PHYTASE FROM *ASPERGILLUS NIGER* EXPRESSED IN *A. NIGER*

Prepared at the 77th JECFA (2013) and published in FAO JECFA Monographs 14 (2013) superseding specifications prepared at the 76th JECFA (2012) and published in FAO JECFA Monographs 13 (2012). An ADI "not specified" was established at the 76th JECFA (2012).

SYNONYMS Phytase, 3-phytase

SOURCES Phytase is produced by submerged fed-batch fermentation of a non-pathogenic and non-toxicogenic genetically modified strain of *Aspergillus niger* which contains the phytase encoding gene derived from *A. niger*. The enzyme is secreted and isolated from the fermentation broth by filtration to remove the biomass and concentrated by ultrafiltration. The enzyme concentrate is subjected to germ filtration and is subsequently formulated and standardized to the desired activity using food-grade compounds.

Active principles: 3-phytase

Systematic names and numbers: Myo-Inositol hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8, CAS 37288-11-2

Reactions catalysed: Hydrolysis of myo-inositol hexakisphosphate (phytate) to inositol pentaphosphate (IP5), and further to give a mixture of myo-inositol di-phosphate (IP2), myo-inositol mono-phosphate (IP1) and free orthophosphate

Secondary enzyme activities: No significant levels of secondary enzyme activities

DESCRIPTION Brownish liquid or yellow to light brown powder.

FUNCTIONAL USES Enzyme preparation.
Used to degrade phytate found in plant derived foods, particularly cereal grains and legumes, in order to improve mineral bioavailability.

GENERAL SPECIFICATIONS Must conform to the current edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Phytase activity: The sample shows phytase activity
See description under TESTS

TESTS

Phytase activity

Principle
This procedure is used to determine the activity of enzymes releasing phosphate from phytate. The assay is based on enzymatic hydrolysis of sodium phytate under controlled conditions by measurement of the amount of inorganic phosphate released. The phytase activity is expressed in phytase units (FTU). One phytase unit (FTU) is defined as

the amount of enzyme that liberates 1 micromole of inorganic phosphorus per minute from 0.0051 mol/l sodium phytate at 37° and pH 5.50 under the conditions of the test.

Reagents and Solutions
NOTE - Ensure the absence of phosphate in all glassware.

Acetate buffer pH 5.50: Dissolve 1.76 g (1.68 ml) glacial acetic acid (C2H4O2), 30.02 g of sodium acetate trihydrate ($C_2H_3O_2Na•3H_2O$), and 0.147 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) in about 900ml of water. Transfer the solution into a 1000 ml volumetric flask, dilute to volume with water, and mix. Adjust the pH to 5.50 ± 0.05.

Substrate solution: Dissolve 8.40 g of sodium phytate decahydrate ($C_6H_6O_{24}P_6Na_{12}•10H_2O$) in 900 ml of acetate buffer. Adjust the pH to 5.50 ± 0.05 at 37 ° by adding 4 M acetic acid. Cool to ambient temperature. Quantitatively transfer the mixture to a 1000 ml volumetric flask, dilute to volume with acetate buffer, and mix. Prepare fresh daily.

Nitric acid solution (27%): While stirring, slowly add 70 ml of 65% nitric acid to 130 ml of water.

Ammonium heptamolybdate solution: Dissolve 100 g of ammonium heptamolybdate tetrahydrate [$(NH_4)_6Mo_7O_{24}•4H_2O$] in 900 ml of water in a 1000 ml volumetric flask. Add 10 ml of 25% ammonia solution, dilute to volume with water, and mix. This solution is stable for 4 weeks when stored at ambient temperature and shielded from light.

Ammonium vanadate solution: Dissolve 2.35 g of ammonium monovanadate (NH_4VO_3) in 400 ml of warm (60°) water. While stirring, slowly add 20ml of nitric acid solution (27%). Cool to ambient temperature. Quantitatively transfer the mixture to a 1000 ml volumetric flask, dilute to volume with water, and mix. This solution is stable for 4 weeks when stored at ambient temperature and shielded from light.

Colour/stop solution: While stirring, add 25 ml of ammonium vanadate solution to 25 ml of ammonium heptamolybdate solution. Continue stirring and slowly add 16.5 ml of 65% nitric acid. Cool to ambient temperature; quantitatively transfer the mixture to a 100 ml volumetric flask, dilute to volume with water, and mix. Prepare fresh daily.

Potassium dihydrogen phosphate solution: Dry a sufficient amount of >99% purity potassium dihydrogen phosphate (KH_2PO_4) in an oven at 105° for 4 h. Cool to ambient temperature in a desiccator over dried silica gel. In two separate 1 L volumetric flasks, weigh accurately 0.245 g of dried potassium dihydrogen phosphate and dilute with acetate buffer to 1 L to obtain potassium dihydrogen phosphate solutions A and B, each containing 1.80 mmol/L of potassium dihydrogen phosphate.

Phytase standard: obtained from DSM, Delft, The Netherlands, with an assigned activity or equivalent.

