related to EC 3.1.3.28), as previously mentioned an HAP-class is also involved, and again the acid phosphatase (related to EC 3.1.3.2).

Plant phytases act preferentially at the C6 carbon, and are 6-phytases. In general, the 4/6-phytases are the most active in weak acidic environments (pH 4~6) with a temperature optimum in the range 40~60 °C. They are usually 50~70 kD and have Michaelis-Menten constants (K_m) in the range of 10^{-5}~10^{-4} mol/L phytic acid. There are exceptions, such as the rather large phytase from tomato roots (164 kD (Li *et al*., 2007)) and the highly active phytase from wheat (K_m 0.5 μmol/L phytic acid (Nakano *et al*., 1999)). Also, some alkaline phytases have been purified, such as the previously mentioned 5-phytase from lily pollen, legume seeds and the phytase from *Typha latifolia* (cattail) pollen (Hara *et al*., 1985; Jog *et al*., 2005; Scott, 1991). Their pH optima lie at pH 8. A list of characterised plant phytases are found in Table 3.

Wheat phytase

Activity of wheat phytases (EC 3.1.3.26) were first reported in (Posternak and Posternak, 1929), where an aqueous extract of wheat bran was used to investigate optically active *myo*-inositol polyphosphate esters that were produced during the degradation of phytate. Attempts to characterise the enzymes were performed by Collatz and Bailey (1921), and Kolobkowa (1936). Both groups found 55 °C to be the optimum temperature for wheat phytase, as a result that was confirmed along with optimum pH of 5.15 (Peers, 1953). Despite these fairly similar results it should however be noted that none of the groups managed to purify the enzymes to homogeneity and that it required additionally 20 years before a pure phytase from wheat was identified.

The number of phytases in wheat is still under investigation. Two enzymes, Phy1 and Phy2, have previously been purified from wheat bran (Lim and Tate, 1973) and two isozymes with the N-terminal amino acid sequence EPAXTLTGPSRPV have also been purified (Nakano *et al*., 1999). Based on amino acid sequence and masses of tryptic peptides, a third enzyme with homology to PAP phytases was cDNA cloned from wheat and its homologue from barley (Rasmussen *et al*., 2007). Recently four cDNAs encoding for MINPPs were also cloned. At least two of these show in vitro phytase activity and they are probably expressed during late seed development and germination (Dionisio *et al*., 2007).

Although a PAP phytase has also been found in soybean (Hegeman and Grabau, 2001), wheat phytase is mainly used as model for characterisation of this type of phytase. It has been documented to be inhibited when the phytate is complexed with Al^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ag^{+}, Hg^{+} or Zn^{2+}, but not salts of Ca^{2+}, Mg^{2+} and Mn^{2+} unless some of the inhibiting ions are present in the solution as well. Organic acids, such as citric acid or oxalate, can to a certain extent circumvent this inhibition, but in too high doses they themselves become inhibitory factors (Nagai and Funahashi, 1962; Tang *et al*., 2006).

The exact structure of PAP phytase has not been elucidated yet, but PAPs usually contain a Fe^{3+}-Me^{2+} centre in their active site, where Me stands for divalent Fe, Mn or Zn (Olczak *et al*., 2003; Strater *et al*., 1995). Five conserved regions containing seven negatively charged amino acids stabilise this structure (Fig.5). The PAPs catalyse the hydrolysis of activated phosphoric acid esters and anhydrides in the pH range from 4 to 7 (Vincent *et al*., 1992) through an interaction between the Me^{2+} and the substrate followed by a

nucleophilic attack on the phosphate group by a Fe^{3+}-coordinated hydroxide ion (Klabunde *et al*., 1996). Three histidines in the conserved regions stabilise the transition state.

Some similar properties are displayed by the investigated wheat phytases (Table 3). All the enzymes characterised so far have been most active at weak acidic to neutral pH. Optimum activity lies between pH 4.5 and 7.2 and there are several reports of an enzyme with the highest activity around pH 6. This suggests that they all are acid phosphatases. Temperature optimum is in the range of 45 °C (Bohn *et al*., 2007; Nakano *et al*., 1999) to 65 °C (Dionisio *et al*., 2007) and there is general agreement concerning inhibition by approximately 3 mmol/L phosphate of some types of phytases but not others. Inconsistencies in the characterisations primarily regard the activities of the enzymes. The K_m vary between 0.48 μmol/L and 830 μmol/L phytic acid and the velocities at maximal concentrations of substrates (V_{max}) vary from 127 to 230 μmol Pi/(mg·min) (Bohn *et al*., 2007; Nakano *et al*., 1999).

