Method for assay of 6-phytase in the additive, vitamin-mineral premixtures and feedingstuffs

Enzyme Reaction:

In the first of a two step assay, phytase reacts with sodium phytate (*myo*-inositolhexakisphosphate) substrate in pH 5.5 citrate buffer for 15 minutes at 37°C to catalyze the release of inorganic phosphate. The phytase reaction is then stopped by the addition of trichloroacetic acid and the amount of free phosphate released is measured spectrophotometrically in a second reaction in which phosphate forms a blue phosphomolydate complex with a maximum absorption coefficient at 820 nm.

Enzyme Unit:

One unit of phytase activity (FTU) is defined as the amount of enzyme that catalyzes the release of 1.0 micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 buffer at 37°C.

Linear Assay Range:

Minimum detection limit = 10 mUnits / ml in solution of purified enzyme or phytase premix; 60 FTU / Kg solids in 1:10 (w/v) extraction mix.

Maximum detection limit = 480 mUnits / ml

Coefficient of

Variation: CV = 5-8%

Method description:

Equipment:

- Lab glassware free of residual phosphate (beakers, graduated cylinders, testtubes including 10 x 75 and 13 x 100 mm)
- Pipettes (0.5, 1.0 and 2.0 ml)
- Vortex mixer
- Water baths (37C and 50C)
- pH Meter
- Spectrophotometer – 820 nm wavelength with 1.0 cm cuvettes
- Centrifuge
- Ggrinding mill with 0.5 mm screen
- Magnetic stir plate and stirrers
- Analytical balance – 0.1 mg sensitivity
- Test tube racks for 10 x 75 mm and 13 x 100 mm tubes

Chemicals:

Sodium citrate, trisodium dihydrate

- Citric acid, monohydrate
- Phytic acid, dodecasodium salt
- Trichloroacetic acid
- L-Ascorbic acid
- Ammonium molybdate-4H2O -
- Sulfuric acid – 18 M
- Potassium phosphate, monobasic,anhydrous
- Deionized water
- Phytase

Reagents:

- Dilution and Extraction Buffer (0.2 M Sodium Citrate pH 5.5): prepare 0.2 M sodium citrate solution by adding 58.82 gm of sodium citrate to a final volume of 1.0 liters of d-H_2O and 0.2 M citric acid by adding 42.02 gm of citric acid to a final volume of 1.0 liters of d-H_2O. The two components are mixed in proportions that give a final solution of pH 5.5. The buffer and buffer components can be stored at 4°C for up to 5 days.

- Tween 20 solution (10.0%): Add 2 ml of Tween 20 to a ~ 18 ml of dH_2O, using a 20 ml volumetric flask. Store at room temperature.

- Feed Extraction & Wash Buffer (0.2 M Sodium Citrate pH 5.5): prepare 0.2 M sodium citrate solution by adding 58.82 gm of sodium citrate to a final volume of 1.0 liters of d-H_2O. Prepare 0.2 M citric acid by adding 42.02 gm of citric acid to a final volume of 1.0 liters of d-H_2O. The two components are mixed in proportions that give a final solution of pH 5.5. Add the 10% Tween 20 solution such that the concentration in the final feed extraction buffer is 0.10%. The buffer and buffer components can be stored at 4°C for up to 5 days.

- Enzyme Substrate Solution (Sodium Phytate): Dissolve 1.0 gram of sodium phytate in 65 ml of pH 5.5 0.2M citrate buffer. Adjust pH to 5.5 by the addition of 0.2 M citric acid and bring final volume to 100 ml with the addition of pH 5.5 0.2M citrate buffer. Prepare fresh daily.

- TCA Stop Solution (15% trichloroacetic acid): prepare 15% TCA solution by adding 150 grams of trichloroacetic acid to 700 ml of dH_2O with stirring and bring final volume to 1.0 liter with additional dH_2O. Store at 25°C for 30 days.

- Ascorbic Acid Solution (10%): prepare solution by adding 10 grams of ascorbic acid to dH_2O and bring to a final volume of 100 ml. Store at 4°C for up to 2 days.

- Ammonium Molybdate Solution (2.5%): prepare solution by adding 2.5 grams of ammonium molybdate to dH_2O and bring to a final volume of 100 ml. Store at 25°C protected from light for up to 14 days.

- Sulfuric acid (1.0 M): prepare by adding 55.5 ml of concentrated sulfuric acid (18M) to 944.5 ml of dH_2O. Store at 25°C.

