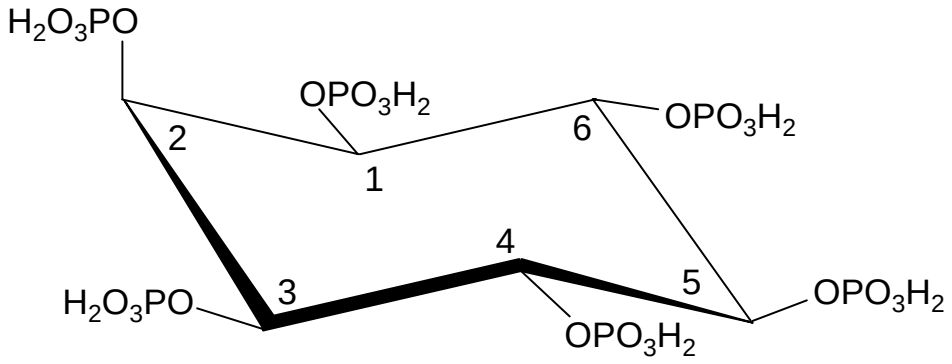
Inositol Phosphates in the Soil–Plant–Animal System: Linking Agriculture and Environment

21st – 24th August 2005
Sun Valley, Idaho, USA

Supported by the Soil Science Society of America

http://striweb.si.edu/inositol_conference/



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Background and Introduction

Inositol phosphates have long been of interest to scientists, because they constitute a considerable fraction of the phosphorus found in the environment. Plants contain large amounts of inositol phosphates, especially in seeds, where they function as a storage compound. Soils also contain inositol phosphates in abundance, some of which exist nowhere else in nature. Monogastric animals cannot digest phytic acid, so considerable research has been directed towards improving the availability of this compound in animal feeds. Such efforts are aimed in part at reducing phosphorus concentrations in animal manures, because the pollution of water bodies by phosphorus in runoff from agricultural land in now one of the most pervasive problems in the 'developed' world.

Biochemical research on inositol phosphates in animal nutrition is at the cutting edge of the discipline, yet the environmental impacts of this technology remain poorly understood. For example, the environmental fate of inositol phosphates in manures, especially in regions with high animal densities, is unknown, as is their impact on eutrophication of receiving waters. Given the widespread adoption of dietary modifications in animal production, there is an urgent need to improve our understanding of the behavior, mobility, and biological availability of inositol phosphates in terrestrial and aquatic ecosystems. This meeting will address this in a multidisciplinary context by focusing on five key topics:

- Analytical procedures for determining inositol phosphates in environmental samples
- Inositol phosphates in animal nutrition and manures
- Function and diversity of plant and microbial phytases
- Synthesis and bioavailability of inositol phosphates in biological systems
- Mobility and reactions of inositol phosphates in the environment

Addressing these issues demands interdisciplinary approaches, but research often remains isolated. For example, significant advances are being made in the analytical technology required to determine inositol phosphates in environmental samples, but these are not always accessible to the scientists who need them. This meeting represents a unique opportunity to address this key deficiency in a multidisciplinary context. By synthesizing perspectives from microbial, plant, animal, and soil scientists, the meeting will advance understanding of inositol phosphates in animal nutrition and the environment, unify directions for future research, and generate collaborative interdisciplinary studies to address key themes.

Bouyoucos Conferences unite scientists with common interests for an intense, highly focused examination of a topic at the frontiers of soil science. They are designed to promote personal relationships and a free exchange of ideas that are not typically achieved at larger meetings. It is in this spirit that we welcome you to Sun Valley for what promises to be a fascinating and productive few days.

Ben Turner
Alan Richardson
Ed Mullaney

The Program at a Glance

Sunday 21st	Monday 22nd	Tuesday 23rd	Wednesday 24th
	7:00 – 8:30 ***Breakfast***	***7:00 – 8:30*** ***Breakfast***	***7:00 – 8:30*** ***Breakfast***
	8:30 – 9:10 Murthy	8:30 – 9:10 Leytem	8:30 – 9:10 George
	9:10 – 9:50 Cooper	9:10 – 9:50 Dao	9:10 – 9:30 Berry
	9:50 – 10:10 Mimura	9:50 – 10:10 He	9:30 – 9:50 Patel
Arrival	***10:10 – 10:40*** ***Break***	***10:10 – 10:40*** ***Break***	***9:50 – 10:20*** ***Break***
	10:40 – 11:20 Raboy	10:40 – 11:20 Turner	10:20 – 11:00 McKelvie
	11:20 – 12:00 Mullaney	11:20 – 11:40 Condron	11:00 – 11:20 Lim
Registration 16:00 – 17:00	12:00 – 12:20 Grabau	11:40 – 12:00 L'Annunziata	11:20 – 11:40 Cade-Menun
	12:20 – 14:00 ***Lunch***	***12:00 – 14:00*** ***Lunch***	11:40 – 12:00 Synthesis/close
Welcome drinks 18:00 – 19:00	14:00 – 14:40 Greiner	14:00 – 14:40 Richardson	***12:00*** ***Lunch***
	14:40 – 15:00 Sandberg	14:40 – 15:20 Hill	**Departure**
	15:00 – 15:20 Forsberg	15:20 – 15:40 Powers	
Dinner 19:00	**15:20 – 16:10** **Break and posters**	***15:40 – 16:10*** ***Break***	
	16:10 – 16:50 Lei	**16:10 – 18:00**	
	16:50 – 17:10 Shears	**Discussion topics**	
	17:10 – 17:30 Johansen		
	Dinner	***Dinner***	

Detailed Program

Sunday 21st August 2005: Arrival and Registration

Welcome drinks at the bar between 18:00 and 19:00.

Monday 22nd August 2005: Morning Session

Section A: Analytical procedures for the determination of inositol phosphates

Chair: Ian McKelvie

8:30 – 9:10: Pushpa Murthy, Michigan Tech., USA
Identification of inositol phosphates by nuclear magnetic resonance spectroscopy: unraveling structural diversity

9:10 – 9:50: William Cooper, Florida State University, USA
Analysis of inositol phosphates by mass spectrometry

9:50 – 10:10: Tetsuro Mimura, Kobe University, Japan
*Inositol hexa*kis*phosphate synthesis in plant suspension-cultured cells and measurement of inositol phosphate isomers with anion chromatography*

10:10 – 10:40: Break

Section B: Inositol phosphates and phytase in animal nutrition

Chair: Cecil Forsberg

10:40 – 11:20: Victor Raboy, USDA–ARS Aberdeen, USA
Inositol phosphates in plants and development of low phytate germplasm

11:20 – 12:00: Ed Mullaney, USDA–ARS New Orleans, USA
Phytases: attributes, catalytic mechanisms, and applications

12:00 – 12:20: Elizabeth Grabau, Virginia Tech., USA
Characterizing steps in phytic acid biosynthesis as targets for manipulating inositol phosphate metabolism in soybean

12:20 – 14:00: Lunch

Monday 22nd August 2005: Afternoon Session

Continuation of Section B: Inositol phosphates and phytase in animal nutrition

Chair: Alan Richardson

14:00 – 14:40: Ralf Greiner, Federal Research Institute for Nutrition, Germany
Current biochemical research on phytase genes in microorganisms and plants

14:40 – 15:00: Ann-Sofie Sandberg, Chalmers University, Sweden
Expression of phytase in yeast: phytate degradation and survival of yeast cells during in vitro *digestion*

15:00 – 15:20: Cecil Forsberg, University of Guelph, Canada
Expression of the Escherichia coli *APPA phytase in the genetically enhanced Enviropig™ breed*

15:20 – 16:10: Break and Poster Session – see page 8 for list of poster presentations

Continuation of Section B: Inositol phosphates and phytase in animal nutrition

Chair: April Leytem

16:10 – 16:50: Xingen Lei, Cornell University, USA
Effects of dietary manipulation on animal nutrition and phosphorus excretion by animals

16:50 – 17:10: Stephen Shears, National Institutes of Health/ NIEHS, USA
The avian MINPP *gene can help to alleviate the planet's "phosphate crisis"*

17:10 – 17:30: Karoline Johansen, Danish Institute of Agricultural Sciences, Denmark
Degradation of phytate in soaked diets for pigs

Close and Dinner

Tuesday 23rd August 2005: Morning Session

Section C: Inositol phosphates in animal manures and soils

Chair: Victor Raboy

8:30 – 9:10: April Leytem, USDA–ARS Kimberly, USA
Environmental implications of inositol phosphates in animal manures

9:10 – 9:50: Thanh Dao, USDA–ARS Beltsville, USA
Ligand effects on phytic acid in animal manures: assessing time dependence of inositol phosphate solubilization and bioactivity

9:50 – 10:10: Zongqi He, USDA–ARS Orono, USA
Synthesis and initial characterization of metal-phytate compounds

10:10 – 10:40: Break

Continuation of Section C: Inositol phosphates in animal manures and soils

Chair: Puspha Murthy

10:40 – 11:20: Ben Turner, Smithsonian Tropical Research Institute, Republic of Panama
Inositol phosphates in world soils: evolutionary paradoxes and the role of the phosphorylated stereoisomers

11:20 – 11:40: Leo Condron, Lincoln University, New Zealand
Quantification and bioavailability of scyllo-*inositol hexa*kis*phosphate in pasture soils*

11:40 – 12:00: Michael L'Annunziata, The Montague Group, USA
Origins and biochemical transformations of soil inositol and inositol phosphate stereoisomers: Review of work during 1967-1977 and ideas for future work with stable and radioactive isotopes

12:00 – 14:00: Lunch

Tuesday 23rd August 2005: Afternoon Session

Section D: Bioavailability of inositol phosphates in the environment

Chair: Ralf Greiner

14:00 – 14:40: Alan Richardson, CSIRO Plant Industry, Australia
Plant access to inositol phosphates in soil

14:40 – 15:20: Jane Hill, Yale University, USA
Microorganisms that utilize inositol phosphates in the environment

15:20 – 15:40: Jennifer Powers, University of Minnesota, USA
Diversity and abundance of inositol phosphate-utilizing bacteria in terrestrial and aquatic ecosystems on Cape Cod, Massachusetts, USA

15:40 – 16:10: Break

16:10 – 18:00: Discussion Session

Chair: Ed Mullaney

Preliminary List of Topics:

1. Nomenclature for inositol phosphates and phytases.
2. Future directions and priorities for phytase and animal nutrition research.
3. Linking agriculture and environment – priorities for understanding the impact of diet modification on soil and water quality.
4. Inositol phosphates and the nutrition of organisms in terrestrial and aquatic ecosystems – current understanding and research priorities.

The results of this discussion session will be disseminated as the final chapter of the conference book, to be co-authored by all delegates at the conference.

Close and Dinner

Wednesday 24th August 2005: Morning Session

Continuation of Section D: Bioavailability of inositol phosphates in the environment

Chair: Leo Condron

8:30 – 9:10: Tim George, CSIRO Plant Industry, Australia
Understanding phytate-phytase interactions in soil

9:10 – 9:30: Duane Berry, Virginia Tech., USA
Development of a novel soil phytase assay: significance, challenges, progress

9:30 – 9:50: Kuldeep Patel, M.S. University of Baroda, India
*Role of organic acid secretion by phytase producing rhizobacteria in release of inorganic phosphate from calcium-phytate and in growth promotion of pigeon pea (*Cajanaus cajan*)*

9:50 – 10:20: Break

Section E: Fate of inositol phosphates in aquatic ecosystems

Chair: Ben Turner

10:20 – 11:00: Ian McKelvie, Monash University, Australia
The fate of inositol phosphates in aquatic systems

11:00 – 11:20: Boon Lim, University of Hong Kong, China
Beta-propeller phytases in the aquatic environment: Characterization of a novel phytase from Shewanella oneidensis *MR-1*

11:20 – 11:40: Barbara Cade-Menun, Stanford University, USA
Temporal and depth trends in inositol phosphates in settling ocean particulate matter from Monterey Bay, California

11:40 – 12:00: Synthesis and Final Comments

12:00: Lunch and Departure

Poster Presentations

Gao, Y., Shang, C., Biyashev, R.M., Buss, G.R., Grabau, E.A., Maroof, M.A.S.
Virginia Tech., USA
Selection of low phytate soybeans and QTL mapping of phytate genes in soybean with a simple, rapid, and quantitative procedure

Greiner, R.
Federal Research Institute for Nutrition, Germany
myo-*Inositol phosphate esters generated by the reaction of a phytate-degrading enzyme from* Raoultella terrigena *on phytate*

Greiner, R., Farouk, A.-E.
Federal Research Institute for Nutrition, Germany, and International Islamic University, Malaysia
Properties of a highly specific phytate-degrading enzyme with an acid pH optimum from a bacterium isolated from Malaysian waste water

Greiner, R., Muzquiz, M., Burbano, C., Cuadrado, C., Perdosa, M.M., Goyoaga, C.
Federal Research Institute for Nutrition, Germany, and SGIT–INIA, Spain
De novo *synthesis of enzymes participating in phytate breakdown during germination of lentils (*Lens culinaris *var. Magda)*

Hansen-Møller, J., Sehested, J.
Danish Institute of Agricultural Sciences, Denmark
Determination of potential phytase activity in digestive fluids with rumen fluid as an example

Leytem, A.B., Turner, B.L.
USDA–ARS Kimberly, USA, and Smithsonian Tropical Research Institute, Republic of Panama
Quantifying the phosphorus composition of animal manures by solution phosphorus-31 nuclear magnetic resonance spectroscopy

Ma, X.-F., Xiao, K., Harrison, M., Bouton, J., Wang, Z.-Y.
The Samuel Roberts Foundation, Ardmore, USA, and Cornell University, USA
Improving phosphate acquisition in white clover by transgenic expression of MtPAP1 *and* MtPHY1

Mehta, B.D., Murthy, P.P.N.
Michigan Tech., USA
Unique alkaline phytase from lily pollen: cloning, characterization, and differential expression

Abstracts

Abstracts of oral presentations appear in chronological order, followed by abstracts of poster presentations in alphabetical order. All abstracts were edited for consistency of style, spelling, and grammar according to the American Society of Agronomy style guidelines. In some cases, abstracts were also edited for to fit the two-page limit.

Identification of inositol phosphates by nuclear magnetic resonance spectroscopy: unraveling structural diversity

Pushpalatha P.N. Murthy

Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, USA

Inositol phosphates are widely distributed in nature. The discovery that they play critical roles in signal transduction and Ca regulation triggered widespread interest in the structures and metabolism of inositol phosphates. This interest has necessitated the need for new methods of separation and structural analysis.

Inositol is a deceptively simple molecule; the inherent stereochemical complexity of inositol and its derivatives results in multiple closely related stereo- and regioisomers. Of the nine isomers of inositol, the *myo*-isomer is the most prevalent; a total of 63 isomers of *myo*-inositol phosphates, from inositol monophosphate to inositol hexa*kis*phosphate, are possible. Therefore, there is a need to distinguish between closely related isomers. An additional complexity is the fact that the metabolisms of inositol phosphates are highly interconnected, so that a change in the concentration of one compound is rapidly transmitted to other phosphorylated derivatives by the quick action of phosphatases and kinases. Establishing the structures of inositol phosphates has been a challenge because of the need to separate and identify the structures of individual inositol phosphates in mixtures containing closely related stereo- and regioisomers.

In classic work, structures of inositol phosphates were established by first purifying the compound (high-performance liquid chromatography, column chromatography, or high voltage paper electrophoresis) and then subjecting the purified compound to elaborate chemical and enzymatic reactions and identifying the product(s) by co-migration with standards. Such indirect methods of analysis are elaborate and time consuming and provide ambiguous evidence. Thus, there is a need for techniques that allow the structural assignment of individual inositol phosphates in mixtures without prior separation.

Nuclear magnetic resonance (NMR) spectroscopy has been extensively used in the study of inositol phosphates because it can rapidly provide conclusive evidence. ^{1}H and ^{31}P NMR spectroscopic techniques are the methods of choice because of the high natural abundance and intrinsic sensitivity of the nuclei. One- and two-dimensional NMR techniques have been employed in numerous investigations, including structure determination of individual inositol phosphates in extracts of mutant corn and barley without prior separation, conformational analysis on inositol phosphates, quantification of inositol phosphates, determination of pK_a values and, more recently, solid-state NMR techniques. An overview of the NMR spectroscopic techniques employed for structural analysis of inositol phosphates, the advantages and disadvantages of the various techniques, and current challenges will be presented.

High-performance chromatographic separations of inositol phosphates and their detection by mass spectrometry

William T. Cooper[1], Matthew Heerboth[1], Vincent J.M. Salters[2]

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[2] *National High Magnetic Field Laboratory and Department of Geological Sciences, Florida State University, Tallahassee, Florida, USA*

Mass spectrometry would appear to be an attractive approach for measuring inositol phosphates in soil, water and plant tissue. These compounds are difficult to detect by conventional UV-Visible spectroscopy because they contain no chromophoric groups. The common approach is to isolate inositol phosphates, oxidize them to release phosphate, and then detect the released phosphate colorimetrically after reaction with molybdate. While this approach is relatively simple and straightforward, it is tedious, time-consuming and subject to interferences. It is also unsuitable for on-line detection in high performance liquid chromatography separation techniques which can distinguish degrees of phosphorylation and isomeric forms of inositol phosphates.

We have shown that inductively coupled plasma (ICP) ionization and high-resolution mass spectrometry (HRMS) is superior to the colorimetric technique for quantifying organic P compounds in water (Cooper et al., 2005). Detection limits are lower, analytical sensitivities are higher, and the technique can be used for both on-line and off-line chromatographic detection. We also demonstrated that electrospray ionization combined with high- and ultrahigh-resolution mass spectrometry can be used for qualitative identification of organic P species (Llewelyn et al., 2002); that is, identification of molecular masses and molecular formulas of individual organic P compounds.

We have now turned our attention to coupling liquid chromatography separations with ICP and ES ionization mass spectrometry for detection of individual inositol phosphates. High performance size exclusion chromatography (HP-SEC), ion pairing reversed phase liquid chromatography (HP-IP-RPLC), and β-cyclodextrin bonded reversed phase liquid chromatography (HP-β-RPLC) have all been evaluated for their ability to separate inositol phosphates based on degree of phosphorylation and isomeric forms of individual inositol phosphates. Unfortunately, none of these techniques can be fully optimized for inositol phosphate separation because of the limitations imposed by the ICP and electrospray ionization steps required for mass spectrometry detection. These ionization techniques require low concentrations of organic modifiers and volatile salts in the spray matrices, greatly limiting the separation potential of the liquid chromatography methods. To date, HP-SEC has proven to be the most versatile if least efficient separation method. Resolution of all six inositol phosphates in less than 30 minutes is possible in a mobile phase that allows efficient ionization by either ICP or electrospray.

In previous studies of dissolved organic P speciation in the Everglades we noted that electrospray ionization was not very efficient for organic P. We therefore carried out a series of optimization experiments in solutions that produced acceptable separations by the HP-SEC method, using a mixture of inositol monophosphate (IP_1; $[C_6H_6(H_nPO_4)_1]$) and inositol hexa*kis*phosphate (IP_6, $[C_6H_6(H_nPO_4)_6]$. We found that the inositol phosphates were

efficiently ionized, but were surprised to observe significant fragmentation of inositol hexa*kis*phosphate, with the 2-5 congeners also appearing in the mass spectrum, as well as some sodium adducts (Fig. 1). We believe that the phosphate groups in inositol phosphates may be relatively labile, and intact molecular ions may not be very abundant. Nevertheless, these results are encouraging, because they indicate that, with sufficient analytical separation of the individual inositol phosphate species, their molecular formulas can be verified by electrospray ionization–mass spectrometry.

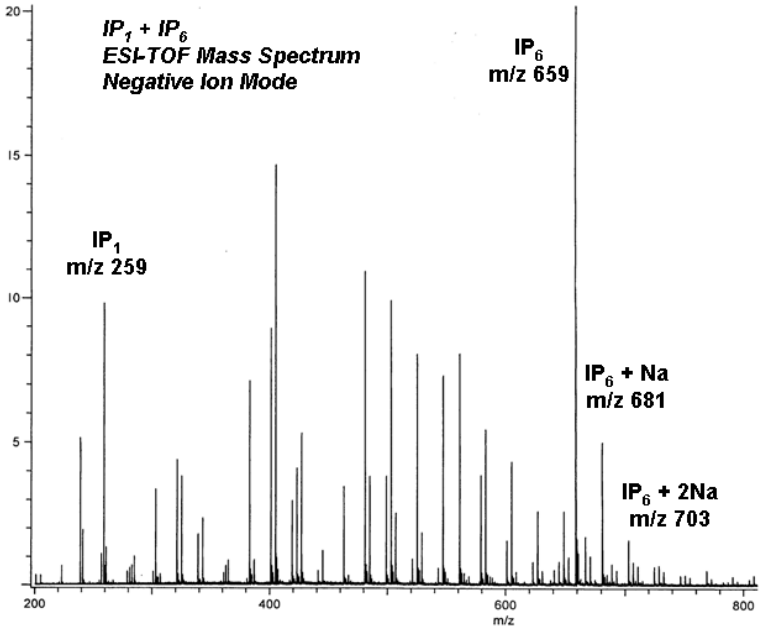

Figure 1. Electrospray ionization–time of flight mass spectrum of a mixture of *myo*-inositol monophosphate (IP_1) and *myo*-inositol hexa*kis*phosphate (IP_6).

HP-SEC chromatograms of inositol phosphates with both ICP-HRMS and ES-TOF MS detection will be the primary focus of this presentation. We will also describe our efforts to further develop the chromatographic separation, using a β-cyclodextrin bonded reverse phase column and micellar mobile phase that has been shown to be effective at separating various isomeric forms of the individual inositol phosphate congeners (Dragani et al., 2004).

References

Cooper, W.T., J.M. Llewelyn, G.L. Bennett, and V.J.M. Salters. 2005. Mass spectrometry of natural organic phosphorus. Talanta 66:348-358.

