An improved method for a rapid determination of phytase activity in animal feed

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ABSTRACT: The current direct colorimetric assay for phytase activity in feeds has interference from high P background and other factors. Our objective was to develop a rapid and reliable spin column method to accurately determine phytase activity in feed ingredients or complete diets. After the feed sample was extracted by stirring in 0.2 M citrate buffer, pH 5.5, for 30 min at room temperature, the oily layer of the supernatant fraction was removed by passing through an acrodisc syringe filter (0.45-μm HT Tuffryn membrane, Gelman Laboratory, Ann Arbor, MI). The filtrate was then loaded onto a spin column (MW cutoff 30,000, Millipore, Bedford, MA) to remove free phosphate before the phytase activity assay. Compared with the direct assay, this new procedure improved both accuracy and reproducibility. When diets contained phytase at 0 to 1,500 U/kg (as fed), the CV for multiple assays of the same samples (n = 6) by the new method ranged from 1 to 6% compared with 28 to 39% by the direct method. A linear relationship was found between the added phytase activity in practical diets and the analyzed activity by the new method (r² = 0.99; P < 0.01). In conclusion, the spin column method is an improved assay for phytase activity in animal feed, and may be used for quality control of phytase supplementation.

Key Words: Feed, Filtration, Phosphorus, Phytase, Spin Column

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Introduction

Phytase (myo-inositol hexakisphosphate phosphorylase, EC 3.1.3.8) catalyses the release of phosphate from phytate (myo-inositol hexakisphosphate; Pandey et al., 2001). Supplemental microbial phytases have been used to improve bioavailabilities of phytate P and other minerals in diets for swine and poultry (Cromwell et al., 1993; Johnston and Southern, 2000; Traylor et al., 2001). Thus, a rapid and accurate assay for phytase activity in animal diets is needed for both phytase research (Liu et al., 1998; Sands et al., 2001; Shen et al., 2002) and application (Radcliffe et al., 1998; Augspurger et al., 2003; Martinez et al., 2004). Usually, phytase is directly extracted from feed samples, and the activity is determined by the color formation between molybdate and the released inorganic orthophosphate from phytate (Cooper and Gowing, 1983; Han et al., 1998; Engelen et al., 2001). However, a rather large variation and poor reproducibility often occurs when this direct method is used to assay for phytase activity in feed ingredients or complete diets. Free or watersoluble P in these samples results in high backgrounds and inhibits the completion of the phytase-mediated hydrolysis of phytate added in the assay mixture (Ullah, 1988). The strong background of color in blanks decreases the color contributions by the enzymatic hydrolysis, resulting in unstable absorbance readings. In addition, the formation of an oily layer on the surface, probably due to fat and carbohydrate complex from the direct extraction of complete diets or feedstuffs, makes it difficult to accurately sample or to completely mix the samples with the enzyme assay solutions. Thus, our objective was to determine whether an addition of filtration and spin column concentration in the direct extraction method was sufficient to remove the high free-P background and the oily layer in the extract and make the procedure a rapid and reliable assay for phytase activity in complete diets.

Materials and Methods

Reagents and Apparatus

The direct extraction method used the same reagents and apparatus (items 1 to 9) as described previously (Han et al., 1999). The spin column method required...
two additional supplies (items 10 to 11): 1) 0.2 M citrate buffer, pH 5.5; 2) 1% (9 mM) sodium phytate (rice-derived) in 0.2 M citrate buffer, pH 5.5; 3) 15% (wt/vol) trichloroacetic acid (room temperature); 4) color reagent (prepared fresh daily): mix three volumes of 1 M sulfuric acid and one volume of 2.5% ammonium molybdate, then add one volume of 10% ascorbic acid (wt/vol) and mix well; 5) 9 mM KH₂PO₄ solution, and then make a series of dilutions: 1:100, 1:200, 1:400, 1:800, and 1:1,600 (corresponding to 90, 45, 22.5, 11.25, and 5.625 µM phosphate, respectively); 6) Oster homogenizer (Oster Corp., Milwaukee, WI); 7) spectrophotometer (Beckman DU 640, Beckman Coulter, Fullerton, CA); 8) centrifuge (Beckman Coulter); water bath (37 °C); 9) acrodisc syringe filter (0.45-µm HT Tufryn membrane, Gelman Laboratory, Ann Arbor, MI); and 8) spin column (Amicon Microcon YM-30 centrifugal filter devices, Millipore Corp., Bedford, MA).