- Potassium Phosphate Standard Solution (9.0 mM): dissolve 0.6124 gram of potassium phosphate (anhydrous) in a final volume of 500 ml of dH_2O. Store at 4°C for up to 14 days.

- Reagent C: Mix thoroughly 3 volumes of 1.0 M sulfuric acid, 1 volume of 2.5% ammonium molybdate and 1.0 volume of 10% ascorbic acid. Prepare fresh daily just prior to use.

Preparation of samples and standards:

Phytase containing materials for assay include Phytase enzyme concentrate (5000-14000 FTU/g), product OPTIPHOS® (4000 FTU/g) and supplemented animal feeds (100-1000 FTU/Kg).

Phytase enzyme concentrate and Product OPTIPHOS® 4000 are dissolved directly in citrate buffer while the supplemented animal feed and the VMP premix samples are first ground to a uniform particle size before the solids are added to citrate FEED extraction & Wash buffer. Extraction of sample materials for phytase assay is performed as follows:

- Phytase enzyme concentrate (5000-14000 FTU/g) – Weigh with an accuracy of 0.1 mg such quantity of the enzyme and dilute it with citrate buffer to obtain 490.0 mU/ml phytase standard stock solution. Dissolve by stirring with magnetic mixer for 30 minr at ambient temperature before further diluting this initial enzyme solution.

- OPTIPHOS® (4000 FTU/g) - Phytase activity in product samples can be measured without grinding of the product if adequate mixing of suspended samples can be accomplished. If grinding is preferred to facilitate suspension of product solids, the following protocols are recommended: 1) grind 5-10 g sample of product in grinding mill (keep cool – avoid overheating) and 2) weigh 2.0 gram of ground sample to within 1.0 mg, suspend in 100 ml of citrate buffer pH 5.5 and stir resulting suspension with a magnetic stirrer for 1.0 hour at 25C. When solubilizing product samples without grinding, follow the procedure outlined in step 2) with vigorous stirring on a magnetic stir plate. Filter (0.45 micron) or centrifuge (5-6000 x g for 10 min) suspended mix to separate solids from soluble enzyme (avoid sampling fatty layer on surface of centrifuged samples). Dilute the Product Extract Solution with citrate buffer to linear range of phytase assay.

- Phytase Supplemented Animal Feed (100-1000 FTU/Kg) – Grind 50-100 gram sample of supplemented feed in grinding mill until entire sample passes through a 0.5 mm sieve (keep cool – avoid overheating). Weigh 5.0 gram of ground sample to within 1.0 mg, suspend in 50 ml of citrate buffer pH 5.5 and stir resulting suspension with a magnetic stirrer for 1.0 hour at 25C. Centrifuge (5-6000 x g for 10 min) the suspended mix to separate solids from soluble enzyme (avoid sampling fatty layer that appears on the surface of many centrifuged samples). To reduce levels of phosphates and other ingredients that contribute to

assay variability, particularly in feeds with low levels of phytase supplementation, centrifuged or filtered feed extracts are first diafiltered to reduced levels of salts and other interfering small molecules using an Ultrafree®- 4 centrifugal ultrafiltration unit (Millipore Corp.) place 2.0 ml of centrifuged or filtered feed extract into the top of the Ultrafree-4 device containing a 10,000 dalton exclusion limit membrane. Add 2.0 ml of 0.2 M Citrate buffer pH 5.5 and centrifuge unit in fixed or swinging bucket centrifuge at 6000xg for 10-35 minutes at 25°C until ~ 0.5 ml of retentate remains in the "pocket" of the concentration unit. Add 1.5 ml of Citrate buffer pH 5.5 to remaining concentrate to bring retentate volume to 2.0 ml (use long tipped pipette to stir resulting mix). Diafiltration removes approximately 85-88% of background sample salts while retaining 95-100% of phytase activity.

Preparation of Sample Dilutions:

- Phytase enzyme concentrate solution (490 mU/ml) – To prepare enzyme dilutions for Phytase enzyme concentrate in the linear range of the assay (15-245 mUnits/ml) dilute the 490 mU/ml phytase concentrate in consequtive two fold dilutions with citrate buffer pH 5.5 to give a 245 mUnits/ml to 15 mUnits/ml standard.

