Tetrachlorofluorescein TInsP$_5$ as a Substrate Analog Probe for Measuring Phytase Activity in Surface Water: Proof of Concept

Duane F. Berry* and Kim Harich

An innovative approach for measuring phytase activity (PA) in surface water is presented. A substrate analog of myo-inositol hexakis(dihydrogen) phosphate (InsP$_6$) is used here, commonly referred to as phytic acid, 1o-myo-5-O-{(1-oxo-[1\'-2",4",7",9",11\'-tetrachloro-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6-yl]-5,8,11-trioxa-2-azatridecan-13-yl)-inositol 1,2,3,4,6-pentakis-O-(dihydrogen) phosphate, referred to as tetrachlorofluorescein TInsP$_5$, has been developed that can be used to monitor the (phytase-catalyzed) phosphorus ester bond-cleavage reaction. Test phytases, (wheat [4-] and Aspergillus niger [3-] phytase) sequentially remove phosphate groups from TET TInsP$_5$, producing dephosphorylated probe species that were readily separated by reversed-phase high-performance liquid chromatography (RP-HPLC). Because dephosphorylated probe species retain the TET group, highly sensitive quantification could be achieved using fluorescence detection (excitation/emission $\lambda$ = 245/540 nm). Calibration curves for TET TInsP$_5$, which could be used as a standard for quantifying all probe species, were linear ($R^2 > 0.999$) over the range of concentrations tested. Phytase-generated dephosphorylated probe species were characterized or identified using RP-HPLC with mass spectrometry. Results of mass spectrometry analysis show that the RP-HPLC system was capable of distinguishing between dephosphorylated probe species at the regiosomeric level. The TET TInsP$_5$ molecular probe was used to successfully measure PA in pond water. We found that the PA associated with the particulate plus water-soluble fraction was greater than that observed for the water-soluble fraction alone. Moreover, it appeared that 4- and 3-phytase were active in pond water based on an analysis of the chromatographic profile (i.e., elution sequence) of dephosphorylated probe species produced. The advent of a fluorescent substrate analog of InsP$_6$ allows environmental scientists with the means to unambiguously quantify an extremely small amount of phytase-generated dephosphorylated probe(s), enabling the measurement of PA over a reasonably short time duration, in an environmental sample containing low concentrations of enzyme.

The accessibility of phosphorus ($P$) in freshwater ecosystems is frequently the limiting factor where primary production is concerned (Schindler, 1978; Jørgensen et al., 2011). The presence of too much bioavailable $P$ in these ecosystems can, however, be problematic. As pointed out by Correll (1998), eutrophication of freshwater streams, reservoirs, lakes, and headwaters of estuarine systems is most commonly caused by excessive $P$.

Orthophosphate is the form of $P$ most readily assimilated by bacterioplankton; however, organic $P$ comprises a moderate to large fraction of the total $P$ pool in many surface waters and associated sediment (Monbet et al., 2009; Jørgensen et al., 2011) and articles cited therein). The degradability of organic $P$ in surface water ranges from susceptible to refractory (Reitzel et al., 2007). Orthophosphate monoesters, including myo-inositol hexakis(dihydrogen) phosphate (InsP$_6$), commonly referred to as phytic acid, are resistant to degradation (Reitzel et al., 2007; Jørgensen et al., 2011). There is evidence suggesting that InsP$_6$ is protected from (bio)degradative processes in surface waters because it is strongly adsorbed to particulate materials (i.e., mineral surfaces and organic matter) (Cade-Menun et al., 2006). Although aquatic microorganisms are capable of producing phytases, which can transform organic $P$ to orthophosphate, little is known about organic $P$ cycling in freshwater ecosystems (Jørgensen et al., 2011).

InsP$_6$-degrading phosphomonoesterases, including phytase (myo-inositol hexakisphosphate phosphohydrolase), presumably play a unique role in converting InsP$_6$ to orthophosphate in aquatic systems. Unfortunately, there is no information available on measurement of phytase activity (PA) in aquatic systems, making the assessment of the role of InsP$_6$-degrading enzymes in the $P$ cycling process problematic. As noted by Turner et al. (2002) and McKelvie (2007), investigating the role of phytases in the biodegradation of inositol phosphates in sediments and surface waters has been complicated by the lack of an artificial substrate that would permit direct measurement of the phytase-catalyzed dephosphorylation reaction.