Dragani, L.K., C.P. Berrie, D. Corda, and D. Rotilio. 2004. Analysis of glycerophosphoinositol by liquid chromatography-electrospray ionization tandem mass spectrometry using a b-cyclodextrin-bonded column. J. Chromatog. B 802:283-289.

Llewelyn, J.M., W.M. Landing, A.G. Marshall, and W.T. Cooper. 2002. Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry of dissolved organic phosphorus species in a treatment wetland after selective isolation and concentration. Anal. Chem. 74:600-606.

Inositol hexa*kis*phosphate synthesis in plant suspension-cultured cells and measurement of inositol phosphate isomers with anion chromatography

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Summary. We have established a new system for studying phytic acid ($InsP_6$) synthesis in suspension-cultured cells of *Catharanthus*. $InsP_6$ and other intermediates of *myo*-inositol phosphate metabolism were measured using a newly developed ion chromatography method. The detection limit for $InsP_6$ was < 50 nM, which was sufficient to analyze inositol phosphates in living cells. Synthesis of *myo*-inositol phosphates was induced by incubation in high phosphate medium. $InsP_6$ accumulated mainly in vacuoles and was enhanced when cells were grown in a high concentration of phosphate with K^+, Ca^{2+}, or Zn^{2+}. However, there was a strong tendency for $InsP_6$ to accumulate in the vacuole in the presence of Ca^{2+} and in non-vacuolar compartments when supplied with Zn^{2+}, possibly due to precipitation of $InsP_6$ with Zn^{2+} in the cytosol. Expression of some enzymes involving inositol phosphate metabolism are investigated.

Results and Discussion. *myo*-Inositol phosphates play crucial roles in both animal and plant cells. In plants, a large amount of $InsP_6$ is synthesized and accumulated in seeds as a P reservoir instead of inorganic phosphates. $InsP_6$ also has various physiological roles other than storage of P, such as mRNA export and chromatin remodeling. Although there are many reports dealing with InsP6 synthesis in plants, we report the development of a more convenient experimental system for the in vivo investigation of the dynamics of synthesis and compartmentation of $InsP_6$ using suspension cultured cells of *Catharanthus*.

Induction of $InsP_6$ accumulation in suspension-cultured cells. When grown in Murashige-Skoog (MS) with 1.25 mM phosphate, the cells depleted phosphate in the medium and were effectively starved after 7 d (low-phosphate cells) and contained negligible amounts of $InsP_6$ (Fig. 1A). If the medium was supplemented with 7.5 mM phosphate at day 3 and day 5, cells then accumulated high concentrations of both phosphate and $InsP_6$ (high-phosphate cells). In addition to $InsP_6$, various other isomers of $InsP_4$ and $InsP_5$ were detected in high-phosphate cells (Fig. 1B), but $InsP_1$, $InsP_2$ and $InsP_3$ were either absent or below the detection limit. $InsP_6$ and other intermediates of inositol phosphate metabolism were measured using the newly developed ion chromatography method.

Cellular localization of inositol phosphates. The sub-cellular localization of inositol phosphates in the cultured cells was investigated by comparing the profiles of inositol phosphates in protoplasts and in vacuoles isolated from the protoplasts. High-phosphate cells contained more than half of the $InsP_6$ in their vacuoles, while vacuoles from low-phosphate cells accumulated a low level of $InsP_6$. Low levels of $InsP_4$ and $InsP_5$ were found in protoplasts of high-phosphate cells, but were absent from the vacuole.

Effect of cations on accumulation of $InsP_6$ in vacuoles. In mature dry seeds, $InsP_6$ is usually bound to Ca^{2+} and Mg^{2+}, forming phytin globoids. Thus, accumulation of $InsP_6$ might be closely related to storage of cations. The effects of cations on accumulation of $InsP_6$ were investigated by growing *Catharanthus* cells in high-phosphate medium with K^+, Ca^{2+}, Mg^{2+}, Zn^{2+} or Mn^{2+} for 7 days. The amount of $InsP_6$ in cells incubated with phosphate plus K^+, Ca^{2+} or Zn^{2+} increased markedly compared to that in cells supplied only with phosphate, but decreased slightly in cells incubated with Mg^{2+} or Mn^{2+}. The location of $InsP_6$ in *Catharanthus* cells was investigated by isolating protoplasts and vacuoles. In high-phosphate + Ca^{2+} cells, $InsP_6$ was mainly accumulated in vacuoles. In contrast, in high-phosphate + Zn^{2+} cells, $InsP_6$ was accumulated predominantly in non-vacuolar compartments. Furthermore, in some inhibitor experiments, vesicle transport system from ER to vacuole may be concerned with $InsP_6$ synthesis. These results suggest that $InsP_6$ is synthesized in cytosol and then incorporated into vacuoles directly or indirectly.

Analysis of enzyme expression in $InsP_6$ synthesis. The level of MIPS, Ins(1,4,5)P_3 6-/3-kinase (Ipk2) and Ins(1,3,4)P_3 5-/6- kinase in *Catharanthus* was investigated with specific antibodies against recombinant *Arabidopsis* proteins. The level of $InsP_6$ does not appear to relate to the levels of any above enzymes.

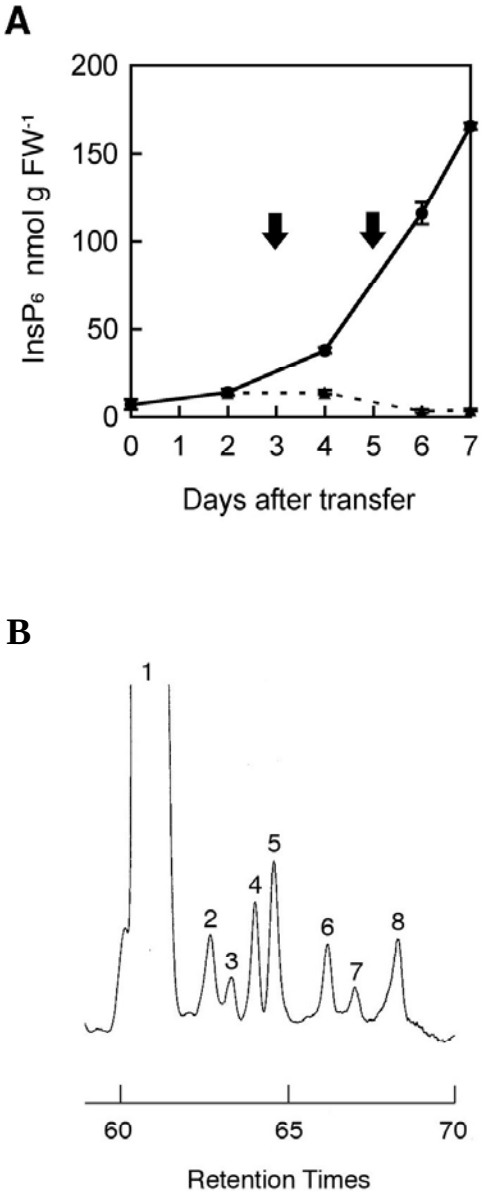

Figure 1. Changes in $InsP_6$ level in suspension cultured *Catharanthus* cells. (A) $InsP_6$ in the extracts was measured by ion chromatography. (B) Assignment of Ins phosphates in high-phosphate cells. Each peak was assigned as follows: (1) $InsP_6$, (2) Ins(2,4,5,6)P_4, (3) D/L-Ins(1,2,4,5,6)P_5, (4) Ins(1,2,3,4,6)P_5, (5) D/L-Ins(1,2,3,4,5)P_5, (6) D/L-Ins(1,4,5,6)P_4, (7) D/L-Ins(1,3,4,5)P_4, (8) D/L-Ins(1,2,4,5)P_4.

References

Mitsuhashi, N., M. Ohnishi, Y. Sekiguchi, Y.-U. Kwon, Y.-T. Chang, S.-K. Chung, Y. Inoue, R.J. Reid, H. Yagisawa, and T. Mimura. 2005. Phytic acid synthesis and vacuolar accumulation in suspension-cultured cells of *Catharanthus roseus* induced by high concentration of Pi and cations. Plant Physiol., in press.

Inositol phosphates in plants and the development of low phytate crops

Victor Raboy

United States Department of Agriculture–Agricultural Research Service, Small grains and Potato Germplasm Research Unit, Aberdeen, Idaho 83211, USA

From 65 to 80% of the total P in mature seeds of the major grain crops is found in phytic acid. For basic cellular housekeeping functions, developing seeds need about 1 mg P g^{-1} dry weight of tissue. During development nearly all additional P is synthesized into phytic acid (Ins P_6). Since mature seed produced by grains and legumes contain 3–5 mg total P g^{-1}, respectively, they therefore typically contain 2–4 mg phytic acid P g^{-1}. This represents 7–15 mg phytic acid g^{-1}. Applied interest in phytic acid primarily concerns its role as the major form of P in seeds and its importance in human and animal nutrition. However, essentially all cells synthesize *myo*-inositol (Ins, the cyclitol backbone of phytic acid) and its phosphorylated derivatives such as phytic acid. With these two metabolites cells do many things important to basic housekeeping, the regulation of development, and signal-transduction. In plants this means that the pathways to Ins and Ins P_6 are active in both vegetative and reproductive tissues, and in addition to roles in seed P storage, are important in processes such as stress response and signal transduction. Therefore, genetic resources useful in manipulating seed phytic acid, such as mutations that block Ins or Ins P_6 synthesis in seeds or transgenic introduction of phytases, might also perturb other functions important to crop productivity. This presentation will review the genetics of seed P and phytic acid, developments in breeding "Low Phytate" crops, and studies of the potential value of Low Phytate crops as human foods and animal feeds.

Seed P can be manipulated by altering the amount of seed total P, its chemical composition, or a combination of the two. While there has been progress in the physiology and genetics of P uptake by the plant, there has been little progress in aspects specific to P transport from the plant to the developing seed. Thus it is well known that increasing or decreasing the supply of P to the developing plant, results in increases or decreases (respectively) in the amount of plant and seed P. Increasing seed total P usually results in an increase in seed phytic acid. There has been substantial interest in P transport functions such as those encoded by the *Phosphate transporter* (*Pht*) genes important to P uptake from the soil, and to P transport within the plant. However, single genes or functions that if perturbed greatly alter the transport of P from the plant to the seed remain unidentified. For example, it might prove useful to identify a function that if perturbed reduces the transport of P from the plant to the seed by 50%, maintaining plant P concentration but greatly reducing seed total P.

In the cell-division phase of seed development, perhaps the first 14 days after pollination of most crops, sufficient P for basic cellular processes is acquired. After this, in normal seeds essentially all additional P is synthesized into phytic acid. In addition to an adequate supply of P, phytic acid synthesis requires seed-specific *de novo* synthesis of Ins and conversion of Ins to Ins P_6. Mutations that block the synthesis of Ins, or its conversion to Ins P_6, reduce the accumulation of seed Ins P_6. For example, Ins is synthesized via a two-step pathway: the conversion of glucose 6-phosphate to *myo*-inositol 3-phosphate (Ins(3)P_1), catalyzed by *myo*-inositol 3-phosphate synthase (MIPS); the breakdown of Ins(3)P_1 to Ins and phosphate catalyzed by Ins monophosphatase (IMP). A mutation (LR33) in a soybean MIPS gene (Hitz et al., 2002) blocks Ins synthesis and phytic acid accumulation. Interestingly, the block in Ins synthesis in LR33 seed also reduces raffinosaccharides, a desirable outcome from a nutritional

standpoint. This is one example of how manipulating Ins or Ins phosphates can have multiple outcomes due to fact that they are part of complex interrelated metabolic pathways that have numerous biological functions. In addition to studying MIPS-encoding genes, we are currently studying IMP-encoding genes in our model system, barley, in order to have a complete tool box in terms of Ins synthesis.

A first example of plant mutations that reduce seed phytic acid and that are in the pathway that converts Ins to Ins phosphates would be maize *low phytic acid* 2-1 (*lpa*2-1; Raboy et al., 2000). *lpa*2-1 is a mutation in an Ins(1,3,4)P_3 5-/6-kinase that converts Ins(1,3,4)P_3 to Ins(1,3,4,5,6)P_5, an intermediate step in phytic acid synthesis (Shi et al., 2003). Plants homozygous for maize *lpa*2, or for one of several barley *lpa* mutations, or for a soybean *lpa* mutation, either have problems with germination and emergence, or are clearly more sensitive to stress. For example, we have developed near-isogenic lines in barley that are either homozygous for wild-type genes, or homozygous for one of four independent *lpa* mutations that reduce seed phytic acid by 50% (barley *lpa*1-1) to 90% (barley *lpa*-M955). In non-stressful (irrigated) production environments barley *lpa*1-1 yields were similar to those of wild-type, and barley *lpa*-M955 yields were reduced by about 13%. However, in stressful environments (dryland production during a drought), barley *lpa*1-1 yield was reduced by about 11% as compared with wild-type, but barley *lpa*-M955 yield was reduced by 34%. Two mutations, barley *lpa*2-1 and barley *lpa*3-1 that cause reductions in phytic acid intermediate between barley *lpa*1-1 and barley *lpa*-M955, had intermediate impacts on yield in non-stressful and stressful environments. The impact on stress tolerance of mutations that reduce seed phytic acid therefore appeared to be proportional to the level of phytic acid reduction. This represents a second example of the pleiotropic effects one observes when manipulating Ins and Ins phosphate pathways.

The maize *lpa*2-encoded Ins phosphate kinase, along with a number of other kinases, synthases like MIPS, phosphatases like IMP or phytases, and genes encoding several other functions such as transporters, all potentially represent targets for molecular engineering of the Low Phytate trait. The goal of this molecular engineering is to improve the nutritional quality of seed while maintaining agronomic quality such as stress tolerance and yield.

All *lpa* mutations isolated to date result in reduced phytic acid accompanied by matching increases in seed inorganic P, resulting in the so-called "High Inorganic P" (HIP) seed phenotype, but have little impact on seed total P. Thus these Low Phytate crops have greatly increased "available P", at least when used in diets for non-ruminant animals such as poultry, swine and fish. A number of studies showed that use of Low Phytate crops in feeds for non-ruminants can help to reduce the level of phosphate or phytase supplementation needed for optimal animal productivity, and can reduce the level of P in animal waste. A series of studies using human subjects have now shown that Fe, Zn, and Ca are 30–50% more available in foods prepared from Low Phytate maize as compared with normal maize, and that at least in the case of Zn, availability is proportional to the level of phytic acid reduction. The current status of nutritional studies using Low Phytate crop varieties, as well as the current status of plant genetics and breeding involving plant and seed phosphate and phytic acid, will be reviewed.

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Phytases: attributes, catalytic mechanisms, and applications

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Phytic acid, which was discovered in 1903 (Posternak, 1903), has been found to be a nearly ubiquitous component in cereals and grains. The widespread occurrence of phytate in the plant kingdom is accompanied by the need for plants, microorganisms and some animals to hydrolyze the compound. A number of phytate-degrading enzymes have now been reported and studied. The detailed characterization of some of these enzymes has revealed that nature did not develop a single catalytic mechanism to cleave phosphate groups from phytate in all these diverse organisms. The recognition that not all phytases are structurally similar or share a common active site has become important for anyone who wishes to fully comprehend research in this field. Today, four distinctly different classes of phytases are recognized: histidine acid phosphatase, β-propeller phytase, cysteine phosphatase, and purple acid phosphatase.

Histidine acid phosphatase (HAP). Phytases belonging to this class are the most widely studied and utilized today. Representatives of this large class of enzymes are known to occur in animals, plants and microorganisms (Wodzinski and Ullah, 1996). A common active site motif, RHGXRXP, is shared by all HAPs (Ullah et al., 1991). The catalytic histidine in this sequence initiates a two-step reaction that results in the hydrolysis of phosphate monoesters. The term Histidine Acid Phytase (HAPhy) has been advanced to designate the HAPs that can accommodate phytate as a substrate (Oh et al., 2004). Both prokaryotic and eukaryotic HAPhys are known and they share little sequence homology other than the conserved active site motif. Among prokaryotic phytases, the one produced by *Escherichia coli* is the best-characterized HAPhy (Greiner et al., 1993). A three-dimensional molecular model of its structure is available, and a eukaryotic version of the enzyme from *Aspergillus niger* has been advanced for use as an animal feed additive. In eukaryotes, HAPhys have been cloned in maize and in a number of fungal isolates. The most widely studied fungal phytases are from *A. niger* and *A. fumigatus*. Theses studies revealed that there are two classes of HAPhys. The first class has broad substrate specificity but a lower specific activity for phytic acid; the second class has narrow substrate specificity but a high specific activity for phytase (Wyss et al., 1999). Evidence from site-directed mutagenesis studies established the importance of certain amino acid residues that make up the substrate specificity site in fungal HAPhys. Mutating these key amino acids leads to changes in substrate affinity and the pH profile (Mullaney et al., 2002). While not directly involved in the catalytic mechanism of HAPhys, the conservation of an eight-cysteine motif appears to be essential to maintain the proper molecular structure necessary for the enzyme activity in fungal phytases (Mullaney and Ullah, 2005). Today, the major application for HAPhys is in the hydrolysis of phytate in cereal and grains in animal feed. Future applications extend from the development of plant cultivars that require less P fertilizer to modification for use as a peroxidase.

β–Propeller Phytase (BPP). A wide range of catalytic functions has been ascribed to proteins possessing the β-propeller molecular architecture (Pons et al., 2003). A 3-

dimensional drawing of BPP molecule shows a shape that resembles a propeller with six blades (Ha et al., 2000). A novel Ca dependent *Bacillus* phytase with this configuration has been cloned and characterized. It lacks the RHGXRXP sequence motif and, therefore, it is not a member of HAP. It requires Ca^{2+} for both activity and thermostability (Kim et al., 1998). This phytase employs two phosphate-binding sites, a cleavage site for substrate hydrolysis and an affinity site to bind the substrate (Shin et al., 2001). β–propeller phytases share an optimum pH range with some alkaline plant phytases. The molecular structure of these plant phytases has yet to be determined, but they display some common traits with β-propeller phytases. They both have a narrow substrate range while requiring Ca for activity and only remove three phosphates from phytic acid to yield inositol triphosphate as a final product. No commercial applications are available thus far for BPP, but it has been advanced as an animal feed additive and as a means to promote plant growth under phosphate limiting conditions.

Cysteine Phosphatase (CP). Recently, another class of phytase has been reported from an anaerobic ruminal bacterium, *Selenomonas ruminantium* (Chu et al., 2004). Its optimum temperature ranged between 50–55°C with optimal activity in the pH range of 4.0–5.0 depending on the buffer used. Lead cations enhance activity, while Fe^{2+}, Fe^{3+}, Hg^{2+}, and Zn^{2+} ions strongly inhibited the enzyme. Sequence homology studies support similarities between this phytase and the catalytic domain found in the cysteine phosphatase superfamily. Its 3-dimensional structure's accession number is 1U24. The structure of this phytase consists of one large and one small domain. Towards the C-terminal, near the edge of the large domain is a shallow pocket containing a two loop structure similar to the active site found in protein tyrosine phosphatase with the catalytically important HCXXGXXR(T/S). This enzyme catalyzes dephosphorylation of phytic acid to *myo*-inositol monophosphate.

Purple acid phosphatase (PAP). Characterization of a soybean (*Glycine max* L. Merr.) phytase has revealed the purple acid phosphatases sequence motif, DXG..GDXXY. .GNH(E,D)..VXXH..GHXH. The *GmPhy* phytase, found in germinating soybean seedlings, apparently contains the catalytic mechanism associated with this large class of metalloenzymes (Hegeman and Grabau, 2001). This and a putative rice (*Oryza sativa*) phytase are the only PAP phytases currently deposited in GenBank. As compared to fungal phytase, this soybean seed phytase has a relatively low specific activity for phytic acid. It has been proposed that the low catalytic activity of *GmPhy* may be advantageous in plant seed where a slow and balanced breakdown of phytate during germination could be efficacious. No 3-D model of soybean phytase is available, and no commercial applicant is envisioned.

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Characterizing steps in phytic acid biosynthesis as targets for manipulating inositol phosphate metabolism in soybean

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Phosphorus in plant seeds is stored primarily as phytate (*myo*-inositol hexa*kis*phosphate), an inositol phosphate compound that is poorly digested by non-ruminant animals. A long-term goal of our research is to alter the phytate content of soybean (*Glycine max* L.) seeds to improve P utilization in feed and reduce P excretion in animal manure. Developing feed components with improved P bioavailability will provide additional tools for managing nutrients associated with animal production and will help prevent entry of agricultural P into critical watersheds.

Phytic acid biosynthesis has been elucidated for a number of organisms including yeast (*Saccharomyces cerevisiae*), slime mold (*Dictyostelium discoideum*), and aquatic duckweed (*Spirodela polyrhiza*). Synthesis can proceed by at least two possible routes: through a phosphatidylinositol pathway (lipid-dependent) as in yeast, or through a series of sequential phosphorylation steps (lipid-independent) as shown for *Dictyostelium* and *Spirodela*. Although individual enzymes have been characterized in several plants, a definitive biosynthetic pathway remains undefined. Taking advantage of a large soybean EST (expressed sequence tag) database, we have used a bioinformatics approach to identify clones encoding candidate enzymes involved in inositol phosphate metabolism.

Based on similarity to sequences from other organisms, we have identified cDNAs for three putative pathway enzymes in soybean, inositol (1,3,4)P_3 5/6-kinase, inositol (1,4,5)P_3 6/3-kinase, and inositol (1,3,4,5,6)P_5 2-kinase (Table 1). We have cloned full-length sequences for the soybean inositol polyphosphate kinases into pGEX vectors for expression of GST-tagged fusion proteins in *Escherichia coli*. Similarity between the four soybean inositol (1,3,4)P_3 5/6-kinase clones and previously characterized sequences from *Arabidopsis thaliana* and *Zea mays* ranges from 51 to 66% at the nucleotide level.

Table 1. Predicted inositol polyphosphate kinases in soybean.