- Product Extract Solution – To further dilute Product extract samples to a concentration within the linear range of the phytase assay, prepare a 1/800 dilution of the Product extract by first adding 1.0 part of extract to 7.0 parts of citrate buffer pH 5.5 and further diluting this concentration by adding 1.0 part of this 1/8 dilution to 99.0 parts of citrate buffer pH 5.5.
  The resulting sample should have approximately 100 mUnits/ml

- Feed Sample Extract (~25-50 mUnits/ ml) – Diafiltered feed extract if necessary dilute with Feed Extraction & Wash Buffer to reach ~25-50 mUnits/ ml.

Preparation of phosphate standard solutions:

To measure ortho-phosphate liberated in the phytase reaction, prepare a series of phosphate standards including the following concentrations: 5.6, 11.3, 22.5, 45.0 and 90.0 µM.

Starting with a 9.0 mM stock phosphate solution, prepare a 90.0 µM standard by adding 2.0 ml of phosphate stock to 198.0 ml of d-H_2O. The remaining standards are created by preparing serial two-fold dilutions starting with the highest 90.0 µM standard, e.g. add 100 ml of 90.0 µM standard to 100 ml of d-H_2O to produce a 45.0 µM standard and 100 ml of 45 µM standard to 100 ml d-H_2O to produce a 22.5 µM standard. Standards should be stored at 4C and can be used for up to 5 days. (Take precautions to use glassware that has been washed free of phosphate-containing detergents.)

Assay Reactions Two

Step Assay:

(i) Phytate Hydrolysis:

- Aliquot 0.5 ml of each sample dilution into 3 test tubes – one blank tube and two tubes for duplicate assay reactions
- Prepare enzyme standards containing 15, 30, 60, 120, and 245 mUnits per ml of phytase activity; aliquot 0.5 ml of each standard into 3 tubes which include one blank and duplicate reaction tubes.
- Equilibrate all tubes above in 37°C water bath for 5.0 minutes and add 1.0 ml of 15% TCA to all blank tubes. Mix by vortexing and remove all blank tubes to test tube rack at room temperature.

- Start reaction in tubes containing sample dilutions by staggered addition of 0.5 ml of phytate substrate (5.1 mM) followed by rapid vortexing. Continue incubation at 37C for 15 minutes and at the end of 15 minutes add 1.0 ml of 15% TCA to each tube to stop reaction.
- Add 0.5 ml of phytate substrate (5.1 mM) to all blank tubes containing TCA plus sample dilution and mix by vortexing.

(ii) Ortho-Phosphate Determination (Phosphomolybdate Complex Measurement)

- Prepare 1:10 dilutions of all phytate hydrolysis reactions by pipetting 0.2 ml of each sample reaction into separate glass tubes followed by 1.8 ml of d-H2O to each.
- Add 2.0 ml of each of the phosphate standards to duplicate tubes. As reagent blanks, add 2.0 ml of d-H2O to two additional tubes.
- Add 2.0 ml of fresh Reagent C to all the tubes, vortex thoroughly and place in 50C water bath for 15 minutes. Stop reaction by removing tubes from water bath and after samples have cooled to room temperature.
- Nullify the spectrophotometer with a reagent blank sample containing distillated water + Reagent C. Take a spectrum of the sample blank and test sample and calculate the net absorbance of the test sample.
- Prepare standard curve by plotting A820 against concentration of phosphate standards or enzyme concentrate standards.
- Phosphate concentration of samples is obtained by subtracting A820 of sample blank from A820 of corresponding sample tube and reading phosphate concentration from standard curve.

Calculation of Phytase Activity In Assay Samples:

Phytase activity (mUnits / ml) of the sample is obtained by dividing the resulting phosphate concentration (nmoles/ml) by the time of hydrolysis (15 minutes) and mutiplying this number times 4 (dilution factor in phytase reaction = 0.5 ml sample in 2.0 ml reaction mix) and times 10 (dilution of phytate hydrolysis reaction in phosphate assay = 0.2 ml + 1.8 ml d-H2O). The resulting figure is then multiplied times the dilution of the original sample to give milli-Units (nmoles of Pi released / min) or converted to Units (nmoles ÷ 1000 = µmoles of Pi released / min) of phytase activity.

$$\textbf{Phytase Activity (mUnits/ml)} = \frac{\textbf{Pi conc. (nmoles/ml) x 4 x 10 x sample dilution}}{\textbf{15 (minute reaction)}}$$