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Zhongqi He
United States Department of Agriculture-Agricultural Research Service, Zhongqi.He@ars.usda.gov

Heidi W. Waldrip
United States Department of Agriculture–Agricultural Research Service

C. Wayne Honeycutt
United States Department of Agriculture–Agricultural Research Service

M. Susan Erich
University of Maine

Zachary N. Senwo
Alabama A&M University

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Enzymatic Quantification of Phytate in Animal Manure

Zhongqi He,[1] Heidi W. Waldrip,[1] C. Wayne Honeycutt,[1] M. Susan Erich,[2] and Zachary N. Senwo[3]

[1]U.S. Department of Agriculture–Agricultural Research Service, New England Plant, Soil, and Water Laboratory, Orono, Maine, USA
[2]Department of Plant, Soil, and Environmental Sciences, University of Maine, Orono, Maine, USA
[3]Department of Plant and Soil Sciences, Alabama A&M University, Normal, Alabama, USA

Abstract: Phytate (inositol hexaphosphate) has been identified as a major organic phosphorus (P) form in soil, animal manure, and other environmental samples. Although a number of methods are available for quantitative isolation and determination of phytate, they are time-consuming and not amenable to routine analysis. We developed a simple, rapid method for enzymatic determination of phytate in animal manure. Animal manure was extracted by H_2O, 1 M hydrochloric acid (HCl), 0.1 M sodium acetate (NaOAc, pH 5.0) with or without 0.05 M ethylenediaminetetraacetate (EDTA), and 0.25 M or 0.5 M sodium hydroxide (NaOH)–0.05 M EDTA. Extracts were diluted (1/10–1/150) and adjusted to pH 5.0 in sodium acetate buffer. The diluted extracts were then incubated at 37 °C for 1 h in the absence and presence of fungal 3-phytase (PHY) and potato acid phosphatase (PAP). Enzymatic hydrolyzable organic P was calculated as the difference in inorganic P (P_i) between the mixtures with and without enzymes. Our data indicated that enzymatic incubation of properly diluted and pH-adjusted HCl or NaOH/EDTA extracts released phytate P. The

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Address correspondence to Zhongqi He, U.S. Department of Agriculture–Agricultural Research Service, New England Plant, Soil, and Water Laboratory, Orono, ME 04469. E-mail: Zhongqi.He@ars.usda.gov

complementary substrate specificity of the two enzymes is considered to enhance the effectiveness of enzymatic hydrolysis. Consequently, we recommend this method of combining PAP and PHY for quantifying phytate P. Additional research is being conducted to verify the effectiveness of this method for general use across a wider range of soils and manures.

Keywords: Poultry manure, dairy manure, phytic acid, phosphorus

INTRODUCTION

Phytate or phytic acid (inositol hexaphosphoric acid, IP6) contains a six-carbon ring with one hydrogen and one phosphate attached to each carbon. Phytate is an important phosphorus (P) storage pool in crop seeds and fruits as the amount of P in phytate is equal to nearly 65% of the elemental P sold worldwide used in mineral fertilizers (Lott et al. 2000). Monogastric animals, such as poultry and swine, lack the ability to digest phytate, leaving significant amounts of phytate as the major organic P form in their excreta (Caldwell and Black 1958; He et al. 2007, He and Honeycutt 2001; He et al. 2006a, 2006b; Turner 2004; Turner and Leytem 2004). Application of low-phytate grains (Bowen et al. 2006; Guttieri, Peterson, and Souza 2006; Raboy et al. 2000) and supplementation with phytase enzymes (Maguire et al. 2004; Park, Choi, and Oh 1999) are two strategies to improve dietary availability of P in animal feedstuffs; however, these practices can result in more unpredictable phytate concentrations in animal manure and thus in the environment. A reliable and convenient analytical method is needed to identify and quantify phytate in animal manure.

High-performance liquid chromatography (HPLC) has been used for analysis of phytate and other inositol phosphates (Woodcock 1997). Because these compounds do not have a characteristic absorption spectrum, their detection by HPLC is limited to methods that monitor refractive index, post-column reaction products, conductivity, or other indirect detection techniques (Woodcock 1997; Xu, Price, and Aggett 1992). Because of this limitation, HPLC is mainly used for "clean" samples in food and biological studies. For animal manure, a traditional analytical method involves hydrochloric acid (HCl) (0.5 M or greater) extraction and anion-exchange chromatographic separation (Caldwell and Black 1958). Novel methods for the quantitative isolation and determination of phytate include enzymatic hydrolysis coupled with sequential fractionation (He, Griffin, and Honeycutt 2004; He et al. 2006b) and solution P-31 nuclear magnetic resonance (NMR) spectroscopy following sodium hydroxide (NaOH)–ethylenediaminetetraacetate (EDTA) extraction (Turner 2004). These methods, however, are

time-consuming because of multiple-step pretreatments, extraction, or concentration. In this work, we report our effort to develop a simple, rapid enzymatic method for routine analysis of phytate in animal manure.