Predicted Enzyme	Open reading frame (bp)	Number of amino acids	Predicted protein size (kD)
Ins(1,3,4)P_3 5/6-kinase	1,020	339	38.4
	948	315	35.0
	1,065	354	40.1
	1,026	341	38.3
Ins(1,4,5)P_3 6/3-kinase	840	279	30.9
Ins(1,3,4,5,6)P_5 2-kinase	1,371	456	51.2

GST fusion proteins were purified by affinity chromatography (Figures 1A and 1B). Purified proteins for Ins(1,3,4)P_3 5/6 kinase showed the ability to phosphorylate [^{3}H]-Ins(1,3,4)P_3 to [^{3}H]-Ins(1,3,4,5)P_4 as detected by high performance liquid chromatography (Fig. 1C). Similar analysis of reaction products indicated the addition of a single phosphate

during the 15 minute incubation period. Additional characterization will establish whether these enzymes add a second phosphate group and determine the ratio of addition of phosphates at the 5 and 6 positions of the inositol ring. The GST fusion protein from the putative soybean Ins(1,4,5)P_3 6/3-kinase sequence also showed kinase activity, however, no activity has been demonstrated from the soybean Ins(1,3,4,5,6)P_5 2-kinase fusion protein to date. Expression of the soybean sequences is being examined among different tissues and stages of soybean seed development. Sequences from all three enzyme classes are being used to generate silencing constructs in the pHELLSGATE vector. Knock-outs of the different genes will be performed in embryogenic soybean cultures to determine the effects on inositol phosphate metabolism.

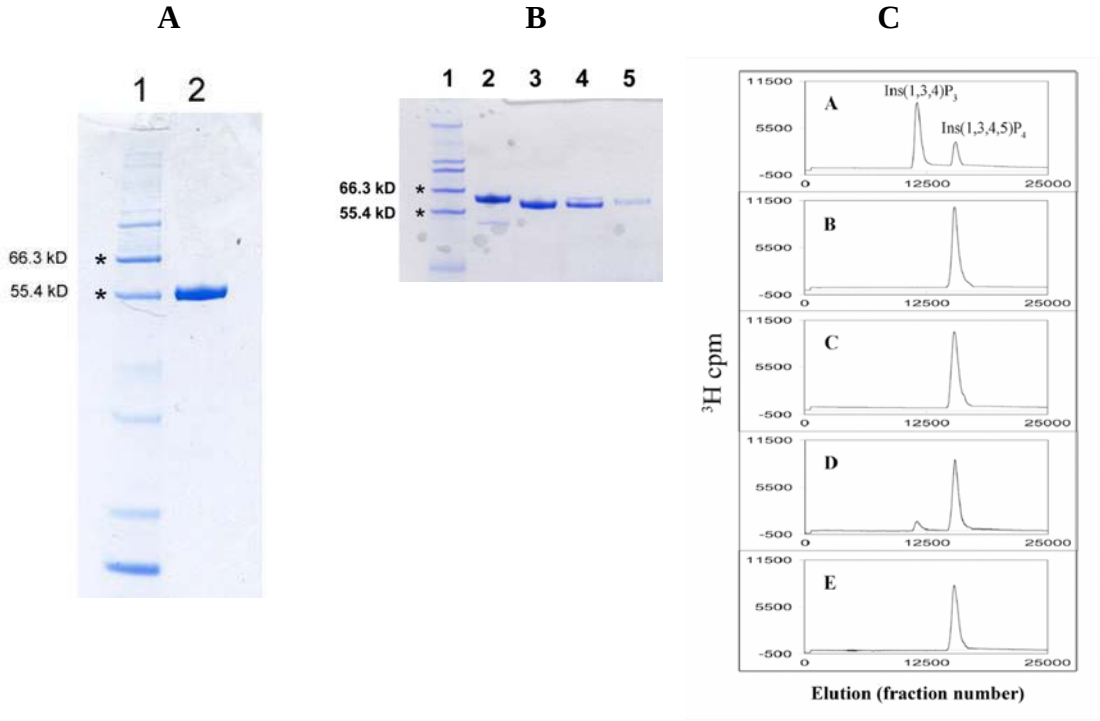

Figure 1. Expression and characterization of GST-kinase fusion proteins. A) GST-tagged fusion protein expressed from a soybean Ins(1,4,5)P_3 6/3-kinase sequence. Lane 1: protein size markers, Lane 2: GST-Ins(1,4,5) P_3 6/3-kinase from soybean. B) GST-tagged fusions proteins expressed from soybean Ins(1,3,4)P_3 5/6-kinase sequences. Lane 1: Protein size markers, Lanes 2-5: four independent GST-Ins(1,3,4)P_3 5/6-kinase fusion proteins. C) High-performance liquid chromatography analysis of kinase reaction products. To generate the mixture of substrates shown in panel 1 (control – without adding kinase), [^{3}H]Ins(1,3,4,5)P_4 was incubated with inositol 5'-phosphatase. Panels 2-5 show the HPLC analyses of the products after a 15 min. incubation with a 30 nM substrate mixture of [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4, with each of the four GST-Ins(1,3,4)P_3 5/6-kinase fusion proteins. High-performance liquid chromatography was performed with a Waters Spherisorb S5 Sax 4.0 x 125 mm column and a 10 mM to 1.02 M ammonium phosphate gradient.

Current biochemical research on phytase genes in microorganisms and plants

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The stepwise release of phosphate from phytate, the major storage form of phosphate in plant seeds and pollen, is initiated by a class of enzymes that have been collectively called phytases. The classification is solely due to the *in vitro* capability of these enzymes to accept phytate as a substrate. Phytase is an inducible enzyme in most microorganisms and its expression is subject to a complex regulation, but phytase formation is not controlled uniformly among different microorganisms. Until now, phytase production was studied in some detail only in *Escherichia coli* (Touati et al., 1987; Greiner et al., 1993), *Raoultella terrigena* (Greiner et al., 1997; Zamudio et al., 2001), and *Saccharomyces cerevisiae* (Andlid et al., 2004). In moulds, phytase production is growth associated; enzyme activity increases from the onset of growth to the beginning of the stationary phase. However, in non-limiting media the formation of many bacterial phytases halts in exponentially growing cells and begins when cultures enter the stationary phase. This suggests that either nutrient or energy limitation, known to occur in the stationary phase, could be responsible for phytase induction.

Among the nutrient limitations tested, only C starvation provoked immediate synthesis of the *R. terrigena* phytase, whereas in *Bacillus* and *E. coli*, phytase synthesis was triggered when bacteria were starved of phosphate; C, N, and S limitation had no effect. A tight regulatory inhibition of phytase formation by phosphate concentration was generally observed in all microbial phytase producers, including moulds, yeast, and bacteria with the exception of *R. terrigena* and the rumen bacteria. Thus, phytase synthesis is regulated at transcription level by derepression. The repression of phytase synthesis by inorganic phosphate seems to be less significant in more complex media, although it is not known which components account for the reduced repression.

In addition, phytase expression depends on medium pH and the nature of the C source used for growth (Konietzny and Greiner, 2004). In the presence of simple sugars, strong repression of enzyme synthesis was observed. In moulds, formation of mycelial pellets was responsible for the low enzyme yields. That cAMP-CAP, rather than the C source itself, are directly involved in the regulation of phytase synthesis was shown in *E. coli*. Synthesis of the phytase in both *E. coli* and *R. terrigena* were reported to be negatively regulated by cAMP. For several *Raoultella* sp. phytate is needed to induce phytase production. Substrate induction was also found in *Mitsuokella jalaludinii*, whereas phytate had no effect on phytase synthesis in *E. coli*. Phytase formation in *Pseudomonas* sp. and *R. aerogenes* was reported to be significantly induced in the presence of *myo*-inositol as the sole C source, but not in other *Raoultella* sp.

The efficient induction or derepression of phytase formation by phosphate starvation in most microorganisms suggests a possible role in providing the cell with phosphate hydrolyzed from molecules such as phytate. This hypothesis is supported by the identification of a phytase in the stalk of *Caulobacter crescentus*, a gram-negative alpha-purple proteobacterium that lives in low-nutrient aquatic environments. Phosphate is the limiting nutrient in the environments where *C. crescentus* is found, and one of the hypothesized functions of the stalk is phosphate uptake. Stalks elongate when phosphate is limiting, increasing the surface area available for

phosphate uptake; the presence of a phytase also would allow the uptake of organically phosphate by the stalk (Ireland et al., 2002). In addition, ruminants seem to digest phytate through the action of phytase produced by microbes in the rumen. In contrast to most other bacteria, anaerobic rumen bacteria can tolerate a high level of phosphate without any negative impact on phytase production. This unique ability leads to a more efficient phytate hydrolysis in the rumen, even under the high phosphate levels in the rumen fluid of ruminants fed concentrated feed. The phosphate generated by splitting of phytate is utilized by both the microbial flora and ruminant host.

Soil microorganisms colonizing the plant rhizosphere and producing extracellular phytase activity (e.g., *Bacillus*, *Enterobacter* ssp., mycorrhizal fungi), could promote plant growth by making phytate available. In addition, phytate-degrading enzymes may play a role in inositol phosphate metabolism, or perhaps microbial pathogenesis. Research into the mechanisms by which pathogens cause disease implicated acid phosphatases and inositol phosphate phosphatases as effectors that help to undermine host cell signalling and defence mechanisms. In particular, inositol phosphate phosphatases from some pathogenic members of the Enterobacteriaceae are important virulence factors after they are injected into the host cell by Type III secretion systems. Once inside the host cell, these phosphatases can interfere with a variety of host cell signalling pathways, including those affecting cellular survival and regulation of intracellular membrane trafficking (DeVinney et al., 2000).

In higher plants, phytases occur predominantly in grains, seeds, and pollen (Konietzny and Greiner, 2002), where they are responsible for phytate degradation during germination to make phosphate, minerals, and *myo*-inositol available for plant growth. Grains, seeds and pollen contain both constitutive and germination-inducible phytases. Although large increases in phytase activity occur in germinating seeds as well as in germinating pollen, the biochemical mechanism leading to this rise in activity is not well understood. In pollen, phytases induced during germination may be synthesized from long-lived, pre-existing mRNA. In cereals and legumes, the cause of the rise in phytate-degrading activity during germination is also disputed. Some studies suggest *de novo* synthesis, whereas others suggest simple activation of pre-existing enzymes. During germination, phytase activity may be controlled by the action of either gibberellic acid or phosphate. Recently, it was shown that gibberellic acid did not stimulate phytase activity in an *in vitro* assay using a phytase from lentils, and that gibberellic acid at least partly stimulated *de novo* synthesis of the lentil phytase(s) during germination. Phosphate directly inhibits phytase, although it was concluded that phosphate also acts at the transcription level, since phosphate added early enough in the germination sequence can repress the increase in phytase activity.

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Expression of phytase in yeast: phytate degradation and survival of yeast cells during *in vitro* digestion

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Objective. To investigate the potential of modified strains of baker's yeast, *Saccharomyces cerevisiae*, to degrade phytate in a meal during *in vitro* digestion at simulated physiological conditions.

Background. Phytate (*myo*-inositol hexa*kis*phosphate) is a naturally occurring compound in plants. Due to chelation of several dietary minerals, it acts as a potent anti-nutrient in foods. Degradation of phytate has previously been found to significantly improve absorption of nutritionally important minerals, such as Fe and Zn, in the human intestine. By removing regulatory genes for phytase expression in *Saccharomyces cerevisiae*, high-phytase strains that constitutively degrade phytate have been constructed. A novel application is to use such strains for the degradation of phytate during gastrointestinal passage, and *S. cerevisiae* can constitute a suitable phytase carrier to the gastrointestinal tract since it has GRAS status (generally considered as safe).

Methodology. The phytate degrading capacity and survival of the yeast strains were tested during *in vitro* digestion. Yeast peptone dextrose growth medium (YPD) or commercial ready-made wheat gruel (~0.2 mM native phytate), with added phytate in known concentrations, were used as simple model meals. During the gastric phase, pepsin solution was added and the meal was submitted to a pH gradient (pH 5.5 to pH 2.0) for 90 minutes. Thereafter, pancreatin/bile solution was added and the pH was increased to 5.0 for 30 minutes, followed by an incubation at pH 6.5 for 60 minutes (intestinal phase). Phytate content and cell concentration was measured before, during, and after digestion.

Results and Discussion. A degradation of 62% (n=6) and 36% (n=5) was found in YPD and gruel, respectively, when phytate (0.5 mM) and high-phytase yeast cells (corresponding to 1 mg mL^{-1} dry weight) was added, as compared to the wild-type yeast that did not show any significant degradation. The major part of the phytate degradation occurred during the gastric phase. A gradually descending gastric pH was however found crucial, since hardly any degradation could be observed when static pH 2 or pH 3 was tested in the gastric phase. No significant degradation was observed in the late intestinal phase, indicating that the very low solubility of phytate at pH 6.5 inhibited enzyme-phytate interactions. Furthermore, the strain was found to withstand acidic pH, bile salts, and digestive enzymes, giving a good survival of the cells during digestion.

Conclusion. It was shown that the modified strain used in the study survived and can degrade phytate at digestive conditions. The level of phytate degradation seems however to be dependent on pH, incubation time and phytate solubility. Even though further studies must be done, our results show the potential of using high-phytase yeast to improve mineral absorption during the gastrointestinal passage.

Expression of the *Escherichia coli* APPA phytase in the genetically enhanced Enviropig™ breed

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The Enviropig™ breed of pigs expresses the APPA phytase gene from *Escherichia coli* under the control of the mouse parotid secretory protein (PSP) promoter. The phytase enzyme is produced primarily in the salivary glands of the pig (Golovan et al., 2001), and is not detected in tissues normally recovered for human consumption. The level of enzyme detected in the saliva differed among the various lines of the Enviropig™ breed as well as among the different phases of growth. Greatest concentration of enzyme per milliliter of saliva usually occurred during the weanling phase and decreased to a base level during the late growing and finishing phases of growth. The phytases produced by different lines of pigs had a similar size of approximately 51 kDa, which were approximately 6 kDa larger than the *E. coli* phytase due to N-linked glycosylation. The purified enzyme from three different lines of the Enviropig™ breed exhibited similar K_m and V_{max} values for phytase and acid phosphatase activities to that of the APPA phytase overexpressed in *E. coli.* These activities were determined by assaying phytate hydrolysis at pH 4.5 and *p*-nitrophenyl phosphate hydrolysis at pH 2.5. The three enzyme preparations also exhibited similar pH and temperature profiles to the *E. coli* phytase. The phytase produced by the Wayne line of Enviropig™ hydrolyzed phytate and produced inositol phosphate intermediates and final products identical to that of the *E. coli* phytase. Analysis of tryptic peptides of the three different preparations of the Enviropig™ phytase indicated the presence of pyroglutamic acid at the N-terminus and documented N-glycosylation at all three potential N-glycosylation sites. The glycosylated phytase, like that of the *E. coli* enzyme, was unstable below pH 2.0 in the absence of proteases, but was stable at pH values 2.5 and 4.5 in the presence of pepsin. It was inactivated at pH 7.0 in the presence of serine proteases. The differential stability of the phytase in the presence of pepsin and its sensitivity in the presence of trypsin may be explained by the limited number of solvent accessible pepsin recognition sites compared to the higher number of solvent accessible trypsin recognition sites.

These characteristics of the phytase ideally suit the dietary requirements for a genetically enhanced monogastric food animal. When the animal begins eating, this is when most phytase is secreted in the saliva, and the pH increases to a pH between 2.5 and 5.0 due to the buffering capacity of the food. The phytase is maximally active in this pH range. Once the enzyme reaches the small intestine and is exposed to serine proteases, it is rapidly degraded.

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Inositol phosphates in animal nutrition: dietary manipulation and phosphorus excretion by animals

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myo-Inositol hexa*kis*phosphate (phytic acid) and its salt (phytate) represent the major form of P present in plant feeds. Because simple-stomached species such as swine, poultry and fish do not have hydrolytic enzymes in their digestive tracts, phytate in feed is poorly digested by these animals and is largely excreted into feces. This results in the need for supplemental phosphate, a non-renewable natural resource, in diets for the simple-stomached animals. More problematically, the excessive manure P excretion in areas of intensive animal production has become a major environmental pollutant, contributing to eutrophication. In addition, phytic acid chelates divalent metals such as Ca, Zn, and Fe, and renders them unavailable for absorption.

Phytase is a phosphohydrolytic enzyme that initiates the stepwise removal of phosphate from phytate. Supplemental microbial phytase in diets for swine and poultry has become an effective tool for improving bioavailability of phytate-P to these food-producing animals, reducing dietary phosphate supplementation, and controlling manure P excretion in complying with environmental regulation. Several commercial phytase enzymes have been available, but none of them is completely satisfactory.

Functional site and impact of phytase. The stomach is the main functional site for both fungal and bacterial phytases to release P from phytate. Little supplemental phytase activity is detectable beyond the upper small intestine. Numerous experiments have shown that supplemental microbial phytases at between 300 and 1,000 units kg^{-1} of feed improve bioavailability of dietary P by between 10 and 35%, and reduce manure P excretion by up to 50%. In production, specific dietary inclusion rates of phytases for different species have been recommended to provide equivalence for the replacement of 1 g of phosphate.

Efficacy determinants and application limitation of phytase. When microbial phytase is supplemented into diets, total dietary Ca levels should be reduced to a Ca:P ratio of approximately 1.2:1. Effectiveness of supplemental phytase may be improved by the presence of organic acids, but could be reduced by the presence of phosphate. The combination of intrinsic phytase activity present in cereal byproducts and supplemental exogenous phytase exhibits additive effect on phytate hydrolysis, but synergistic effects of exogenous microbial phytases with different biochemical properties remain unclear. Supplemental phytase with other feed enzymes such as proteases or cell wall-degrading enzymes has been shown to improve phytase efficacy and the overall nutritive utilization of the feed. Because of the acidic pH optimum and great pepsin resistance, bacterial phytase (AppA2) is three to four-fold more effective than fungal PhyA phytase in both diets for swine and poultry. The inability of phytase to tolerate heat inactivation from feed pelleting is the main constraint for the wide application of phytase in animal production. When stored under cool and dry conditions, phytase preparations are stable for 4–12 months in powder form or up to 6 months in liquid form. Caution should be taken when storing phytase with mineral and vitamin premixes, as some of their components may have deleterious effects on enzyme stability. The extra cost

associated with supplemental phytases can only be justified in areas where regional environmental policies impose a maximum allowable level of manure P.

Biotechnology of phytase. Early biotechnology of phytase focused on screening for novel phytase genes. That effort resulted in the isolation and characterization of several new phytase genes from bacteria (*Escherichia coli; Bacillus sp.*) and fungi (*Aspergillus fumigatus; Peniophora lycii*). These novel phytases offer a wide range of enzymatic properties for developing effective phytases targeting different species or feed processing conditions. Most recent molecular research of phytase has been shifted to developing efficient enzyme production systems and optimizing catalytic efficiency and thermostability of phytase. Different expression systems of bacteria, yeast, and plants have been compared for low cost of large scale of phytase fermentation. Crystal structures of several phytases have been solved and provided the structural basis for site-directed mutagenesis with desirable functions. This approach has been successfully employed to shift the optimal pH of *A. niger* PhyA phytase from 5.5 to 3.5 and made the enzyme more effective in the digestive tract of pigs than the wild-type enzyme. Directed evolution such as error-prone PCR has been used to improve the thermostability of commercially important phytases. A heat-stable consensus phytase has been synthesized based on primary sequence homology of different fungal phytases.

Future perspective. Developing catalytically efficient, protease-resistant, heat-stable, and economic phytases, or "ideal phytases", for different species and types of animals would overcome the current constraint of phytase application. A wide application of phytase in animal diets would alleviate manure P pollution worldwide. Transgenic plants over-expressing phytase in leaves and seeds have recently been developed and may offer another economic source of phytase for animal or even human nutrition. Meanwhile, low-phytate grains have been identified, which can provide an appreciable level of available P to animals. Transgenic pigs with over-expressed phytase in their saliva could relieve their requirements for phosphate supplements in plant-based diets, because they excrete approximately 70% less of fecal P than control animals. Hopefully, these novel technologies can be combined to effectively improve dietary phytate utilization by animals and to minimize environmental pollution of their manure P excretion.

The avian *MINPP* gene can help to alleviate the planet's "phosphate crisis"

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The chicken intestinal tract lacks phytase, so dietary phytate (IP_6) is not digested, yet this contains 80% of organic phosphate in poultry feed. Inorganic phosphate is added to the feed, which consumes the planet's reserves of a major fertilizer ingredient. Some of this phosphate, together with undigested phytate, is excreted; runoff from the manure imperils adjacent aquatic systems with phosphate-driven eutrophication (Abelson, 1999). Efforts to ameliorate this "phosphate crisis" (Abelson, 1999), by adding microbial phytase to animal feed, are costly (Ward, 2001), and represent a high-risk factor for occupational asthma and rhinitis in farm workers (Baur et al., 2002). A novel solution arises from our discovery that chickens already have an active, intracellular phytase, previously named MINPP (Multiple INositol Polyphosphate Phosphatase) that resides in the endoplasmic reticulum (ER).

Mammalian MINPP has not previously been considered a member of the phytase family because of its very weak activity (6 nmol/mg protein/min; microbial phytases are 10^3- and 10^4-fold more active; Mullaney et al., 2002). MINPP from *Gallus gallus* (Romano et al., 1998; Chi et al., 1999), with an added C-terminal poly[His] epitope tag, was expressed in *Pichia pastoris*, and purified by Ni-agarose affinity chromatography. At pH 5.0, recombinant avian MINPP hydrolyzed IP_6 with a K_m of 0.14 mM and an unexpectedly high V_{max} of 715 nmoles mg^{-1} protein min^{-1} (>100-fold greater than the phytase activity of mammalian MINPP; Nogimori et al., 1991). Glycosylation did not affect activity. A pH profile of MINPP showed a peak at pH 5.0, although the enzyme retained near-maximal activity over a broad range of pH 4.5 to 7.5, which also spans the pH of much of the chicken's intestinal tract. An end-point assay indicated that 80% of IP_6 phosphate was released by MINPP. Knowledge of the three-dimensional structure of microbial phytases has permitted the rational design of mutants with improved capacity to digest IP_6 (Tomschy et al., 2000). The structure of MINPP has not previously been determined, but we now predict a model structure that has some similarities to microbial phytases. This model attributes function to two amino-acid residues (T27; Q78) not previously thought part of MINPP's catalytic site. We verified this model by site-directed mutagenesis: A T27A mutant was 96% less active, and a Q78A mutant had 2-fold higher catalytic efficiency (i.e., K_m / V_{max}).

