

Original Research Article

SPECTROPHOTOMETRIC DETERMINATION OF PHYTASE HYDROLYSABLE PHOSPHORUS (PHP), TOTAL DISSOLVED PHOSPHORUS (TDP), FILTERABLE REACTIVE PHOSPHORUS (FRP) AND DISSOLVED ORGANIC PHOSPHORUS (DOP) IN NATURAL WATERS

ABSTRACT

This work investigates the hydrolysis of phytic acid using soluble phytase. Phytic acid (*myo-inositol hexakis dihydrogen phosphate*) is an important organo-phosphorus compound. It is the main form in which phosphorus is stored in seeds and is therefore available in natural environment like water and soil and can be hydrolysed by phytases which also occur naturally in soil and water. Water samples were collected from different parts of Abakaliki metropolis, Nigeria within a period of 5 days in October, 2012. The samples were digested with 0.04M peroxydisulphate and 0.01M H₂SO₄ at 121°C for 45 minutes in an autoclave. After digestion, the presence of PHP (phytase hydrolysable phosphorus), FRP (filterable reactive phosphorus), TDP (total dissolved phosphorus) and DOP (dissolved organic phosphorus) were investigated in the water samples using a spectrophotometer at wavelength of 820 nm. Results obtained showed varying concentrations of PHP, FRP, TDP and DOP ranging from 0-46.5 µg L⁻¹P, 204.7-228.0 µg L⁻¹P, 297-432 µg L⁻¹P and 81-148 µg L⁻¹P respectively. Recovery studies using Adenosine-triphosphate (ATP) and the calcium salt of phytic acid (PTA) as model organic phosphorus compounds gave quantitative recoveries ranging from 92-96%. The technique was effective in digesting both the model compounds and the real samples. Also, the soluble phytase was effective in hydrolyzing phytic acid and phytase hydrolysable phosphorus (PHP) present in the water samples.

Keywords: phosphorus, phytase, organo-phosphorus, peroxydisulphate, spectrophotometry, water

1. INTRODUCTION

Phosphorus is an essential nutrient required by plants and animals for growth and development. Deficiency can retard growth and therefore can be used to monitor metabolic processes. In the aquatic environment, several species are available, but the activity prevalent in the area could have an overall influence on the concentrations of the various species. The determination of phosphorus species in the environment provides essential data for checking the health of ecosystems, investigating biogeochemical processes and

26 monitoring compliance with legislation. Phosphorus export from both points at catchments
 27 scale and diffuse sources can bring about increased primary production and eutrophication,
 28 for seasonal development of toxic algal blooms which can have a major impact on the global
 29 water quality (Mckelvie, 2005; Adeyemo et al., 2008; Mckelvie, 1999). Organic phosphorus
 30 compounds take part in terrestrial and aquatic biological phosphorus cycles and are
 31 distributed in nature.
 32 Phytic acid also known as *myo-inositol hexakis dihydrogen phosphate* is a naturally
 33 occurring non-toxic phosphoric ester that is found in cereals, pollens, legumes, seeds and
 34 plants as well as aquatic environment. It is the major form of storage of phosphorus in seeds
 35 averaging 60-90% (Cosgrave, 1980). Phytic acid is considered an anti-nutritional factor since
 36 it chelates magnesium, zinc, calcium and may also react with proteins thus, decreasing their
 37 bioavailability and important mineral nutrients. In natural environments (waters), it is
 38 hydrolyzed by phytases to the less phosphorylated forms (inositol polyphosphates and
 39 orthophosphoric acid). There are two types of phytases which can be distinguished by their
 40 position or carbon of attack and these are 3-phytases and 6-phytases (plant derived and
 41 animal derived respectively) (Cosgrave, 1980; International Union of Biochemistry, 1984;
 42 Omaka, 2006; Tadash et al., 1999, Omaka et al., 2008; Harland and Oberleas, 1980).
 43 Concentration of phosphorus in natural waters fluctuates with changes in physicochemical
 44 conditions and biological activities. Phosphorus species are found in dissolved, colloidal and
 45 particulate fractions as inorganic and organic compounds and in biotic and abiotic particles
 46 (Clesceri et al., 1988). Accurate determination of dissolved organic phosphorus (DOP) is of
 47 importance for the study of phosphorus cycling, algal assimilation and terrestrial fluxes
 48 towards the sea. These measurements are based on the conversion of complex forms of
 49 phosphates by hydrolysis and oxidation of molecules containing P-O-P, C-O-P and C-P
 50 bonds (Mckelvie, 2005; Clesceri et al., 1988; Aminot and Kerousel, 2001).