The sequential dephosphorylation of PA by wheat phytase has been studied by several groups (Bohn *et al*., 2007; Lim and Tate, 1973; Nakano *et al*., 2000; Tomlinson and Ballou, 1962), and it has been determined that it acts through both C6 and C3 to yield *myo*-inositol (Fig.6). This is also the case when isolated phytate globoids are used as the substrate, although the activity of the wheat phytase decreases approximately 30% on globoids as compared to when acting on pure PA (Bohn *et al*., 2007).

Table 3 Phytases purified from plants and their properties. The lower part of the table describes the properties of cereal phytases; the top part of the table shows examples of other plant phytases in alphabetical order. If more than one phytase is referred to, the individual characteristics of the enzymes are separated with semicolon. Blank space means no value reported

Phytase source	pH	Temp. (°C)	K_m (mmol/L)	*M* (kD)	Reference
Buttercup squash	4.8	48		67	Goel and Sharma, 1979
Canola seed	4.5~5	50	0.36; 0.25	70	Kim and Eskin, 1987
Faba beans	5	50	0.148	65	Greiner *et al*., 2001b
Hazel seed	5		0.162	72	Andriotis and Ross, 2003
Legume seeds	8				Scott, 1991
Lily pollen	8	55	0.081	88	Jog *et al.*, 2005
Lupin seeds	5.0	50	0.08; 0.3; 0.13	57~64	Greiner, 2002
Mung beans	7.5	57	0.65	160	Mandal *et al.*, 1972
Navy beans	5.3	50	0.018		Lolas and Markakis, 1977
Peanut	5	55		22	Gonnety *et al.*, 2007
Rapeseed	5.2	50			Mahajan and Dua, 1997
Scallion leaves	5.5	51	0.2		Phillippy, 1998
Soybean seeds	4.5~4.8; 4.5~5	55; 58	0.05; 0.061	119; 72~130	Gibson and Ullah, 1988; Hegeman and Grabau, 2001
Sunflower	5.2	55	0.29		Agostini and Ida, 2006
Tomato roots	4.3	45	0.038	164	Li *et al.*, 1997
Typha latifolia pollen	8		0.017		Hara *et al.*, 1985
Barley	5; 6	45; 55	0.072; 0.19	67	Greiner *et al.*, 2000
Maize	5	55	0.02; 0.03; 0.04; 0.117	71; 76	Hubel and Beck, 1996; Laboure *et al.*, 1993
Oat	5.0	38	0.030	67	Greiner and Alminger, 1999
Rice	4.4; 4.6	40	0.17; 0.09	66; 61	Hayakawa *et al.*, 1989
Rye	6	45	0.3	67	Greiner *et al.*, 1998
Spelt	6	45	0.4	68	Konietzny *et al.*, 1994
Wholemeal wheat	5.15	55	0.3		Peers, 1953
Wheat bran	5		0.49		Nagai and Funahashi, 1962
Wheat bran	5.6; 7.2		0.02; 0.2	47	Lim and Tate, 1973
Wheat bran	6; 5.5	45; 50	0.0005; 0.0008	68; 66	Nakano *et al.*, 1999
Crude extract wheat	6	45	0.83	65	Bohn *et al.*, 2007