Phytases originate from a diverse group of organisms including fungi, bacteria, and plants (Irving, 1980). These
enzymes catalyze hydrolysis of the phosphate ester bond(s) of InsP<sub>i</sub>, resulting in the production of orthophosphate and a series of partially dephosphorylated phosphoric esters of myo-inositol (Cosgrove, 1980; Mitchell et al., 1997; Konietzny and Greiner, 2002). Four classes of phosphatases, each containing InsP<sub>i</sub>-degrading members, are currently recognized: (i) histidine acid phosphatases, (ii) β-propeller phytases, (iii) cysteine phosphatases, and (iv) purple acid phosphatases (Mullaney and Ullah, 2007). The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, www.chem.qmul.ac.uk/iupac/) classifies phytase(s) as 3-phytase (EC 3.1.3.8), 4-phytase (EC 3.1.3.26), or 5-phytase (EC 3.1.3.72) depending on the specific position of the initial dephosphorylation reaction. Although 4-phytase appears to be associated primarily with higher plants, microorganisms are thought to be the primary source of 3-phytase (Cosgrove, 1980; Greiner, 2007).

Our goal is to develop a reliable, unambiguous, and highly sensitive method for measuring PA in environments such as surface waters that contain low concentrations of enzyme. This method used the novel fluorescent substrate analog of InsP<sub>i</sub>, 1-0-my-5-O-(1-oxo-1-(2',4,7,7'-tetrachloro-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6-yl)-5,8,11-trioxa-2-azatridecan-13-yl)-inositol 1,2,3,4,6-pentakis-(dihydrogen) phosphate, referred to as tetrachlorofluorescein (TET) tethered InsP<sub>i</sub> (T)InsP<sub>i</sub> (FLD) and to characterize or identify phytase-generated TET TInsP<sub>i</sub> (i) using reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection (FLD) and to characterize or identify phytase-generated TET TInsP<sub>i</sub> species using RP-HPLC coupled with visible detection (VIS) and mass spectrometry (MS), (ii) to provide experimental evidence demonstrating that (i) is highly specific for phytase, and (iii) to evaluate efficacy of (1) as a means to assay PA in surface water.

**Materials and Methods**

**Chemicals and Reagents**

TET TInsP<sub>i</sub> (1) (undecaammonium salt form, purity 91.3%) and 6-carboxy TET (6-carboxy-2',4,7,7'-tetrachlorofluorescein, purity 94.6%) were obtained from Berry and Associates, Inc. Crude preparations of wheat (Triticum aestivum L.) phytase, Aspergillus ficuum (niger) phytase, potato (Solanum tuberosum L.), and sweet potato (Ipomoea batatas) acid phosphatase (EC 3.1.3.2) were purchased from Sigma-Aldrich Chemical Co. along with InsP<sub>i</sub> dodecasodium salt. A 500 μmol L<sup>-1</sup> stock solution of (1) was prepared in 0.2 mol L<sup>-1</sup> sodium acetate—buffered solution (pH 5.2) and stored at −20°C. A 890 μmol L<sup>-1</sup> stock solution of 6-carboxy TET was prepared in 0.1 mol L<sup>-1</sup> phosphate (pH 6.0) methanol solution (85:15 v/v) and stored at −20°C. Tetrachlorofluorescein TInsP<sub>i</sub> (1), and 6-carboxy TET standards were prepared by diluting probe stock solution with 0.1 mol L<sup>-1</sup> phosphate-buffered (pH 6.0) methanol solution (85:15 v/v). The standards could be stored at 4°C for 16 wk. Ultra-pure water (1.82 kΩ m<sup>-1</sup>) and analytical-grade chemicals were used to prepare buffers and reagents. The ACS-certified potassium dihydrogen phosphate used in phosphate-buffered solutions was oven dried before use.

**Instrumental Analysis**

Quantitative analysis of probe species was accomplished using RP-HPLC-FLD. An Agilent Technologies 1200 series HPLC system, consisting of a binary pump, microvacuum degasser, thermostatted column compartment, and fluorescence detector was used to analyze (1), dephosphorylated probe species and 6-carboxy TET. Detector output was processed using Agilent Technologies LC 2D Chemstation software. A Model 7725i Rheodyne valve, fitted with a 20-μL PEEK flex loop, was used to deliver samples onto the column. PEEK tubing connected the Rheodyne valve, column, and detector. Isocratic separation of the analytes was achieved on a heated (37°C) Supelcosil LC-18-T stainless steel column (3 μm, 15 cm × 4.6 mm) (Sigma-Aldrich) using a mobile phase consisting of

![Chemical structure of tetrachlorofluorescein (TET) TInsP<sub>i</sub> (1) molecular probe.](image)

Fig. 1. Chemical structure of tetrachlorofluorescein (TET) TInsP<sub>i</sub> (1) molecular probe.

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<th>Abbreviation</th>
<th>Molecular probe species</th>
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† TET, tetrachlorofluorescein.

Table 1. Molecular probe species.
0.1 mol L$^{-1}$ phosphate-buffered (pH 6.0) methanol solution (85:15 v/v) at a flow rate of 0.9 mL min$^{-1}$. Components of the mobile phase were filtered using a 0.2-µm nylon membrane. The optimal excitation/emission wavelength for (I), determined to be 245/540 nm, was obtained using the Agilent 1200 Series fluorescence detector in scan mode. Dephosphorylated probe species and 6-carboxy TET were quantified using the external standards procedure with (I) and 6-carboxy TET serving as the respective standards. Calibration curves for (I) and 6-carboxy TET were linear ($R^2 > 0.999$) over the range of concentrations tested.