While MINPP has a number of useful properties as a phytase, its most practical attribute is that it is already endogenous to the chicken. Thus, we next considered what strategies might be used to deliver MINPP to its required site of action *in vivo*. Avian MINPP, like its mammalian counterparts, is normally an ER luminal resident (Romano et al., 1998; Chi et al.,

1999). We transiently over-expressed full-length MINPP in the LMH immortalized chicken liver cell line under the control of the CMV promoter. Much of the MINPP accumulated inside cells in an inactive form, possibly because it was misfolded. To avoid this problem, we next transiently expressed a truncated mutant [)ADEL-MINPP] from which the C-terminal ER-retention tetrapeptide was removed. This construct retained full activity, escaped the ER, and entered the secretory pathway; in cell cultures, 92% of activity was extracellular. While in transit through the membrane-delimited secretory pathway, the enzyme was insulated from cytosolic inositol phosphates. Thus, cellular levels of the MINPP substrates in control liver cells (IP_5 = 12.6 ± 2.4, IP_6 =12.2 ± 1.8) were similar to those in cells expressing)ADEL-MINPP (IP_5 = 11.8 ± 2.7. IP_6 = 13.9 ± 3.3). The)ADEL-MINPP expression did not affect either cell viability or proliferation.

We propose that a transgenic chicken expressing)ADEL-MINPP in intestinal epithelial cells would constitutively secrete the enzyme into the digestive tract, improve hydrolysis of dietary IP_6, and reduce the need for inorganic phosphate feed supplementation, reduce pollution, and thereby ease the planet's phosphate crisis. Public perception that it might be hazardous to introduce "foreign" proteins into food has contributed to the delay in the commercial exploitation of other transgenic animals expressing exogenous, microbial phytase (Forsberg et al., 2003; Hollingworth et al., 2003). We anticipate that the consumer can more readily accommodate genetic modifications that alter expression of an endogenous chicken gene, because it is conceptually akin to the acceptable practice of breeding.

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Degradation of phytate in soaked diets for pigs

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Introduction. Up to 80% of P in cereals and seeds widely used in pig feed is present as phytic acid (*myo*-inositol hexakisphosphate, IP_6). Phytate-P is poorly available for absorption by monogastric animals, so additional phosphate is often supplied in feed to meet the animals' requirements. Consequently, more than half of the ingested P is often excreted in feces, resulting in serious environmental concern. Removal of phosphate groups from the inositol ring by plant (e.g., Pointillart et al., 1984; 1987) or microbial phytases (e.g., Pallauf et al., 1994a; 1994b) increases the digestibility of phytate-P. However, the effect of microbial phytase supplementation depends strongly on the composition of the diet and the plant phytase activity (Johansen and Poulsen, 2003). In experimental diets without feed phosphate addition, the maximal digestibility of P after microbial phytase supplementation rarely exceeds 60–65% in dry feed even at high levels of supplemented phytase. At present, the use of liquid feed is becoming more common in pig production. A few studies have shown a substantial reduction in phytate content by soaking, but only a limited effect on the digestibility of P (Näsi and Helander, 1994; Skoglund et al., 1997; Larsen et al., 1999). A series of experiments has been started in order to study the effect of diet composition, heat-treatment of the diets, phytase activity, time of soaking, and temperature on (i) the degradation of phytate in soaked feed (part 1) and (ii) the quantitative amount of generated degradation products of inositol phosphates (IP_6–IP_2) in soaked feed (part 2). The hypothesis is that it is possible to improve the digestibility of P above 60–65% by a pre-digestion of phytate-P in liquid feeding compared with dry feeding, because phytase requires a moist, acid, and warm environment to optimise the hydrolysis of phosphate groups on the inositol ring.

Materials and methods. The study was conducted *in vitro*. The diets were based on barley and soybean meal or wheat and soybean meal that were either untreated or heat-treated and pelleted at minimum 81°C. Each of the four basic diets (barley or wheat based, non-heat-treated or heat-treated) was supplemented with no phytase or 500 FTU kg^{-1} (Natuphos, BASF). The diets were soaked with water (1:2.75) in 1-L fermentors (closed glass jars) and incubated at 10, 20 or 38°C. At 10 and 20°C, samples of 60 mL were taken at 4, 8, 12, 24, 48 and 72 h. At 38°C, samples were taken at 2, 4, 6, 8, 10, 12 and 24 h of soaking.

Part 1. pH and dry matter was measured. Phosphorus was analyzed by the colorimetric vanadomolybdate procedure (Stuffins, 1967), phytate-P according to the method of Haug and Lantzsch (1983), and phytase activity by the method of Engelen et al. (1994).

Part 2. Further studies were conducted to distinguish between the different inositol phosphates present in the soaked feed, using high-performance liquid chromatography.

Results and discussion. *Part 1.* In general, phytate degradation was slower in heat-treated diets without phytase supplementation compared with non-heat-treated diets as the heat-processing reduced the plant phytase activity by 75–83%. Table 1 shows the results on barley based diets as an example. When the heat-treated diets were supplemented with phytase the phytate degradation increased to the same levels as the non-heat-treated diets. On the other hand, addition of phytase to non-heat-treated diets did not improve phytate degradation as the

non-heat-treated diets contained a considerable amount of plant phytase. At 10 or 20°C, between 17 and 79% of the total amount of phytate in the diets were degraded within the first 8 h of soaking, with the greatest degradation rate at 20°C. However, at 38°C a comparable amount was degraded within 2 h of soaking.

Increasing the temperature resulted in a decrease in pH mainly due to an increase in lactic acid bacteria. Microbial phytase appeared to be considerably more active at lower pH values than plant phytase, which showed no activity at pH < 5. More details were reported by Carlson and Poulsen (2003).

Table 1. Phytate degradation (g phytate-P kg^{-1} dry matter) in the barley-based diets as affected by temperature and time of soaking (hours).

	10°C							20°C						
	0	4	8	12	24	48	72	0	4	8	12	24	48	72
Non-heat-treated	3.3	2.0	1.7	1.5	1.2	1.1	0.9	3.3	1.7	1.5	1.0	0.9	0.4	0.2
Non-heat-treated + phytase	3.3	1.4	1.3	1.2	1.3	1.0	0.9	3.3	1.4	1.3	1.3	1.2	0.0	0.0
Heat-treated	3.3	2.9	2.8	2.6	2.2	1.8	1.5	3.3	2.7	2.4	2.1	1.7	1.1	1.0
Heat-treated + phytase	3.3	1.6	1.2	1.1	0.9	0.8	0.5	3.3	1.4	1.1	1.1	0.9	0.2	0.0

Part 2. Currently, the quantitative amount of inositol phosphates (IP_6-IP_2) in the diets is studied in order to compare the amount of different inositol phosphates as affected by diet composition, heat-treatment, phytase activity, time of soaking, and temperature. The accumulation of the different degradation products will furthermore show at which stage the release of the phosphate groups on the inositol ring is lowered or stopped.

Conclusion. Phytate degradation is slower in heat-treated diets compared with non-heat-treated diets, because heat processing inactivates the phytase activity to some extent. Addition of phytase to non-heat-treated diets has no effect on phytate degradation, whereas phytase addition to heat-treated diets increases phytate degradation to the same level as in non-heat-treated diets. The rate of phytate degradation increases markedly when the temperature is increased from 10 to 38°C. Microbial phytase is far more active at lower pH values compared with plant phytase, which showed no activity at pH < 5.

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Environmental implications of inositol phosphates in animal manures

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Animal production in the United States is valued at over $98.8 billion and has consolidated significantly over the past twenty years, with a larger number of animals being produced on an increasingly smaller land area. Manure generated from these industries is estimated to exceed 335 million tons of dry matter per year. Recycling manure-derived nutrients to crops not only reduces the need for commercial fertilizers, but can improve soil physical properties. However, over-application or mis-management of manure can degrade surface and groundwater quality with excess nutrients. Long-term manure application to agricultural land leads to soil P accumulation and an acceleration of P transfer in runoff to water bodies. This can contribute to eutrophication in freshwater ecosystems, and there are numerous examples of water quality impairment associated with P pollution from animal operations. The environmental fate of P in manures is determined partly by its chemical composition, yet few studies have examined the P composition of manures and its effect on P reactivity in soils.

Animal manures contain significant amounts of P (6.7–29.1 g P kg^{-1} dry wt for several species of animals; Barnett, 1994). This is composed of inorganic and organic fractions, the latter varying from 10 to 80% of the total and decreasing as the manure ages (Peperzak et al., 1959). The large range in organic P content is a function of diet and animal physiology. Barnett (1994) published the most recent comprehensive research on organic P compounds in animal manures by sequential fractionation. Inorganic P was found to constitute a large proportion of total P, followed in descending order of magnitude by residual, acid soluble organic P, and small amounts of phospholipids. More recently, manure P speciation by solution ^{31}P nuclear magnetic resonance spectroscopy has been applied to a variety of manures (e.g., Turner and Leytem, 2004). The technique allows multiple compounds to be quantified simultaneously with minimal sample preparation and handling. The results indicate that in many cases manure P is predominately inorganic phosphate, with smaller amounts of phosphate monoesters, phospholipids, pyrophosphate, DNA, and in some cases phosphonates. The phytic acid (*myo*-inositol hexa*kis*phosphate) content of manures ranges from non-detectible in many ruminants and in some monogastric animals up to 80% in manures from monogastrics fed corn based diets.

In most cereal grains, P is present primarily as phytic acid. Monogastric animals are inefficient in utilizing phytic acid because they do not possess the digestive enzyme phytase that is required to hydrolyze the phytic acid molecule. Mineral phosphate supplements are commonly added to monogastric diets to prevent P deficiency, although this can lead to high P concentrations in manure, which accumulates in soil and can contribute to the pollution of water bodies. To address this, strategies involving dietary manipulation are being widely adopted to reduce P concentrations in manures. For monogastric animals that cannot digest phytic acid, such strategies include the isolation of mutant grains having low phytic acid concentrations and supplementation of animal diets with microbial phytase to increase phytic acid hydrolysis in the gut. In addition to reducing the concentrations of P in manure, dietary modification is

expected to influence manure P composition, which may have implications for the environmental fate of manure P. Compared with phosphate, which is relatively soluble in manure, phytic acid reacts strongly in soils to form insoluble complexes that are unlikely to be lost in runoff. Attempts to modify manure P composition through dietary manipulation could therefore affect the risk of P transfer in runoff to water bodies.

Maguire et al. (2004) examined the impact of phytase addition to poultry diets on P forms and solubility in litter and litter amended acidic soils. They found no consistent trends in P solubility of manure amended soils when manures were applied at the same total P rate, suggesting that dietary modification has little effect on extractable P in these soils. Although the range of phytic acid in the manures was wide (26–56% of total P), this did not influence P solubility in soil. Leytem and Westermann (2005) found a negative relationship between water soluble (r^2=0.38) and bicarbonate extractable P (r^2=0.69) and the amount of phosphate monoesters applied to a calcareous soil. Although it appears as if the organic P fraction impacted extractable P, the amount of C added with the manure treatments was a better predictor of increases in soluble P with manure additions. For poultry manures with a range of phytic acid contents (2–80%), a negative relationship existed between added phosphate monoesters and bicarbonate extractable P in the calcareous soils (r^2 = 0.63), but no significant relationship for acidic soils (unpublished data). When manures having similar C:P ratios were considered separately (to eliminate the effect of added C), the relationship between both phosphate monoesters and phytic acid additions and increases in bicarbonate extractable P improved (r^2 = 0.75) for the calcareous soils, while there was still no significant relationship for the acidic soil.

It has been demonstrated that the amount of inositol phosphate excreted by animals is dependent both on species and diet. While these differences could potentially impact P retention in soils, and therefore affect P losses from soils, there is little conclusive evidence to suggest that differences in inositol phosphate concentrations are the dominant factor affecting P solubility in manure amended soils. Other manure properties, such as the C:P ratio, may have a greater influence than the inositol phosphate content of the manures.

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Ligand effects on phytic acid in animal manures: assessing time dependence of inositol phosphate solubilization and bioactivity

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In areas of intensive animal production, agricultural soils can become enriched in P because of continuous manure applications over the years. The near-surface zone accumulates and shows elevated levels of inorganic and organic P. Animal manures are major sources of the organic P and are attracting a great deal of interest because of the inefficiency of animal digestive systems in absorbing or retaining P, in particular *myo*-inositol hexa*kis*phosphate (phytic acid).

Although there have been many studies on the composition and fate of inorganic P in manure and manure-amended soil, speciation of organic P compounds is poorly understood. The knowledge gap hampers the understanding of the environmental behavior and transformations of inositol phosphates, in particular *myo*-inositol hexa*kis*phosphate in animal feed, manure, soil, water, and mixed environmental media. Thus, it affects the development of comprehensive strategies for mitigating agricultural P transfer to aquatic systems.

In this presentation, I will examine the factors that affect the processes of solubilization and dephosphorylation of dissolved and insoluble complexes of *myo*-inositol hexa*kis*phosphate to replenish the water-soluble P fraction and will elaborate on a mild *in situ* ligand-based enzymatic method used to obtain insights about the biological stability of inositol phosphates in animal manure. Selected polydentate ligands and fungal phytases are found able to differentiate the various reserve pools that contribute to the solution-phase phosphate concentration of dairy manure and manure-amended soils. In such C-rich media, and in the ubiquitous presence of monoester phosphohydrolases, biological and biochemical mechanisms may more adequately reflect the availability of manure P pools to microorganisms and plants over time. Moreover, these mechanisms can reveal the underlying potential for the timed release and dephosphorylation of inositol phosphates in complex media to the environment.

Synthesis and initial characterization of metal-phytate compounds

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Recently, spectral techniques, such as, X-ray absorption near edge structure (XANES) spectroscopy, solid-state ^{31}P nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared spectroscopy (FT-IR), and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS), have been applied to investigate the metal-P species in animal manure and soil. However, it is hardly possible to resolve these spectral data on metal-organic P compounds partly due to the lack of model metal-organic P compounds for reference. Phytic acid (*myo*-inositol hexa*kis*phosphate, IP6) has been identified as a major organic P form in soil, animal manure and other environmental samples. It contains a six-C ring with a H and a phosphate attached to each C moiety. Each of the six phosphate groups is attached in an ester linkage and retains two replaceable H atoms. This compound, thus, occurs in plants mainly in the forms of the Ca-Mg salt. Phytic acid can also exist in the salt forms with other metals (such as Fe and Al) in soil and manure. In this work, we synthesized phytate metal compounds in order to yield spectral information of these solid model compounds which would facilitate characterizing metal-organic P interactions in the environment.

Table 1. Composition of synthetic metal phytate compounds.

Metal-IP6	P (%)	C (%)	Metal (%)	P:C:Metal (mole)	Purity* (%)
Ca_6IP6	16.15	6.73	19.28	1:1.06:0.91	77.2
Mg_6IP6	17.07	7.33	12.95	1:1.11:0.96	72.9
Cu_6IP6	13.01	5.02	26.80	1:1.00:1.00	72.0
Mn_6IP6	15.53	6.63	27.40	1:1.10:1.10	81.6
Al_4IP6	17.7	6.54	10.95	1:0.96:0.72	72.0
Fe_4IP6	15.23	6.19	23.44	1:1.06:0.86	65.9
$Fe_{4-x}Al_x$IP6	15.59	6.42	17.3 (Fe) 2.96(Al)	1:1.08:0.84	70.2

*: Based on the difference in the P contents detected in the synthetic compounds and calculated from the theoretical formulas.

Synthesis of metal-IP6 compounds. Seven metal-phytate compounds were synthesized from Na_{12}IP6 and metal chlorides (Table 1). The theoretical mole ratio of P:C:metal is 1:1:1 for metal(II)-IP6, and 1:1:0.67 for metal(III)-IP6. Element analysis indicated that compounds with Ca, Mg, Cu, Mn, and Al were obtained near stoichiometrically (± 10%). The last two

compounds containing Fe(III) were 25–28% higher in metal content, indicating some Fe hydroxide was present in the two compounds. The presence of Fe hydroxide might also partly explain the lowest purity of the two Fe-containing synthetic compounds.

FT-IR spectra. All seven synthetic compounds and $Na_{12}IP6$ showed a weak absorption band around 1680 cm^{-1} (Fig. 1) and a broad absorption band at 3400 cm^{-1} (data not shown). These two bands did not disappear when these samples were dried for 1 h at 105°C. These observations demonstrated the existence of O-H bonds in these compounds which could be due to the crystalline H_2O, impurity metal hydroxides, and unbounded P-O-H bonds although the last two should not be parts of these compounds theoretically. A sharp absorption band at 972 cm^{-1} and a broad band with trailing at 1106 cm^{-1}appeared in the spectrum of $Na_{12}IP6$. The band at 972 cm^{-1} shifted to 996 cm^{-1} with Ca_6IP6, and 1010 cm^{-1} with Mg_6IP6. Both compounds showed the broad band around 1135 cm^{-1} with less trailing character. In the spectra of Cu and Mn compounds, the broad band split to two overlapped but distinguishable bands at 1086 cm^{-1} and 1133 cm^{-1} or nearby with the sharp band at 997 cm^{-1}. The spectral characteristics of trivalent metal (Fe and Al) compounds were a shoulder band at 1007 cm^{-1} with a strong band at 1092 or 1166 cm^{-1}. Thus, the absorption bands from 900 to 1200 cm^{-1} in FT-IR could be used to classify metal phytate compounds into three groups: (1) light divalent metal (Ca and Mg) phytate compounds with a sharp band and a broad band, (2) heavy divalent metal (Cu and Mn) compounds with splitting broad bands, and (3) trivalent peaks with a shoulder band.

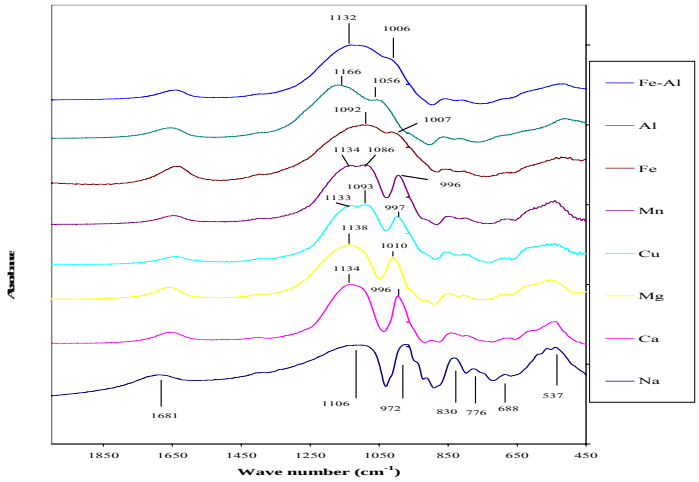

Figure 1. Fourier-transform infra-red spectra of metal-IP6 compounds.

Enzymatic hydrolysis. The metal-phytate compounds (2 mM P) were suspended in 100 mM glycine-HCl (pH 2.5) or 100 mM sodium acetate buffer (pH 5.0) with 3-phytase (0.1 U mL^{-1}). The reaction mixtures were incubated at an end-over-end shaker (40 rpm) at 22°C for 20 h. Whereas less than 0.1 mM of phosphate was detected at the end of incubation in the reaction in mixtures without 3-phytase, nearly all Na, Ca, Mg, Mn, and Cu phytates were enzymatically hydrolyzed at either pH. No enzymatic release of P from Fe and Al phytates were detected.

Inositol phosphates in soil: the enigma of the phosphorylated stereoisomers

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Inositol phosphates are the most abundant form of organic P in most soils, and occur in forms that exist nowhere else in nature (Turner et al., 2002). The origin and ecological function of these compounds remain largely unknown, which is remarkable given the importance of P in biological processes.

Four stereoisomeric forms of phosphorylated inositols occur in soils (Fig. 1). Of these, the *myo* isomer is most common, which is unsurprising as it occurs widely in plants. In contrast, highly phosphorylated forms of the other three isomers occur extremely rarely elsewhere.

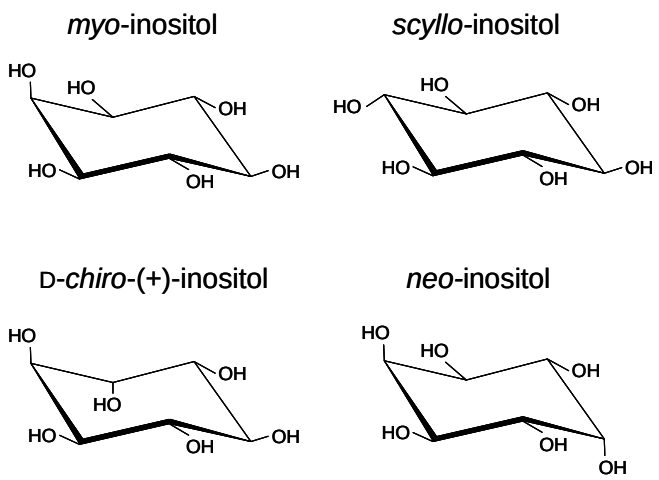

Figure 1. The four stereosimeric forms of phosphorylated inositols detected in soils.

