LITERATURE REVIEWS

Phytic acid

Phytic acid (myo-inositol-1, 2, 3, 4, 5, 6 – hexakisphosphate, or $InsP_6$) is a unique reserve compound of phosphorus (P) in cereal seeds and oilseeds. Typically about 60 to 90% of all the phosphorus in these seeds is present as phytic acid P (Cheryan, 1980). The structure of phytic acid is a hexaphosphate of myo-inositol. The anionic nature of the six phosphate groups on inositol will be strongly negatively charged (Figure 1). It imparts a potential to bind positively charged molecules such as cations, proteins and carbohydrate. Due to its multiplicity of reactive phosphate groups, phytic acid can complex a cations within phosphate group itself, between two phosphate groups of a molecule, or between phosphate groups of different phytic acid molecules. Thus, phytic acid fits probably the classic definition of a chelating compound (Cheryan, 1980; Harland and Morris, 1995). The term phytin implies a calcium, magnesium, iron, zinc or potassium salt of phytic acid, whereas phytate would mean the mono to dodeca anion of phytic acid (Maga, 1982; Raboy, 2001).

Seed phytins are primarily deposited in storage microvacuoles or "protein bodies" as discrete inclusions referred to as "globoids" (Raboy, 2001). Phytin is found only in tissues that remain alive during desiccation, e.g. cereals. It is present in the protein bodies of aleurone layer cells but it is not detected in the protein bodies of starchy endosperm cells (Cochrane, 2000). In other plant tissues and organs found that $InsP_6$ accumulates nutrient stores for subsequent redistribution, such as pollen, roots and tubers. The abundance of $InsP_6$ in seeds, pollen and other plant tissues is probably the main reason why derivatives of this compound are the most abundant form of organic phosphorus in soils (Raboy, 2001).

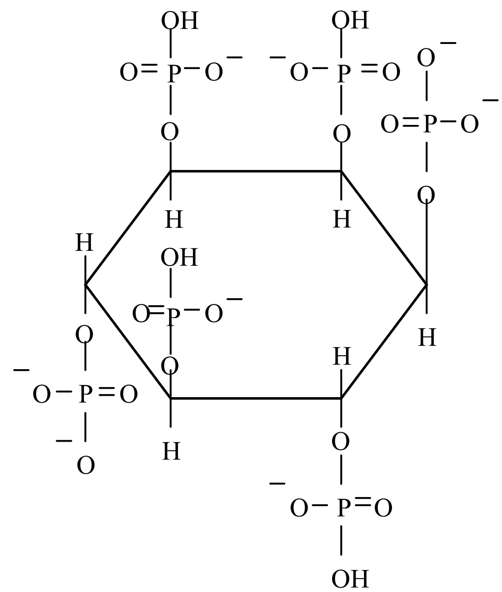

Figure 1 Structure of phytic acid

Source: Cheryan (1980)

Occurrence of phytic acid

Phytic acid constitute about 1 to 2 % by weight of many cereal seeds (Cheryan,1980; Lott *et al*., 2000). In 1982, Maga concluded that phytic acid is found in numerous cereals and legumes such as barley, dry beans, corn, cotton seed, oats, peanut, peas, rapeseed, rice, sesame, sunflower and wheat. Deposition of phytic acid found in small-grained cereals, ~ 90% of the seed phytic acid is found in the aleurone layer and the remaining 10% in the scutellum. In maize kernels found that ~ 90% of phytic acid is accumulated in scutellum and 10 % in aleurone layer. In dicots, phytic acid is deposited in the endosperm and cotyledons (Brinch-Pedersen, 2002).

Williams (1970) reported that phytic acid was not detected in the endosperm. There was a rapid build-up of phytic acid in the embryo plus scutellum and aleurone fractions of the wheat grain.

In rice, Juliano (1972) described that phytin is important constituent of the outer layers of the cereal grain. At least 80% of the phosphorus content of brown rice and 40% of the phosphorus in milled rice is phytin phosphorus. About 90% of the phosphorus in rice bran is phytin phosphorus. Reported of phytin phosphorus contents are brown rice ≈ 0.2%; milled rice ≈ 0.04-0.06%; bran-germ ≈ 2.2-2.6%; and embryo ≈ 0.8%. Phytin phosphorus represents only 0.16% of starch content in milled rice. Phytin increased rapidly during ripening in rice grain.

In 1972, O'Dell *et al*. found that the major proportion of phytic acid in the total kernel existed in the outer layers.

Kennedy and Schelstraete (1975) reported that phytic acid was found in the outer layers of rice grain and no detectable phytic acid in endosperm.

Yoshida *et al*. (1999) reported that phytin was synthesized in rice seeds. Phytin-containing particles called as globoids. It appeared in the scutellum and aleurone layer four days after anthesis and increased gradually in the both tissues.

<u>The role of phytic acid</u>

Phytic acid is a hexaphosphate of myo-inositol. Its mainly function is a storage form of phosphorus. The anionic of phosphate groups on myo-inositol will be strongly negative charged, indicating tremendous potential for a complex or binding of positively charged molecules such as cations or protein (Cheryan, 1980). A mixed cation salts of phytic acid called as phytate or phytin. It is important mineral storage compound in seeds. Then, phytic acid may be causing for decrease the bioavailability of minerals from plant foods (Cheryan, 1980).