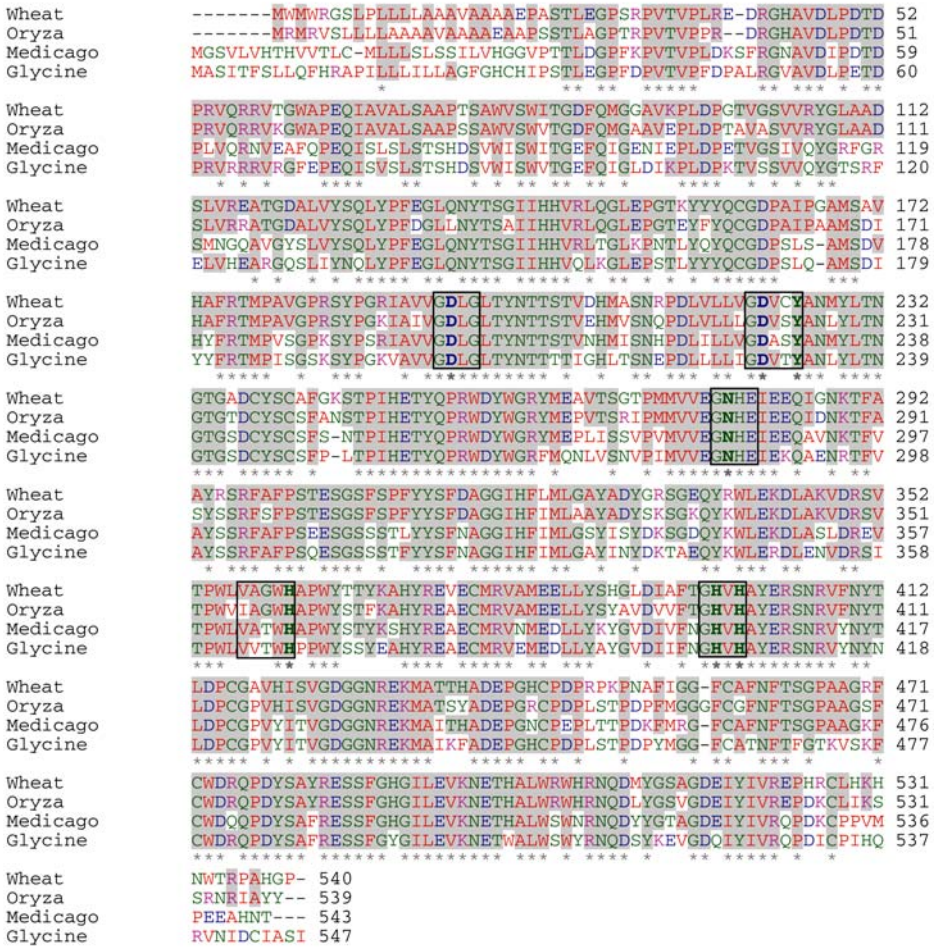

Fig.5 Alignment of wheat phytase to related PAPs in other plants: *Orysa sativa*, *Medicago truncatula* and *Glycine max*. The five conserved regions (black boxes) contain the seven invariant amino acids (bold) involved in coordination of the metals in the binuclear center (Klabunde *et al*., 1995). Asterisks (*) mark invariant amino acid residue between the aligned sequences and numbers to the right are residue numbers

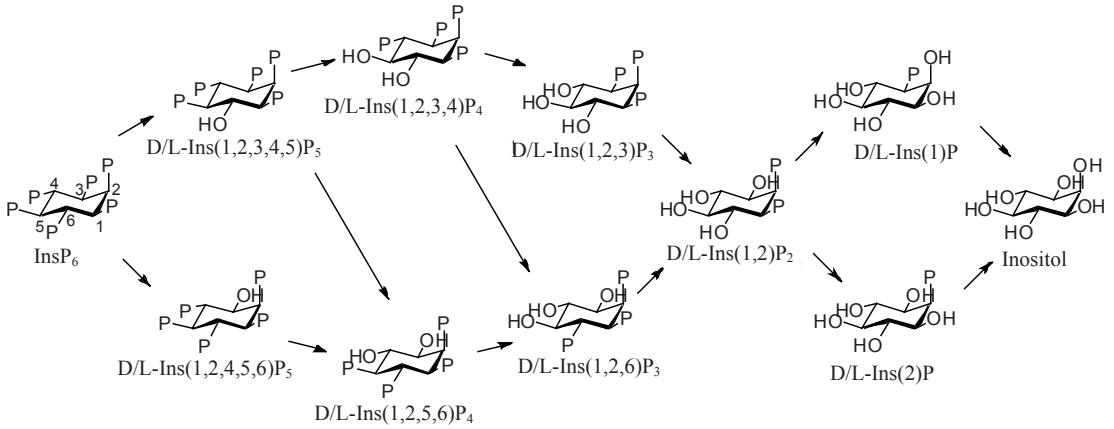

Fig.6 Dephosphorylation of phytic acid by 3/6-wheat phytase according to most recent publications. Hydrolysis of phytic acid initiates on C6 or C3 of the inositol ring (Bohn *et al*., 2007) and the adjacent phosphates are removed consecutively. End product is inositol (Nakano *et al*., 2000). In this figure, P represents inorganic phosphate and the rest of the molecules have been omitted for clarity (software: ChemDraw)

PHYTASE IN APPLICATIONS

Degradation of PA and the release of phosphorous and minerals have as previously described been of great interest to human and animal nutritionists as well as ecologists. The development of the most optimal method for applying phytase into food and feed is an on-going process with multiple pathways. The first commercially available phytase was from *Aspergillus niger* (NatuphosTM, BASF) in 1991, but now several phytases are on the market, from e.g., *Peniophora lycii* (RonozymeTM, DSM/Novozymes), *Escherichia coli* (QuantumTM, Diversa/Syngenta) and *Schizosaccharomyces pombe* (PhyzymeTM, Diversa/Danisco). The major problem in production of plant phytases is that a cost-effective and efficient production of the enzymes is yet to be developed. The higher pH and thermal stability of microbial phytases compared to plant phytases have made the microbial phytases more investigated for industrial purposes.

