Megazyme

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PHYTASE

ASSAY PROCEDURE

K-PHYTASE 04/21

100 Assays per Kit

INTRODUCTION:

Phytases are a class of phosphatase enzymes which catalyse the sequential hydrolysis of phytic acid (InsP6, *myo*-inositol-(1,2,3,4,5,6)-hexakisphosphate) to less-phosphorylated *myo*-inositol derivatives and inorganic phosphate. Phytases are important enzymes in the animal feed industry and are used to increase phosphate availability for monogastric animals (such as pigs and poultry) with sufficient endogenous phytase activity to fully degrade phytates in feed. Animal feeds are now often supplemented with phytase enzymes which remove the requirement to directly supplement phosphate, and also reduce phytic acid interference with the nutritional uptake of important minerals, proteins and amino acids.[1]

Phytase supplementation is also seen as a growing sector within the functional foods market,[2] as the role of phytic acid in humans is complex. High phytate intake has been shown to reduce the risk of osteoporosis[3] but is also linked to reduced absorption of dietary nutrients.[4]

Existing methodology for the detection of phytase activity is not ideal. Methods, such as the molybdenum based colourimetric assay, are cumbersome, requiring the creation of a phosphate standard curve and the use of toxic chemicals. Other methods such as the fluorometric based glucose detection method require costly reagents, expensive instruments and consumables and are time inefficient as a result of a lengthy procedure and the requirement to perform a standard curve with every experiment.

The Phytase assay (**K-PHYTASE**) method is a simple, quantitative method which can be used to measure phytase activity. Results are measured using a standard UV-VIS spectrophotometer and do not require the creation of a standard curve. This method has been used to measure phytase activity in cereal, fungal and bacterial phytases, and analysis of animal feed samples has shown good correlation with, and improved sensitivity over, ISO 30024 (Animal feeding stuffs - Determination of phytase activity).[5]

PRINCIPLE:

The assay is based on the hydrolysis of phytic acid by phytase, thus releasing inorganic phosphate (P_i) (1). Upon termination of this reaction, the amount of phosphate is quantified in a second reaction (2). The phosphate quantification assay is based on the purine nucleoside phosphorylase (PNPase) mediated conversion of 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to 2-amino-6-mercapto-7-methylpurine and ribose-1-phosphate in the presence of free inorganic phosphate. This reaction causes a shift in the UV absorbance maximum of the MESG substrate from 330 nm to 360 nm. The rate of release of phosphate relates directly to the rate of hydrolysis of phytic acid by phytase and can be used to determine the activity of the phytase.

(1) Phytic acid + H_2O $\xrightarrow{\text{phytase}}$ *myo*-inositol (phosphate)$_n$ + P_i

(2) P_i + MESG $\xrightarrow{\text{PNPase}}$ ribose 1-phosphate + 2-amino-6-mercapto-7-methylpurine

The assay principle is shown in more detail in Figure 1 (page 9).

INTERFERENCE:

Interference caused by the sample matrix can be identified by performing recovery experiments, i.e. by adding a known amount of the supplied *Aspergillus niger* phytase standard to the sample in the test. Quantitative recovery of this standard would be expected.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 100 assays in manual format are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Phytic Acid, Lyophilised powder.
Stable for > 2 years below -10°C.

Bottle 2: Stopping reagent (11 mL) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 3: Buffer (20 mL, pH 7.75) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 4: (x2) MESG, Lyophilised powder.
Stable for > 5 years below -10°C.

Bottle 5: PNPase suspension (1 mL).
Stable for > 2 years at 4°C.

Bottle 6: Phytase standard solution from *Aspergillus niger* (5 mL, see label for exact value) in glycerol solution containing BSA and sodium azide (0.02% w/v).
Stable for > 4 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

1. Dissolve the contents of bottle 1 in 21 mL of distilled water. Use immediately or divide into appropriate volume aliquots and store in polypropylene tubes below -10°C.
   Stable for > 2 years below -10°C.
2. Use the contents of bottle 2 as supplied.
   Stable for > 2 years at 4°C.
3. Use the contents of bottle 3 as supplied.
   Stable for > 2 years at 4°C.
4. Dissolve the contents of one of bottle 4 in 5 mL of distilled water. Use immediately or divide into appropriate volume aliquots and store in polypropylene tubes below -10°C. Do not re-freeze once thawed. Do not dissolve the contents of the second bottle until required.
   Stable for > 2 years below -10°C.
5. Use the contents of bottle 5 as supplied. Before opening for the first time, swirl the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position.
   Stable for > 2 years at 4°C.
6. With a positive displacement pipette, dispense 0.5 mL of the contents of bottle 6 into a 100 mL volumetric flask and accurately make to volume with Extraction/Dilution buffer (B). Mix thoroughly before use.

