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

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The paucity of information on the transformation of myo-inositol hexakisphosphate (myo-IP₆), 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 will 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-IP₆ in soil extracts was recently achieved using solution ³¹P 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, 5-0-[6-(benzoylamino)hexyl]-D-myoinositol-1,2,3,4,6-pentakisphosphate (T-IP₅ (1)), that permits direct measurement of the phytase-catalyzed phosphate ester bond-cleavage reaction using high-performance liquid chromatography (HPLC) (Berry and Berry, 2005).

T-IP₅ was designed as a substrate for 3-phytase (EC 3.1.3.8, myo-inositol hexakisphosphate 3-phosphohydrolase) (Berry and Berry, 2005). The ether linkage, attaching the C₆ 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 C₆ 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 hexakisphosphate 6-phosphohydrolase) due to steric hindrance.

Our initial investigation revealed that T-IP₅ can serve as a substrate for 3-phytase (Aspergillus ficuum, Sigma) and 6-phytase (wheat, Sigma) (Berry and Berry, 2005). Dephosphorylation of T-IP₅ results in production of the phosphatidylinositol intermediates, T-IP₄ and T-IP₃, which are readily quantified using reversed phase HPLC analysis. In our assay, the rate of T-IP₅ dephosphorylation was a measure of phytase activity. We observed buildup of T-IP₃ subsequent to its disappearance, indicating that phytase-catalyzed dephosphorylation of T-IP₃, forming T-IP₂, is a rate-controlling step (Berry and Berry, 2005). In a phytase assay involving an environmental sample, the dephosphorylation rate of T-IP₃ (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 ³¹P NMR study elucidating the chemical structure of T-IP₃.

Chromatographic analysis of T-IP₅ (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-IP₅ to serve as a substrate for phytase, (2) susceptibility of the T-IP₅ amide linkage, that secures the benzamido chromophore to the linker, to protease attack, and (3) resistance of the T-IP₅ probe toward dephosphorylation by non-specific phosphatases.

In summary, a novel tethered phytic acid probe, T-IP₅ has been synthesized that can serve as a chromophoric substrate for phytase. Phytase-catalyzed P-group removal from T-IP₅ is readily quantified using reversed phase HPLC with UV detection.

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References