Qualitative analysis of probe species was accomplished using RP-HPLC-VIS-MS. Probe species were analyzed using an Agilent Technologies 1200 series HPLC system connected in series to a UV-visible variable wavelength detector (set to 522 nm) and a linear ion trap quadrupole LC-MS/MS mass spectrometer (Model 3200 Q Trap; Applied Biosystems—SCIEX Instruments). PEEK tubing connected the system as previously described. Mass detection was accomplished by electrospray ionization (ESI) with the instrument operating in the enhanced positive ion mode. We scanned from 750 to 1350 m/z at a rate of 1000 amu s$^{-1}$. Analyst, version 1.4.2, was used to process detector output. The MS chromatograms are presented in terms of total ion current. Isocratic separation of (I) and dephosphorylated probe(s) was achieved on a heated (32°C) Agilent Technologies ZORBAX Eclipse XDB-C18 column (5 µm, 15 cm × 4.6 mm) using a mobile phase consisting of 0.04 mol L$^{-1}$ ammonium hydrogen carbonate (ammonium bicarbonate), pH adjusted to 6.7 with 0.2 mol L$^{-1}$ acetic acid, and acetonitrile (96:4 v/v) at a flow rate of 1.0 mL min$^{-1}$ (adapted from Asakawa et al., 2008). Components of the mobile phase were filtered using a 0.2-µm nylon membrane. Although the bicarbonate-acetonitrile mobile phase was compatible with the operational aspects of MS, we did need to passivate the stainless steel surfaces along the flow-path by injecting a 0.1 mol L$^{-1}$ citrate-buffered (pH 6.0) sodium InsP$_6$ (1.0 mmol L$^{-1}$) solution onto the column twice before delivery of an analytical sample (50 µL). Sodium InsP$_6$ was added to analytical samples to reduce peak tailing of phosphorylated probe species (Berry et al., 2011). The concentration and pH of the bicarbonate mobile phase were above the levels recommended by Agilent Technologies for their silica-based ZORBAX Eclipse XDB-C18 column. In fact, the bicarbonate mobile phase shortened the lifetime of the ZORBAX column.

Characterization of Phytase-Generated Dephosphorylated Probe Species

Time-course experiments were performed (i) to create a chromatographic profile of the dephosphorylated probe species formed during phytase-catalyzed dephosphorylation of (I) and (ii) to produce dephosphorylated probe(s) for identification purposes. These experiments were performed in 10-mL screw-capped Erlenmeyer flasks.

The first wheat phytase time-course experiment, conducted for the purpose of creating a chromatographic profile of dephosphorylated probe species and for identification, was initiated by adding 4.5 mU of enzyme to 2 mL of 50 mmol L$^{-1}$ glycine-HCl–buffered solution (pH 2.6) containing 200 nmol (I). The control mixture consisted of glycine-HCl buffered (1). The active and control mixtures were incubated at 37°C on a rotary shaker at 150 rpm. The active mixture was sampled after 20 s, 150 min, and 300 min of incubation. The control mixture was sampled after 20 s and 300 min of incubation. Diluted samples were analyzed by RP-HPLC-FLD as previously described. In preparation for RP-HPLC-VIS-MS analysis, samples were heat deactivated, and particulates were sedimented as previously described. An aliquot of the supernatant was diluted with 0.04 mol L$^{-1}$ ammonium bicarbonate (pH 6.7) methanol solution (75:25 v/v) containing 0.6 mmol L$^{-1}$ sodium InsP$_6$.

Stock enzyme reagent was prepared by adding freeze-dried wheat- and A. niger phytase to 0.2 mol L$^{-1}$ sodium acetate buffer (pH 5.2) and 50 mmol L$^{-1}$ glycine-HCl buffer (pH 2.6), respectively. Enzyme reagents were mixed on a stir plate for 15 min and filtered using a 33-mm Millex-GV syringe filter with a 0.22-µm PVDF Durapore membrane (Millipore).

Acid Phosphatases and Tetrachlorofluorescein TInsP$_6$ Reactivity

The procedure used to assay potato and sweet potato acid phosphatase activities was based on methods described in the Sigma-Aldrich product information guide. The assays, which consisted of 22.5 mU of enzyme and 50 nmol of (I) in 1 mL of 50 mM citrate-buffered solution (pH 4.8), were incubated at 37°C on a rotary shaker for 3 h. Control assays consisted of buffered (1) solution without enzyme. After incubation, samples of the assay were diluted with 0.1 mol L$^{-1}$ phosphate-buffered (pH 6.0) methanol solution (85:15 v/v) and analyzed using RP-HPLC-FLD.