MATERIALS AND METHODS

Manure Samples

A frozen wet poultry manure (PM) from a commercial Maine egg farm (Dail et al. 2007), a dried poultry litter (PL) from a broiler house in Alabama (He et al. 2006b), and a frozen wet dairy manure (DM) from a New York dairy farm (He et al. 2006c) were used in this study. Selected properties of the three samples are listed in Table 1.

Extraction

Two grams of wet manure samples or 0.3 g of dry litter sample were suspended in 25 mL of extractant. Extraction was performed at 22 °C by placing the sample tubes horizontally on the platform of an orbital shaker (250 r min^{-1}). After 4 h of extraction, the samples were centrifuged at 12,000 × g for 20 min. The supernatants were then carefully decanted and filtered through a 0.45-μm membrane to obtain the extracts. The extraction was performed in triplicate with six extractants, H_2O, 100 mM sodium acetate buffer (NaOAc, pH 5.0), 100 mM NaOAc/0.05 M disodium EDTA, 1.0 M HCl, 0.25 M NaOH/0.05 M EDTA, or 0.50 M NaOH/0.05 M EDTA. In 1 mL of each extract, an appropriate amount of 400 mM unbuffered sodium acetate or 2.5 M acetic acid was added with H_2O and/or 100 mM NaOAc buffer to a final volume of 10 mL and pH 5.0.

Table 1. Characteristics of animal manures used in this study

Sample	Solid (%)	P (g kg^{-1} dry matter)	Fe (g kg^{-1} dry matter)	Al (g kg^{-1} dry matter)	Ca (g kg^{-1} dry matter)	Mg (g kg^{-1} dry matter)	C (g kg^{-1} dry matter)
PM	33.2	13.9	1.14	0.53	153	6.89	335
PL	N/A[a]	24.6	1.08	1.2	33.0	7.2	325
DM	14.3	6.9	0.66	0.40	16.7	6.4	521

[a]No data available.

Enzymes and Enzymatic Incubation

Acid phosphatase (EC 3.1.3.2) type IV-S from potato (PAP) and phytase (EC 3.1.3.8) from *Aspergillus ficuum* (PHY) were purchased from Sigma (St. Louis, Mo.). One unit (U) of enzyme activity was defined as liberation of 1.0 μM of relevant product from appropriate substrates at appropriate incubation conditions based on the supplier's information. The stock solutions of acid phosphatase and phytase were then dispensed as 1-mL aliquots into microcentrifuge tubes and stored at −20 °C until use.

For enzymatic hydrolysis, the diluted and pH-adjusted extracts were further diluted 4- to 15-fold with distilled water or NaOAc buffer to maintain a final sodium acetate buffer concentration of 100 mM, pH 5.0. All enzymatic incubations were carried out at 37 °C for 1–4 h in a refrigerator–shaker (250 rpm). Each incubation mixture (4 or 5 mL) contained potato phosphatase and/or fungal phytase, separately or in combination (0.25 U mL^{-1} each). Controls were also included where either the enzyme or substrates were omitted.

Phosphorus Determination

Soluble inorganic orthophosphate (P_i) in the incubation mixtures (0.1–0.2 mL each) was directly quantified by a molybdate blue method (He and Honeycutt 2005). The P_i concentration in control samples was designated as inorganic P. Enzymatically hydrolyzable organic P was calculated as the increase in P_i concentration after enzymatic incubation. Total P in each fraction was determined after persulfate–sulfuric acid digestion (He et al. 2006a).