Phytase standard solution: In duplicate, accurately weigh an adequate amount of phytase standard and dissolve and dilute in acetate buffer to obtain a solution containing 0.06 ± 0.01 phytase units per 2.0 ml. Quantify the activity of the phytase standard according to procedure described below.

Procedure for quantifying the activity of the phytase standard

Using 6 individual 20×150 mm glass test tubes add to one tube 2.00 ml of the phytase standard solution, add to 3 tubes 2.00 ml of potassium dihydrogen phosphate solution A, and to the remaining 2 tubes 2.00 ml of potassium dihydrogen phosphate solution B. Place the tubes into a 37.0 ± 0.1° water bath, at regular time intervals and allow their contents to equilibrate for 5 min.

At time equals 5 min, in the same order and within the same time intervals as the tubes were added, add 4.0 ml of substrate solution (previously equilibrated to 37.0±0.1°), to each of the tubes. Mix the tubes, and replace in the 37.0 ± 0.1° water bath.

At time equals 35 min, in the same order and within the same time intervals, terminate the incubation by adding 4.0 ml of colour/stop solution to each of the tubes. Mix, and cool to ambient temperature.

Prepare an enzyme blank by adding 2.00 ml of phytase standard solution into one 20 ×150 mm glass test tube. Prepare reagent blanks by adding 2.00 ml of water into a series of five separate 20 ×150 mm glass test tubes. Add 4.0 ml of colour/stop solution to all blank tubes and mix. Next add 4.0 ml of substrate solution, and mix.

Determine the absorbance of all solutions at 415 nm in a 1 cm path-length cell with a spectrophotometer, using water to zero the instrument.

Calculation of the activity of the phytase standard

Calculate the corrected absorbances (AR) for each sample preparation (absorbance of the standard phytase solution minus the corresponding absorbance of the blank) and for each potassium dihydrogen phosphate solution, Ap (absorbance of the potassium dihydrogen phosphate solution minus average absorbance of the reagent blanks). Calculate C, the phosphate concentration of each potassium dihydrogen phosphate solution:

$$C\ (\text{mmol}/2\ \text{ml}) = (W \times 1000 \times 2)/MW.$$

Calculate the absorbances (D) for each potassium dihydrogen phosphate solution after correction for the amount of potassium dihydrogen phosphate weighed:

$$Ap/C = D\ (\text{absorbance units/mmol of phosphate per 2 ml})$$

Calculate the average of results D, giving E (maximum allowable difference, 5%).

Calculate the activity for the phytase standard:

$$FTU/g = (AR \times f)/(30 \times R \times E)$$

where,

AR is the corrected absorbance of the phytase standard solution;
f is the total dilution factor of the standard preparation;
30 is the incubation time, in min;
R equals sample weight, in g;
E is average of D factors;
W is the weight of potassium dihydrogen phosphate, in g;
MW is the molecular weight of potassium dihydrogen phosphate, 136.09 (g/mol).

Determination of phytase activity in samples

Sample preparation: Suspend or dissolve and dilute accurately weighed amounts of sample in acetate buffer so that 2.0 ml of the final solution will contain between 0.01 and 0.08 phytase units.

Preparation of phytase standard curve: Weigh, in duplicate, with an accuracy of ± 1 mg, an amount of phytase standard based on the activity which corresponds to about 20,000 phytase units in 200 ml volumetric flasks. Dissolve in and dilute to volume with acetate buffer, and mix. Use this stock solution and dilute with acetate buffer to obtain standard solutions containing approximately 0.01, 0.02, 0.04, 0.06, and 0.08 phytase units per 2.0 ml.

Add 2.00 ml of each phytase standard solution and 2.00 ml of the sample solution into separate 20-×150-mm glass test tubes. Place the tubes into a 37.0±0.1° water bath, at regular time intervals and allow their contents to equilibrate for 5 min. At time equals 5 min, in the same order as the tubes were added, add 4.0 ml of substrate solution (previously equilibrated to 37.0 ± 0.1°) to the each of the test tubes. Mix, and replace in the 37.0 ± 0.1°water bath. At time equals 35 min, in the same order and within the same time intervals, terminate the incubation by adding 4.0 ml of colour/stop solution to each of the tubes. Mix, and cool to ambient temperature.

Prepare enzymes blanks as described for quantifying the activity of the phytase standard. Centrifuge all test tubes for 5 min at 3000 ×g. Determine the absorbance of each solution at 415 nm in a 1-cm path-length cell with a spectrophotometer, using water to zero the instrument.

Calculation of phytase activity in samples

Calculate the corrected absorbance (sample minus blank) for each sample preparation and phytase standard solution. Plot the calculated phytase activity (FTU per 2 ml) of each phytase solution against the corresponding absorbance. From the curve, determine the phytase activity in each sample preparation (FTU per 2 ml):

$$\text{Activity (FTU/g)} = (\text{FTU per 2 ml} \times \text{dilution})/\text{sample weight}$$