We determined Michaelis constant, $K_M$, values for wheat and A. niger phytase. The PA assays were performed in 2-mL screw-capped microcentrifuge tubes and incubated at 37°C on a rotary shaker at 220 rpm. Reactions (total volume per assay was 500 µL) were initiated by adding 0.1 mU of wheat- or A. niger phytase to 0.2 mol L$^{-1}$ sodium acetate–buffered solution (pH 5.0) or 50 mmol L$^{-1}$ glycine-HCl–buffered solution (pH 2.5), respectively. Wheat PA assays, containing 10, 4, 1, 0.25, or 0.1 nmol of TET...
TInsP₅ (1), were sampled at \( t = 0 \) (i.e., within 4 s of mixing the assay components) and after 360 s. The \( A. \ niger \) PA assays, containing 5, 2, 0.8, 0.32, or 0.1 nmol of (1), were sampled at \( t = 0 \) and after 180 s. In preparation for RP-HPLC-FLD analysis, samples (125 µL) of the reaction mixture were immediately diluted with 875 µL of ice-cold 0.1 mol L\(^{-1}\) phosphate-buffered (pH 6.0) methanol solution (85:15 v/v) containing 10 mol L\(^{-1}\) urea and 3.43 mmol L\(^{-1}\) sodium TInsP₅. The concentration of urea required to quench phytase activity was experimentally determined for wheat phytase and \( A. \ niger \) phytase. The urea-sodium TInsP₅ reagent effectively inhibited wheat and \( A. \ niger \) phytase catalyzed dephosphorylation of (1) and was compatible with the LC system. The initial velocity, \( v_i \), of wheat phytase-catalyzed dephosphorylation of (1) was determined by measuring appearance of TET TInsP₅ over time (i.e., \( v_i = \frac{\text{TET TInsP}_5}{\Delta t} \), where \( \Delta t = 360 \) s). Tetrachlorofluorescein TInsP₅ was not detected at \( t = 0 \). The \( v_i \) of \( A. \ niger \) phytase catalyzed dephosphorylation of (1) was determined by measuring the disappearance of (1) over time (i.e., \( v_i = \frac{\left( \text{TET TInsP}_5 \right)_0 - \left( \text{TET TInsP}_5 \right)}{\Delta t} \), where \( \Delta t = 180 \) s). Two completely independent assays were conducted for each of the two phytases. Kinetic data were analyzed using Lineweaver-Burk plots. The average \( K_M \) value is reported along with the % RE: \( [\text{replicate} \text{ } K_M - \text{mean } K_M]/[\text{mean } K_M] \times 100 = \% \text{ RE} \). Stock enzyme reagents were prepared as previously described.

**Measuring Phytase Activity in Pond Water**

Surface water was collected in September 2011 from a pond located on the Virginia Tech campus in Blacksburg, Virginia. The 2-acre Virginia Tech Duck Pond supports seasonal and year-round populations of waterfowl. Stoubles Creek, a tributary of the New River, recharges the pond. Stream water exiting the pond is considered moderately impaired.

Approximately 1 L of pond water was collected on two separate sampling trips (i.e., an initial and a follow-up investigation). In the initial investigation, pond water was transported back to the lab and immediately split into two equal batches. The first batch was not altered in any way before testing (designated “unaltered pond water”). The second batch was autoclaved for 20 min (designated “autoclaved pond water”). In the follow-up investigation, pond water was transported back to the lab and split into two equal batches, which were handled as previously described. Half of the unaltered water was filtered through a Durapore membrane syringe filter (designated “filtered pond water”). An aliquot of the filtered water was autoclaved for 20 min and refiltered through a Durapore syringe filter (autoclaved filtered pond water). Phytase activity assays were conducted on the day that the pond water was collected. The pH of the pond water was 7.7.

Pond water PA assays, conducted in 10-mL enclosed Erlenmeyer flasks, contained 167 (initial experiment) or 500 nmol L\(^{-1}\) (1) (follow-up investigation) and 10% ethanol, which was used as a biostatic agent (Berry et al., 2009). We also ran a series of background controls for each treatment (i.e., pond water without (1)). The pond water assays were incubated for 24 h at 37°C on a rotary shaker at 150 rpm. In preparation for RP-HPLC-FLD analysis, a 900-µL aliquot of the pond water was (i) diluted with 270 µL methanol, (ii) vortexed 2 min, (iii) diluted with 630 µL 50 mmol L\(^{-1}\) EDTA-sodium TInsP₅ reagent, (iv) vortexed 2 min, and (v) filtered through a Durapore syringe filter. Phytase activity was ascertained by quantifying production of dephosphorylated probe species for a given time period.