Dissolved organic phosphorus constitutes a significant portion of the bio-available phosphorus (BAP). It is the difference between total dissolved phosphorus (TDP) and filterable reactive phosphorus (FRP), i.e.

$$\text{DOP} = \text{TDP} - \text{FRP} \quad \text{Eq. (1)}$$

Bio-available phosphorus is the sum of immediate available phosphorus and phosphorus that can be transformed into available forms by naturally occurring processes such as: physical, chemical and biological processes. This fraction of the total phosphorus is important for understanding the biogeochemical processes prevalent in any aquatic ecosystem (Mckelvie, 2005; Clesceri et al., 1988; Aminot and Kerousel, 2001; Worsfold et al., 2005; Hosmi and Sudo, 1986).

Many oxidizing reagents have been used in the degradation of complex organic molecules in aqueous solution. Peroxydisulphate ($\text{S}_2\text{O}_8^{2-}$) is one of the most commonly used. Peroxodisulphate oxidation is favoured by increased temperature which leads to the generation of highly reactive sulphate and hydroxyl radicals by photolysis in the aqueous phase (Mckelvie, 1999; Aminot and Kerousel, 2001; Worsfold et al., 2005; Hosmi and Sudo, 1986).

1.1 STEPS INVOLVED IN ACID SOLUTION

The acid peroxodisulphate method generally gives good recoveries for model compounds and is simple and easy to use and is therefore recommended for TP (total phosphorus) and TDP (total dissolved phosphorus) determinations in natural waters and particularly, soil solutions (Mckelvie, 1999; Worsfold et al., 2005).



This work was aimed at investigating the effect of soluble phytase on the hydrolysis of phytic acid and phytase hydrolysable phosphorus (PHP) and applies same to the determination of

78 phytic acid or PHP in natural waters within Abakaliki metropolis of Ebonyi State, Nigeria.

79 Also the concentrations of TDS (total dissolved solid) and DOP (dissolved organic
80 phosphorus) in the same samples were determined after digestion with acidified
81 peroxydisulphate.

82 2. METHODOLOGY

83 2.1 REAGENTS

84 All reagents were of analytical grade and were prepared using deionised water. All glass
85 wares and high density polyethylene (HPDE) bottles and containers used were first cleaned
86 with a phosphate free detergent, rinsed three times with deionised water and soaked in 10%
87 (v/v) HCl overnight (12 hours) and finally rinsed with deionised water three times. The dried
88 containers were stored in air tight clean polyethylene bags to prevent contamination by dust
89 (Omaka, 2006; Worsfold et al., 2005; Gardolinski et al., 2001). Except otherwise stated, all
90 reagents were stored at 4°C and brought about for at least one hour before use.