The catabolism of phytin releases phosphate, cations and myo-inositol, which is a precursor in the synthesis of membrane components and cell wall material. It is also possible that the myo-inositol released from phytin becomes involved in signal transduction mechanisms operating during differentiation of seedling tissues (Cochrane, 2000; Lott, 1995). Therefore, phytic acid is vital for seed/grain development and successful seedling growth (Lott, 1995; Lott et al., 2000).

Erdman (1981) suggested that phytic acid has affected to bioavailability of zinc and iron from cereal and legume-based food.

In 1982, Maga reviewed the nutritional significance of phytic acid with regard to mineral binding abilities. This review concluded that phytic acid could bind to essential dietary minerals as zinc, calcium, iron and reduced bioavailability of these minerals.

Sanberg *et al.* (1989) studied effect of inositol tri-, tetra-, penta- and hexaphosphates on iron availability. *In vitro* study indicated that inositol hexaphosphates and inositol pentaphosphates have a negative effect on iron availability.

In 1999, Sanberg *et al.* reported that inositol hexaphosphate is a inhibitor of iron absorption. The effect of inositol tri-, tetra-, and pentaphosphate ($InsP_3$, $InsP_4$ and $InsP_5$, respectively) on iron absorption in humans found that $InsP_5$ has an inhibitory effect on iron absorption, whereas $InsP_3$ and $InsP_4$ in isolated form have no such effect in processed food. $InsP_3$ and $InsP_4$ contribute to the negative effect on iron absorption, presumably by binding iron between different of inositol phosphate. To improve iron absorption from cereals and legumes, degradation of inositol phosphates needs to be to less phosphorylated inositol phosphate than $InsP_3$.

The effect of phytic acid to iron uptake in an in vitro digestion/Caco-2 cell culture model found that iron was less available at high phytic acid levels. As in human studies, heme iron was less inhibited by phytic acid than non heme iron (Glahn and Worthley, 2002).

The study of iron bioavailability from 15 rice genotypes by using an in vitro digestion/Caco-2 cell culture model found that the Azucena genotype demonstrated one of the highest Fe bioavailability levels and also contained the lowest ratio of phytic acid to Fe. The Azucena genotype may have greater Fe bioavailability under meal conditions. It would be easier for promoters such as meat and ascorbic acid to overcome the inhibitory effects of phytic acid (Glahn *et al.*, 2002).

Owing to, phytic acid is a strong chelator of mineral cations such as calcium, iron and zinc, forming mixed salts that being sparingly soluble. There is a strong concern that consumption of food products rich in phytates may lead to borderline malnutrition, due to reduced mineral absorption. Phytic acid is considered to be an important anti-nutritional factor, which can interfere with absorption or utilization (bioavailability) of micronutrients into the body. Hence, mineral-binding capacity of phytic acid may be result in mineral deficiencies in animals and humans. It is the most important factor to hindering the uptake of a range of minerals. (Harland and Morris, 1995; Harland and Narula, 1999; Lott *et al.*, 2000; Raboy, 2001; Brinch-Pedersen *et al.*, 2002).

The beneficial roles of phytic acid have evidence that it was anti-carcinogen in colon but the mechanism of action was not understood. Phytic acid serves dietary antioxidant by complex with Fe^{+3} iron for preventing it from catalyzing hydroxyl radical formation. (Harland and Morris, 1995; Harland and Narula, 1999).

Adachi *et al*. (2006) reported that the removal efficiency of Fe^{3+} by rice bran has focused on the chelating properties of phytic acid.

Phytic acid biosynthesis

Lott *et al.* (1995) suggested that phytic acid biosynthesis occurs in cisternal endoplasmic reticulum and the product is subsequently deposited in phytin granules.

Phytic acid is generated by a stepwise phosphorylation of myo-inositol. The sole *de novo* route to myo-inositol is the conversion of D-glucose-6-phosphate to D-myo-inositol-3-phosphate (Ins (3) P1) by D-myo-inositol-3-phosphate synthase (MIPS) or in part via phosphatidyl inositol phosphate (Ptd InsP) intermediates (Loewus and Murthy, 2000; Raboy, 2001; Raboy *et al.*, 2001; Brinch-Pedersen, 2002).

