

Determination of Phytase Activity—Molybdate-Blue Method

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A1. PRINCIPLE

Determination of phytase activity is based on the colorimetric quantification at 700nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdate as color reagent.

1 U is the amount of enzyme that liberates 1 μmol inorganic orthophosphate per minute under test conditions (pH 5.0; temperature 37°C; and substrate concentration, sodium phytate at 0.0051 mol/L).

A2. REAGENTS

All the reagents used are of analytical grade. Detergents containing phosphate should not be used in washing container.

A2.1. Water—Distilled water, or equivalent.

A2.2. Buffer solution (0.1 mol/L)—Dissolve 5.742 g sodium acetate, 0.5 g Triton X-100 and 0.5 g bovine serum albumin in 900 mL water; adjust to pH 5.0 with acetic acid (100%), and dilute to 1 L with water.

A2.3. Substrate solution—Dissolve 577.4 mg sodium phytate ($\text{C}_6\text{H}_6\text{O}_{24}\text{P}_6\text{Na}_{12}$) from rice (Cat. No. P-3168, Sigma Chemical Co., St. Louis, MO) and 574.2 mg sodium acetate in 90 mL water, adjust the pH to 5.0 with acetic acid (100%), and dilute to 100 mL with water. Prepare this solution fresh daily.

A2.4. Reaction stop solution—Trichloroacetic acid (5%).

A2.5. Ammonium heptamolybdate stock solution (Solution A)—Dissolve 7.5 g ammonium heptamolybdate ($\text{N}_6\text{H}_{24}\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$) in 400 mL distilled water, slowly add 22 mL sulfuric acid (98%), and dilute to 500 mL with water. This solution may be kept at 4°C shielded from light for 1 month.

A2.6. Ferrous sulfate stock solution (Solution B)—Ferrous sulfate (2.7%). This solution may be kept at 4°C and shielded from light for 1 month.

A2.7. Color mix—Mix 100 mL solution A and 25 mL solution B. Prepare this solution fresh daily.

A2.8. Potassium dihydrogen phosphate stock solution—Prepare potassium dihydrogen phosphate to constant weight at 60°C before dissolving it to a final concentration of 4.0 mmol/L using buffer solution (A2.2). Prepare this solution fresh daily.

A3. APPARATUS

A3.1. Waterbath: thermostatically controlled to $37.0 \pm 0.1^\circ\text{C}$ by circulating water.

A3.2. Ultraviolet-visible spectrophotometer

A3.3. Centrifuge: provided with inserts for 12 centrifuge tubes of 7 mL each and used at a

relative centrifugal force of 3000g

A3.4. pH meter

A4. PREPARATION OF STANDARD SOLUTIONS AND CURVE

Prepare working standards of 0.0、 0.8、 1.6、 2.4、 3.2、 4.0mmol/L potassium dihydrogen phosphate solution by serial dilution of stock solution (A2.8). Carry out the procedure as described in Table A1, and then plot the absorbance difference of the standard solutions (X-axis) against the corresponding exactly calculated amount of potassium dihydrogen phosphate (Y-axis). Draw the best fitting curve through the origin and give the regression equation ($Y=KX+B$).

A5. PREPARATION OF SAMPLE

Dilute the weighted sample in duplicate (sample and blank) with buffer solution to a phytase activity within 0.03-0.08 FTU/mL.

A standard sample with exactly calculated activity is recommended to be determined as the same procedure to test the accuracy.

A6. ASSAY

The assay is carried out as the following procedure (Table A1). In this procedure, interval of adding reagents to every tube should be completely coincident after the substrate is added to the reaction mixture.

Table A1

Procedure	Sample, Standards	Sample blank	Standards blank
Sample solution (mL)	0.2	0.2	—
Buffer solution (A2.2) (mL)	—	—	0.2
5min at 37°C	√	√	√
Substrate solution (A2.3) (mL)	0.8	0.8 (the second step)	0.8 (the second step)
Mixing	√	√	√
30min at 37°C	√	—	—
Stop solution (A2.4) (mL)	1.0	1.0 (the first step)	1.0 (the first step)
Color reagent (A2.7) (mL)	1.0	1.0	1.0
Mixing	√	√	√
Total volume (mL)	3.0	3.0	3.0

Centrifuge all the tubes for 10 min at 4,000 rpm before standing for 10 min at room temperature. Measure the absorbance of sample (A) and its blank (A_0) at 700 nm with the spectrophotometer after zeroing the instrument with standards blank of. Determine the enzyme activity by reading the corrected absorbance difference for the sample ($A-A_0$) and calculating the released phosphorus. Enzyme activity is expressed in activity units (FYU). 1

FYU is the amount of enzyme that liberates 1 μmol inorganic orthophosphate per minute under test conditions (pH5.0; temperature 37°C; and substrate concentration, sodium phytate [C₆H₆O₂₄P₆Na₁₂] at 0.005 mol/L).

A7. CALCULATION AND EXPRESSION

Activity of sample (U) is calculated as follows:

$$U = \frac{K \times (A - A_0)}{S \times m \times 30} \times F$$

In which: *U*—Activity of sample, U/g;

K—Slope of standard curve;

F—Dilution multiple;

S—Determination amount of sample; *S*=0.2 (mL) in Table A1;

m—Sample weight, g;

30—Time of reaction, min.

The final result is from two average values, and should be expressed by whole number.

A8. DEVIATION PERMITTED

The relative deviation of two parallel values from one sample should be less than 8%.