Phytase Extraction and Filtration

Grind feed samples with an Oster homogenizer (or a coffer grinder) in a cold room (4°C) until all material passes through a 1-mm sieve. Weigh a 5-g sample (triplicates for each sample) into a 125-mL flask. Add 50 mL of 0.2 M citrate buffer (pH 5.5) into the flask and extract phytase by constant stirring (magnetic stir bar) at room temperature for 30 min (or selected time). Transfer the mixture into a 50-mL Falcon tube (Becton Dickinson, Oxnard, CA) and centrifuge it at 4°C and 15,000 × g for 20 min with the GA-20 rotor (Beckman Coulter or with a microcentrifuge in a cold room). Transfer the supernatant fraction (use a 25-mL disposable pipette) to a conical tube. Pass the supernatant through an acrodisc syringe filter using a 10-mL syringe, and collect all filtrate into a new tube. Pipette 0.5 mL of filtrate into the sample reservoir of the spin column and assemble the reservoir into the vial following the manufacturer’s instruction. Centrifuge the assembly at 14,000 × g at 4°C up to a final volume of approximately 50 µL (approximately 12 to 20 min). Weigh a new vial using an analytic balance and record the weight to the nearest 1 mg. Place the sample reservoir upside down in the vial and spin briefly to transfer the retentate to the vial. Place the vial on the balancer and add 0.2 M citrate buffer, pH 5.5, into the vial to make a final volume of exactly 0.5 mL by weighing the solution (1 mL of citrate buffer = 1.0298 g). Mix the resultant solution thoroughly for the subsequent phytase activity assay.

Phytase Activity Assay

Take two aliquots (0.2 mL each) of samples (after Steps 5 and 12 from the above procedure for the direct and spin method, respectively; Figure 1) into 10-mL test tubes. Incubate the test tubes in 37°C water bath for 5 min. Add 0.2 mL of 1% (wt/vol) sodium phytate in the selected buffer and pH to start the enzymatic hydrolysis of phytate, and incubate for 15 min at 37°C. Stop the reaction by adding 0.4 mL of 15% trichloroacetic acid (room temperature). Centrifuge the mixture at 2,000 × g for 10 min and transfer the supernatant fraction to a new tube. Mix 0.2 mL of supernatant fraction with 1.8 mL of nanopure water (18 MΩ-cm, Barnstead-Thermolyne, Dubuque, IA). Add 2.0 mL of fresh color reagent to each tube and mix well. Incubate the mixture at 50°C for 15 min and take the tubes to room temperature. Read the absorbance of each sample solution at 820 nm, using water as the blank and the series diluted potassium phosphate solutions as standards. Calculate phytase activity per gram of feed. One unit of phytase is defined as the amount of enzyme required to release 1 µmol of inorganic P/min from sodium phytate at 37°C. Because 5 g of diet is extracted in 50 mL of buffer, the dilution factor is 10. The equivalent phytase activity (dilution is considered) in each of the phosphate standard solutions is listed in Table 1.

Sources of Phytase Samples

Two phytases were tested in the present study. Natuphos, a recombinant enzyme of Aspergillus niger with a specific activity of 5,000 U/g of product (as-fed basis for all phytase activity levels), was kindly provided by BASF (Mt. Olive, NJ). A bacterial phytase (EcoPhos, Phytex, L.L.C., Portland, ME), derived from Escherichia coli as an AppA2 enzyme (Rodriguez et al., 1999), was expressed in Pichia pastoris yeast (Stahl, 2001). The expressed AppA2 phytase was concentrated by ultrafiltration, mixed with wheat middlings, and freeze-dried to make a dry preparation containing 4,000 U/g of product. Both phytases were added into diets at the time of mixing.