Inositol phosphates challenge our ecological understanding of soil biogeochemical cycles and the nutrition of plants and organisms, because they accumulate in soils and are considered to be of limited availability to organisms. However, they persist in environments in which productivity is strongly limited by P availability. This seems paradoxical given the remarkable ability of evolution to equip organisms with the tools to exploit resources in their environment. Soil inositol phosphates may therefore have a non-nutritional function in soils, although precisely what this function is remains unclear.

Given that highly phosphorylated inositols other than the *myo* isomer have not been detected in the tissue of plants, nor in any soil organisms, their origin in soil remains unknown. It seems likely that they have a microbial source, but it is unclear why organisms should synthesize stereoisomers other than the *myo* form that is so prevalent in nature.

Analytical methodology has limited studies on inositol phosphates in soils. For example, problems with conventional chromatography mean that much of the older literature must be considered with caution. Bromine oxidation is an important procedure for quantifying higher inositol phosphates in soils by solution ^{31}P NMR spectroscopy (Turner and Richardson,

2004). The improvement in resolution and signal identification that can be achieved using bromine oxidation of alkaline soil extracts is demonstrated in Fig. 2.

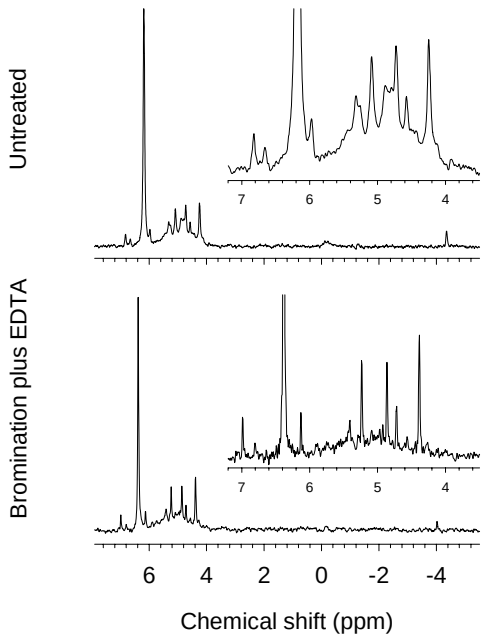

Figure 2. Solution ^{31}P NMR spectra of an NaOH–EDTA extract of a temperate pasture soil, analyzed directly (top) or after bromine oxidation of organic matter other than the inositol phosphates.

Despite several comprehensive studies of inositol phosphates in many soils, few factors have emerged as key regulators of their concentrations or contribution to the soil organic phosphate. From my own studies, it is clear that inositol phosphates can account for anything from none to almost all of the organic phosphate extractable in alkali. Some soils contain more *scyllo*-inositol phosphates than the *myo* forms, but it is unclear why this is the case. It is also noteworthy that inositol phosphates were not detected in submerged organic soils from the Florida Everglades, perhaps due to anaerobic conditions (Turner and Newman, 2005). When soils under similar vegetation, climate, and land-use are analyzed, there is evidence that the hexaphosphates accumulate in soils with low N:P ratios (Turner et al., 2003; 2005), suggesting that organisms that can degrade inositol phosphates may be favored in soils with low P availability.

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Quantification and bioavailability of *scyllo*-inositol hexa*kis*phosphate in pasture soils

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Most soils contain *scyllo*-inositol phosphates, yet quantitative data is scarce due to the analytical difficulties involved in their determination in soils. We used the recent identification of *scyllo*-inositol phosphates in alkaline soil extracts by solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy to re-analyze spectra from two published studies. Our aim was to quantify *scyllo*-inositol hexa*kis*phosphate in permanent lowland pasture soils and to assess its potential bioavailability to growing plants.

The first study involved twenty-nine permanent pasture soils from England and Wales (Turner et al., 2003). The soils were acidic (pH 4.4–6.8) with relatively large amounts of clay (22–68%), and contained a wide range of C (2.9–8.0%), N (0.28–0.87%), and P (0.04–0.20%). The second study involved a pot experiment with six New Zealand grassland soils in which either ryegrass (*Lolium perenne* L.) or pine seedlings (*Pinus radiata* D. Don) were grown for 10 months (Chen et al., 2004). The soils were acidic (pH 5.2–6.5) with relatively small amounts of clay (11–26%), but contained a wide range of C (3.9–9.3%), N (0.31–0.85%), and P (0.04–0.27%). In both studies, P was extracted in a solution containing 0.5 M NaOH and 50 mM EDTA (ethylenediaminetetraacetate) and analyzed by solution ^{31}P NMR spectroscopy. We identified *scyllo*-inositol hexa*kis*phosphate in re-analyzed spectra as the signal appearing close to 4.2 ppm following spectral deconvolution. Concentrations were calculated by multiplying the proportion of the spectral area assigned to *scyllo*-inositol hexa*kis*phosphate by the total P concentration of the initial extract.

Concentrations of *scyllo*-inositol hexa*kis*phosphate in twenty-nine temperate pasture soils from England and Wales ranged between 11 and 130 mg P kg^{-1} soil and accounted for between 4 and 15% of the soil organic P. Concentrations of *scyllo*-inositol hexa*kis*phosphate were correlated positively with those of organic P and *myo*-inositol hexa*kis*phosphate and negatively with the C-to-organic P and N-to-organic P ratios ($p<0.001$). Concentrations were also positively correlated with bicarbonate-extractable organic P ($p<0.01$).

In a 10 month pot experiment with six grassland soils from New Zealand, growth of pine seedlings decreased *scyllo*-inositol hexa*kis*phosphate concentrations by between 10 and 46%. Growth of ryegrass decreased *scyllo*-inositol hexa*kis*phosphate in three low-nutrient soils by 5–21%, but increased it in three other soils by 11–16%. The different responses to the growth of ryegrass appeared to be linked to nutrient status, because decreases in *scyllo*-inositol hexa*kis*phosphate occurred in soils with smaller nutrient concentrations (N ≤0.38%; organic P ≤0.05%), whereas increases occurred in soils with larger nutrient concentrations (N ≥0.48%, organic P ≥0.06%).

scyllo-Inositol hexa*kis*phosphate is therefore an important component of soil organic P with potential ecological significance. Inositol phosphates are stabilized strongly in soils through interaction with clay, organic matter, and metal oxides. The strong stabilization of inositol phosphates in soil also renders them relatively unavailable to plants and microorganisms, while *scyllo*-inositol hexa*kis*phosphate is expected to be especially inaccessible due to it resistance to hydrolytic attack by phytase. However, our results indicate that *scyllo*-inositol hexa*kis*phosphate is bioavailable under certain conditions, as reported previously for *myo*-inositol hexa*kis*phosphate. Decomposition by pine seedlings was mediated presumably by ectomycorrhizal fungi, which form strong associations with pine roots and secrete extracellular phosphatases enzymes that can catalyze the hydrolysis of inositol phosphates.

Results from both re-analyzed datasets provide tentative evidence that nutrient status regulates *scyllo*-inositol hexa*kis*phosphate in soil. In particular, it was inversely correlated with N-to-organic P ratios and was degraded by ryegrass only in low-nutrient soils. This suggests that P limitation favours organisms that can access recalcitrant inositol phosphates in the soil, such as *Aspergillus ficuum* or *Pseudomonas* spp. Conversely, accumulation of *scyllo*-inositol hexa*kis*phosphate following the growth of ryegrass in high nutrient soils indicates synthesis by microbes under P-sufficient conditions.

In summary, *scyllo*-inositol hexa*kis*phosphate was confirmed as a large and potentially bioavailable component of the organic P in temperate pasture soils. Analysis of a broader range of soils and detailed mechanistic experiments are now required to probe the origins and function of this intriguing compound.

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Origins and biochemical transformation of soil inositol and inositol phosphate stereoisomers: review of work during 1967–1977 and ideas for future work with radioactive and stable isotopes

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A review of work carried out during 1967–1977 will be presented together with ideas for new research work on biochemical transformations of soil inositol phosphates with the use of radioactive and stable isotopes. During 1967–1969 inositol phosphates were extracted as barium salts from leaf litter on the soil surface and the underlying soil organic matter of a forest and desert soil by the method of Anderson (1956). The phosphate groups were removed from the inositol cyclohexanehexol rings by hydrolysis to facilitate the separation and isolation of the inositol stereoisomers by preparative paper chromatography and their subsequent recrystallization and structural identification by electron impact–mass spectrometry, infrared- and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (L'Annunziata, 1970, L'Annunziata and Fuller, 1971, 1972). The objective of the study was to investigate the origin(s) of soil inositol phosphates by determining if there were any similarities or dissimilarities with the stereochemistry of the inositol phosphates in leaf litter on the soil surface and in the organic matter of the underlying soil. The only type of mass spectroscopy available to us in the 1960s was electron-impact mass spectrometry. The electron-impact mass spectra for the inositol stereoisomers were all identical as expected, but provided initial evidence for the basic inositol structure by the molecular ion peak at m/e 180 and the characteristic base peak at m/e 73. The most powerful NMR spectrometers available to us in the 1960s were a 60-MHz and 100-MHz instrument. The *myo*-and D-*chiro*-inositol phosphates were identified in Ponderosa Pine needles and the underlying forest soil as well as in the dry leaves of Velvet Mesquite and the underlying desert soil. The D-*chiro*-inositol was identified by its specific rotation of +56° measured by a recording polarimeter. The inositol stereoisomers were identified by fingerprint NMR spectra compared to known standards. All of the inositol phosphate isomers were not identified due to the small amounts (3–5 mg) of the compounds isolated and to the limited power of the spectrometers during the 1960s. The similarities of two inositol phosphate stereoisomeric structures (*myo*- and D-*chiro*-inositol phosphates) in the plant litter on the soil surface and in the underlying soil pointed to plant residue as one of the origins of the inositol phosphate isomers.

Microbial activity of the soil was subsequently suggested as another origin of the soil inositol phosphate stereoisomers (L'Annunziata, 1975). During 1975–1977 work was carried out on the soil microbial metabolism of uniformly labeled ^{14}C(U)-*myo*-inositol, ^{14}C(U)-phytic acid, and ^{14}C(U)-Fe(III) phytate. The radioisotope-labeled compounds were incubated in a microbially-active and sterilized forest soil in Bartha and Pramer (1965) incubation flasks. The flasks permitted determination of the soil microbial metabolism rates by measurement of the evolution rates of $^{14}CO_2$ trapped by 0.1 M KOH in an attached side arm. The results showed that 61% of *myo*-inositol C, 1.9% of phytic acid C, and 0% of Fe(III)phytate C was oxidized to CO_2 in 12 d of incubation. No C whatsoever from *myo*-inositol, phytic acid or Fe(III) phytate was oxidized to CO_2 in sterile soil. The rate of oxidation of ^{14}C-phytic acid to CO_2 rapidly dropped from 0.99% after day 1 to 0.17% after day 2 to 0.05 % after day 12

indicating a rapid fixation of soil organic P into a form unavailable to the microorganisms (L'Annunziata and Gonzalez-Iturbe, 1977). The epimerization of ^{14}C-*myo*-inositol to ^{14}C-*chiro*-inositol was demonstrated (L'Annunziata et al., 1977).

Future work is proposed with radioisotope-labeled *myo*-inositol and its phosphates including ^{3}H, ^{14}C and ^{32}P labels together with the hyphenated instrumental techniques of HPLC-FSA-MS-NMR for the on-line separation and analysis of isotope-labeled metabolites (L'Annunziata, 2003). The use of dual-labeled *myo*-inositol and inositol phosphates together with isotope ratios (e.g., ^{3}H/^{14}C, ^{14}C/^{32}P) are suggested as possible tools to determine mechanisms of microbial transformations. The use of uniformly-labeled ^{13}C(U)-*myo*-inositol and its phosphates is suggested to permit the *in situ* ^{13}C NMR analysis of inositol phosphate metabolites in soil microbial cultures as a function of time. In light of the demonstrated soil microbial epimerization of ^{14}C-*myo*-inositol to ^{14}C-*chiro*-inositol in the soil (L'Annunziata et al., 1975) the structures of the four inositol stereoisomers found in soils as phosphates are reviewed (i.e., *myo*- D-*chiro*-, *scyllo*-, and *neo*-inositol). Further studies with ^{14}C(U)-*myo*-inositol and/or ^{13}C(U)-*myo*-inositol are suggested to possibly demonstrate the soil microbial epimerization of *myo*-inositol to *scyllo*- and *neo*-inositol. The following are considered as origins to the soil inositol phosphate stereoisomers: (1) plant and animal residue or remains, (2) soil microbial epimerization at a *myo*-inositol C atom, and (3) soil microbial synthesis.

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Plant access to inositol phosphates in soil

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Phosphorus deficiency is a major constraint to the growth of plants in many natural and agricultural ecosystems throughout the world. Plants have therefore developed mechanisms to enhance the availability of soil phosphate. These include changes to root structure, association with mycorrhizal fungi and other soil microorganisms, and biochemical processes at the root-soil interface (Jakobsen et al., 2005). Mineralization of phosphate from soil organic P by phosphatase enzymes is of particular significance, as organic P accounts for a major proportion (generally 40–80%) of the total P in most soils, occurring primarily as inositol phosphates (Turner et al., 2002). However, our understanding of the utilization of specific organic P substrates in soil, and the role of plant and/or microbial phosphatases to organic P mineralization is limited (Richardson et al., 2005). Similarly, it is unclear whether mycorrhizae provide plants increased access to soil organic P above that of their benefit in increasing soil exploration (Joner et al., 2000). Importantly, and despite the predominance of inositol phosphates in most soils, we have little understanding of their biological availability.

A number of studies using controlled growth conditions have shown that inositol phosphates are a potential source of P for plants. However, their contribution is dependent on the P-fixation capacity of the soil or growth media used (Adams and Pate, 1992; Findenegg and Nelemans, 1993; Hayes et al., 2000). Inositol phosphates are subject to adsorption and precipitation reactions in soil, which are major factors in determining their availability. Using a range of species grown in sterile culture, we have also shown that plants have limited ability to directly access P from inositol phosphates, which is associated with insufficient extracellular phytase activity of roots (Richardson et al., 2000). Growth and P nutrition of plants was improved significantly by the addition of either purified phytase or inoculation with phytase-producing microorganisms (Hayes et al., 2000; Richardson et al., 2001a). Transgenic plants (arabidopsis, tobacco, potato and subterranean clover) that express heterologous phytase genes (e.g., *phyA* from *Aspergillus*), and release extracellular phytase from their roots, also show significantly increased ability to acquire P from inositol phosphates when supplied as an available substrate (Richardson et al., 2001b; Zimmermann, e*t al*., 2004; George et al., 2004; George et al., 2005a).

However, the importance of extracellular phytase for improving the P nutrition of plants grown in soil is less evident. Whilst some studies have shown that plant utilization of inositol phosphates can be improved by microbial inoculation, other studies have indicated little or no benefit (Tarafdar and Marschner, 1995; Martin 1973; Richardson et al., 2001a). Using transgenic subterranean clover that express *phyA*, no P nutrition response was observed when plants were grown in an unamended acidic and P-deficient pasture soil (George et al., 2004). Transgenic tobacco similarly showed no response in this soil when unamended, but had 14–32% greater P content relative to control plants when grown in P-fertilized soil. The P nutrition of tobacco was also increased by up to 52% when inositol phosphate was added (as Ca-phytate) to the soil, although absolute growth of the plants was still significantly less than that for plants that received an equivalent amount of phosphate (George et al., 2005a). The

importance of substrate availability to the P nutrition of plants that express *phyA* has further been investigated using soils with differing organic P contents. In separate experiments, consistently greater P accumulation by plants that express *phyA* has been observed in soils with naturally high organic P contents (George et al., 2005b).

Mineralization of inositol phosphates in the rhizosphere of plants has recently been demonstrated using nuclear magnetic resonance spectroscopy (Chen et al., 2004). Although the extent of mineralization was dependent on soil type, greater depletion was observed in soils under radiata pine compared to ryegrass, and it was suggested that this may be due to association with ectomycorrhizal fungi. We have also observed specific depletion of phosphate monoesters in the rhizosphere of transgenic clover plants that release extracellular phytase (George et al., 2005c). Interestingly, depletion of organic P by the plants appeared to be greatest from recently synthesized organic P, as determined by radioactive tracer. This suggests the microbial synthesis of inositol phosphates and that plants exuding the microbially-derived phytase have increased capacity to access this P. Such dynamics may also account for the greater response observed when transgenic plants were grown in fertilized soils, but further work to verify this is required.

Clearly there is a need to better understand the biological cycling of inositol phosphates in soil and the factors that affect their synthesis and degradation. The importance of mycorrhizae and other soil microorganisms and their interaction with plant roots, and role of root exudates, requires more detailed investigation. Better understanding of the efficacy of inositol phosphate-phytase interactions in soil is needed (see paper by George et al.), as is the need to apply appropriate analytical procedures to measure 'biologically-relevant' pools of inositol phosphates. Better knowledge of the behavior of inositol phosphates in soil may ultimately improve P-fertilizer management and provide economic and environmental benefit for a more sustainable agriculture.

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Microorganisms that utilize inositol phosphates in the environment

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Phytic acid – *myo* inositol hexa*kis*phosphate – is the main storage form of P in plants, accounting for between 70 and 80% of the total P in the seeds of cereal and legume crops. Intensive agricultural operations, such as pig and poultry feedlots, use grain as the primary source of macro and micronutrients. Monogastric animals however, are unable to degrade phytate and 95% is excreted directly into the manure. Additionally, passage through the gastrointestinal tract leads directly to a number of nutritional problems; for example, complexation of the compound with metals such as Fe can lead to anemia, while complexation with proteins can inhibit protein digestion.

Manure from intensive agricultural operations is commonly composted and then used as a crop fertilizer. Over-application of P is typical and the phytate in the manure is not bioavailable to most plants directly. Excess P loading from these non-point pollution sources into freshwater bodies via erosion and surface runoff causes eutrophication. Algal blooms (some harmful), anoxia, and catastrophic fish kills are all hallmarks of such excess nutrient addition.

The hydrolysis of phytate to phosphate and inositol (or to inositol monophosphate) is achieved enzymatically by phytase. Organisms producing phytase have been obtained from such diverse locations as soil, cattle rumen, cattle shed floors, soybean mash, seawater, culture collection repositories, and plant seeds, which suggests that the ability to degrade phytate might be widely distributed in a variety of ecosystems. The described organisms range from common soil (e.g., *Pseudomonas* spp., *Bacillus subtilis*, and *Klebsiella* spp.) and anaerobic (e.g., *Escherichia coli* and *Mitsuokella* spp.) bacteria, to fungi (e.g., *Aspergillus* spp. and *Penicillium* spp.) and plants (e.g., barley and wheat). No study, however, has investigated the prevalence of phytate-degrading organisms across an ecological gradient, and monogastric farms, which produce an abundance of manure-derived phytate, have not been used as a potential source of microbes that can degrade the compound.

Soil and water strata extend through several redox conditions, which give rise to unique ecological societies. In this study, a spatial transect of a poultry manure-enriched region in the Chesapeake Bay, provides samples from which concurrent degradation of phytate and thiosulfate occurs. Organisms from each sampling site were able to degrade phytate. *Pseudomonad*, *Arthrobacter*, *Staphylococcus*, and *Bacillus* species were identified and their phytate-degrading activity assessed. An exploration of the potential advantages exhibited by bacteria living in a thiosulfate lifestyle will be shared, along with preliminary characterization data from the phytase enzyme of novel species of *Pseudomonad*, *Staphylococcus*, and *Arthrobacter*.

Diversity and abundance of inositol phosphate-utilizing bacteria in terrestrial and aquatic ecosystems on Cape Cod, Massachusetts USA

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Inositol phosphates constitute the majority of the organic P in most soils, and also occur in freshwater and marine sediments, produced *in situ* or delivered as sediments in runoff from terrestrial ecosystems. Inositol phosphates accumulate in soils because they sorb strongly to clays and form insoluble precipitates with metal ions. This limits their availability to plants and microbes, and the higher inositol phosphates are conventionally considered to be of low bioavailability. However, *myo*-inositol hexa*kis*phosphate can be bioavailable under certain circumstances and is apparently quickly mineralized in coastal and marine ecosystems, where it may contribute to eutrophication.

Many organisms including bacteria, fungi, animals and higher plants express the enzyme phytase that liberates ester-linked phosphates from the inositol molecule. Although some plants produce phytase, their ability to utilize inositol phosphates is greatly increased in the presence of soil bacteria, suggesting that the ability to use inositol phosphates is not a universal trait and that microorganisms may be largely responsible for inositol phosphate mineralization in most ecosystems. Moreover, there is evidence that the ability to mineralize inositol phosphates depends upon environmental conditions as well as the microbial community. For example, the dephosphorylation of inositol phosphates in marine sediments was reported to be twice as fast under anaerobic conditions compared to aerobic.

To date, there have been no comprehensive surveys of community-level diversity of microorganisms that use inositol phosphates, or studies of how diversity varies among habitats. Understanding the diversity of microorganisms that can use inositol phosphates and the conditions under which this is favorable is an important step for understanding the global P cycle. The objective of this study was to compare the abundance and taxonomic richness of bacteria that can utilize inositol phosphates as a sole P source in three different ecosystems on Cape Cod, Massachusetts, USA: an upland forest soil, a freshwater marsh, and a marine marsh. We expected that the three ecosystems would have opposing gradients of inositol phosphate stocks (forest soils > freshwater marshes > marine marshes) and capacity for inositol phosphate utilization as measured by abundance and diversity of microbial communities (forest soils < freshwater marshes < marine marshes).

To test this hypothesis, we collected environmental samples from these three ecosystem types and cultured bacteria on appropriate agar media containing *myo*-inositol hexa*kis*phosphate as the sole P source. To understand how variation in the abiotic environment might influence the abundances of inositol phosphate-utilizing microbes, samples from each ecosystem were cultured under different conditions. We assumed that the

majority of the microbes in surface soils of forests may be cultured aerobically, but have different pH ranges, so forest soil samples were incubated in media at pH 5.4 and 7.0. We further assumed that surface sediments from freshwater and marine marshes that experience fluctuating redox conditions, so samples from these habitats were incubated both aerobically and anaerobically in media at pH 7.0. Abundance of culturable inositol phosphate-utilizing bacteria was estimated via most probable number counts, and phylogenetic diversity was assessed by amplifying, cloning, and sequencing 16S rRNA genes from the communities of bacteria recovered on the plates.