Therefore, the biochemical pathway of phytic acid can be summarized as consisting of two parts. The early pathway showed that glucose-6-phosphate is converted to Ins(3)P1. The later pathway, phytic acid is synthesized from myo-inosiol via a series of phosphorylation steps. The biochemical pathway of phytic acid showed in figure 2. In figure 2, numbers at arrows indicate the following enzymatic activities: (1) D-myo-inositol(3)P1 synthase (MIPS); (2) D-Ins(3)P1 monophosphatase, or Ins monophosphatase; (3) D-Ins_3-kinase or Ins kinase; (4) Ins P or polyP kinases; (5) Ins(1,3,4,5,6)P_5 2-kinase or phytic acid-ADP phosphotransferase; (6)

phytases and phosphatases; (7) $InsP_6$ or pyrophosphate-forming kinases; (8) pyrophosphate-containing Ins PolyP-ADP phosphotransferases; (9) phosphatidyl-inositol (PtdIns) synthase; (10) PtdIns and PtdIns P kinases; (11) PtdIns P-specific phospholipase C; (12) $InsP_6$ 5-phosphatase; (13) Ins-$(1,2,3,4,6)P_5$ 5-kinase; (14) Ins P6 3-phosphatase; (15) $InsP_6$ 3-kinase. The late pathway represented Ins poly P metabolism (Raboy, 2001; Raboy *et al.*, 2001; Raboy, 2003)

In rice, Yoshida *et al.* (1999) reported that relationship between the biosynthesis of phytic acid and the formation of D-myo-inositol-3-phosphate by MIPS. By Yoshida's teams isolated cDNA clone, pRINO1 of rice (*Oryza sativa L.*), that is highly homologous to MIPS of yeast and other plants. In situ hybridization of developing rice seeds showed that the transcript of RINO1 were accumulated to high levels in embryos but were undetectable in shoots, roots and flowers. Trasncription of RINO1 first appeared in the apical region of globular-stage embryos at two days after anthesis. Strong signals were detected in the scutellum and aleurone layer at four days, increased until seven days after anthesis, and gradually decreased thereafter. Phytin-containing globoids appeared at four days after anthesis in the scutellum and aleurone, coinciding with the presence of RINO1 transcript. The temporal and spatial patterns of accumulation of RINO1 transcripts and globoid formation in rice suggest that MIPS is directs phytin biosynthesis in rice seeds.

<u>Phytic acid degradation</u>

Copeland and McDonald (1985) described that the insoluble mixed potassium, magnesium of phytin and calcium salt of myo-inositol hexaphosphoric acid or phytic acid was hydrolyzed by phosphatase. During the germination of seeds, phosphatases that hydrolyze phytin increase several fold. The phytase activity was highest in the scutellum and aleurone layers. Since a large proportion of the seed's phosphate,

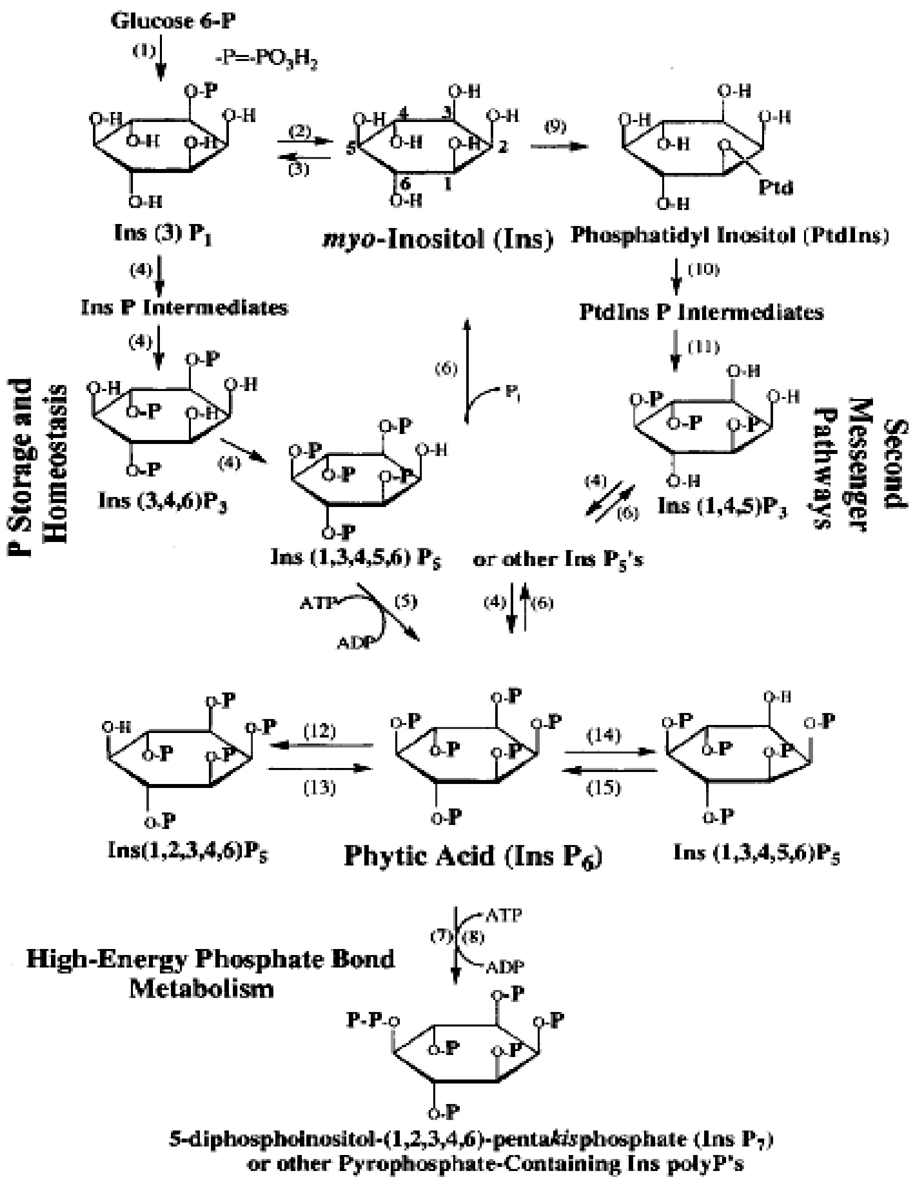

Figure 2 A summary of biochemical pathways involving phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate or Ins P6) in the eukaryotic cell.