Determination of Extraction Time

A corn–soybean meal diet (provided by United Feeds, Sheridan, IN) for swine containing AppA2 at 1,250 U/kg of feed was used to determine the optimal extraction time. Triplicate feed samples (5 g) each were extracted with 50 mL of 0.2 M citrate buffer, pH 5.5 at room temperature for 0, 5, 10, 15, 30, 45, 60, 90, and 120 min. After the extractions, duplicate samples collected at each time point were assayed for phytase activity by the direct and spin column methods.

Determination of Phytase Recovery in Swine Diet

A corn–soybean meal diet (Crowe, 2003) for swine supplemented with AppA2 at 500 U/kg of feed was used as the baseline to determine the recovery of the assay. Before the extraction, 25 and 50 µL of concentrated AppA2 phytase (100 U/mL) were added into the flasks containing 5 g of the swine diet to double (1,000 U/kg) and triple (1,500 U/kg) the activity, respectively. One flask (5 g of diet) was added with buffer to serve as the control. All samples were extracted for 30 min at room temperature before the subsequent steps.
Figure 1. Flow chart of the direct and the spin column methods for determining phytase activity in feeds. RT = room temperature.

**Determination of AppA2 and Natuphos Activities in Swine and Poultry Diets**

Phytase activities in the corn-soybean meal diets for swine supplemented with AppA2 or Natuphos at 500 U/kg of feed were assayed by the two methods to determine whether the new method gave similar improvement for both bacterial and fungal phytases in the swine diet. To test the validity of the new method, corn–soybean meal diets (Applegate et al., 2003) for turkey poults supplemented with AppA2 at 0, 250, 500, and 750 U/kg of feed were assayed. In all assays, triplicate feed samples (5 g each) were used and extracted for 30 min at room temperature.

**Statistical Analyses**

Data were expressed as means ± SE (n = 6). The reproducibility of the two methods was compared by the CV. The relationship between the calculated and the assayed phytase activity in diets containing graded levels of phytase activity was analyzed by regression with SAS (SAS Inst., Inc., Cary, NC).

**Results**

**Extraction Time**

As shown in Figure 2, phytase was nearly completely extracted after 15 min for both methods. After that time
A new assay for feed phytase activity

Figure 2. Effects of extraction time on the phytase activity of a corn-soybean meal swine diet supplemented with AppA2 at 1,250 U/kg of feed. The extraction was conducted at room temperature and each bar represents the mean ± SE (n = 6).

point, the spin column method gave a fairly consistent activity regardless of the extraction time, whereas there was more variation (25 to 72 U/kg of feed) and some activity loss (10.4 and 8.9%; \( P < 0.05 \)) from the samples taken after 90 and 120 min of extraction by the direct method. Overall, an extraction time of 30 min seemed to be adequate for both methods.

**Determination and Recovery of AppA2 Activity in Swine Diets**

When the swine diet containing AppA2 phytase at 500 U/kg was tested by the spin column and direct methods, the former gave a mean of 521.7 U/kg, with a SE of 8.8 (n = 6), whereas the latter resulted in a mean of 459.5 U/kg, with a SE of 73.9 (n = 6). The recovery for the addition of 500 and 1,000 U of concentrated AppA2 phytase to the swine diet was 90.0 and 100.5% by the direct method, and was 111.6 and 117.4% by the spin column method, respectively. For the three levels of phytase activity (500, 1,000, and 1,500 U/kg), the CV for the direct assay was 39.4, 28.9, and 28.2%, respectively. In contrast, the CV for those two diets using the spin column method was only 4.1 and 5.8%, respectively. The spin column method gave a lin-