- 91 • ~~Sodium acetate (anhydrous) $C_2H_5O_2Na$, MW 82.03g/mol (Analar BDH):~~ 0.2M
92 ~~solution was prepared by dissolving 0.8g in 50 ml of deionized water and 0.1M~~
93 ~~buffer solution of pH 5.5 was prepared by dilution of the 0.2M acetic acid. The buffer~~
94 ~~solution was stored at room temperature.~~
- 95 • ~~Acetic acid, CH_3COOH , MW 60.05g/mol and specific gravity, 1.09 g/L (Analar~~
96 ~~BDH):~~ 0.2M stock solution was prepared by dissolving 0.6g in 50ml of deionised
97 ~~water. Stock solution was stored in the dark.~~
- 98 • ~~Phytase (EC 3.1.3.8) crude from *Aspergillus ficuum* (2-5 units per mg solid)~~
99 ~~from Sigma-Adrich. This was stored at 0 °C until ready for use.~~
- 100 • ~~Phytic acid, calcium salt, PTA-Ca. $C_6H_6O_{24}P_6Ca$, MW 698.1 g/mol (Sigma 90%):~~
101 ~~0.1M stock was prepared by dissolving 0.16 mg in 25 ml of deionised water.~~
102 ~~Working standards were prepared by serial dilution of the stock.~~
- 103 • ~~Adenosine-5⁺ triphosphate disodium salt ATP, $C_{40}H_{50}O_{13}P_3Na_2$, MW 551.5 g/mol~~
104 ~~(BDH, 99%):~~ A 1000mg/L as P stock was prepared by dissolving 0.15g in 25 ml of

deionised water. Working standard of $1000 \mu\text{g L}^{-1}$ P was prepared by serial dilution of the stock.

- **Ammonium molybdate** ($(\text{NH}_4)_6\text{Mo}_{12}\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (Merck, 99.0%), MW 1235.86 g/mol: 0.1M stock was prepared by dissolving 6.0 g of ammonium heptamolybdate in 150 ml of deionised water.
- **Sulphuric acid**, MW 98g/mol and specific gravity 18.4 g/dm^3 (Analar BDH): 0.2M solution was prepared by measuring 2.27 ml and diluted to a 250 ml flask with deionised water. The stock was stored at room temperature in the cupboard.
- **L-Ascorbic acid**, $\text{C}_6\text{H}_8\text{O}_6$, MW 176 g/mol (Analar BDH 99.7%): 2.6g of ascorbic acid was dissolved in 150ml deionized water.
- **EDTA (Disodium salt)**, $\text{C}_{10}\text{H}_{14}\text{Na}_2\text{O}_8\text{N}_2 \cdot 2\text{H}_2\text{O}$, MW 372.92 g/mol (Baker 99.5%): 40mg was dissolved in the ascorbic acid stock.
- **Antimony Potassium (+) tartrate**, KSbOC_4O_6 (Analar BDH, 99%), MW 324.92 g/mol: Stock was prepared by dissolving 0.14 g in 20mL of deionised water. Dilutions were made when required.
- **Potassium phosphate dibasic trihydrate**, $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$, MW 189.99 g/mol (Sigma 99%): 1000 mg L^{-1} P was prepared by dissolving 1.53g in 250mL of deionised water. $1000 \mu\text{g L}^{-1}$ P was prepared by dilution of the stock. The acidified stock was stable at 4°C for several weeks.
- **Potassium peroxodisulphate**, $\text{K}_2\text{S}_2\text{O}_8$, FW 270.33, (Sigma 99%): 0.1M stock solution was prepared by dissolving 2.703g in 100 ml of deionised water.

2.2 APPARATUS

The apparatus for the work consisted of the following: A Hanna UV-VIS grating spectrophotometer (Model 8610) equipped with 4.0 ml quartz type), pipettes (1ml, 2ml and 5ml) refrigerated centrifuge and Asatel autoclave digester.

2.3 SAMPLING

Water samples were collected from five different locations within the metropolis as grab samples. These were filtered in-situ using 0.4µm filter paper (cellulose acetate type). The filtered samples were brought in water coolers containing ice blocks to the laboratory and stored at 4°C until the time for analysis. Important physiochemical parameters that will aid in the interpretation of results like pH, DO (dissolved oxygen), TDS (total dissolved solid), conductivity and temperature were measured in-situ using probes (Worsfold et al., 2005; Gardolinski et al., 2001).