Source: Raboy *et al*. (2001)

magnesium and potassium were present in phytin. The seed's subsequent metabolism was dependent on the hydrolysis of phytin and the concomitant release of magnesium and potassium ions.

The enzyme responsible for hydrolysis of phytic acid phytases (myo-inositol-(1, 2, 3, 4, 5, 6)-hexakisphosphate phosphohydrolase), a special class of phosphatase that catalyze the stepwise hydrolysis of phytic acid to orthophosphate, a series of lower phosphate esters of myo-inositol. Phytase can be roughly categorized as 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). Both enzymes hydrolyze phytic acid to Ins (2) P1. In general, plant acid phytases are considered to be 6-phytase, whereas phytases from microorganisms are often referred to as 3-phytase (Brinch-Pedersen *et al.*, 2002 ; Loewus and Murthy, 2000).

In 2002, Greiner *et al*. studied pathway of dephosphorylation of phytic acid by phytases, which were purified from legume seeds. The experiment used a combination of high-performance ion chromatography analysis and kinetic studies. The data demonstrated that phytase of Faba bean seeds and the phytase LP2 of lupine seeds degrade phytate by sequential removal of phosphate groups via D-Ins (1, 2, 3, 5, 6) P_5, D-Ins (1, 2, 5, 6) P_4, D-Ins (1, 2, 6) P_3 and D-Ins (1,2) P_2 to finally Ins (2) P, whereas the phytase LP11 and LP12 from lupine seeds generate the final degradation product Ins (2) P via D-Ins (1, 2, 4, 5, 6) P_5, D-Ins (1, 2, 5, 6) P_4, D-Ins (1, 2, 6) P_3 and D-Ins (1, 2) P_2.

Characterization of phytase in plants

Several reports described characterization of phytase in many plants. In legume, phytase was isolated from germinated mung bean cotyledons *Phaseolus aures.* Phytase was further purified and migrated as a single protein band in polyacrylamide gel electrophoresis. The molecular weight (MW) obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is 158000 (Maiti *et al.*, 1974). Maiti and Biswas (1979) found that phytase was purified from germinated mungbean cotyledons of *Phaseolus aureus*. It is more active with myo-inositol hexaphosphate.

In 1987, Gibson and Ullah reported that phytase was purified from 10-day-old germinating cotyledons of soybean. The purified enzyme exhibited two closely migrating bands on SDS-PAGE of approximately 59 and 60 kDa.

The study of phytase in Faba beans (*Vicia faba* Var. Alameda) found that in during germination, maximum activity of phytase was reached after 4 days. Analysis of purified Faba bean phytase by SDS-PAGE indicated that molecular mass of enzyme was ~ 65 kDa and as a monomeric enzyme. It exhibits a single pH optimum at 5, optimum temperature at 50 $^{\circ}$C and Km = 148 μmol L^{-1} (Greiner *et al.*, 2001)

The purification and characterization of phytase in *Zea mays* seedlings found that the native protein has a molecular mass ~ 76 kDa. It appears to be a dimer of a 38 kDa subunit. Composition of amino acid indicated a high hydrophobicity. Western-blot analyses clearly demonstrate that the increasing of phytase activity observed clearing the first 7 days of germination. The pH and temperature optima were 4.8 and 55 $^{\circ}$C, respectively (Laboure *et al*. 1993).

The study of purified phytase from maize root found that three phytase isoforms were purified from maize roots (*Zea may*). The molecular mass of the native protein was 71 kDa. Each of the three isoforms consisted of two subunits with a molecular mass of 38 kDa. The study of histochemical localization of phytase found that its activity was strictly confined to the endodermis of the primary root (Hubel and Beck, 1996)

In cereal grains such as rice, Akazawa (1972) described that during the germination of rice seeds, phytin was hydrolyzed by phytase because most Pi was liberated in the process. They were presumably derived from phytin. This report referred to Ory and Henningsen's working. They reported that protein bodies were isolated from ungerminated barley seeds. The protein bodies were associated with acid phosphatase which, was specific for phytin, but not active on β-glycerol-P, glucose-1-P, fructose-1, 6-diphosphate and ATP.

Muckherji *et al.* (1971) reported that the phytase activity rose steadily during germination of rice seeds while phytin was degraded which it corresponded with increasing of inorganic phosphate. The pH optimal of phytase was at 4.0 and 9.0. This report suggested that existing of two phytases hydrolyzed phytin. The enzyme at pH 4 optimum hydrolyzed phytin more activity than pH 9 optimum.