RESULTS AND DISCUSSION

Recovery of P with Different Extractants

Water extracted 4,828 mg P kg^{-1} of dry matter in PM, 3,213 mg P kg^{-1} of dry matter in PL, and 4,280 mg P kg^{-1} of dry matter in DM, which accounted for 34.8%, 13.1%, and 62.1% of total P in the three manure samples, respectively (Table 2). It seems that the higher the total P content in a manure, the less P was extracted by water. Extraction with 100 mM NaOAc buffer (pH 5.0) effectively improved the recovery of P from manure. Phosphorus extracted with NaOAc was 55.4% of total P for PM, 53.1% for PL, and 96.9% for DM. Inclusion of EDTA in NaOAc buffer further raised the recovery of P to 95.4% of total P in PM

Table 2. Recovery of P in animal manure with different extractants

Sample	H_2O	100 mM NaOAc, pH 5.0	100 mM NaOAc, 50 mM EDTA, pH 5.0	1.0 M HCl	0.25 M NaOH, 50 mM EDTA	0.50 M NaOH, 50 mM EDTA
Poultry manure	4828 ± 84[a] (34.8%)	7687 ± 334 (55.4%)	13250 ± 611 (95.4%)	14518 ± 708 (104.5%)	13458 ± 310 (96.9%)	14318 ± 557 (101.8%)
Poultry litter	3213 ± 155 (13.1%)	13053 ± 1576 (53.1%)	15487 ± 550 (63.0%)	15126 ± 844 (61.5%)	15682 ± 877 (63.7%)	15679 ± 880 (63.7%)
Dairy manure	4280 ± 769 (62.1%)	6671 ± 1244 (96.9%)	6490 ± 503 (94.2%)	5258 ± 1182 (76.3%)	6706 ± 314 (97.4%)	6322 ± 503 (91.8%)

[a]Mean ± standard deviation (n = 3) in $mg\,kg^{-1}$ of dry matter. Values in parenthesis are the % of total P extracted from each sample.

and to 63.0% of total P in PL. Turner (2004) found that inclusion of 0.05 M EDTA in 0.25 M NaOH raised the recovery of P from three manures from 32–53% to 80–96%. In this study, there was no further improvement in P extraction with either 0.25 or 0.50 M NaOH with EDTA, as compared with the recovery of P by NaOAc with EDTA (Table 2). Combining this work with the findings of Turner (2004), it seems that EDTA is more critical than NaOAc or NaOH in improving the extraction efficiency.

Hydrochloric acid (1.0 M) also extracted P in PM and PL as effectively as sodium acetate and NaOH with 50 mM EDTA. However, only 76.3% of P was extracted from DM by 1.0 M HCl, lower than the recovery of DM P by NaOAc and NaOH. Previously, Turner (2004) reported that 1.0 M HCl extracted less P from cattle and swine manures than NaOH/EDTA, whereas both extractants recovered equal amounts of P from boiler litter; therefore, NaOH or NaOAc with EDTA both appear superior to HCl for extracting manure P.

Enzymatic Release of Organic P

Enzymatic incubation slightly increased P_i concentrations by 1, 5, and 5% for PAP, PHY, and PAP + PHY, respectively, in H_2O extracts, and 1, 8, and 8% in NaOAc extracts of PM (Figure 1). Based on the substrate specificity of the two enzymes (He et al. 2007; He, Griffin, and Honeycutt 2004), these data indicate there were only negligible amounts of easily soluble simple monoester P and small amounts of easily soluble phytate in the layer hen manure. Enzymatic hydrolysis markedly increased P_i concentrations in NaOAc/EDTA, HCl, and NaOH/EDTA extracts. The increases in P_i were in the ranges of 7–15, 24–37, and 37–38% for PAP, PHY, and PAP + PHY, respectively (Figure 1). There was no difference in P released by PHY and the combination of PAP + PHY except in the

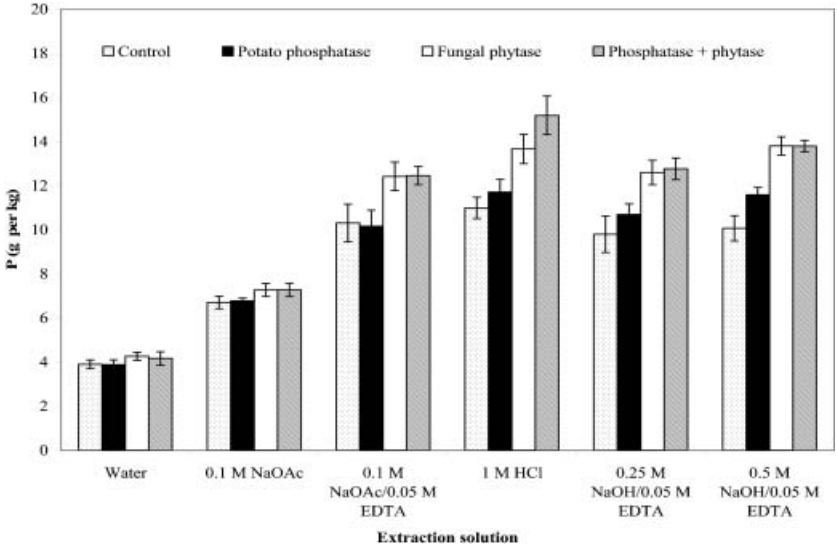

Figure 1. Enzymatic release of organic P in poultry manure.