2.4 PROCEDURE FOR THE DIGESTION OF NATURAL WATER SAMPLES

The five natural water samples were prepared for digestion using 0.004M peroxodisulphate spiked with 0.01M H₂SO₄. Final volume of the container was 50ml (reagents plus the filtered water). As a control, two samples, one containing 100 µg PTA as P each were spiked with 80 µg L⁻¹ P and 100 µg ATP as P with 100 µg L⁻¹ P. Digestion reagents were also added and made up to the 50ml mark of the digestion bottle. All the samples were digested in the autoclave at 121°C for 45 minutes (Worsfold et al., 2005; Hosmi and Sudo, 1986). After digestion, all the samples were cooled at room temperature and pH adjusted to 7.0 -7.5.

2.5 PROCEDURE FOR THE EXTRACTION OF THE PHYTASE

5mg of the phytase was weighed and dissolved in 5ml of buffer (pH 5.5). The solution was centrifuged at 5 x 1000 for 15 minutes at 4°C until when required for use (International Union of Biochemistry, 1984; Omaka, 2006; Tadash et al., 2006; Omaka et al., 2008). Two sets of the five natural water samples were prepared for the phytase activity. Each bottle contained 50ml of the filtered water: batches (A and B) and 800 µg of the soluble phytase was spiked into each bottle. Batch A was incubated in a water bath maintained at 37 °C for 40 Minutes and batch B was incubated in the cupboard at room temperature <32 °C for seven days. As control, two samples containing 200 µg PTA as P each were spiked with 80 µg L⁻¹ P and 100 µg L⁻¹ P and also with 800 µL of phytase and later incubated at 37 °C.

2.6 SPECTROPHOTOMETRIC DETERMINATION OF PHOSPHORUS (P) AS ORTHOPHOSPHATE

158 Acidified phosphomolybdate was reduced with ascorbic acid (Omaka, 2006; Murphy et al.,
159 1962) containing antimony tartrate as a catalyst. 12.4 ml of the water sample was added, 1.8
160 ml molybdate reagent and 0.8 ml ascorbic acid containing 0.0001M EDTA were also added
161 and mixed in a test tube. The reaction vessel was allowed to stand for 30 seconds and
162 absorbance reading taken using the spectrophotometer set at 820 nm. Before use, the
163 spectrophotometer was allowed to attenuate for 30 minutes and deionised water used to run
164 the blank. Similar procedure was employed for the determination of FRP, TDP, and PHP.

165 3. RESULTS AND DISCUSSION

166 A calibration was performed with the standard phosphorus stock and results obtained are
167 shown in Table 1. The calculated limit of detection was $0.01 \mu\text{g L}^{-1}\text{P}$ (James and Jane, 2000).
169 Tables 2, 3, 4 and 5 are the results obtained after analysis of FRP, digested samples,
170 recovery samples and PHP respectively.
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172 **Table 1: Analytical Characteristics of the method**

Parameter	Value
Linear range $\mu\text{g L}^{-1}\text{P}$	5-500
Slope	0.0003
Intercept	0.002
Correlation coefficient	0.9912
LOD (calculated) $\mu\text{g L}^{-1}\text{P}$	0.01
RSD (%), n=3	2
Determination temperature $^{\circ}\text{C}$	30-32

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176 **Table 2: Physiochemical data of the samples and FRP**

Station	Time (9am)	Temperature($^{\circ}\text{C}$)	Conductivity ($\mu\text{g cm}^{-1}$)	TDS (mg/L)	pH	DO (mg/L)	FRP ($\mu\text{g L}^{-1}\text{P}$)
1	8.00	28	119	60	7.30	7.6	232.6
2	8.45	28	29	14	7.00	8.0	223.3
3	9.25	29	50	24	7.30	8.1	284.0
4	9.50	28	140	68	7.12	10.9	232.6
5	10.20	29	55	26	7.16	10.1	204.7