In 1973, Palmiano and Juliano found that phytase activity was significantly higher in the germinating seed than in the ungerminated seed after steeping for 1 day. It was essentially constant after 4 days of germination.

Yoshida *et al.* (1975) isolated aleurone particles of rice grain. They found that orthophosphates were released from aleurone particles. The pH and Tm optimum of phytase estimated at 4.2 and 45 $^{\circ}$C, respectively. The results showed that dephosphorylation of phytic acid occurred in aleurone particles. The enzyme which, dephosphorylated phytic acid was also associated with aleurone particles.

Acid phosphatase four fractions were purified from rice bran of *Oryza sativa* L. *Japonicas* called F1, F2, F3, F4. Fraction F1 and F2 had phytase activity, but F3 and F4 did not. Because of F1 and F2 were active against all phytic acid and p-nitrophenyl phosphate (NPP), which were substrate. F3 and F4 were active against only Npp. The optimum pH of F1 and F2 for phytic acid were 4.4 and 4.6, respectively and those of F1, F2, F3,and F4 for Npp were 5.1, 5.2, 5.4 and 6.0 respectively. Their molecular weights were estimated to be about 59-70 kDa by SDS polyacrylamie gel electrophorsis (Hayakawa *et al.*, 1989).

The study of phytase gene in plants

There has been a significant growing progress in the study of plant phytase gene. In maize, Maugenest *et al.* (1997) proposed that during germination, maize seedlings expressed a phytase which able to hydrolyse the large amount of phytin in dry seed. In experiment, total RNA was purified from frozen embryos (scutellum and embryonic axis) of 3-to-4 days-old maize seedlings and synthesized cDNA. Cloning cDNA were used an antibody against the purified maize phytase for screening a maize seedling cDNA expression library. Several positive clones containing an insert of about 1400 bp were isolated. The nucleotide sequence of the insert of one of these cloned has been established. This cDNA called *phyS11*, was 1335 bp long and contained an open reading frame of 387 amino acids. Analysis of the expression of the phytase gene at various stages of maize seed germination found that no transcript was present in dry seeds. The mRNA accumulated during the first day of germination, to reach a maximum after 2 days and then decreased in young seedlings. Genomic southern blot analyses suggested that the existence of at least two genes and genetic mapping revealed two loci separate by 1 cM on chromosome 3 of maize.

Maugenest *et al.* (1999) reported that [^{32}P]-labelled *Eco*RI / *Not*I phyS*11* insert was used as a probe for screen genomic DNA library of maize seedlings. The results found two distinct genes (*PHYT I* and *PHYT II)* were isolated and sequenced. The transcribed sequences of these two genes presented a strong homology whereas the untranscribed upstream and downstream sequences appeared very difference. Northern blot analysis showed that after 1 day of sowing, a strong accumulation of phytase mRNA occurred in coleoptile, coleorhiza and radicle, whereas no signal was detected with RNA from scutellum or from embryo leaves. The strongest accumulation was observed in coleoptiles at 2 and 4 days of germination, then the signal decreased by 7 days. In the radicle, phytase mRNA amount was high during the two first days of germination and then decreased to a low level over the following days. In the scutellum, low levels of transcript were detected between 2 and 4 days. No significant signal was observed with RNA from leaves. RNA extracted from 1-month-old maize seedlings and analysis by Northern blot. It revealed low but signify-cant levels of phytase mRNA in roots. In situ hybridizations on root cross-sections found that phytase mRNA localized in rhizodermis, endodermis and pericycle layers. Immunolocation analysis showed that phytase was accumulated at the same sites as its mRNA. A RT-PCR approach was used to discriminated between the transcripts from each gene. In different situations found that in coleorhiza appeared to contain of transcripts from both phytase genes. In coleoptiles and radicles found that in these organs, both genes appeared to be expressed, but the levels of transcripts from *PHYT II* were always lower than from *PHYT I.* Only *PHYT I* is expressed in adult roots. This suggested that signals responsible for phytase gene expression in roots were different from those responsible for gene expression during germination.

Novel phytase was purified from cotyledons of germinated soybean (*Glycine max*) seeds. The pH optimum of soybean phytase was estimated between pH 4.5 and 5. The Km value for phytate was 61 µM and Tm optimum at 58 °C. Peptide sequence data generated from the purified enzyme facilitated the cloning of the phytase sequence (*GmPhy*) employing a polymerase chain reaction strategy. The soybean phytase sequence contained a 1,644 bp. RNA-blot analysis analyzed the expression of *GmPhy* in soybean cotyledons at 4, 6, 8, 10 and 12 days after germination. The highest steady state RNA levels were detected at 8 days after germination, which preceded maximal enzyme activity. Then, phytase was synthesized early in germination for several days in the cotyledons. The inducing of *GmPhy* into soybean tissue culture found that phytase increased activity in transformed cells, which confirmed the identity of the phytase gene. No homology was revealed to previously reported phytase sequences from maize or microbes. The soybean phytase sequence exhibited a high degree of similarity to purple acid phosphatases, a class of metallophosphoesterases (Hegeman and Grabau, 2001).