NOTE: The phytase standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample.

PREPARATION OF BUFFERS (NOT SUPPLIED):

(A) Concentrated Buffer

(Sodium acetate buffer, 1 M, pH 5.5)

Add 60 g of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water. Adjust the pH of this solution to 5.5 by the addition of 5 M (20 g/100 mL) NaOH solution. Adjust the volume to 1 L.
Stable for > 2 years at room temperature.

(B) Extraction/Dilution Buffer

(Sodium acetate buffer, 100 mM, pH 5.5)

Add 100 mL of concentrated buffer (A) to 850 mL of distilled water. Adjust the pH to 5.5 by dropwise addition of 2 M HCl or 2 M NaOH and adjust the volume to 1 L.
Stable for > 1 year at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL capacity)
2. Disposable polypropylene tubes (13 mL capacity) (1 cm light path, 1.5 mL).
3. Disposable plastic cuvettes (1 cm light path, 1.5 mL).
4. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
5. Positive displacement pipettor, e.g. Eppendorf Multipette®.
   - with 5.0 mL Combitip®.
   - with 25 mL Combitip®.
6. Analytical balance.
7. Spectrophotometer set at 360 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2.
9. Spectrophotometer set at 360 nm.
10. Thermostated water bath set at 40°C.
11. Stop clock.
12. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.

CONTROLS AND PRECAUTIONS:

A blank should be prepared for each sample. To obtain this value, add 0.1 mL of Stopping Reagent to 0.2 mL of phytic acid solution and then add 0.2 mL of diluted enzyme preparation. A single sample blank determination is sufficient for each sample. If the value A_2-A_1 for the sample blank exceeds 0.5, then the sample should be desalted to remove free phosphate following the phosphate removal procedure (Example C described on page 7) and should then be re-analysed.

ASSAY PROCEDURE

STEP 1: Enzyme/substrate reaction

1. Dispense 0.2 mL aliquots of Phytic acid (bottle 1) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate suitably diluted enzyme at 40°C for 5 min in a separate vessel. Sample preparation examples are supplied on page 7.
3. To each tube containing Phytic acid solution (0.2 mL), add 0.2 mL of the pre-incubated enzyme extract directly to the bottom of the tube. Mix thoroughly and incubate the mixture at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add exactly 0.1 mL of Stopping Reagent and mix the contents vigorously. Take a 0.1 mL aliquot of this reaction mixture into Step 2 for phosphate detection.

NOTE: The volume of reaction mixture taken for analysis in Step 2 can be increased to 0.4 mL to increase the sensitivity of the assay. The volume of distilled water in the cuvette should be reduced accordingly. See 'Troubleshooting' on page 8.

STEP 2: Phosphate detection

Wavelength: 360 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 22°C
Final volume: 1.01 mL
Sample solution: 0.1-10 µg of phosphate per cuvette (in 0.1-0.4 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Sample Blank	Sample
distilled water (at ~ 22°C)	0.60 mL	0.60 mL
sample from Step 1	-	0.10 mL
sample blank from Step 1	0.10 mL	-
bottle 3 (buffer)	0.20 mL	0.20 mL
bottle 4 (MESG)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reaction by addition of:		
bottle 5 (PNPase)	0.01 mL	0.01 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (~ 20 min).		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the sample blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{phytase}$.

In order to achieve sufficiently accurate results the value of $\Delta A_{phytase}$ should as a rule be at least 0.1 absorbance units, and not more than 0.8 absorbance units. See 'Troubleshooting' on page 8.

One unit of Phytase activity is the amount of enzyme that generates 1.0 µmol of phosphate per min, at pH 5.5 at 40°C.