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**Determination of AppA2 and Natuphos Activities in Swine and Poultry Diets**

Both methods gave similar mean values of phytase activities in the corn-soybean meal diets that were close to the supplemented AppA2 or Natuphos activities (Figure 3). However, the direct method yielded CV of 39.4 and 28.2%, for the AppA2 and PhyA supplemented diets, respectively. In contrast, the CV for those two diets using the spin column method was only 4.1 and 5.8%, respectively. The spin column method gave a lin-

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**Figure 3.** Determination of phytase activity in swine diets supplemented with AppA2 or Natuphos (PhyA) at 500 U/kg of feed by the direct and spin column methods. Each bar represents the mean ± SE (n = 6).
ear regression ($y = 1.15x - 20.3; r^2 = 0.99, P < 0.01$) between the added phytase ($x$, 0 to 750 U/kg feed) and the assayed activity ($y$; Figure 4). However, the relationship was not significant ($P = 0.40$) for the direct method.

**Discussion**

Results from the present study indicate that the addition of a filtration and a spin column step to the commonly used preparation of phytase samples for the activity assay significantly improved the accuracy and reproducibility of the assay. The filtration with the 0.45-μm membrane effectively removed the white creamy layer that is supposed to be protein or lipid complexes binding with phytic acid and its hydrolytic derivatives (Maga, 1982). Contamination of the phytase assay sample by this white creamy layer results in fluctuating absorbance values in the color reaction of the phytase assay (Harland and Morris, 1995). The spin column step decreased the free-P background in blanks by 85% (data not shown), ensuring the phytase-mediated hydrolysis of phytate P as the major contributor to the color reaction. The regressions between the supplemented and the analyzed phytase activities were significant for the spin column method. The new method had CV for the multiple assays of the same samples ranging from 1.3 to 5.8%, compared with 28.2 to 39.4% for the direct method.

Our data also show that the new assay worked effectively for both bacterial AppA2 and fungal PhyA phytases supplemented either in swine or poultry diets. The additional cost for the filter and the spin column used in the new assay are well justified by the improved reproducibility, accuracy, and reliability for the phytase activity assay in the complete diets or feed ingredients. Because of the presence of relatively high levels of soluble P in the complete diets, even in those without inorganic P supplementation, the direct extraction method has been unsatisfactory. For example, feed extracts in 10 volumes of buffer (wt/vol) may give photometric readout at 820 nm from 3 to 4 (approximately 260 μM phosphate), which nearly exceed the photometric readout limits of most spectrophotometers. These types of constraints associated with the direct method might have resulted in uncertainty for the research findings from previous phytase studies.

For both methods, extraction of phytase from samples for 30 min seemed to be sufficient. This is because phytase activity assayed by both methods reached a plateau after only 15 min of extraction. In addition, there was no significant difference in extraction rate and enzyme stability between 4°C and room temperature (25 to 30°C; data not shown). Because the molecular weight of AppA2 phytase expressed in *P. pastoris* is approximately 50 to 55 kDa (Rodriguez et al., 1999), all of the phytase was recovered in the retentates of both membranes, with the cut off of molecular size at 10 and 30 kDa, respectively, without detectable activity in the filtrate (data not shown). Because the passing rate of the membrane is proportional to its molecular size cut-off, the 30-kDa cut off membrane is recommended for the assays of AppA2, PhyA, and other enzymes with similar or larger molecular sizes. As the phytase extractions from the feed samples can be concentrated at the spin column step in the new method, it may allow for the assay of feed samples with low phytase activity.

In summary, we have added two steps of filtration and spin column concentration in the sample preparation for the colorimetric assay of phytase activity in feeds. Compared with the currently used direct extraction method, these additional steps remove the oily layer of the extraction of feed samples and decrease the P background. The improved accuracy and reproducibility over the direct method has been shown with samples of complete diets for swine and poultry supplemented with bacterial or fungal phytases.

**Literature Cited**