Strategies for solving the phytic acid content

Because of phytic acid is considered to be an important anti-nutritional factor, preventing the uptake of a range of important minerals. It is excreted by non-ruminants. Phytic acid phosphorus contribute to water pollution.

Harland and Narula (1999) suggested that hydrolysis of phytic acid or myo-inositol hexakisphosphate to tri, tetra, and penta inositol phosphate have much less affinity or less potential for reducing the bioavailability of minerals. Therefore, decreasing of anti-nutrients such as phytic acid is one alternative for improved mineral nutritional in crop plants.

Decreasing phytic acid by genetic engineering approaches

Phytase from *Aspergillus niger* increases the availability of phosphorus from feed for monogastric animals by releasing phosphorus from the substrate phytic acid. In plants can be transformed for increased phytase production such as in tobacco leaves. A cDNA fragment encoding the mature *Aspergillus niger* phytase gene was transferred to tobacco (*Nicotiana tabacum*) plants showed that a chimeric phytase gene gave rise to transgenic tobacco plants expressing high level of phytase (Verwoerd *et al.*, 1995)

Lucca *et al.* (2001) introduced a thermotolerant phytase from *Aspergillus fumigatus* into the rice endosperm. The phytase level in the grains about 130-fold, giving a phytase activity sufficient to complete degrade phytic acid in a simulated digestion experiment.

Mutants with altered phytic acid levels

The isolation of cereal low phytic acid (lpa) mutants provides a novel approach to studying the biology of seed phytic acid and to dealing with environmental and nutritional problems associated with it. Seed produced by *Ipa* lines contain normal levels of total phosphorus (P), but greatly reduced levels of phytic acid P. Two phenotypically distinct types of *lpa* mutants have been isolated in maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.). In "lpa1-like" mutants, seed phytic acid P reductions ranging from 50% to 95% (in comparison with levels typical of non-mutant seed) are largely matched by corresponding increases in inorganic P. In "lpa2-like" mutants, seed phytic acid P reductions ranging from 50% to 75% are matched by increases in both inorganic P and in myo-inositol (Ins) phosphates containing five or fewer P esters (compared with phytic acid 's six P esters). In all cases the sum of seed Ins phosphates (including phytic acid) and inorganic P remains constant and similar to that in normal seeds. Some *lpa* alleles are lethal as homozygotes, others have a negative effect on plant or seed growth and function but are viable, still others have little effect and are being used to breed "low phytate" maize and barley types (Raboy *et al.*, 2001).

Isolating of low phytic acid mutant (lpa) is one approach to the improvement of the nutritional quality of crops. Raboy's teams at the US Department of Agriculture-Agricultural Research Service (USDA-ARS) has been isolating low phytic acid mutants (Table 1). Seeds homozygous for lpa mutant contain normal levels of seed total phosphorus but greatly reduced levels of phytic acid phosphorus (Raboy, 2000).

Barley low phytic acid mutant

Larson *et al.* (1998) described the inheritance and linkage map position of two low phytic acid barley (*Hordeum vulgarae*) mutations, lpa1-1 and lpa2-1 that reduce phytic acid content in grain and increase inorganic phosphorus (P) seed. The barley lpa1-1 and lpa2-1 mutation were mapped to sites on barley chromosome 2H and 7H, respectively.

Soybean low phytic acid mutant

Wilcox *et al*. (2000) generated a soybean mutation line by treatment with ethyl methanesulfonate (EMS). Seeds were planted. M3 seeds were tested for HIP phenotype and two progenies were classified putative low phytic acid mutation (M153 and M766). M3 seeds which high in inorganic P of M2 plant produced progenies high in inorganic P through the M6 plant generation. The both of these low phytic acid indicate that two independent, heritable and nonlethal mutants are phenotypically similar to the low phytic acid 1-1 muatnts.

Maize low phytic acid mutant

In 2000, Raboy *et al.* generated a population of ethylmethane sulphonate for induced mutants in maize and screening it for mutants whose seed contained substantial reductions in phytic acid phosphorus. Seed phenotype showed two non-lethal maize low phytic acid mutants (lpa1-1 and lpa2-1). Low phytic acid mutant indicated two loci map to two sites on chromosome 1S. Seed phytic P is reduced in these mutants by 50% to 66% but seed total P is unaltered. The decrease in phytic acid P in mature lpa1-1 seeds is accompanied by a corresponding increase in inorganic phosphate (Pi). In mature lpa 2-1 seed it is accompanied by increase in Pi and at least three other myo-inositol phosphates. In both cases the sum of seed Pi and myo-inositol phosphate (including phytic acid) is constant and similar to that observed in normal seeds. In both mutants P chemistry appears to be perturbed throughout seed development. Homozygosity for either mutant results in a seed dry weight loss, ranging from 4% to 23%. These results indicate that phytic acid metabolism during seed development is not solely responsible for P homeostasis and indicate that the phytic acid concentration typical of a normal maize seed is not essential to seed function.

