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APPENDIX

Appendix 1:

Enzymatic Assay of Phytase (Heinonen et al., 1981)

1. Objective

Determine the activity of phytase,

2. Scope

The scope of this procedure includes the following reaction:

Phytic Acid + H2O Phytase ------- > p-myo-inositol 1, 2, 4, 5, 6-PKP + Pi

3. Definitions

3.1. Purified Water – Water from a deionizing system

3.2. p-myo-inositol 1,2,4,5,6-PKP = p-myo-inositol 1,2,4,5,6-Pentakisphosphate

3.3. Phytic Acid = Myo-Inositol Hexakis (dihydrogen phosphate)

3.4. Pi = Inorganic Phosphate

4. Discussion

4.1. Phytic Acid + H_2O Phytase -------> p-myo-inositol 1, 2, 4, 5, 6-PKP + Pi

4.2. Method = Colorimetric Determination, Temperature (T) = 37°C, pH = 2.5, A420nm, Light path = 1cm

5. Procedure

5.1 Reagents

5.1.1 200 mM Glycine Buffer, pH 2.8 at 37°C (Buffer)

5.1.1.1. Prepare 500 ml in purified water using Glycine.

5.1.1.2. Adjust to pH 2.5 at 37°C with 1M NaOH or 1M HCl.

5.1.2. 0.5478 mM Phytic Acid Solution, pH 2.5 at 37°C (Phytic Acid)

5.1.2.1. Prepare 500 ml in Reagent 5.1.1 using Phytic Acid, Dipotassium.

5.1.2.2. Adjust to pH 2.5 at 37°C with 1M HCl.

5.1.3. 20% (w/v) Ammonium Molybdate Solution (Amm Moly)

Prepare 100 ml in hot water using Molybdic Acid, Ammonium Tetrahydrate Salt.

5.1.4 70% nitric acid.

5.1.5 1% (w/v) ammonium vanadate

Prepare 100 ml in hot water using Molybdic Acid, Ammonium Tetrahydrate Salt.

5.1.6 Color Reagent Solution (CRS)

5.1.6.1 Prepare 1000 ml by adding 100 ml of Reagent 5.1.3 to 140 ml of Reagent 5.1.4 and 100 ml of Reagent 5.1.5.

5.1.6.2 Mix and use immediately.

5.1.6.3 Prepare fresh.

5.1.7 2.8 mM Potassium Phosphate Solution (P Std.)

Prepare 250 ml in deionized water using Potassium Phosphate, Monobasic.

5.1.8 Phytase Enzyme Solution (Enzyme)

Immediately before use, prepare a solution containing 0.5 – 2.0 units/ml of Phytase in cold Reagent 5.1.1.

5.2. Procedure – Blank/Test Preparation

5.2.1. Pipette (in milliliters) the following reagents into a 4 dram vial:

	Test	**Blank**
Reagent 7.1.2 (**Phytic Acid**)	15	15

5.2.2. Equilibrate to 37°C, then add:

Reagent 5.1.8 (**Enzyme**)	0.5	----
Reagent 5.1.1 (**Buffer**)	----	0.5

5.2.3. Immediately mix by inversion and incubate at 37°C for exactly 20 minutes. Then add:

Reagent 5.1.6 (CRS)	12.50	12.50
Purified Water	22	22

5.2.4. Mix by swirling, then transfer to suitable cuvettes and record the A420nm for both the test and blank using a suitable spectrophotometer.

5.3. Procedure – Standard Curve

5.3.1 Prepare by pipetting (in milliliters) the follow reagents into suitable containers:

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std. Blk.
Purified Water	7.5	6.25	5	3.75	2.5	1.25	-----	8.75
Reagent 5.1.7 (**P Std.**)	1.25	2.5	3.75	5.00	6.25	7.5	8.75	------
Reagent 5.1.2 (**Phytic Acid**)	15	15	15	15	15	15	15	15

5.3.2 Mix by swirling and incubate at 37°C for 20 minutes. Then add:

Reagent 5.1.6 (**CRS**)	12.5	12.5	12.5	12.5	12.5	12.5
Purified Water	13.75	13.75	13.75	13.75	13.75	13.75

5.3.3 Mix by swirling, then transfer to suitable cuvettes and record the A_{420nm} for both the standards and the standard blank using a suitable spectrophtometer.

5.4. Calculations

5.4.1 Standard Curve

5.4.1.1 ΔA_{420nm} Standard = A_{420nm} Standard - A_{420nm} Standard Blank

5.4.1.2 Prepare a standard curve by plotting ΔA_{420nm} versus µmoles of phosphate.

5.4.2 Sample Determination

5.4.2.1 ΔA_{420nm} Test = A_{420nm} Test - A_{420nm} Test Blank

5.4.2.2 Determine the µmoles of phosphate liberated using the standard curve.

5.4.2.3 Units/ml enzyme = $\frac{(\mu\text{moles of phosphate released})\ (df)}{(20)(0.5)}$

df = dilution factor

20 = Time (in minutes) of assay per the Unit Definition

0. 5 = volume (in milliliters) of enzyme used

5.4.2.4 Units/mg solid = $\frac{\text{units/ml enzyme}}{\text{Mg solid/ml enzyme}}$