178 **LEGEND:** 1=Stream at Presco Campus; Ama stream at Afikpo Road; 3=Onu-Ebonyi River
179 at Ogoja Road; 4=Iyi-udele stream at Nna Street; 5=Iyi-Okwu Stream at Hope High School.
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Tables 2 and 3 show the results obtained after the analysis. In Table 2, the FRP distribution was significantly affected by the activities prevalent in the area. The highest observed concentration of $284.0 \mu\text{g L}^{-1}$ P was as a result of drainage and fertilizer application on peripheral farm lands. The lowest concentration of $204.7 \mu\text{g L}^{-1}$ P had less farming activity thus, atmospheric deposition of dust particulates at the upper part due to quarrying was likely the major source of orthophosphate (Mckelvie, 2005; Omaka et al., 2008). Use of fertilizers and compost manures was very prevalent in the areas under investigation. A similar trend was also observed in table 3. Apart from farming, domestic use of soaps and detergents which contained high concentrations of sodium tripolyphosphate was the likely additional source of contamination. As can be seen in Table 4, recovery studies performed using ATP and PTA gave quantitative recoveries of about 92 – 96%.

Table 3: Total dissolved phosphorus (TDP), filterable reactive phosphorus (FRP) and dissolved organic phosphorus (ODP)

Location	TDP($\mu\text{g L}^{-1}\text{P}$)	FRP ($\mu\text{g L}^{-1}\text{P}$)	DOP = TDP-FRP ($\mu\text{g L}^{-1}\text{P}$)
1	360.0	232.6	127.4
2	304.7	223.3	81.4
3	432.0	284.0	148.0
4	352.0	232.6	119.4
5	297.1	204.7	92.4

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Table 4: Recovery studies using Model Compounds

Samples after autoclave digestion ($\mu\text{g L}^{-1}$ P)	Percentage Recovery
100 μg ATP as P + 100	92
100 μg PTA as P + 100	93
Samples after phytase incubation	
200 μg PTA as P + 80 ($\mu\text{g L}^{-1}$ P)	
200 μg PTA as P + 100 ($\mu\text{g L}^{-1}$ P)	96
200 μg PTA as P + 80 ($\mu\text{g L}^{-1}$ P)	92

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Table 5: Phytase Hydrolysable Phosphorus (PHP) after incubation at 37°C (A) and at room temperature after seven days (B)

Location	PHP (A) $\mu\text{gL}^{-1}\text{P}$	PHP (B) $\mu\text{gL}^{-1}\text{P}$
1	0.00	-9.3
2	44.0	76.7
3	-42.0	-18.4
4	13.9	38.4
5	46.5	60.9

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210 In Table 5, the areas closer to shrubs and flower gardens had higher concentrations of PHP
211 e.g. the Ama and Iyi-Okwu streams (Tadash et al., 1993). The negative values depicted
212 inhibition probably caused by heavy metals e.g. mercury and ions such as sulphides
213 (Omaka, 2006). Both batches were effective but increased temperature and length of
214 exposure resulted to faster rate of hydrolysis as has been observed earlier (Omaka, 2006).

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4. CONCLUSION

218 The technique was effective in digesting both the model compounds and real samples. Also
219 the soluble phytase was effective in hydrolyzing phytic acid and phytase hydrolysable
220 phosphorus (PHP) present in the water samples. Recovery study gave quantitative results of
221 about 92-96%. Future studies should investigate the effect of seasons and the
222 concentrations of TP, TDP, and DOP in these streams and other rivers in the state and also
223 determine the ion or ions that caused the inhibitory effect and thereafter apply the method for
224 the determination of phytic acid in seeds, flowers and fruits. A collaborative study is currently
225 ongoing to isolate and characterize phytases prevalent in the locality.

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COMPETING INTERESTS

229 Authors have declared that no competing interests exist.

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