The three ecosystems differed substantially in soil and sediment chemistry. The forest soils were significantly more acidic (mean pH 4.4), than the aquatic ecosystems (mean pH 6.5–6.7). The freshwater marsh had three times as much organic C (30.2%) compared to the forest soil (8.0%), and almost twenty times higher concentrations than the salt water marsh (1.5%). Total P concentrations were tenfold higher in the freshwater marsh compared to the forest soil and the marine marsh, but the forest soil and the marine marsh were similar. Phosphate monoesters were the dominant organic phosphates, constituting between 40 and 54% of the extracted P. The presence of *myo*-inositol hexa*kis*phosphate was detected in the forest and freshwater marsh soils. However, inositol hexa*kis*phosphates were not detected in the salt marsh sediment, in which phosphate monoesters were mainly alkaline hydrolysis products of phospholipids and RNA. Phosphate diesters constituted between 6 and 10% of the extracted P in the freshwater marsh and forest soils, respectively, mainly in the form of DNA. However, only a trace of phosphate diesters was detected in the salt marsh sediment.

Differences in the abundance of inositol phosphate-utilizing bacteria paralleled the differences in total P among the ecosystems. Inositol phosphate-utilizing bacteria were most abundant in the freshwater sediments (2.2×10^6 colony forming units g^{-1} dry weight), and nearly an order of magnitude less abundant in forest soils and marine sediments (1.1×10^5 and 2.3×10^5 CFU g^{-1} dry weight, respectively). Within ecosystems, there were no statistically significant differences in abundance between cultivation conditions, although the plates incubated anaerobically consistently yielded higher counts in both the marine and freshwater sediments.

A comparison of the sequenced 16S rRNA genes to a large database revealed that the majority of the sequences fell within the proteobacteria division, with 46% of clones representing gamma proteobacteria, and 16% falling within the beta proteobacteria. The bacterial communities differed among ecosystems. The forest soil community represented a distinct assemblage compared to the marine and freshwater marshes, whereas the marine community was a subset of the taxa found in the freshwater marsh.

Improving our understanding of P transformations within and among ecosystems will require a better understanding of microbial transformations of organic P compounds. Our study suggests that the potential for mineralization of inositol phosphates varies among ecosystems. Freshwater marshes contain an abundant and diverse community of bacteria that can use one of the most important forms of organic P, and thus may serve as an important hotspot for P transformations at the landscape scale.

Interactions between phytase and soil constituents: implications for hydrolysis of inositol phosphates

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A large proportion of endogenous soil P occurs in organic forms, of which derivatives of inositol phosphates constitute a major fraction (Richardson et al., 2005). The bioavailability of inositol phosphates depends on their mineralization by extracellular phytases (*myo*-inositol-hexa*kis*phosphate phosphohydrolases; EC 3.1.3.8 and EC 3.1.3.26), which have many biological sources. However, we currently have a poor understanding of the interactions between phytases and inositol phosphates in soil. While availability of inositol phosphates is clearly a major issue, factors affecting the stability and efficacy of extracellular enzymes in soils are important. These include: (i) proteinase and microbially-mediated degradation, (ii) partial or total deactivation by adsorption onto soil particles and (iii) deactivation via interaction with polyvalent anions, metal ions and microbial metabolites (Quiquampoix and Mousain, 2005). These factors differentially affect phytase activity depending on both the characteristics of the soil environment and the source and biochemical properties of the discrete phytase.

Adsorption of enzymes to soil solid constituents is frequently reported, and demonstrated to be a result of both enthalpic (intermolecular) and entropic (intramolecular) forces (Quiquampoix, 2000). Such adsorption tends to reduce enzyme affinity for substrates and thus reduce their effective activity, which in some cases may be complete and irreversible (Quiquampoix and Mousain, 2005). However, adsorption may be important for the long-term persistence of soil enzymes, by protecting them against degradation. Generally, it is evident that heterogenous surfaces, such as those found in soils, are less inhibitory to enzyme activity upon adsorption than pure clays or some organic materials (Rao et al., 1994). Moreover, inhibition upon adsorption of phytase is soil specific, particularly in terms of soil texture and mineralology. For example, after being adsorbed for 28 days, more than 40% of added phytase remained active in a sandy soil compared to just 5% in a soil dominated by clay (George et al., 2005a).

The inhibition of phytase upon adsorption also appears dependent on the evolved function of the phytase. For example, intracellular phytases are completely adsorbed and inhibited by clay particles, while extracellular phytases retain significant catalytic activity in the presence of clay (Matumoto-Pintro and Quiquampoix, 1997). Species specific variability in the biochemical properties of the extracellular enzymes is also important. Phosphohydrolases of one ectomycorrhizal fungus (*Pisolithus tinctorius*) showed no adsorption on montomorilonite, while those of other strains (*Cenococcum geophilum, Hebeloma cylindrosporum*) were strongly adsorbed and inhibited (Quiquampoix and Mousain, 2005).

Regardless of whether extracellular phytases are inhibited by adsorption, immobilization to the solid phase will presumably limit their interaction with inositol phosphates in soil, particularly if these substrates are also immobilized through adsorption or precipitation. With this in mind, we have observed an effect of the rhizosphere on phytase adsorption, in that

greater activity was retained in rhizosphere soil solution compared to bulk soil (George et al., 2005a). This may be attributed to the changed biochemical environment in the rhizosphere, most notably modified pH or the presence of organic anions and proteins competing for adsorption sites. Changes in rhizosphere pH may also affect the adsorption reactions of phytase in soils. Phytase collected from the roots of plants expressing a phytase gene (*phyA*) from *Aspergillus niger*, was completely adsorbed in a range of soils at pH 4.5. In contrast, adsorption was decreased with increasing pH, such that all remaining activity was recovered in solution at pH 7.5 (George et al., 2005a). Partitioning of enzyme activity to the solution phase with increased pH is attributed to enthalpic forces causing greater electrostatic repulsion above the isoelectric point (pI) of the protein, which for the *A. niger* phytase was pH 4.8. Importantly, there were major differences in adsorption characteristics of two phytases with different pI in acidic soils. Phytase with a relatively low pI (pH 3.6 from *P. lycii*) remained in solution in a range of soils (pH 4.5–5.0), whereas phytase with a higher pI (pH 4.8; from *A. niger*) was totally adsorbed. Moreover, we have found that the phytase with low pI is approximately twice as effective in hydrolyzing inositol phosphates in soil suspensions.

The biochemical characteristics of phytases produced by different organisms have obvious implications for their behaviour in soil environments and thus their efficacy for mineralization of inositol phosphates. Maintaining phytases in solution apparently enhance their ability to interact with immobilized substrates. Therefore, increasing pH in acidic soils could be a useful management tool for making phytase more effective in the soil environment. In fact, we have recently shown that the P nutrition of transgenic plants that exude phytase was greatest in soils at neutral pH, where the ability of inositol phosphates and phytases to interact would likely be optimized (George et al., 2005b). Future work will concentrate on expressing genes in plants for the production of phytases with biochemical traits that effect their interaction with soil, such as low pI. This will allow better understanding of the factors that contribute to the biological availability of inositol phosphates in soil and potentially improve the ability of plants to acquire P from this source.

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Development of a novel soil phytase assay: significance, challenges, progress

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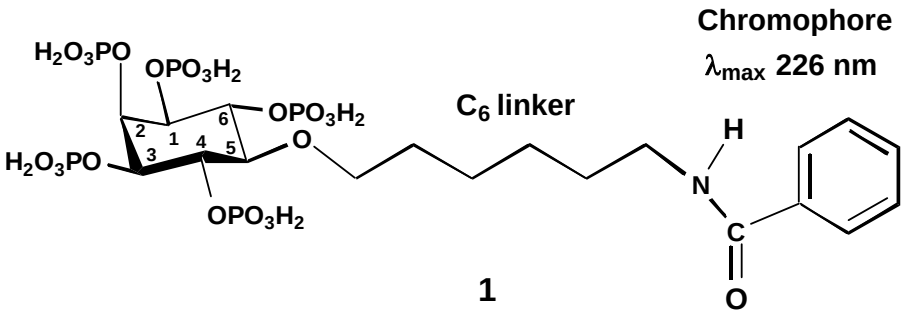
The paucity of information on the transformation of *myo*-inositol hexa*kis*phosphate (*myo*-IP_6), otherwise referred to as phytic acid, to plant nutrient P presents the biggest challenge to a thorough understanding of the P-cycling process in natural ecosystems, including P-burdened manure amended soils. In high P soils, rates of phosphate release in excess of crop uptake lead to an increase in P transport to nearby surface waters and/or groundwater. Over the long term, increased rates of phytase-catalyzed P release will influence the amount of P transported from high P soils to open waterways accelerating eutrophication.

The soil chemistry of inositol phosphates has been reasonably well investigated over the past quarter century (Turner et al., 2002). Quantification of *myo*-IP_6 in soil extracts was recently achieved using solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy (Turner et al., 2003). Despite these advances, little progress has been made in characterizing soil phytases. Complete understanding of the P-cycling process in natural environments, including high P soils, will not be achieved until an accurate and accessible phytase assay is developed.

Our research goal is to develop a highly specific, sensitive and convenient phytase assay capable of measuring, with absolute certainty, dephosphorylation of phosphatidylinositol substrate in (samples collected from) complex (bio)chemical environments (e.g., soil, sediment, or rumen). To this end, we have created a new chromophoric substrate analog of phytic acid, 5-*O*-[6-(benzoylamino)hexyl]-D-*myo*-inositol-1,2,3,4,6-pentakisphosphate (T-IP_5 (**1**)), that permits direct measurement of the phytase-catalyzed phosphate ester bond-cleavage reaction using high-performance liquid chromatography (HPLC) (Berry and Berry, 2005).



1
T-IP_5

T-IP_5 was designed as a substrate for 3-phytase (EC 3.1.3.8, *myo*-inositol hexa*kis*phosphate 3-phosphohydrolase) (Berry and Berry, 2005). The ether linkage, attaching the C_6 linker to the *myo*-inositol moiety, is hydrolytically stable and resistant to enzymatic (i.e., hydrolase) activity. A hydrolytically resistant amide bond connects the benzoyl and C_6 linker. Attaching the linker to the 5-position, placing it "meta" to the initial site of the reaction (i.e., the 3-position), minimizes the likelihood that the linker (plus chromophore) would

interfere with the preferred active site of the 3-phytase. Positioning the linker "ortho" to the 6-position may, however, interfere with the preferred active site of 6-phytase (EC 3.1.3.26, *myo*-inositol hexa*kis*phosphate 6-phosphohydrolase) due to steric hindrance.

Our initial investigation revealed that T-IP_5 can serve as a substrate for 3-phytase (*Aspergillus ficuum*, Sigma) and 6-phytase (wheat, Sigma) (Berry and Berry, 2005). Dephosphorylation of T-IP_5 results in production of the phosphatidylinositol intermediates, T-IP_4 and T-IP_3, which are readily quantified using reversed phase HPLC analysis. In our assay, the rate of T-IP_5 dephosphorylation was a measure of phytase activity. We observed buildup of T-IP_3 subsequent to its disappearance, indicating that phytase-catalyzed dephosphorylation of T-IP_3, forming T-IP_2, is a rate-controlling step (Berry and Berry, 2005). In a phytase assay involving an environmental sample, the dephosphorylation rate of T-IP_3 (tentatively identified in our initial investigation) may serve as a measure of both phytase and non-specific phosphatase activity. We will report on the results of a recently completed ^{31}P NMR study elucidating the chemical structure of T-IP_3.

Chromatographic analysis of T-IP_5 (λ_{max} 226 nm) and dephosphorylated intermediates was performed on an analytical HPLC system consisting of a LDC analytical multiple-solvent delivery pump connected to a variable wavelength detector. Separation of the T-phosphatidylinositol intermediates was achieved using a Hamilton PRP-1 5 μm reversed phase column and an ion-pair (tetrabutylammonium hydroxide) mobile phase. PEEK tubing was used to connect the Rheodyne valve, PRP-1 column (heated 45°C) and detector (Berry and Berry, 2005).

Successful application of our tethered probe in the development of a phytase assay for environmental samples will depend upon several factors including: (1) the ability of T-IP_5 to serve as a substrate for phytase, (2) susceptibility of the T-IP_5 amide linkage, that secures the benzamido chromophore to the linker, to protease attack, and (3) resistance of the T-IP_5 probe toward dephosphorylation by non-specific phosphatases.

In summary, a novel tethered phytic acid probe, T-IP_5 has been synthesized that can serve as a chromophoric substrate for phytase. Phytase-catalyzed P-group removal from T-IP_5 is readily quantified using reversed phase HPLC with ultraviolet detection.

Acknowledgements

We thank Catherine A. Waltham Sajdak for conducting ^{31}P NMR analysis of T-IP_3.

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Role of organic acid secretion by phytase producing rhizobacteria in release of inorganic phosphate from calcium-phytate and in growth promotion of pigeon pea (*Cajanaus cajan*)

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Phytates (salts of inositol phosphates; IP_x, where x = 1, 2,…6) are a class of organic compounds found widely in the natural environment, particularly in soil as an organic P pool that is largely unavailable to plants. In the present investigation, different rhizospheric bacteria that could grow on laboratory media using Na-phytate as a sole P source were isolated on glucose as the C source and pH 8 was maintained with 0.1 M Tris-HCl to approximate soil pH. Phytase activity and other properties of the isolates are shown in Table 1. The bacterial isolates differed in the nature of phytase activity (whether extracellular or cell-associated, and whether active at acidic or alkaline pH). A number of isolates showed organic acid secretion and ability to solubilize mineral P; these isolates displayed a zone of clearance on phytate containing plates.

Table 1. Characteristics of phytate utilizing rhizospheric isolates.

Isolate	Extracellular phytase activity		Cell associated phytase activity		Growth on Na-phytate	Growth on Ca-phytate	Growth on Na-phytate with Methyl Red	P-solubilization on Pikovskaya agar
	pH 4.5	pH 8.0	pH 4.5	pH 8.0				
TOM-1	-	-	-	-	+	+	+RR	+ZZ
TOM-2	-	+	-	-	+	+	+	+
TOM-3	+	-	+	+	+	+	+	+
TOM-4	+	+	+	+	+	+	+R	+Z
TOM-5	+	+	+	-	+	+	+R	+Z
SP-1	+	+	+	-	+	+	+R	+Z
SP-2	-	+	-	+	+	+	+	+
SP-3	+	+	-	+	+	+	+	+
SP-4	-	+	-	-	+	+	+	+
Tuver	-	+	-	+	+Z	+Z	+RR	+Z
ST-10	-	+	-	-	+	+	+R	+Z
DHRSS	+	+	-	-	+Z	+Z	+RR	+ZZ
Psi-3	+	+	-	-	+	+	+R	+Z

+ : Growth
R : Zone of pink coloration indicating acid secretion (RR indicates bigger zone size than R)
Z : Zone of clearance indicating mineral phosphate solubilization (ZZ indicates bigger zone size than Z)

To understand the role of organic acid in phytate hydrolysis, three isolates (Tuver, DHRSS & Sp-4) were checked for their ability to release P from phytate in buffered media. Isolates that could acidify the medium (Tuver and DHRSS) were proficient at releasing P from Ca-phytate regardless of whether the enzyme was extracellular or intracellular. On the other hand, isolate Sp-4, which did not acidify media and produced extracellular phytase, was

unable to liberate P. This effect of the organic acid may not be due to the acid optima of the phytase enzyme since one of the isolates (Tuver) showed exclusively alkaline (pH 8) phytase activity. Solubilization of Ca-phytate particles, which was visibly observed upon acid production, may account for the synergistic action of organic acid on phytate hydrolysis by phytase. High-performance liquid chromatography of the culture supernatant showed that isolate DHRSS secreted (in decreasing order of concentration) gluconic, acetic and pyruvic acids, while Tuver secreted gluconic, pyruvic and an unidentified acid. 16S rDNA amplification and partial sequencing allowed the isolates DHRSS, Tuver, and SP4 to be identified tentatively as species of *Citrobacter*, *Pantoea*, and *Pseudomonas*, respectively.

Pot inoculation studies were carried out to find out the effect of the above three isolates on growth promotion of C*ajanus cajan* plants under gnotobiotic conditions. Inoculated and uninoculated plants were monitored for root and shoot dry weight and P content in shoots. Inoculation of the organisms led to significant increase in shoot mass in case of all three isolates. Shoot to root biomass ratio increased significantly in case of DHRSS and to a lesser extent in Sp-4. To check the effects of enriching the soil with phytate-rich manure, another set of pots were amended with poultry manure and a similar inoculation study was carried out. However, in this case none of the organisms proved effective in increasing the plant biomass. Interestingly, the shoot P concentration and shoot to root biomass ratio was highest in case of one of the organic acid producing isolate *viz.* Tuver.

In summary, our results show that in laboratory media organic acid secretion by bacteria has a synergistic effect with phytase in the hydrolytic release of P from Ca-phytate. The results of the pot inoculation studies however showed all three selected isolates to be effective in increasing plant biomass regardless of whether they secrete organic acid or not. Isolate Tuver (secreting mainly gluconic acid) brought about an increase in the P concentration of the shoot in presence of the phytate-rich manure, indicating that one of the mechanism of its plant growth promotion to be due to increase in plant-available P.

Inositol phosphates in aquatic systems

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While there is a considerable body of research on the abundance and behavior of inositol hexa*kis*phosphate in soils, much less is known regarding its behaviour in aquatic systems. The observed physico-chemical behavior of this organic P species suggests that in marine and freshwaters, it should be complexed and precipitated by major ions such as Ca, Mg, Fe or Al, and thus immobilized in the sediments (de Groot and Golterman, 1993). Furthermore inositol hexa*kis*phosphate is known to be un-amenable to hydrolysis by exocellular alkaline phosphatase or phosphodiesterase (Turner et al., 2002a) and is therefore regarded as biologically unavailable to phytoplankton. These observations tend to reinforce the classical, and quite understandable view, that inositol hexa*kis*phosphate exists in aquatic systems as an insoluble, refractory, immobile and biologically unavailable P species.

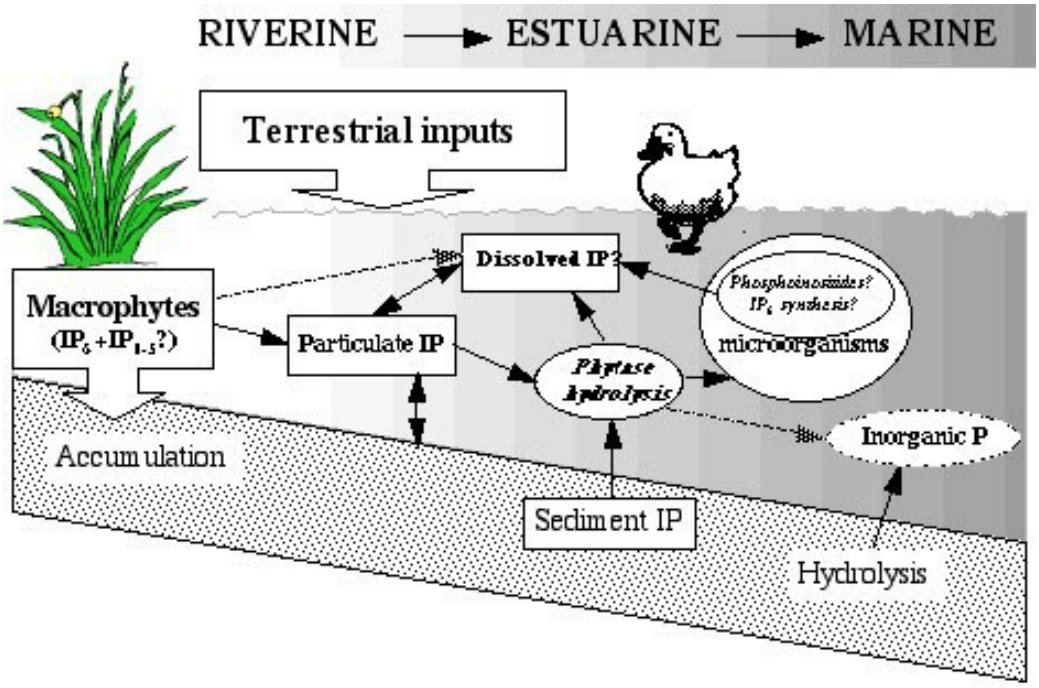

Figure 1. Suggested sources, pathways and transformations of inositol hexa*kis*phosphate in aquatic ecosystems (Turner et al., 2002b)

However, in contradiction to this widely held view, it is observed that substantial concentrations of inositol hexa*kis*phosphate can occur in the filterable fraction of sediment pore waters and overlying waters (McKelvie, 2005). Some workers have shown that terrestrially derived inositol hexa*kis*phosphate deposited in coastal marine sediments undergoes fairly rapid hydrolysis to lower inositol phosphates and free phosphate and thus may constitute an important source of bioavailable phosphate (Suzumura and Kamatani,

1995). The implication of these observations is that inositol hexa*kis*phosphate or lower inositol phosphates and phosphate may be released from sediments. That this is more prevalent under anaerobic than aerobic conditions is possibly due to enhanced solubility of Fe-associated inositol hexa*kis*phosphate under conditions of anoxia.

With respect to bioavailability, there is evidence to suggest that some cyanobacteria possess surface phosphomonoesterases capable of hydrolyzing inositol hexa*kis*phosphate (Livingstone et al., 1983). However the importance of this mechanism for the hydrolysis and release of bioavailable P, by benthic algae for example, is as yet unknown.