HCl extract. This difference is to be expected, as phytate P is the major organic P in animal manure (He et al. 2007). Further incubation up to 24 h did not increase P_i detected in these reaction mixtures (data not shown).

Similar results were observed in the water extracts of PL (Figure 2). Only small amounts of P were water extractable, and very little extractable P was organic. Unlike results observed in PM, PHY and PAP + PHY released a large amount of organic P in the NaOAc extract, increasing the P_i detected by 170% to 190%. The amounts of P_i detected in the other four extracts were quite consistent: incubation of the four extracts with PHY and PAP + PHY increased P_i in solution by approximately 300%.

Enzymatic incubation also released organic P in DM extracts (Figure 3). The highest increase in P_i was still observed in the incubation with PAP + PHY. However, the observed increase was much lower than that of PL. PAP + PHY increased P_i in HCl extracts of DM by 50%, but just 10 to 29% in other extracts as compared to the DM control. Incubation with PAP increased P_i in all the extracts to a larger extent in DM than in PM and PL. For instance, the increase in P_i due to PAP hydrolysis was 12% in 0.1 M NaOAc extract and 11% in 0.25 M NaOH/0.05 M EDTA. Data in Figures 1–3 show that the combination of PAP and PHY consistently released equal or higher amounts of organic P than either enzyme alone from all extract and manure combinations. This is due to the complementary substrate specificity of the two enzymes, which

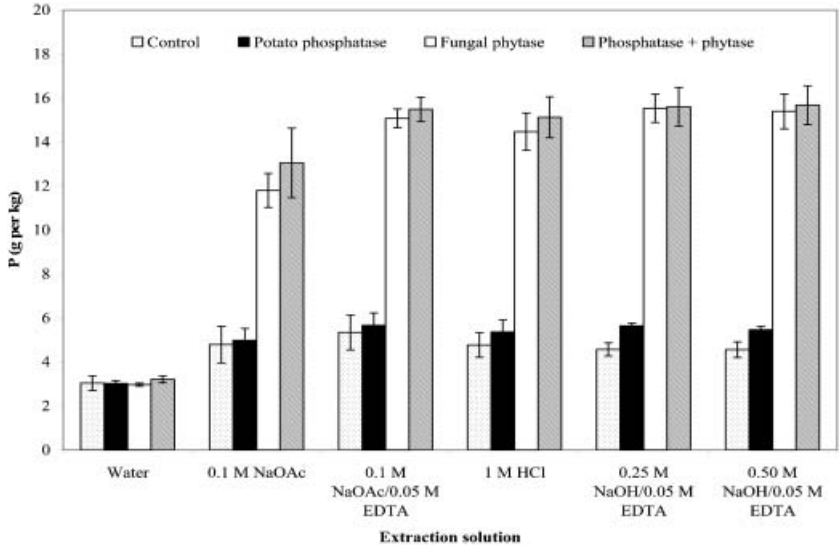

Figure 2. Enzymatic release of organic P in poultry litter.

enhances the effectiveness of enzymatic hydrolysis (He et al. 2007; He, Griffin, and Honeycutt 2004). We therefore recommend this method of combining PAP and PHY for quantifying phytate P.