### Results and Discussion

**Characterization of Phytase-Generated Dephosphorylated Probe Species**

Wheat phytase preferentially initiates removal of a phosphate group from the \( \text{d}-4 \) (1-6) position of InsP₆, producing \( \text{d-Ins(1,2,3,5,6)P}_6 \) (Nakano et al., 2000; Greiner, 2007). Wheat phytase from Sigma (labeled 6-phyase) is a 4-phytase based on criteria recently recommended by the NC-IUBMB (see Berry et al., 2009 for explanation). The \( A. \ niger \) phytase is referred to as a 3-phytase because it preferentially removes phosphate from the C-3 position of InsP₆, producing \( \text{d-Ins(1,2,4,5,6)P}_6 \) (Chen and Li, 2003). We expect 4- and 3-phytase to behave in a manner that is consistent with their biochemical nature when it comes to dephosphorylating the substrate analog of InsP₅ (1). Previously we determined that wheat (4-) and \( A. \ niger \) (3-) phytase catalyzed removal of phosphate from \( \text{d-InsP}_3 \) ([O-6-[benzoylamino]hexyl]-d-nyeo-inositol-1,2,3,4,6-hexakisphosphate) in a manner that is consistent with their biochemical nature and that a decrease in the number of phosphate groups on the nyeo-inositol moiety generally increases lipophilicity of the probe species, which increases the chromatographic retention time (\( t_R \)) when using a phosphate-buffered mobile phase in conjunction with RP-HPLC (Berry et al., 2007; 2011).

Wheat phytase catalyzed hydrolysis of (1) producing several putative dephosphorylated probe species not including TET TInsP₃ (2), which is an impurity (at 1.85%) in the TET TInsP₅ (1) preparation (Fig. 2). The dephosphorylated probe species produced included TET TInsP₃₁₈₆ (3), TET TInsP₃₈₉₉₉₅ (4), TET TInsP₃₋₁₃₅ (5), TET TInsP₃₋₂₀₃ (6), TET TInsP₃₋₁₃₅ (7), TET TInsP₃₋₃₁ (8), and TET TInsP₃₋₃₈ (9) (Fig. 2). Examination of the chromatographic profile of dephosphorylated probe species generated by 4-phytase reveals that as the reaction progressed there was an initial buildup of (3) (Fig. 2b), which was the only dephosphorylated probe species that could be detected in the early stages of the reaction, followed by a transitory accumulation of (6) (Fig. 2c) [i.e., the major pathway for phosphate group removal by 4-phytase proceeds from (1) to (3) and then (6)].

\( A. \ niger \) phytase catalyzed hydrolysis of (1), producing several putative dephosphorylated probe species, including TET TInsP₃₋₁₄ (10), TET TInsP₃₋₂₂ (11), TET TInsP₃₋₂₆₅ (12), TET TInsP₃₋₂₇ (13), and TET TInsP₃₋₂₅₇ (14) (Fig. 3). Examination of the chromatographic profile of dephosphorylated probe species over time reveals that (10) and (11) were the first to build up (Fig. 3b), followed by a transitory accumulation of (11) and (13) (Fig. 3c) [i.e., the major pathway for phosphate group removal by 3-phytase proceeds from (1) to (10) and then (11)]. Based on comparable chromatographic \( t_R \) for (6) and (11), it appears that phytase-catalyzed dephosphorylation of (1) progressed through a common probe species. Moreover, (5) and (10) appear to be the same probe species based on chromatographic \( t_R \). Given that (i) wheat (4-) phytase sequentially removes phosphate groups from the C-4 and C-3 positions (Nakano et al., 2000; Chen and Li, 2003) of InsP₆, (ii) \( A. \ niger \) (3-) phytase sequentially dephosphorylates the C-3 and C-4 positions of InsP₆ (Chen and Li, 2003), and (iii) both wheat (4-) and \( A. \ niger \) (3-) phytase behave in a manner that is consistent with their biochemical nature, we reasoned that (6) (Fig. 2) and (11) (Fig. 3) are TET TIns(1,2,6)P₃ and that (3) (Fig. 2) and (10) (Fig. 3) are TET TIns(1,2,3,6)P₄ and TET TIns(1,2,4,6)P₄, respectively.
Fig. 2. Chromatographic profile of dephosphorylated probe species (tetrachlorofluorescein [TET] TInsP) produced by wheat (4-) phytase catalyzed hydrolysis of TET TInsP5 (1) after 10 s (a), 80 min (b), and 180 min (c) of incubation. The control mixture (d) was incubated for 300 min. Tetrachlorofluorescein TInsP were separated on a Supelcosil LC-18-T column using phosphate buffered-methanol mobile phase. Dephosphorylated probe species are TET TInsP\(_{tR=9.6}(3)\) (i = 4), TET TInsP\(_{tR=11.9}(4)\) (i = 3), TET TInsP\(_{tR=20.3}(6)\) (i = 3), TET TInsP\(_{tR=26.5}(12)\) (i = 3), TET TInsP\(_{tR=27.7}(13)\) (i = 2), and TET TInsP\(_{tR=38}(9)\) (i = 2). Assignment of parenthetical i values, where i equals the number of phosphate groups, is based on MS data (see Fig. 4 and 5). The \(t_R\) values represent chromatographic retention times.