Production of phytase in transgenic plants

The first hurdle in the use of phytase as means for decreasing PA concentration is the production of phytase. In planta production of phytases can be used to reduce the amount of PA in feed for animals, but one of the problems in this regard is the stability of the enzyme. In wheat and barley the WT *Aspergillus fumigatus* phytase has been expressed (Brinch-Pedersen *et al*., 2003) as well as a heat stable engineered phytase. Although the native *Aspergillus* phytase had high regeneration abilities after heat treatment, it was found that high temperature stability was a more reliable approach to generating low phytic acid feed (Brinch-Pedersen *et al*., 2006).

In several experiments it has been confirmed that "biofarming" of the phytase is a cost-effective approach to its production. Native *Aspergillus ficuum* phytase has been expressed in tobacco, alfalfa and potato leaves. The most common approach is to use the Cauliflower Mosaic Virus (CaMV) 35S promoter for the construct, and the results are enzymes with almost the same characteristics as the fungal phytase, although minor changes in pH optima and sizes have been observed (Ullah *et al*., 1999; 2002; 2003). Similar experiments with a heat stable *A. fumigatus* phytase expressed in tobacco leaves and *Pichia pastoris* have also shown great potential. Especially regarding feed production, the relatively high heat resistance of the *A. fumigatus* enzyme is an important asset because the enzyme will then be able to withstand the elevated temperatures employed during feed pelleting processes (Wang *et al*., 2007).

Purification of the phytase is not always necessary for applications: a transgenic strain of *Bacillus mucilaginosus*, a rhizosphere soil organism able to express high phytase activity extracellularly and degrade PA in the soil, has proven to be able to promote tobacco growth and increase phosphorous content in the plant, thereby potentially limiting eutrophication (Li *et al*., 2007). Of more nutritional relevance are experiments performed on pigs that were fed fresh rice leaves expressing yeast phytase. The leaves were ground and mixed with a grain-based diet, and PA was degraded using up to twelve weeks old leaves, indicating that the yeast phytase is quite resistant to denaturation when expressed in rice (Hamada *et al*., 2006).

Phytase as feed additive

Exogenous phytase in feed has multiple benefits, mainly in increasing mineral, phosphorous and energy uptake and thereby decreasing the necessity to fortify the fodder with these substances. The increased availability of phytic acid phosphorous at the same time decreases phosphorous excretion and hence reducing the phosphate load in water supplies in regions with intensive rearing of animals. Comparison of 4 commercially available phytases as fortifiers of pigs feed revealed that none of them satisfied all of the criteria of an ideal phytase for feed production, such as resistance to denaturation under extreme temperatures and pH (Boyce and Walsh, 2006). Nonetheless, supplemental microbial phytase increased P availability by 38%, 12% and 15% in pig diets containing maize, wheat and triticale, respectively (Dungelhoef *et al*., 1994), and up to 60% reduction in manure P due to phytase supplementation has been reported (Nahm, 2002). Experiments with growing pigs have also shown that the Zn supply can be reduced to approximately 1/3 of the otherwise required amount in a diet based on maize and soybean when microbial phytase is added (Revy *et al*., 2006). Furthermore, average daily energy intake from feed actually increases with the addition of phytase, making it necessary to reduce the amount of feed offered

to the pigs in order to prevent them from producing too much fat in the muscle tissue (Brady *et al*., 2003; Revy *et al*., 2006).

In broiler chickens, supplementing with exogenous phytase has reduced the excretion of endogenous amino acids, calcium, sodium, phytate phosphorus and sialic acid significantly (Cowieson *et al*., 2004; Nahm, 2002). As in pigs, increased weight gain from a phytase supplemented diet is also reported for broilers. It has been predicted that in both cases this response will be declining with time, due to improvements in animal strains, feeds and management techniques (Selle and Ravindran, 2007).

Exchanging a meat based protein-rich diet with a lower cost plant protein diet would be desired by the industry of aquaculture. However, fish in general have rather short gastrointestinal tracts, and are therefore quite sensitive to the inhibited micronutrient utilisation. Dephytinisation of the plant material is consequently an important prerequisite to this application. The effects on phosphate utilization and growth of fish using phytase treated fodder are to date inconsistent and species-related. Some fish have a basic environment (pH 8) in the gastrointestinal tract which does not correspond to the conditions for optimal phytase activity, and although the potential in dephytinisation is there, for once the acidic microbial phytases may not be the first choice of enzyme (Cao *et al*., 2007).