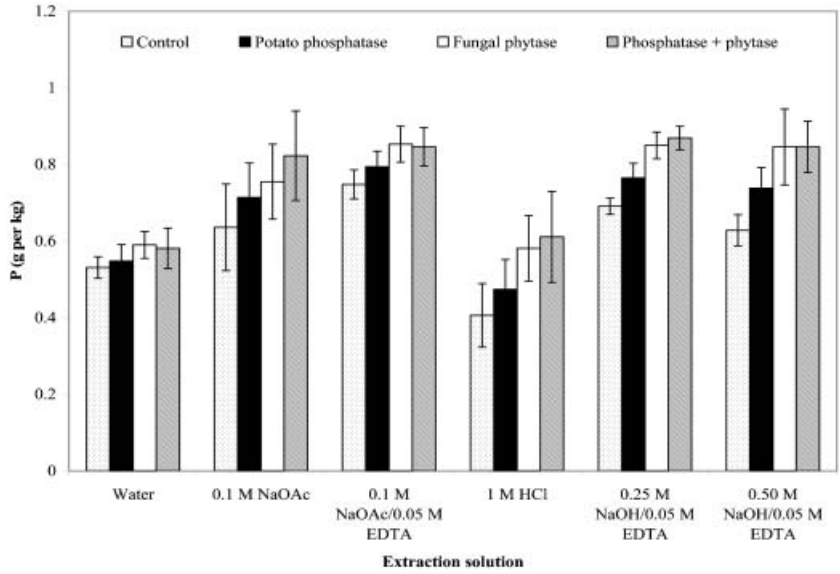

Figure 3. Enzymatic release of organic P in dairy manure.

With the procedure described in the materials and methods section, we could assign three types of P in the three manures: inorganic P (P_i determined in control), simple monoester P (P_i increased after incubation with PAP), and phytate P (difference in P_i after incubation with PAP + PHY compared to PAP alone). The composition of the three P types changed greatly with type of extractants used in these three manure samples (Table 3). In a trend similar to the results for total P, the concentration of the three P forms increased in the order of H_2O < NaOAc (pH 5.0) < NaOAc/EDTA ≈ 0.25 M or 0.5 M NaOH/EDTA ≈ HCl and was consistent with the extractant power of these solutions. In fact, the concentrations of phytate determined in the extracts of NaOAc and NaOH with EDTA were not statistically significantly different for PM and PL (Table 3). Interestingly, even though, compared to NaOAc and NaOH, 1.0 M HCl extracted relatively less total P from DM, the level of phytate in the HCl extract was high. The difference was due to lower levels of inorganic P extracted by HCl. This observation implies that previous methods using HCl for extracting phytate P in animal manure (Caldwell and Black 1958) are still valid even though total P may not always be fully recovered.

Phytate P was the major P form in PL. Its concentration doubled the inorganic P concentration in the 63% of total P extracted. This is

Table 3. Concentrations ($mg\,kg^{-1}$ dry matter) of inorganic P (P_i), simple monoester P (P_{sm}), and phytate P (P_{ip6}) extracted by different extractants

Sample	H_2O	100 mM NaOAc, pH 5.0	100 mM NaOAc, 50 mM EDTA, pH 5.0	1.0 M HCl	0.25 M NaOH, 50 mM EDTA	0.50 M NaOH, 50 mM EDTA
Poultry manure						
P_i ($mg\,kg^{-1}$)	3900a[a]	6705b	10307c	10992c	9799c	10067c
P_{sm} ($mg\,kg^{-1}$)	0a	115ab	61a	725b	895bc	1522d
P_{ip6} ($mg\,kg^{-1}$)	218a	458a	2092b	3484c	2075b	2205b
Poultry litter						
P_i ($mg\,kg^{-1}$)	3043a	4795b	5346b	4775b	4525b	4565b
P_{sm} ($mg\,kg^{-1}$)	100a	230a	340a	590a	1122b	901b
P_{ip6} ($mg\,kg^{-1}$)	70a	8028b	9800b	9120b	9963b	10190b
Dairy manure						
P_i ($mg\,kg^{-1}$)	3715a	4448a	5230b	2836a	4827c	4386c
P_{sm} ($mg\,kg^{-1}$)	126a	544ab	319bc	471bc	522bd	771bd
P_{ip6} ($mg\,kg^{-1}$)	291a	767a	389a	965b	723b	858b

[a]Concentrations followed by the same letter within rows are not significantly different ($\alpha = 0.05$).

consistent with the previous observation (He et al. 2006b). The concentrations of phytate P in DM were relatively low with more simple monoester P present. That is, near one third to one half of hydrolyzable organic P was simple monoester P. Based on these data, for practical application of this method, a control and a combination of PAP and PHY could be used to determine the phytate P concentration in extracts of animal manure. Incubation with PAP is recommended for more accurate determination or in distinguishing phytate P from other simple monoester P.

CONCLUSIONS

Enzymatic incubation of properly diluted and pH-adjusted NaOAc or NaOH/EDTA extracts released organic P in animal manure. The complementary substrate specificity of potato acid phosphatase (PAP) and fungal phytase (PHY) enhanced the effectiveness of enzymatic hydrolysis; consequently, we recommend this method of combining PAP and PHY for quantifying phytate P. An additional incubation with PAP alone should be included for distinguishing other simple monoester P from phytate P if needed. Additional research is being conducted to verify the general use of this method across a wider range of soils and manures.

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