The activity of phytase can be calculated as follows:

$$c = \frac{V_1 \times V_2 \times D}{\varepsilon_{\mu M} \times d \times v_1 \times v_2 \times 1000 \times t} \times \Delta A_{phytase} \quad [U/mL]$$

where:

V_1 = final volume from Step 1 [mL]

V_2 = final volume from Step 2 [mL]

D = dilution factor (e.g. if sample is diluted 10-fold, D = 10)

$\varepsilon_{\mu M}$ = micromolar extinction coefficient of MESG at 360 nm

= 0.0084 [1 x μmol^{-1} x cm^{-1}]

d = light path [cm]

v_1 = sample volume from Step 1 [mL]

v_2 = sample volume from Step 2 [mL]

1000 = conversion from L to mL

t = reaction time from Step 1 [min]

It follows for phytase:

$$c_{phytase} = \frac{0.5 \times 1.01 \times D}{0.0084 \times 1 \times 0.2 \times 0.1 \times 1000 \times 10} \times \Delta A_{phytase} \quad [U/mL]$$

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (U/g) is calculated from the amount weighed as follows:

$$= \frac{c_{phytase}\ [U/mL\ sample\ solution]}{weight_{sample}\ [mg/mL\ sample\ solution]} \times 1{,}000{,}000 \quad [U/Kg]$$

where:

1,000,000 = Conversion from U/mg to U/Kg

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

SAMPLE PREPARATION EXAMPLES:

A. Determination of phytase in industrial enzyme preparations:

1. **Liquid enzyme preparations:** Add 1.0 mL of liquid enzyme preparation to 49 mL of the Extraction/Dilution Buffer (B) using a positive displacement dispenser (these solutions can be very viscous) and mix thoroughly. Further dilute in Extraction/Dilution Buffer (B) if required until a dilution suitable for assay is achieved. For calculation purposes a dilution factor (D) of 50 is used for the initial extraction, and any further dilution of the extract should be taken into account by multiplication.

2. **Powder enzyme preparations:** Add 1.0 g of powder enzyme sample to 50 mL of the Extraction/Dilution Buffer (B) and gently stir the slurry over a period of approx. 15 min or until the sample is completely dispersed or dissolved. Clarify this solution by centrifugation (1,000 *g*, 10 min) or by filtration through Whatman No. 1 (9 cm) filter circles. Further dilute in Extraction/Dilution Buffer (B) if required until a dilution suitable for assay is achieved. For calculation purposes a solid sample concentration of 20 mg/mL is used for $weight_{sample}$ [mg/mL sample solution]. Take any further dilution of the extract into account by adjusting the dilution factor (D).

B. Determination of phytase in animal feeding stuffs:

Mill the feed sample to pass a 0.5 mm screen. Weigh 1.0 g of milled sample into a 13 mL polypropylene tube, record the exact weight, and add 5 mL of the Extraction/Dilution Buffer (B). Mix thoroughly by vortex and leave at ambient temperature for 15 min with occasional mixing. Clarify this solution by centrifugation (1,000 *g*, 10 min) or by filtration through Whatman No. 1 (9 cm) filter circles. Where the sample contains high levels of free phosphate (> 60 µg phosphate per mL or gram of appropriately diluted sample) it should be taken through the phosphate removal procedure (**Sample Preparation Example C**). For calculation purposes a solid sample concentration of 200 mg/mL. is used as $weight_{sample.}$. Take any further dilution of the extract into account by adjusting the dilution factor (D).

C. Removal of free phosphate:

Prepare a PD-10 desalting column (columns containing Sephadex G-25 Medium are commercially available from GE Healthcare Cat. no GE17-0851-01) by washing with ~ 20 mL of Extraction/Dilution Buffer (B). Apply 2 mL of sample (from **Sample Preparation Example B**) to the column and allow to percolate through until the top of the column no longer contains any liquid. Wash with

1 mL of Extraction/Dilution Buffer (B). Collect the total eluent (3 mL) and discard. Apply a further 3 mL of Extraction/Dilution Buffer (B) to the column and collect the eluent (3 mL). This is the sample solution ready for use in the enzyme/substrate reaction. For calculation purposes this step should be treated as a 1.5-fold dilution of the extract and the dilution factor (D) should be adjusted to include this.

TROUBLESHOOTING:

Recommendations on how to adjust the assay if absorbance values are not satisfactory.

Issue	Recommendation
Sample blank $A_2 - A_1$ > 0.5	After sample extraction remove free phosphate using sample preparation example C on page 7.
$\Delta A_{phytase}$ > 0.8	Appropriately dilute the sample with Extraction/ Dilution Buffer (B) and repeat the assay from Step 1. Adjust the dilution factor (D) in the calculation accordingly.
$\Delta A_{phytase}$ < 0.1	- Dilute the sample less strongly if possible. Adjust the dilution factor (D) in the calculation accordingly. - Increase the time of incubation in Step 1: Enzyme/ substrate reaction. Adjust the time of incubation (t) in the calculation accordingly. - Increase the volume of sample used in Step 2: Phosphate determination to 0.4 mL. Reduce the volume of distilled water in the test proportionately. Adjust the value for v_2 in the calculation accordingly.