Shi *et al*. (2003) have reported that the maize (*Zea mays*) low phytic acid mutant is caused by mutation in an inositol phosphate kinase gene by using a reverse genetic approach to generate loss of function alleles. Mutant displayed a recessive low phytic acid phenotype which the low phytic acid phenotype is caused by *Mutator (Mu)* insertion in the maize inositol phosphate kinase (ZmIpk) gene. The ZmIpk *Mu* insertion lines accumulated myo-inositol, $InsP_3$, $InsP_4$ and $InsP_5$ in kernels and in embryos of ZmIpk *Mu* insertion mutant had higer myo-inositol content than non-mutant. Because of the ZmIpk *Mu* insertion knockout and the lpa2 mutation in maize are related because they have a very similar phenotype. The homozygous plants carrying the ZmIpk mutant allele were crossed with the recessive lpa2-1 and lpa2-2 alleles. Individual F1 seeds from both crosses displayed the high Pi phenotype in a rapid Pi test. These experiments demonstrated that the lpa2 mutants carry mutation in ZmIpk gene. Cloning and sequencing of the ZmIpk gene from lpa2-2 showed that the lpa2-2 allele has a nucleotide mutation that causes immature termination of the ZmIpk open reading frame. Southern-blot analysis in the lpa2-1 mutant found that the lpa2-1 allele is causes by the genomic sequence rearrangement in the ZmIpk locus and no mRNA expression was detected. Then, the mutant lpa2 in maize carries a mutation in the ZmIpk gene.

In 2005, Shi *et al*. identified a low phytic acid mutant, lpa3, in maize. The Mu-insertion mutant has a phenotype of reduced phytic acid, increased myo-inositol and lacks significant amounts of myo-inositol phosphate intermediates in seeds. The gene responsible for the mutation encodes a myo-inositol kinase (MIK). Maize MIK protein contains conserved amino acid residues found in pfkB carbohydate kinases. The maize lpa3 gene is expressed in developing embryos, where phytic acid is actively synthesized and accumulates to a large amount. Characterization of the lpa3 mutant provides direct evidence for the role of myo-inositol and MIK in phytic acid biosynthesis in developing seeds. Recombinant maize MIK phosphorylates myo-inositol to produce multiple myo-inositol monophosphates, Ins(1/3)P, Ins(4/6)P and possibly Ins(5)P. The characteristics of the lpa3 mutant and MIK suggest that MIK is not a salvage enzyme for myo-inositol recycling and that there are multiple phosphorylation routes to phytic acid in developing seeds. Analysis of the lpa2/lpa3 double mutant implies interactions between the phosphorylation routes.

Rice low phytic acid mutant

In the work of Larson *et al.* (2000) isolated low phytic acid mutant. Grain from 3632 rice M2 lines, derived from gamma-irradiated seed was screened for the *lpa* phenotype. Two mutation, one lethal and one non-lethal were identified. The non-lethal mutation is phenotypically similar to maize and barley *lpa*1 mutants and was designated as rice lpa 1-1. Homozygosity for rice lpa1-1 reduces the phytic acid portion of seed (from 71 to 39% and increases the inorganic portion of seed P from 5 to 32%, with little effect on total seed P). The study of inheritance and genetic mapping of the first rice lpa1 mutation found that the rice lpa1 mutation was mapped to a 2.2 CM interval on chromosome 2L. The reexamination of the MIPS / lpa1 candidate gene hypothesis in rice found a single-copy rice MIPS gene was mapped to a locus on rice chromosome 3 that is orthologous to MIPS loci on maize chromosome 1S (near maize lpa1) and barley chromosome 4H. Unlike maize lpa1, the rice and barley lpa1 mutation loci are clearly distinguishable from this canonical MIPS gene (Figure 3).

In whole grains, embryos and rest-of-grain portions of wild-type and lpa1 seed were analyzed total P, phytic acid P, K, Mg, Ca, Fe, Mn and Zn. The levels of total P, Ca, Mn and phytic acid P in whole grains of lpa1 seed were lower than the wild-type seed, while the levels of K, Mg and Fe were similar and that of Zn was higher. These results indicate that changes in phytic acid affect the distribution of important micronutrients within the seed, which may have a significant impact on the nutritional value of lpa1 rice (Liu *et al*. 2004).

<u>Investigation of qualitative analysis of low phytic acid phenotypic characteristic</u>

High Inorganic Phosphorus (HIP) phenotype

The HIP phenotype is associated with a homozygous low phytic acid mutation. Normal seeds have low level of inorganic phosphorus, typically less than 0.46 µgP. A simple color assay has been developed using a Chen reagent (Chen *et al*., 1956) that detects the different concentrations of inorganic phosphorus allowing selection of HIP genotypes in plant breeding programs (Raboy, 2000)

Low phytic acid mutant screening

Rasmussen and Hatzack. (1998) suggested the method for identified low phytic acid of barley (*Hordeum vulgare* L.) grains using Thin Layer Chromatography (TLC). TLC was used to display free phosphate and phytic acid content simultaneously and found two characteristic low phytic acid phenotypes (A and B) could be distinguished. A-type grains contained very high levels of free phosphate, low levels of phytic acid and trace amounts of other phosphate containing compound but not observed in wild-type. Migration of these novel P compound on TLC plates was similar to phosphoinositols with less than six phosphate groups. B-type grains found increasing in free phosphate and decreasing in phytic acid content. Genetic tests showed that at least three recessive alleles caused low phytic acid of phenotype A while recessive locus was responsible for phenotype B.