Another approach to the degradation of PA by monogastric animals is to create transgenic livestock. A transgenic pig that constitutively secretes microbial phytase from their salivary glands has been generated and it shows up to 75% reduction in phosphorous excretion (Golovan *et al*., 2001a; 2001b). Furthermore, its requirements for inorganic phosphorous supplementation are decreased to almost zero. Similarly, another group has experimented with expressing an avian MINPP phytase in chickens. This approach would overcome public scepticism towards "foreign" proteins in the food (Ward, 2001) and still decrease phosphorous demands of the transgenic line (Cho *et al*., 2006).

Phytase as food additive

Degradation of PA during breadmaking has been known to effect mineral bioavailability for many years (Mollgaard, 1946). Several breadmaking procedures designed to diminish the phytate content have therefore been reported. These include the addition of commercial phosphoesterases from wheat (phytase or phosphatase) to whole wheat flour (Knorr *et al*., 1981) and the activation of the naturally occurring phytase by soaking and malting the grain.

Phytase shows potential as a breadmaking improver, with two main advantages: first, the nutritional improvement produced by decreasing phytate content, and second, all the benefits produced by α-amylase addition (increase in bread volume and improvement in crumb texture) can be obtained by adding phytase, which releases calcium and thereby promotes the activation of endogenous α-amylase (Haros *et al*., 2001a). The changes in pH values during leavening of bread have been measured to approximately 0.2 pH-units. The pH values ranged between 6.3 and 6.1 in yeast-doughs (Leenhardt *et al*., 2005) and 5.6 and 5.4 in sourdoughs (Haros *et al*., 2001b). Wheat phytase has an optimal pH of 6.0 (Bohn *et al*., 2007), and its activity diminishes markedly as the pH is moved more than 0.5 pH-units from optimum. Conversely, *Aspergillus niger* phytase has two pH optima, one at 5.0 and the other at 2.5~3.0 (Turk and Sandberg, 1992). Therefore, as the pH decreases along the leavening, higher phytase activity is observed using the *Aspergillus* phytase (Haros *et al*., 2001b). Enhancing the degradation by using an organic acid such as citric acid to acidify the dough is a possibility, although leavening for at least 2 h is required for maximal phytate hydrolysis by phytase (Porres *et al*., 2001). Consequently, degradation of PA in sourdoughs with long leavening times, such as rye bread, is preferable, and its degradation is almost completed by endogenous phytase in this type of bread, leaving $InsP_3$ as the dominating inositol phosphate (Nielsen *et al*., 2007). Unless they are able to complex with higher inositol phosphates, $InsP_3$s do not inhibit iron uptake in humans (Brune *et al*., 1992; Sandberg *et al*., 1999) and the rye bread should therefore contain bioavailable iron for consumption.

However, although the non-heme iron absorption in humans almost doubles from wheat bread rolls treated with fungal phytase compared to bread untreated with phytase (Sandberg *et al*., 1996), one should be aware that a minor reduction of phytic acid is not enough to maintain iron status in the long run. Whole grain bread, where dephytinisation is not

completed even after long leavening (Nielsen *et al*., 2007), should therefore not be consumed to all main meals (Bach Kristensen *et al*., 2005) to avoid constant mineral uptake inhibition.

Iron absorption from porridges based on flours from rice, wheat, maize, oat, sorghum and wheat-soy flour blend have been tested on humans. The results show that phytate degradation improves iron absorption from cereal porridges prepared with water but not with milk, and that addition of ascorbic acid actually is a better tool for enhancing iron absorption in baby food than addition of phytase. Adding amylase to the porridge in combination with phytase makes the solution more liquid, and probably because of this, the absorption of Fe increases another 3-fold (Hurrell *et al*., 2003).

Interestingly enough, phytate in tofu is actually considered a positive feature, since phytate inhibits chrystal formation of calcium oxalate—also known as kidney stones. In general, oxalate-rich soy foods also contain higher concentrations of phytate, but most commercially available tofu is categorised as low oxalate/phytate food. Anderson and Wolf (1995) published a review on the changes in phytate concentration related to the processing of soy beans, and the most recent research showed that soy flour is the type of soy-bean product containing the highest amount of phytate (Al-Wahsh *et al*., 2005).

In summary, phytases have potential for improving mineral bioavailability in food applications. Due to the higher stability of fungal phytases, these would probably be the most efficient in degradation of phytic acid, and the activity of e.g., *Aspergillus niger* would even be increased in the acidic environment in the stomach. One should however note that in bread applications with long leavening time, exogenous phytase is not necessarily required for phytate degradation, unless whole grains are added to the mixture.

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