This paper reviews the known sources of inositol hexa*kis*phosphate and examines likely transport paths and transformations that may occur in aquatic systems (Fig. 1). Suggested mechanisms for the release and transport of both inorganic and organic P from sediments are reviewed, and some speculative interpretation of the apparently contradictory release and/or hydrolysis and bioavailability of inositol hexa*kis*phosphate is provided.

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Beta-propeller phytases in the aquatic environment: characterization of a novel phytase from *Shewanella oneidensis* MR-1

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Although a substantial amount of phytate is carried from the terrestrial to aquatic systems, it is a minor component of organic P in coastal marine sediments **(**Suzumura and Kamatani, 1995). The ephemeral nature of phytate implies rapid hydrolysis of phytate under aquatic conditions. Among the four classes of known phytases identified from terrestrial organisms, only β-propeller phytase-like sequences can be identified in the aquatic environment. A putative β-propeller phytase gene (*physS*) from *Shewanella oneidensis* MR-1 shows ~30% sequence identities in protein level to *Bacillus* phytases (Tye et al., 2002). Culturing *S. oneidensis* in medium deficient in phosphate induced the expression of a ~62 kDa protein in the cell fraction (Fig. 1). The full length phyS protein has 661 amino acid residues, which is nearly twice the number of residues in *Bacillus* phytases (~380 aa). Alignment and homology modelling analyses (Fig. 2) demonstrated that the phyS protein consists of two domains that resemble the fold of the *Bacillus* phytase TS-phy. Domain II (323-661 aa) is structurally more similar to TS-phy than domain I (36-322 aa). Activity assays on recombinant domains I & II showed that while domain II is the catalytic domain, domain I is also required for full enzymatic activity (Table 1). The full length PhyS and its domain II subunit demonstrated similar biochemical properties, such as Ca dependent catalytic activity, neutral optimal pH and high specificity to phytate, to that of the *Bacillus* phytases. These results confirm that the *physS* gene encodes a functional β-propeller phytase.

The amino acid sequence of *physS* shares 80% identity with an environmental sequence that was collected from a seawater sample (Venter et al., 2004), which indicates that the *physS* gene is present in the other members of the *Shewanella* genus. Three *Shewanella* species have been isolated from the intestines of sea animals, including goby, black porgy, and saury (Satomi et al., 2003). Whether the phytases that are produced by these *Shewanella* species, if any, assist these animals in phytic acid assimilation, is a worthy subject for future study.

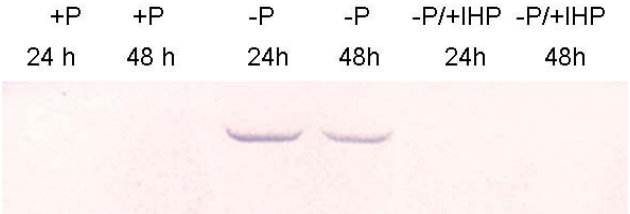

Figure 1. Western blotting of *Shewanella* proteins using an antisera against recombinant *physS*.

Phylogenetic analysis grouped *physS* and several homologs from *Caulobacter crescentus*, *Microbulbifer degradans*, *Azobacter vinelandii*, *Pseudomonas syringae* pv. *tomato*, and *Idiomarina loihiensis* (Fig. 3). Sequence alignment shows the conservation of all amino acid residues that are involved in the phosphate and Ca binding sites of beta-propeller phytases in these putative phytases. Like phyS, these proteins consist of two domains that resemble the fold of the *Bacillus* phytase. Among these bacteria, *C. crescentus* and *M. degradans* have been isolated from freshwater and salt marsh grass. Some closely related strains of *C. crescentus* and *M. degradans* have also been isolated from sea animals: the hind gut of a millipede in the case of *C. crescentus*, and the gland of shipworm in the case of *M. degradans*.

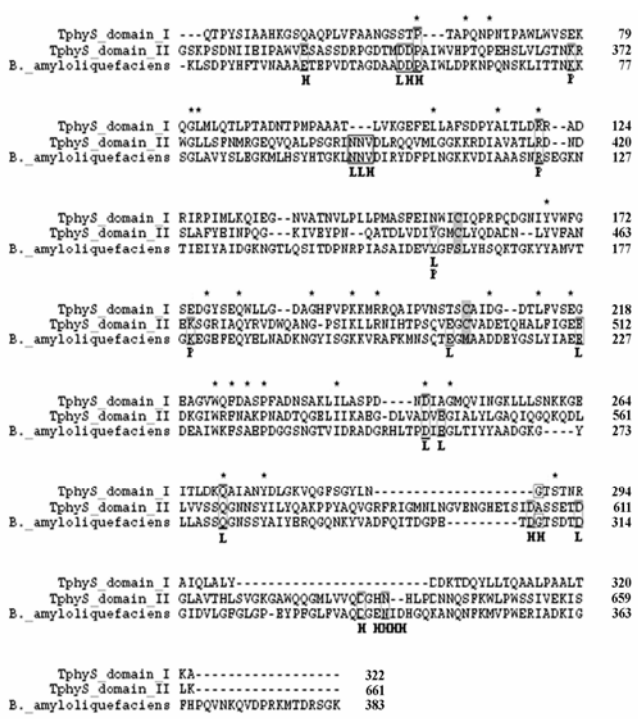

Figure 2. Alignment of the a.a. sequences of PhyS with beta-propeller phytase.

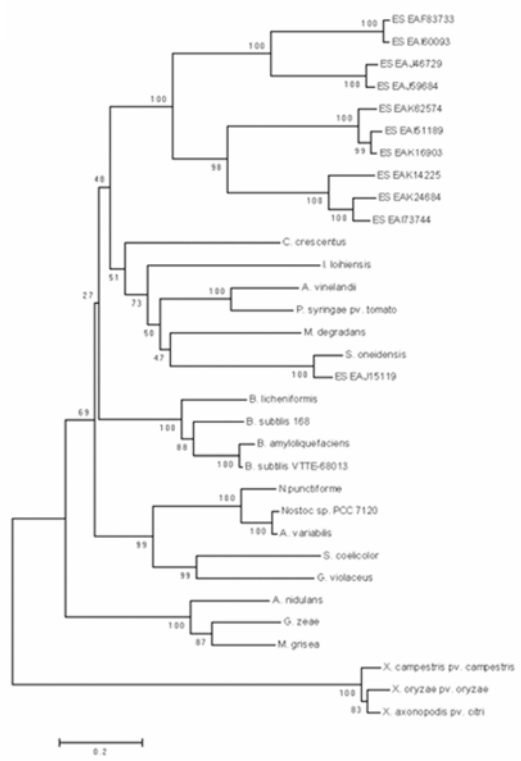

Figure 3. Phylogenetic analysis.

Table 1. Kinetic analysis with sodium phytate as substrate.

Enzyme[a]	K_m mM	K_{cat} min^{-1}	K_{cat}/K_m mM^{-1} min^{-1}
TphyS domain II	0.474 ± 0.041	10.65 ± 0.37	22.46 ± 0.79
His-tagged FLphyS	0.083 ± 0.007	175.92 ± 5.03	2130.51 ± 60.9
Bacillus phytase	0.198 ± 0.022	360.64 ± 29.1	1820.23 ± 146

[a] 37°C in 100 mM Tris-maleate buffer, pH 6.0 (n=3).

In summary, the presence of multiple sequences with a high similarity to *phyS* in environmental samples and the widespread occurrence of *Shewanella* species in water imply that the beta-propeller phytase family is the main class of phytases in the aquatic environment, which may play an important role in the mineralization of phytate in aquatic systems.

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Temporal and depth trends in inositol phosphates in settling ocean particulate matter from Monterey Bay, California

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Phosphorus is recognized as a limiting nutrient in some oceanic systems, and may be the macronutrient most limiting marine productivity over long time scales. Understanding the temporal and depth trends in P concentration and forms is crucial if we are to understand P bioavailability and the role of P in marine productivity. Little is known about organic P cycling in oceans, and less about specific organic P forms such as inositol phosphates. The origins of inositol phosphates in marine environments are largely unidentified. They may be formed *in situ* by aquatic organisms, or they may be transferred from terrestrial environments. The role of inositol phosphates in the marine P cycle is equally uncertain; because they are strong ligands due to high anionic charge, and the phosphate ester linkages are stable in basic conditions, they may be relatively unavailable to aquatic organisms.

We examined the chemical nature of P in settling ocean particulate matter from Monterey Bay, California, using surface plankton tows (25 m depth), IRS trap samples collected biweekly for 6 months at 200 m depth, and Honjo trap samples collected biweekly for 6 months at 1200 m depth. Samples were analyzed by chemical analyses, solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy, enzyme hydrolysis, and solution ^{31}P NMR spectroscopy. Inositol phosphates were identified as the peaks remaining in the orthophosphate monoester region of ^{31}P NMR spectra after alkaline bromination.

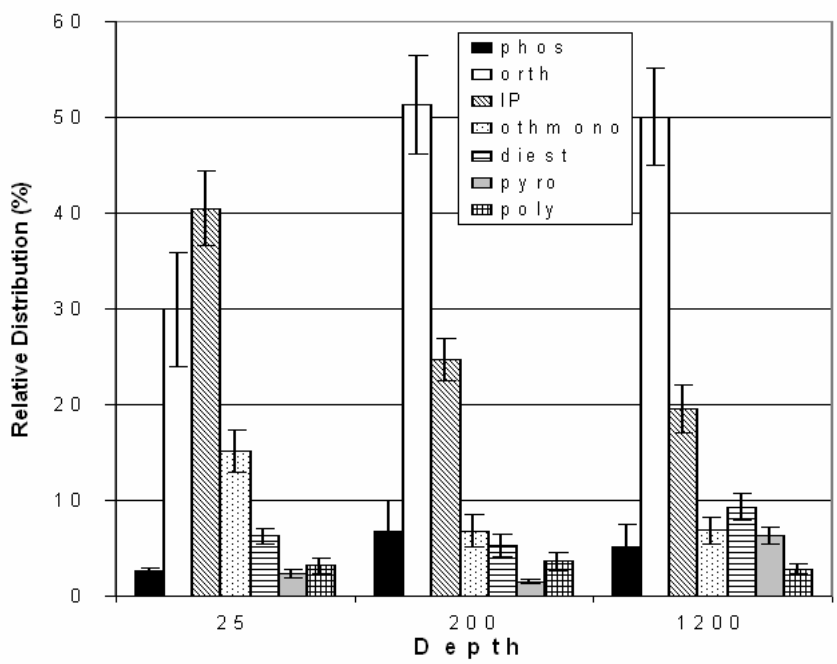

Figure 1. Relative distribution of P forms with depth. Phos is phosphonates, orth is orthophosphate, IP is inositol phosphates, orthmono is other orthophosphate monoesters, diest is orthophosphate diesters, pyro is pyrophosphate, poly is polyphosphate.

Preliminary results show that inositol phosphates were the largest proportion of P forms (40%) in plankton tows at 25 m, and decreased with depth to 25% at 200 m, and 19% at 1200 m (Fig. 1). Some temporal variation in the relative proportion of inositol phosphates was also observed in the 200m and 1200 m samples, but this was generally small compared with variations in the relative proportions of orthophosphate and phosphonates. Inositol phosphates were positively correlated with other monoesters, total C, total N, $\delta^{13}C$, and carbonyl groups (determined by ^{13}C NMR), and were inversely correlated with orthophosphate and with depth.

These preliminary results suggest that inositol phosphates in particulate samples from Monterey Bay are produced by marine organisms and are biodegraded at the surface but become stabilized at depth.

Abstracts of Poster Presentations

De novo synthesis of enzymes participating in phytate breakdown during germination of lentils (*Lens culinaris* var. Magda)

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The phytate-degrading activity of grains, seeds and pollen is responsible for phytate degradation during germination to make phosphate, minerals and *myo*-inositol available for the purpose of plant growth and development (Reddy et al., 1989). Usually there is a good correlation between the rapidity of phytate breakdown and phytate-degrading activity of the seed or seedling. Although large increase in phytate-degrading activities have been extensively reported in germinating seeds as well as pollen, the biochemical mechanism leading to this rise in phytate-degrading activity is not well understood. In pollen, phytate-degrading enzymes induced during germination may be synthesized from long-lived, pre-existing mRNA (Jacksoon and Linskens, 1982). In cereals and legumes, some studies suggest d*e novo* synthesis (Bianchetti and Sartirana, 1967), while others suggest activation of pre-existing enzymes (Eastwood and Laidman, 1971). In addition, several molecular forms of phytate-degrading enzymes, which may be regulated in different ways, have been identified in a certain plants (Greiner, 2002).

With 16.91 mU g^{-1} dry weight, non-germinated lentils (*Lens culinaris* var. Magda) showed only a very low phytate-degrading activity. During germination for 6 days at 20°C in the dark a 17.5-fold increase in extractable and a 2.5-fold increase in total phytate-degrading activity determined at pH 5.0 with a concomitant reduction in phytate content (66%) was observed. The increase in phytate-degrading activity was inversely correlated with the disappearance of phytate. No accumulation of *myo*-inositol penta*kis*-, tetra*kis*-, and trisphosphates occurred during germination. *myo*-Inositol bis- and monophosphates were not detected. Lentils exhibited no phytate-degrading activity at pH 8.0 as reported for other legumes (Scott, 1991), so alkaline phytase activity does not seem to play a role in phytate degradation during germination. Since the non-extractable phytate-degrading activity (pH 5.0) was not significantly affected by germination, only the extractable phytate-degrading activity was suggested to be responsible for phytate breakdown during germination of lentils.

Table 1. Effect of cylcoheximide and actinomycin D on phytate-degrading activity and phytate reduction after 6 days of germination when applied after 24 h (first column) and 72 h (second column) of germination.

	Phytate-degrading activity [mU g^{-1} dry wt]		Phytate content
	extractable	total	[µmol g^{-1} dry wt]
control	296.3 ± 1.0	548.7 ± 4.6	1.12 ± 0.1
cycloheximide			
1 µM	114,9 ± 0.6 / 162.5 ± 0.8	362.2 ± 3.3 / 401.2 ± 3.4	2.27 ± 0.6 / 1.95 ± 0.8
5 µM	61.9 ± 0.3 / 75.1 ± 0.6	303.1 ± 2.9 / 322.6 ± 2.8	2.53 ± 0.3 / 2.53 ± 0.6
10 µM	22.9 ± 0.3 / 50.5 ± 0.5	273.7 ± 1.8 / 300.9 ± 3.8	2.80 ± 0.3 / 2.65 ± 0.6
actinomycin D			
50 µM	100.6 ± 0.8 / 225.9 ± 1.4	345.9 ± 4.3 / 480.5 ± 4.1	2.36 ± 0.1 / 1.61 ± 0.4
100 µM	39.6 ± 0.2 / 177.5 ± 0.9	281.7 ± 2.4 / 417.3 ± 2.7	2.71 ± 0.3 / 1.92 ± 0.7

Cycloheximide, a competent inhibitor of translation in eukaryotes, and actinomycin D, which fblocks transcription in eukaryotes, were applied to germinating lentil seeds after 24 h and 72 h of germination, respectively. A significant reduction in the rise of extractable as well as total phytate-degrading activity, and a reduced drop in phytate content, was observed (Table 1). The observed effects were more pronounced at higher concentration of cycloheximide and actinomycin D and by administration of these compounds earlier in germination. Application of cycloheximide or actinomycin D after 96 h of germination had no significant effect on phytate-degrading activity or phytate hydrolysis compared to the control.

During germination phytate-degrading activity may be controlled by the action of gibberellic acid and phosphate, respectively. The action of gibberellic acid on the phytate-degrading enzymes of the germinating seed is not well understood. It is claimed that gibberellic acid merely increase the secretion of phytate-degrading enzymes but does not stimulate their synthesis (Gabard and Jones, 1986), thus giving phytate-degrading enzymes access to phytate, and that gibberellic acid stimulates phytate-degrading activity (Srivastava, 1964). In lentils, administration of gibberellic acid during germination remarkably enhanced extractable phytate-degrading activity (1.4-fold at 1 μM, 1.6-fold at 5 μM, 1.8-fold at 10 μM) and stimulated phytate degradation (1.2-fold at 1 μM, 1.3-fold at 5 μM, 3-fold at 10 μM) in a dose dependent manner. These effects were reduced by 79–84% (phytate-degrading activity) and 52–66% (phytate degradation) with 5 μM cycloheximide or 50 μM actinomycin D.

Two main mechanisms appear to be involved in the regulation of phytate-degrading activity by phosphate. First, phosphate directly inhibits phytase activity. Second, it was concluded that phosphate also acts at the transcription level, since phosphate added early enough in the germination sequence can repress the increase in phytate-degrading activity (Sartirana and Bianchetti, 1967). The addition of up to 10 mM phosphate during germination of lentils did not significantly affect phytate-degrading activity and phytate degradation as compared to the control.

We conclude that the rise in phytate-degrading activity during germination of lentils is due to *de novo* synthesis of the corresponding enzyme(s). Inhibition of transcription as well as inhibition of translation reduced the increase in phytate-degrading activity and the disappearance of phytate during germination. Gibberellic acid, but not phosphate, seems to control phytate breakdown within the lentil seedling. Since the stimulating effect of gibberellic acid on phytate-degrading activity and phytate breakdown was inhibited by cycloheximide and actinomycin D, we conclude that this effect of gibberellic acid is at least partly due to a stimulation of *de novo* synthesis of phytases. Neither addition of cycloheximide and actinomycin D nor administration of gibberellic acid significantly affects non-extractable phytate-degrading activity, which further suggests that non-extractable phytate-degrading activity does not contribute to phytate degradation during germination.

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Properties of a highly specific phytate-degrading enzyme with an acid pH optimum from a bacterium isolated from Malaysian waste water

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The stepwise release of phosphate from phytate [*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate], the major storage form of phosphate in plant seeds and pollen, is initiated by a class of enzymes collectively termed phytases. Since an ideal phytase for all application does not exist, there is still interest in an enzyme more suitable for the intended use. In this report we present the properties of a phytase purified from a bacterium isolated from Malaysian waste water, which might find application as an animal feed supplement.

In the bacterial cells a significant phytate-degrading activity was detectable during all stages of growth and the activity did not increase significantly after the cells reached the stationary phase. Therefore, this phytase is a constitutive enzyme and, in contrast to other bacteria, its synthesis is not triggered by nutrient or energy limitation (Konietzny and Greiner, 2004). No phytate-degrading activity was detectable in the dialyzed culture medium of growing bacterial cells. As no inhibitors were present in the culture medium there was no question of an extracellular phytase. During an osmotic shock procedure more than 90% of the phytate-degrading activity obtained after lysis of the bacterial cells was released. Therefore, the phytase must be regarded as a periplasmatic enzyme.

The phytase was purified about 180-fold to apparent homogeneity by ion-exchange chromatography and gel-filtration with a recovery of 10% of the phytate-degrading activity in the crude extract. The enzyme had an activity of about 1106 U mg^{-1}. Gel filtration of the native enzyme on a calibrated Sephacryl S-200 column gave a molecular mass of 42.000 ± 1.500 Da, with elution position being measured by determination of enzyme activity. Lower molecular mass species or higher molecular mass aggregates were not observed. The phytase appeared homogeneous by polyacrylamide gel electrophoresis under non-denaturing conditions at pH 8.3 and pH 4.8 and gave a single protein band upon SDS gel electrophoresis after Coomassie staining of the gels. These results indicate that the phytase could be regarded as homogeneous. According to the estimated molecular masses after SDS-PAGE, the protein band corresponds to a molecular mass of 41.500 ± 2.500 Da. The enzyme is therefore a monomeric protein.

The standard phytase assay was performed using a buffers from pH 1.0 to 9.0. The purified enzyme had a single pH optimum at pH 4.5 and was virtually inactive below pH 7.0. At pH 3.0 only 40% of the activity at optimal pH was observed, while at pH 2.5 this value was 20%. Within 14 days the phytase did not lose activity between pH 3.0 to 8.0, but at pH values below 2.0 a rapid decline in activity was observed. At pH 1.5, 72% and at pH 9.0, 65% of the initial activity was lost during 24 h. The temperature optimum was 65°C. The apparent activation energy was estimated at pH 4.5 from the slope of log V_{max} versus 1/T. The data showed excellent linearity between 15 and 65°C. The Arrhenius activation energy for the hydrolysis of phytate was 37.5 kJ mol^{-1}. To check thermal stability, the purified enzyme was incubated at different temperatures, cooled to 4°C and assayed using the standard phytase assay. The enzyme lost no

activity in 10 minutes at temperatures up to 65°C. When exposed for 10 minutes at 70°C it retained 50% of the initial activity, while at 80°C it retained 12%.

To determine the substrate selectivity of the purified phytase, several phosphorylated compounds (GTP, ATP, ADP, AMP, NADP, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, 1-naphthylphosphate, 2-naphthylphosphate, pyrophosphate, phosphoserine, 2-glycerophosphate, pyridoxalphosphate), in addition to phytate, were used for K_m and V_{max} estimation by detecting the release of the phosphate ion during hydrolysis using formation of a soluble phosphomolybdate complex in an acidic water/acetone mixture. Only phytate was identified as a substrate. All other compounds tested were not significantly hydrolyzed by the purified enzyme. The kinetic parameters for the hydrolysis of phytate were determined to be $K_m = 0.15$ mmol L^{-1} and $k_{cat} = 1164$ sec^{-1} at pH 4.5 and 37°C.

Like other bacterial phytate-degrading enzymes, the purified enzyme showed a substrate inhibition (Konietzny and Greiner, 2002). The activity of the purified enzyme was inhibited at substrate concentrations > 7 mM. The study of the effect of metal ions on enzyme activity showed that none of them had an activating effect when used at a concentration between 10^{-4} and 10^{-3} M. Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ag^{+}, Hg^{2+}, Cu^{2+} had little or no effect on enzyme activity, while Zn^{2+}, Fe^{2+}, and Fe^{3+} showed strong inhibitory effects. The reduced phytate-degrading activity in the presence of Fe^{2+} and Fe^{3+} is attributed to a lower phytate concentration in the enzyme assay because of the appearance of a Fe-phytate precipitate. When compounds which tend to chelate metal ions, such as *o*-phenanthroline, EDTA, oxalate, citrate or tartrate, were tested for their effect on enzyme activity, none were inhibitory at concentrations between 10^{-4} to 10^{-3} M. Fluoride, a known inhibitor of different phytate-degrading enzymes from bacteria and the hydrolysis product phosphate as well as its structural analogous molybdate, wolframate and vanadate were strong inhibitors of the purified enzyme. Fluoride inhibited the hydrolysis of phytate with a K_i value of 112 μmol L^{-1}.