In 1999, Hatzack and Rasmussen developed method for the analysis of inositol mono- to hexakisphosphates on cellulose precoated plates by using high-performance thin-layer chromatography (HPTLC) method. HPTLC cellulose precoated glass plates (without fluorescent indicator) were used for inositol phosphate analysis. Plates were developed in 1-propanol-25% ammonia-water (5:4:1) and substance quantities as low as 100-200 pmol were detected by molybdate staining. This method is high detection sensitivity which suitable for micro-analysis and can be applied to single grain analysis. The advantages of the HPTLC system are most ideally exploited in situations where technical convenience and high sensitivity.

Table 1 Description of selected low phytic acid

Species and phytic acid mutant	Approximate reduction in phytic acid phosphorus	Other observations
Maize		
lpa1-1	55%–65%	Reduced phytate matched solely by increased inorganic phosphorus
lpa2-1	50%	Reduced phytate matched by increased inorganic phosphorus and other inositol phosphates
M2 91286-15	95%	Lethal as homozygote. Reduced phytate matched solely by increased inorganic phosphorus
M2 92166-3	95%	Lethal as homozygote. Reduced phytate matched solely by increased inorganic phosphorus
Barley		
lpa1-1	50%	Reduced phytate matched solely by increased inorganic phosphorus
lpa2-1	50%	Reduced phytate matched by increased inorganic phosphorus and other inositol phosphates
M2 635	75%	Reduced phytate matched solely by increased inorganic phosphorus
M2 955	95%	Viable as homozygote. Reduced phytate matched solely by increased inorganic phosphorus

Table 1 (Continued)

Species and phytic acid mutant	Approximate reduction in phytic acid phosphorus	Other observations
Rice		
1pa1-1	45%	Reduced phytate matched solely by increased inorganic phosphorus

Source: Raboy (2000)

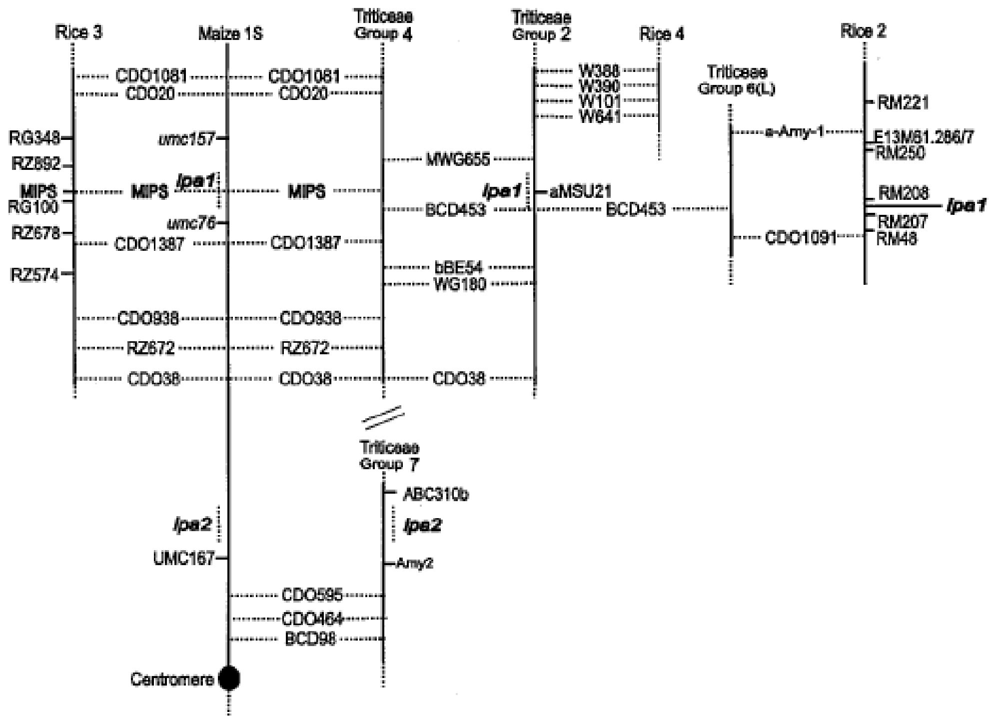

Figure 3 Diagrammatic map of chromosomal regions containing the *lpa1* and *lpa2* loci and *myo*-inositol (3)P1 synthase (MIPS) genes of maize, barley and rice. This map summarizes results of three earlier studies that mapped the position of *lpa* and MIPS loci (Larson *et al*. 1998, 2000; Larson and Raboy 1999).

Source: Raboy *et al*. (2001)