Tetrachlorofluorescein TInsP\(_{tR=17.3}(2)\) (i = 3) is an impurity.

Fig. 3. Chromatographic profile of dephosphorylated probe species (tetrachlorofluorescein [TET] TInsP) produced by A. niger (3-) phytase catalyzed hydrolysis of TET TInsP5 (1) after 20 s (a), 150 min (b), and 300 min (c) of incubation. The control mixture (d) was incubated for 300 min. The high-performance liquid chromatography analysis procedure described in Fig. 2 was employed to separate probe species. Dephosphorylated probe species are TET TInsP\(_{tR=14}(10)\) (i = 4), TET TInsP\(_{tR=21}(11)\) (i = 3), TET TInsP\(_{tR=26.5}(12)\) (i = 3), TET TInsP\(_{tR=27.7}(13)\) (i = 2), and TET TInsP\(_{tR=37.5}(14)\) (i = 2). Assignment of parenthetical i values is based on MS data (data not shown). Tetrachlorofluorescein TInsP\(_{tR=17.3}(2)\) (i = 3) is an impurity.
Phytase-generated dephosphorylated probe(s) were successfully characterized with RP-HPLC-VIS-MS. The key to analyzing dephosphorylated probes with LC-MS was selection of a mobile phase that was compatible with on-line ESI-MS and would separate phosphate compounds. Although an ammonium bicarbonate-acetonitrile mobile phase was compatible with on-line ESI-MS, it did not satisfactorily separate the analytes. Resolution of component peaks was greatly improved when the stainless steel surfaces along the flow path were passivated with sodium InsP6 immediately before sample injection.

The phosphate and ammonium bicarbonate-buffered mobile phases produced comparable chromatographic profiles for the dephosphorylated probe species from the wheat phytase catalyzed hydrolysis of (1) (compare Fig. 4a and 4b). The chromatographic profile of probe species shown in Fig. 4a, obtained using FLD, closely matched the chromatographic profile of probe species shown in Fig. 4b (VIS) and 4c, where the mass spectrometer served as the LC detector. For (1) (m/z 1252; Fig. 5a) and for a majority of the dephosphorylated probe species, the protonated molecular species ([M + H]+) was the major ion observed. All probe species formed adduct ions with Na and K yielding [M + Na]+ and [M + K]+ ions and yielded a (MH– – H2O) product ion. Based on the MS data (see Fig. 5), we identified (3) (example MS data set for a TET TInsP4 species) and (5) in Fig. 4 as regioisomers of TET TInsP5 (m/z 1172); (4), (2) (impurity), and (6) (example MS data set for a TET TInsP4 species) as regioisomers of TET TInsP4 (m/z 1092); and (7) (example MS data set for a TInsP4 species), (8), and (9) as regioisomers of TET TInsP5 (m/z 1012). These results establish that the RP-HPLC system is capable of distinguishing between fluorescent probe species at the regioisomeric level. Similar LC results were observed for the chromatographic profile of dephosphorylated probe species generated by A. niger phytase (LC-MS chromatogram not shown). On the basis of the MS data, we identified (10) as a regioisomer of TET TInsP5 (m/z 1172), (11) and (12) as regioisomers of TET TInsP4 (m/z 1092), (13) and (14) as regioisomers of TET TInsP5 (m/z 1012) (Fig. 3).

Peak tailing of (1) is significant on new Supelcosil LC-18-T columns when using a phosphate-buffered methanol mobile phase. To minimize peak tailing, new columns should be equilibrated with the phosphate-buffered methanol mobile phase for up to 6 h and treated with several injections of 0.1 mol L−1 phosphate-buffered (pH 6.0) methanol solution containing 10 mmol L−1 sodium InsP6. Substituting acetonitrile for methanol in the mobile phase substantially reduces peak tailing of (1). Isocratic separation of (1) (tR = 6.4 min) and dephosphorylated probes was achieved on a heated (37°C) Supelcosil LC-18-T column using a mobile phase consisting of 0.1 mol L−1 phosphate-buffered solution (pH 6.0) and acetonitrile (94.5:5.5) at a flow rate of 1.0 mL min−1. The chromatographic profile of phytase-generated dephosphorylated probe species is not affected when acetonitrile is used in place of methanol (data not shown).

**Acid Phosphatases and Tetrachlorofluorescein TInsP5 Reactivity**

There is evidence supporting phytase specificity for (1). We found that the biodegradability of (1) by potato and sweet potato phytase was negligible. These two nonspecific acid phosphatases (EC 3.1.3.2) exhibit broad substrate specificity. As alluded to in the Sigma-Aldrich product information guide, the sweet potato phytase preparation catalyzes the dephosphorylation of p-nitrophenyl phosphate, fructose-1,6-diphosphate, β-glycerophosphate, α-glycerophosphate, 5′-ADP, 5′-AMP, glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, pyridoxal phosphate, and NADP+ (Sugita et al., 1981), whereas the potato phytase preparation catalyzes dephosphorylation of p-nitrophenyl phosphate, phenylphosphate, α-naphthylphosphate, β-naphthylphosphate, methylumbelliferonyl phosphate, 5′-ATP, and inorganic pyrophosphate (Kruzel and Morawiecka, 1982).