Phytate-degrading enzymes used as feed additives should be effective in hydrolyzing phytate in the digestive tract, stable to resist inactivation by heat from feed processing and storage, and cheap to produce. The purified phytase shares many enzymatic properties in common with other histidine acid phytases. However, some properties make the enzyme attractive for an application as a feed supplement. Compared to the commercially available phytases from *Aspergillus niger* and *Peniophora lycii*, the purified enzyme exhibit a higher thermal stability (Ullah and Sethumadhavan, 2003). Like the phytase of *A. niger*, but in contrast to the *P. lycii* enzyme, the purified phytase showed considerable activity below pH 3.0. In addition, the purified phytase is more resistant to pepsin and pancreatin than the commercially available *A. niger* phytase. Its protease resistance is comparable with that of the phytase from *Escherichia coli*. The enzyme is specific for phytate and dephosphorylates phytate at least to *myo*-inositol bisphosphate. The high affinity for phytate and the high specific activity are also desirable for the intended application. The K_m values are comparable to those of the phytases from *A. niger* and *P. lyccii* and the specific activity is in the same magnitude as the specific activity of the phytase from *P. lycii*.

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myo-Inositol phosphate isomers generated by the action of a phytate-degrading enzyme from *Raoultella terrigena* on phytate

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The properties of *myo*-inositol phosphates strongly depend on the number and distribution of the phosphate residues on the *myo*-inositol ring. *myo*-Inositol phosphates containing the 1,2,3-trisphosphate cluster, for example, are Fe binding anti-oxidants (Phillippy and Graf, 1997), and D-*myo*-inositol (1,2,3,6) tetra*kis*phosphate [D-Ins(1,2,3,6)P_4] is moderately effective in opening Ca channels (Burford et al., 1997). In addition, D-Ins(1,2,6)P_3 has anti-inflammatory and anti-secretory properties (Claxon et al., 1990). Until now, the diversity and unavailability of individual *myo*-inositol phosphate intermediates precluded investigation of their bioactivity. Phytate-degrading enzymes, especially the histidine acid phytate-degrading enzymes (Konietzny and Greiner, 2002), are good candidates for the production of lower *myo*-inositol phosphates from phytate, because they release only one major *myo*-inositol penta*kis*-, tetra*kis*-, tris-, -bis-, and -monophosphate (Greiner and Konietzny, 1996). Only the alkaline phytate-degrading enzymes from *Bacillus* species have been demonstrated to degrade phytate using a dual pathway (Kerovuo, 2000). To exploit the full potential of naturally occurring phytate-degrading enzymes, identification of the *myo*-inositol phosphates generated during enzymatic phytate degradation is of great importance.

The phytate-degrading enzyme of *Raoultella terrigena* is a cytoplasmatic enzyme belonging to the group of histidine acid phosphatases (Greiner et al., 1997). The final product of enzymatic phytate degradation was identified by gas chromatography coupled with mass spectrometry as Ins(2)P, with phosphate released only slowly from this compound. The full sequence of phytate hydrolysis by the phytate-degrading enzyme from *R. terrigena* was elucidated using a combination of high-performance ion chromatography (HPIC) and kinetic studies. The enzyme generated a single *myo*-inositol penta*kis*phosphate identified as D/L-Ins(1,2,4,5,6)P_5. Kinetic studies revealed that D-Ins(1,2,4,5,6)P_5 was the first intermediate of enzymatic phytate degradation (Table 1). Therefore, the phytate-degrading enzyme from *R. terrigena* is a 3-phytase (E.C: 3.1.3.8).

Table 1. Kinetic constants for enzymatic *myo*-inositol phosphate dephosphorylation.

Substrate	K_m [µmol L^{-1}]	k_{cat} [sec^{-1}]
Ins(1,2,3,4,5,6)P_6	300 ± 23	180 ± 13
D-Ins(1,2,4,5,6)P_5	345 ± 21	165 ± 8
D-Ins(1,2,3,5,6)P_5	715 ± 19	45 ± 3
D-Ins(1,2,3,4,5)P_5	721 ± 35	48 ± 3
D-Ins(1,2,5,6)P_4	372 ± 15	143 ± 9
Ins(2,4,5,6)P_4	384 ± 12	149 ± 12
D-Ins(1,2,6)P_3	415 ± 18	128 ± 7
Ins(2,4,6)P_3	no degradation	observed
InsP_5*	352 ± 11	162 ± 14
InsP_3*	419 ± 14	131 ± 9

*Generated by the phytate-degrading enzyme from *Raoultella terrigena*.

Hydrolysis of D-Ins(1,2,4,5,6)P_5 resulted in equimolar amounts of two *myo*-inositol tetra*kis*-, tris-, and -bisphosphate intermediates, suggesting two alternative pathways. The *myo*-inositol tetra*kis*phosphates were identified by HPIC as Ins(2,4,5,6)P_4 and D-Ins(1,2,5,6)P_4. The existence of two alternative pathways was established by using D-Ins(1,2,5,6)P_4 as a substrate. Its enzymatic dephosphorylation resulted in a single *myo*-inositol trisphosphate [D-Ins(1,2,6)P_3] and a single *myo*-inositol bisphophosphate [D-Ins(1,2)P_2]. Therefore, one pathway of hydrolysis of D-Ins(1,2,4,5,6)P_5 proceeds via 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 (Fig. 1).

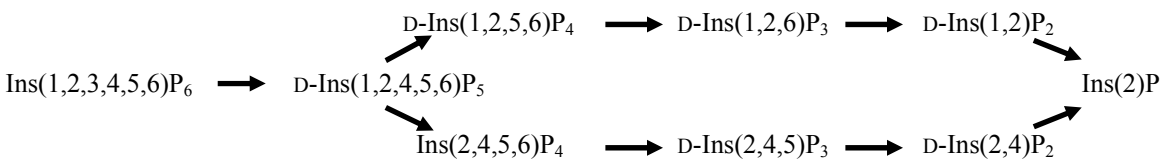

Figure 1. Degradation pathway of phytate by a phytate-degrading enzyme from *Raoultella terrigena*.

Dephosphorylation of Ins(2,4,5,6)P_4, the second *myo*-inositol tetra*kis*phosphate generated by the action of the phytate-degrading enzyme from *R. terrigena* on phytate, could be degraded via: (a) D-Ins(2,4,5)P_3 and D-Ins(1,2)P_2, (b) D-Ins(2,5,6)P_3 and D-Ins(2,6)P_2 and/or Ins(2,5)P_2,or (c) D-Ins(2,4,6)P_3 and D-Ins(2,4)P_2 and/or D-Ins(2,6)P_2. D-Ins(2,4,6)P_3 has to be excluded as an intermediate of enzymatic phytate degradation, since it was not accepted as a substrate by the phytate-degrading enzyme from *R. terrigena* (Table 1) and an accumulation of a *myo*-inositol trisphosphate during phytate degradation did not occur. Discrimination between D-Ins(2,4,5)P_3 and D-Ins(2,5,6)P_4 [= L-Ins(2,4,5)P_3] by HPIC was not possible, since these compounds are an enantiomeric pair and their kinetic evaluation was not possible due to their unavailability in pure form. However, the degradation pathway from Ins(2,4,5,6)P_4 can be rationalized by noting the similarities of the structures of "inverted" Ins(2,4,5,6)P_4 and D-Ins(1,2,5,6)P_4. Provided these similarities satisfy the requirements for substrate recognition and that the nature and orientation of the C-2 and C-3 substituents are not critical determinants of substrate recognition, the 1-hydroxy, 6-phosphate, 5-phosphate, and 4-phosphate of "inverted" Ins(2,4,5,6)P_4, respectively, mimic the configuration of the 4-hydroxy, 5-phosphate-, 6-phosphate, and 1-phosphate of D-Ins(1,2,5,6)P_4. Therefore, it was suggested, that enzymatic hydrolysis of Ins(2,4,5,6)P_4 proceeds via D-Ins(2,4,5)P_3, and D-Ins(2,4)P_2 to finally Ins(2)P (Fig. 1).

This is the first report of a histidine acid phytate-degrading enzyme with two alternative pathways for the hydrolysis of phytate, resulting in one *myo*-inositol penta*kis*phosphate [D-Ins(1,2,4,5,6)P_5] and two *myo*-inositol tetra*kis*- [D-Ins(1,2,5,6)P_4, Ins(2,4,5,6)P_4], tris- [D-Ins(1,2,6)P_3, D-Ins(2,4,5)P_3], and bisphosphate [D-Ins(1,2)P_2, D-Ins(2,4)P_2] intermediates.

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Determination of potential phytase activity in digestive fluids using rumen fluid as an example

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Degradation of phytic acid in the digestive tract of farm animals is catalyzed by phytase. The origin of the phytase may be microbial. Traditionally the determination of phytase activity involves a direct extraction procedure and the activity is then determined by measuring the liberated phosphate colorimetrically (Engelen, 2001). However, this procedure has several limitations. The high background of phosphate generally present in digestive fluids makes it difficult to measure the phytase activity accurately and the presence of different minerals like Fe^{3+}, Fe^{2+}, Cu^{2+} and Zn^{2+}, while buffering agents like citric acid may inhibit phytase activity (Yanke et al., 1999).

The phytase enzyme is a relative large molecule, with a molecular weight above 36-38 kDa. This makes it possible to separate the phytase from interfering phosphate, minerals and phytic acid by a simple filtration through a membrane with a cut off value of 30 kDa, as suggested by Kim and Lei (2005) for feed samples.

In the present study, the isolation of phytase from rumen fluid using membrane filtration will be discussed. The influence of different color reagents for determination of orthophosphate will be illustrated and discussed as well as the influence of buffering agents and minerals on the phytase activity.

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Quantifying the phosphorus composition of animal manures by solution phosphorus-31 nuclear magnetic resonance spectroscopy

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Pollution of water bodies by P in runoff from soil amended with animal manures is one of the greatest threats to water quality in developed countries. The environmental fate of manure P is determined in part by its chemical composition, yet extraction procedures to assess this are poorly developed and provide no structural information.

We used solution ^{31}P NMR spectroscopy to quantify P compounds in sequential extracts of three contrasting manures (broiler litter, beef-cattle manure, swine manure). Using a procedure originally developed for soils, but commonly applied to manures, P was extracted sequentially with deionized water, 0.5 M $NaHCO_3$, 0.1 M NaOH, and 0.5 M HCl.

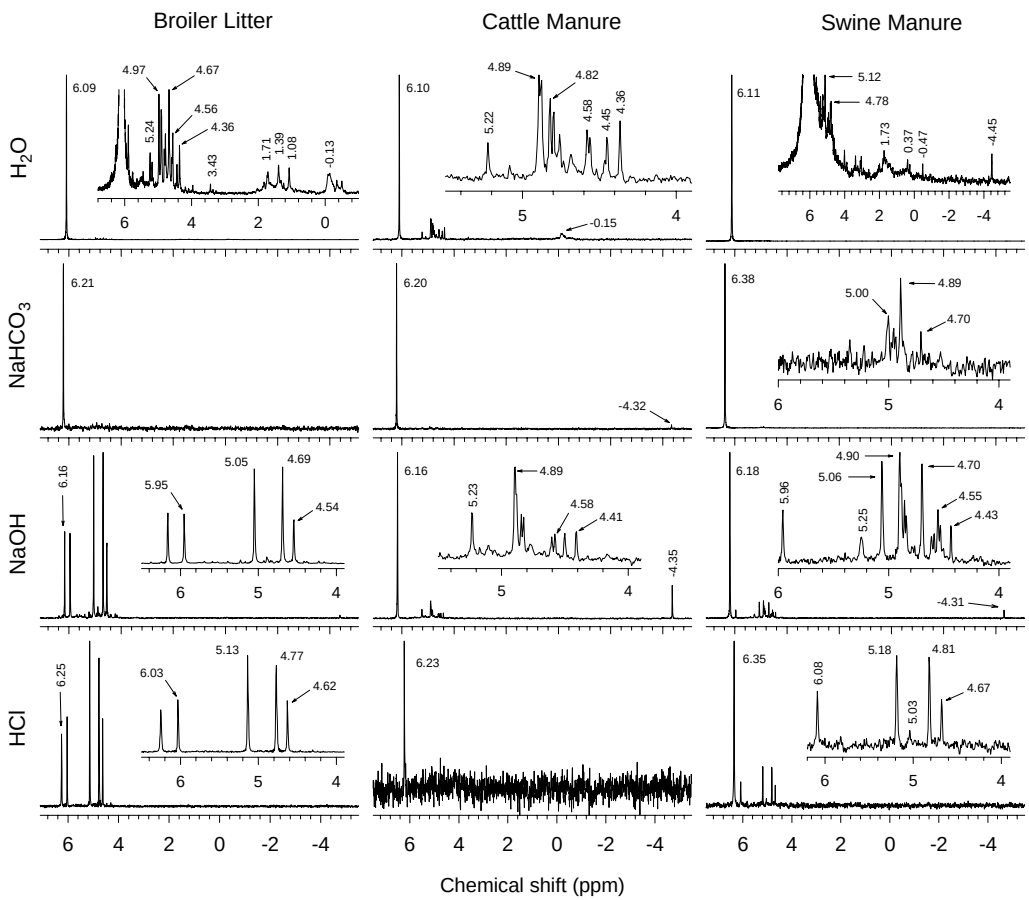

Figure 1. Solution ^{31}P NMR spectra of sequential extracts of a broiler litter, a cattle manure, and a swine manure, using a revised Hedley fractionation procedure (Hedley et al., 1982). The main spectra are scaled to the full height of the phosphate signal at approximately 6.1 ppm, while inset spectra are scaled to the height of the largest phosphate monoester signal. All spectra are plotted with 1 Hz line broadening except the inset spectrum of the water extracts (2 Hz).

Solution ^{31}P NMR spectra are shown in Fig. 1. Water and $NaHCO_3$ extracted readily-soluble compounds, including phosphate, phospholipids, DNA, and simple phosphate monoesters, which are mobile in soil and biologically available. In contrast, NaOH and HCl extracted poorly-soluble compounds, including phytic acid (*myo*-inositol hexa*kis*phosphate). The latter is immobile in soil and of limited biological availability.

Based on these results, we developed a simplified two-step fractionation procedure involving extraction of readily-soluble P in 0.5 M $NaHCO_3$ followed by extraction of stable P in a solution containing 0.5 M NaOH and 50 mM EDTA. The revised procedure separates manure P into structurally-defined fractions with environmental relevance and will facilitate research on this important aspect of environmental science. These results are reported fully elsewhere (Turner and Leytem, 2004). The NaOH–EDTA solution can also be used as an efficient single-step extractant for manures (Turner, 2004). This has been used to characterize the P composition of swine manure few low phytic acid barleys, which demonstrated that almost all the phytic acid is hydrolyzed within the animal, even though the animal may gain no nutritional benefit (Leytem et al., 2004).

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Selection of low phytate soybeans and QTL mapping of phytate genes in soybean with a simple, rapid, and quantitative procedure

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Phytic acid, *myo*-inositol hexa*kis*phosphate ($InsP_6$) is the major storage form of P in seeds. Lacking sufficient phytase, non-ruminant animals cannot digest phytate in soybean (*Glycine max* (L.) Merr.) meal and excrete undigested phytate in animal waste. The low P bioavailability of animal-feeds and land application of the animal manure cause excess accumulation of P in soil and water. Breeding soybeans with low phytate and correspondingly high inorganic P content provides a practical strategy to avoid these problems.

In this study, we have developed a reliable, convenient phytate assay in selecting low phytate soybean breeding lines and QTL mapping of the genes conditioning the low phytate trait. The colorimetric procedure (Vaintraub and Lapteva, 1988), based on the reaction between ferric chloride and sulfosalicylic acid, was modified and standardized to measure crude soybean phytate extracts. The important changes include overnight extraction for consistency and convenience, and the addition of neutral salts to remove protein interference, followed by centrifugation. The modifications allow the use of an ordinary low-speed centrifuge and have increased the applicability of the original method.

We have generated 122 $F_{2:4}$ breeding lines from a cross of CX1834-1-6 (a low phytate line; Wilcox et al., 2000) with V99-3337 (high phytate line). Association between phytate data obtained by our method and the segregation of SSR (simple sequence repeat) marker alleles in the 122 $F_{2:4}$ families agrees with the map location of phytate-controlling QTLs reported by Walker et al. (2002). A breeding line selected for low phytate using QTL markers proved to have the lowest phytate content with our phytate assay.

Phytate content assessed by our procedure was significantly correlated (r = 0.96) with those obtained by high-performance liquid chromatography (Kwanyuen and Burton, 2005), yet our protocol is simple, inexpensive, and high throughput. It provides a suitable method for efficient screening of low phytate breeding lines from large populations and for developing molecular markers closely linked to low phytate genes for marker-assisted selection.

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Unique alkaline phytase from lily pollen: cloning, characterization and differential expression

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Although acid phytases from fungal sources have been extensively studied, there is a paucity of information regarding alkaline phytases. These enzymes have unique biochemical and structural properties, and have been identified in plants and bacteria. The alkaline pH and thermal stability of alkaline phytases suggest that they may be good candidates as animal feed supplements. The lack of structural and mechanistic information about alkaline phytase from plant tissues is partially due to the challenges in purifying phytases from plants including low endogenous levels, low stability and contamination from non-specific phosphatases. The availability of cDNA expression clones of alkaline phytase will allow us to gain knowledge on the structural, molecular and mechanistic aspects of enzymes. Purification of an alkaline phytase from the pollen of *Lilium longiflorum* was achieved in our laboratory by a multi-step procedure. The cloning and characterization of alkaline phytase cDNA from lily pollen will be described.

Six internal peptides from purified lily pollen alkaline phytase were sequenced by a combination of Edman degradation and electrospray ionization-tandem mass spectroscopy methods. The amino acid sequence data was used to design degenerate oligonucleotide primers. The complete cDNA sequence of alkaline phytase was obtained by using a combination of reverse transcriptase–polymerase chain reaction (RT-PCR), 5' "rapid amplification of cDNA ends" (RACE) and 3' RACE techniques. Three isoforms of alkaline phytase transcripts from lily pollen were sequenced. *LlAlp1*, *LlAlp2*, and *LlAlp3* encodes for peptides with 487, 408, and 511 amino acids, respectively. All the isoforms possess the conserved active site motifs, 'RHGXRXP' and 'HD', characteristic of histidine acid phosphatases. However, lily pollen alkaline phytase shows low identity (<25%) to all known phytases including fungal histidine acid phosphatases and multiple inositol polyphosphate phosphatases. Phylogenetic analysis of histidine phosphatases revealed that the histidine phosphatase family of enzymes can be divided into four subgroups. *LlAlp1*, *LlAlp2* and *LlAlp3* are more closely related to the multiple inositol polyphosphate phosphatase containing subgroup rather than the maize and fungal acid phytases.

The expression level of the three isoforms was investigated in leaves, stem, petals and pollen of *L. longiflorum* using semi-quantitative reverse-polymerase chain reaction. These studies suggest that the LlALP gene is expressed in all tissues investigated; however, differential expression of the three isoforms was observed. Expression of one isoform was observed during the early stages of flower development, yet all three isoforms were expressed during later stages. Maximum expression of all three isoforms was found in mature pollen grains.

This is the first report of the cloning of alkaline phytase cDNAs from plants. Availability of the cDNA clones will allow us to further our understanding of the molecular characteristics, structure activity relationships and physiological role of alkaline phytases.

Improving phosphate acquisition in white clover by transgenic expression of *MtPAP1* and *MtPHY1*

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Phosphate, a key substrate in biological activities, is one of the least available macronutrients restricting crop production in many ecosystems since the majority of the soil P is immobilized and not ready for plant uptake. This issue, plus the problem of eutrophication resulting from phosphate pollution in certain areas, makes it particularly important to develop crops with the ability of utilizing soil organic phosphate as P resource. Two genes, a purple acid phosphatase (*MtPAP1*) and a phytase (*MtPHY1*) were isolated from *Medicago truncatula* and cloned into a binary vector, pCAMBIA3301, under the control of the constitutive CaMV35S and the root-specific MtPT1 promoters. Transgenic expression of the genes in *Arabidopsis* resulted in improved utilization of organic P in the rhizosphere. These gene constructs were used for *Agrobacterium*-mediated cotyledon transformation of white clover (*Trifolium repens* L.), which is one of the most nutritious and widely distributed forage legumes worldwide. In total, more than 30 transgenic plants were produced and about half of them showed expression of the MtPAP1 and MtPHY1 genes. The individual plants with high expression levels of the transgenes are being used for further analyses regarding phosphate acquisition from soil.

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Acknowledgements

The meeting would not have been possible without generous support from the Soil Science Society of America, through the Bouyoucos Conference fund. Bouyoucos Conferences were established to facilitate intense, highly focused examination of a topic of critical importance to soil science. Scientists with common goals and interests are brought together in a forum that is not typically possible at large scientific meetings, with the aim of establishing personal relationships and promoting the free exchange of ideas. We hope this latest Bouyoucos Conference fulfills these ideals.

Additional funding was provided by the Agricultural Research Service of the United States Department of Agriculture. Sun Valley generously provided their conference facilities for the meeting without charge, and we thank Andy Christensen, Emily Price, and Kate Wood for their help and support in hosting the conference. We also thank staff of the Smithsonian Tropical Research Institute, Republic of Panama, for help in the planning and administration of the meeting. In particular, Audrey Smith handled registration and payments, and Harold Maduro designed and managed the conference website.