APPENDIX:

A. Assay Principle - Phytase dectection

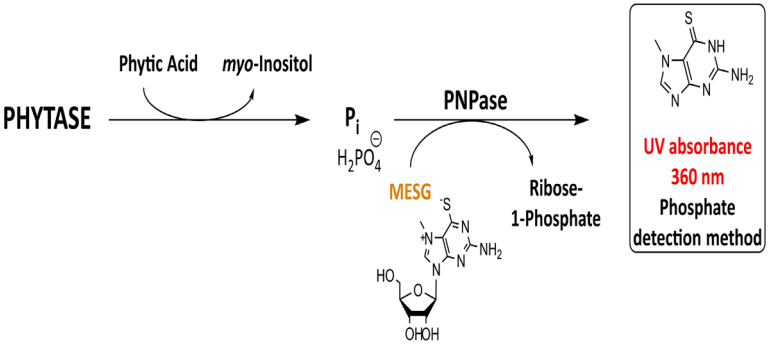

Figure 1. Theoretical basis of the phytase assay.

B. Linearity of assay

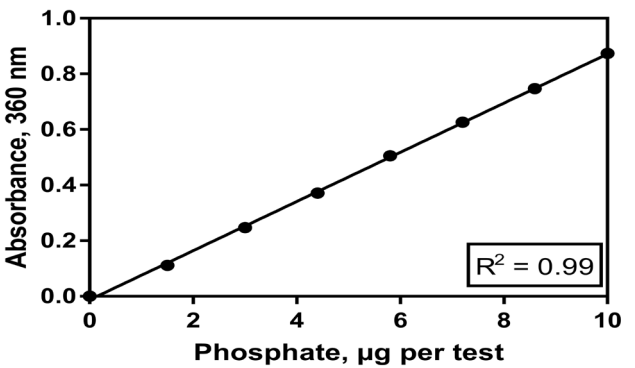

Figure 2. Linearity of the phosphate detection step.

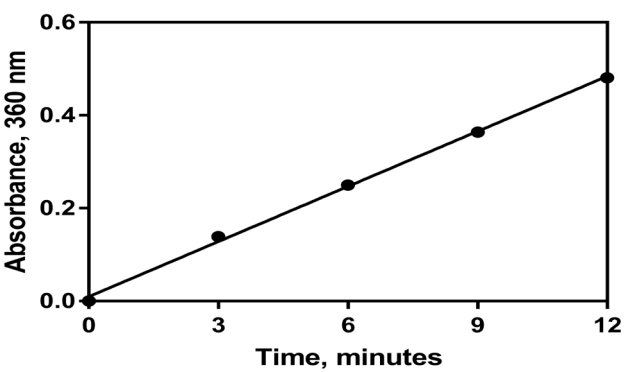

Figure 3. Linearity of Step 1: enzyme/substrate reaction over time for highly purified phytase from *Aspergillus niger,* at pH 5.5 and 40°C.

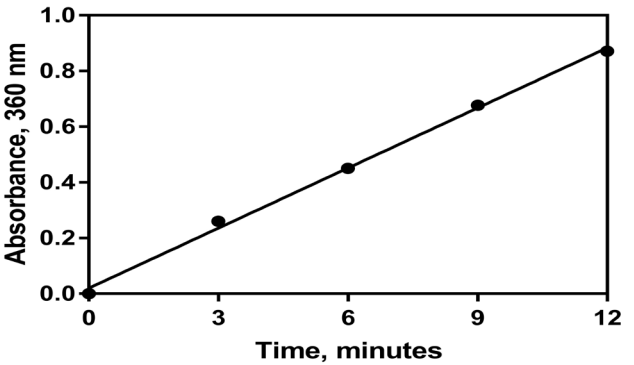

Figure 4. Linearity of Step 1: enzyme/substrate reaction over time for a commercial phytase from *Aspergillus oryzae,* at pH 5.5 and 40°C.

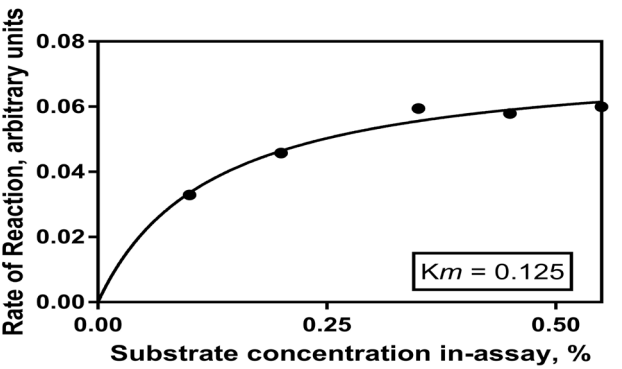

Figure 5. Michaelis-menten kinetics for a highly purified phytase from *Aspergillus niger,* with varying substrate concentrations in Step 1: enzyme/substrate reaction, at pH 5.5 and 40°C.