The Kd values determined for wheat (4-1) phytase– and A. niger (3-) phytase–catalyzed dephosphorylation of (1) were 1.0 (20%) and 1.5 (23%) μmol L−1, respectively. The R2 values

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**Fig. 4. Reversed-phase high-performance liquid chromatography analysis of wheat (4-) phytase–catalyzed hydrolysis of tetrachlorofluorescein (TET) TInsP5 (1) after 4 h of incubation.**

Fluorescence detection analysis of dephosphorylated probe species (TET TInsP) after separation on a Supelcosil LC-18-T column using a 0.1 mol L−1 phosphate-buffered methanol mobile phase (a). Visible (b) and then mass spectrometry (c) analysis of TET TInsP species after separation on a ZORBAX Eclipse XDB-C18 column using a 0.04 mol L−1 ammonium bicarbonate-acetonitrile mobile phase. Dephosphorylated probes are TET TInsP (Tf, 7.5), TET TInsP (Tf, 7.4), TET TInsP (Tf, 7.3), TET TInsP (Tf, 7.2), TET TInsP (Tf, 6.7), TET TInsP (Tf, 6.5), TET TInsP (Tf, 6.3). Parenthetical Tf values represent chromatographic tf from chromatograms (b) and (c). Tetrachlorofluorescein TInsP (tB = 17.5, tC = 21.3) (2) is an impurity.
for data fit to the Lineweaver-Burk plots were >0.98. The $K_M$ values reported in the literature for wheat and \textit{A. niger} phytase–catalyzed degradation of InsP$_6$ (Lim and Tate, 1973; Ullah, 1988; Dionisio et al., 2011) are an order of magnitude larger than the $K_M$ values reported in this investigation for phytase-catalyzed degradation of (1) using comparable assay conditions (i.e., the same pH and incubation temperature).

Because InsP$_6$–degrading enzymes generally exhibit broad substrate specificity (Konietzny and Greiner, 2002), we anticipate that a majority of phytases will be capable of catalyzing the dephosphorylation of (1). However, some InsP$_6$–degrading enzymes are characterized as having narrow substrate specificity for InsP$_6$, including histidine acid phosphatases from \textit{A. niger} and \textit{Aspergillus terreus} (Wyss et al., 1999). Because \textit{A. niger} phytase exhibits a reasonably high affinity for (1), we are confident that a proportionally large number of the phytases exhibiting narrower substrate specificity will be capable of catalyzing dephosphorylation of (1).

Measuring Phytase Activity in Pond Water

Results from the initial pond water assay indicated that detection of PA was possible. The concentration of dephosphorylated probe species (3) was markedly greater in unaltered pond water (Fig. 6a) than the autoclaved control (Fig. 6b). Although the concentration of (3) was high enough to be detected, it was not quantifiable. Pond water–generated (3) was tentatively identified by comparing its respective chromatographic $t_R$ with the $t_R$ of wheat phytase–generated dephosphorylated probe species (see Fig. 7c). About 82% of (1) was recovered from the autoclaved (control) pond water after incubation. The amount of (1) recovered from the unaltered pond water in the initial assay was low (32%). Several factors undoubtedly influenced recovery of the probe, including (i)
the effectiveness of the extracting reagent, (ii) the amount and characteristic(s) of the particulate material (e.g., clay and humic substances), and (iii) the initial concentration of (1). The Durapore membrane is capable of adsorbing a small amount of probe material from aqueous phase samples. Adsorption of TET TInsP<sub>4,5</sub> and TET TInsP<sub>1,3</sub> by the membranes was reduced to <8% and <3%, respectively, with the addition of methanol (25%) to aqueous phase samples (data not shown).

TET TInsP<sub>i</sub> species were recovered from the pond water using a citrate-buffered EDTA-sodium InsP<sub>i</sub> reagent. This reagent was used because it was shown to be highly effective in extracting TInsP<sub>i</sub> species from soil (Berry et al., 2009). Methanol was added to pond water samples to help with solubilization of TET TInsP<sub>i</sub> species.

Based on the data for recovery of (1), we could have reasonably expected to recover ≥32% of any TET TInsP<sub>i</sub> species produced in the unaltered pond water, including the only observed species, (3). We suspected that sorption of the probe by particulates was largely responsible for reduced recovery rates of the parent and dephosphorylated probes. Furthermore, it appears that the binding sites associated with particulates were adversely affected by autoclaving. Given that there was a finite number of high-affinity binding sites, we presumed that if the concentration of (1) in the PA assay was increased, the recovery rate of probe species would increase. Although we suspected that sorption of the probe adversely affected its recovery rate in the unaltered pond water, the possibility that the amide bond was hydrolyzed by the action of arylamidase needed to be assessed.

Results of a follow-up investigation provide further evidence that PA in pond water can be measured using (1). Quantifiable amounts (i.e., ≥2 nmol L<sup>-1</sup> of TET TInsP<sub>i</sub>, given a 20 µL injection volume) of the dephosphorylated probe species (3) (12.6 nmol L<sup>-1</sup>) and (5) (3.2 nmol L<sup>-1</sup>) were produced in the unaltered pond water (Fig. 7a-I). We also detected the presence of (6). The amount of dephosphorylated probe species formed in the autoclaved control was negligible (Fig. 7a-II).

The data indicate that 4-phytase (0.5 nmol L<sup>-1</sup> h<sup>-1</sup> = 12.6 nmol L<sup>-1</sup> per 24 h) had five times the activity of 3-phytase given that 4- and 3-phytase were primarily responsible for producing dephosphorylated probe species (3) and (5), respectively, and that (5) and (10) are equivalent species. If we consider that aquatic bacteria produce β-propeller phytases, which initially remove a phosphate group from the C-3 position of the myo-inositol ring (Greiner et al., 2007), as reported by Lim et al. (2007) in their bioinformatics study, our data suggest that organisms other than bacterioplankton are actively involved in producing InsP<sub>6</sub>—degrading enzymes in pond water. Bacterioplankton are expected to play a major role in InsP<sub>6</sub> degradation in surface water (Lim et al., 2007), giving rise to the possibility that an uncharacterized group of microorganisms in pond water produce 4-phytase.

Probe species (3), (5), and (6) were observed in filtered pond water (Fig. 7b-I), indicating that active phytases were present in the soluble fraction. Phytase activity associated with the particulate plus water-soluble fraction (i.e., unaltered pond water; Fig. 7a-I) was greater than that observed for the water-soluble fraction alone (filtered pond water; Fig. 7b-I), which indicates that phytases were associated with particulate materials. Dephosphorylated probe species were not observed in the autoclaved filtered pond water (Fig. 7b-II). Pond water—generated TET TInsP<sub>i</sub> and TET TInsP<sub>i</sub> species were tentatively identified by comparing the respective chromatographic t<sub>R</sub> with the t<sub>R</sub> of wheat phytase—generated probe species (see Fig. 7c). We did not observe chromatographic signals corresponding to the t<sub>R</sub> of (3), (5), or (6) in unaltered pond water (Fig. 7b-I).
water without (1) (i.e., background control; Fig. 7a-III) or the autoclaved filtered pond water (Fig. 7b-II) or the filtered pond water background control (Fig. 7b-III).

The probe’s amide bond remained intact, as indicated by the fact that no appreciable amount of the hydrolysis product 6-carboxy TET ($K_M = 2.6$ min) was produced in the unaltered pond water (Fig. 7a-I) or in the filtered pond water (Fig. 7b-I). The evidence suggests that the probe’s amide bond is resistant to the hydrolytic activity of arylamidase.

The amount of (1) recovered from the unaltered pond water was 74%, a substantial improvement over the recovery rate observed in our initial pond water assay study. Increasing the concentration of (1) from 167 to 500 nmol L$^{-1}$ apparently increased the amount of extractable probe from unaltered pond water. About 75% of (1) was recovered from the autoclaved (control) pond water after incubation. Greater than 89% of (1) was recovered from the filtered and autoclaved filtered pond water assays.

Conclusions

Phytase catalyzes dephosphorylation of (1), producing TET TInsP$_5$, TET TInsP$_4$, and TET TInsP$_3$, which are readily separated and quantified by RP-HPLC-FLD. Wheat (4-) phytase and A. niger (3-) phytase exhibit a reasonably high affinity for the substrate analog probe, as indicated by our finding that $K_m$ values for (1) are an order of magnitude less than the $K_m$ values reported in the literature for InsP$_6$. Furthermore, the evidence supports phytase specificity for (1).

TET TInsP$_5$ (1) and dephosphorylated probe species can be quantified down to a level of 40 fmol (20 μL injection volume) using RP-HPLC-FLD. This specialized analytical approach was successfully used to measure PA in pond water. The results of our pond water investigation revealed that PA associated with the particulate plus water-soluble fraction was greater than that observed for the water-soluble fraction alone. Furthermore, the evidence suggests that 4- and 3-phytase were active in pond water. No appreciable amount of the hydrolysis product, 6-carboxy TET, was produced in pond water PA assays, which indicates that the probe’s amide bond is resistant to the hydrolytic activity of arylamidase.

Although the TET TInsP$_5$-based PA assay was successfully used to measure PA in pond water, further investigation is necessary to determine if the method can be extended to measurement of PA in streams, rivers, reservoirs, lakes, and estuarine systems.

References