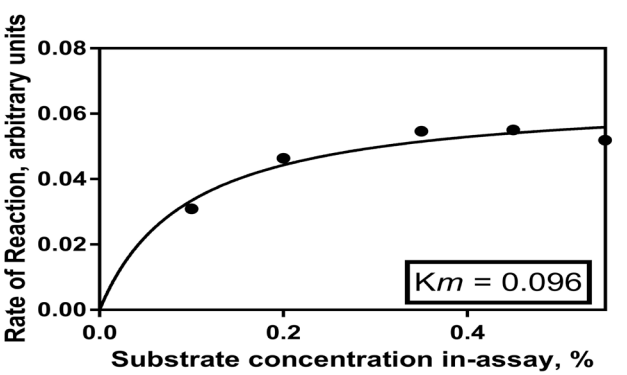

Figure 6. Michaelis-menten kinetics for a commercial phytase from *Aspergillus oryzae,* with varying substrate concentrations in Step 1: enzyme/substrate reaction, at pH 5.5 and 40°C.

C. Repeatability

	Absorbance 360 nm, $\Delta A_{phytase}$	
	2.8 mU/test	22.4 mU/test
Extract 1	0.05	0.354
Extract 2	0.052	0.397
Extract 3	0.049	0.371
Extract 4	0.052	0.355
Extract 5	0.051	0.396
Extract 6	0.048	0.352
Extract 7	0.049	0.383
Extract 8	0.042	0.359
n (extractions)	8	8
MEAN	0.049	0.370875
ST. DEV	0.00	0.02
RSDr (%)	6.57	5.09

Table 1. The repeatability of the assay for liquid enzyme preparations was determined by analysis of 1 highly purified sample of phytase at 2 concentrations (prepared as described in Example A - liquid preparations on page 7) over a period of 3 days, with 8 separate extractions of enzyme analysed in duplicate as per the standard procedure.

	Absorbance 360 nm				
	Sample blank (A_2-A_1)	Sample (A_2-A_1)		$\Delta A_{phytase}$	U/Kg
Extract 1	0.243	0.633	0.638	0.393	886.00
Extract 2	0.285	0.634	0.631	0.348	784.55
Extract 3	0.280	0.619	0.638	0.349	786.81
Extract 4	0.283	0.613	0.617	0.3325	749.61
Extract 5	0.296	0.688	0.683	0.39	879.24
Extract 6	0.278	0.61	0.617	0.3355	756.37
Extract 7	0.242	0.632	0.637	0.3925	884.88
Extract 8	0.251	0.601	0.61	0.3545	799.21
				n (extractions)	8
				MEAN	815.83
				ST. DEV	58.22
				RSDr (%)	7.14

Table 2. The repeatability of the assay for feed samples was determined by analysis of 1 commercially available sample of chicken feed (prepared as described in Examples B and C on page 7) over a period of 3 days, with 8 separate extractions of enzyme analysed in duplicate as per the standard procedure.

D. Comparison to ISO Method

	U/Kg					
	Sample 1		Sample 2		Sample 3	
	ISO 30024	K-PHYTASE	ISO 30024	K-PHYTASE	ISO 30024	K-PHYTASE
Extract 1	728.63	617.7	2629.66	1717.9	909.42	394.53
Extract 2	1271.00	662.8	2333.82	1451.87	679.33	384.76
MEAN	999.82	640.25	2481.74	1584.89	794.38	389.65
ST. DEV	383.51	31.89	209.19	188.11	162.70	6.91
RSDr (%)	38.36	4.98	8.43	11.87	20.48	1.77

Table 3. Commercially available animal feed samples were analysed as per ISO 30024:2009(E) and **K-PHYTASE** with two extractions of sample.

Using ISO 30024 - One Unit of activity is defined as the amount of enzyme that catalyses the release of one µmole of inorganic phosphate per minute from 5.1 mM sodium phytate, at pH 5.5 and 37°C.

Using **K-PHYTASE** - One Unit of activity is defined as the amount of enzyme that catalyses the release of one µmole of inorganic phosphate per minute from 4.5 mM phytic acid, at pH 5.5 and 40°C.

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