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ProductInformation

SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of α-AMYLASE[1] Inhibitor

Prod. No. A1520, A-3410,and A 3535

PRINCIPLE: Starch + H_2O $\xrightarrow{\alpha\text{-Amylase}}$ Reducing Groups (Maltose)

CONDITIONS: T = 20°C, pH = 6.9, A_{540nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

A. 20 mM Sodium Phosphate Buffer with 6.7 mM Sodium Chloride, pH 6.9 at 20°C
(Prepare 100 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751, and Sodium Chloride, Sigma Prod. No. S-9625. Adjust to pH 6.9 at 20°C with 1 M NaOH.)

B. 1.0% (w/v) Soluble Starch Solution (Starch)
(Prepare 25 ml in Reagent A using Starch Potato Soluble, Sigma Prod. No. S-2630. Facilitate solubilization by heating the starch solution in a glass beaker directly on a heating/stir plate using constant stirring. Bring to boil and maintain the solution at this temperature for 15 minutes. Allow the starch solution to cool to room temperature with stirring. Return the starch solution to its original volume (25 ml) by the addition of water and dispense samples for assay while stirring.)

C. Sodium Potassium Tartrate Solution
(Dissolve 12.0 grams of Sodium Potassium Tartrate, Tetrahydrate, Sigma Prod. No. S-2377, in 8.0 ml of 2 M NaOH. Heat directly on a heating/stir plate using constant stirring to dissolve. **DO NOT BOIL**.)

D. 96 mM 3,5-Dinitrosalicylic Acid Solution
(Prepare 20 ml in deionized water using 3,5-Dinitrosalicylic Acid, Sigma Prod. No. D-0550. Heat directly on a heating/stir plate using constant stirring to dissolve. **DO NOT BOIL**. Maintain at 45°C to 50°C throughout assay.)

E. Color Reagent Solution (Clr Rgt Soln)
(With stirring, slowly add Reagent C to Reagent D. Dilute to 40 ml with deionized water. If not completely dissolved, the reagents should dissolve when mixed. The solution should be stored in an amber bottle at room temperature. The Color Reagent Solution is stable for 6 months.)

F. 0.2% (w/v) Maltose Standard Solution
(Prepare 10 ml in deionized water using Maltose, Monohydrate, Sigma Prod. No. M-5885.)

G. 50 mM Sodium Phosphate, 50 mM Sodium Chloride, 0.5 mM Calcium Chloride,0.1% Boive Serum Albumin pH 6.9 at 20°C (Buffer)
(Prepare 100 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751, and Sodium Chloride, Sigma Prod. No. S-9625, Calcium Chloride,Dihydrate, Sigma Prod. No. C-3881, and Albumin, Bovine Serum, A-4503 Adjust to pH 6.9 at 20°C with 1 M NaOH.)

H. α-Amylase Solution (HAYS)
(Immediately before use, prepare a solution containing 40 unit/ml of α-Amylase in cold deionized water α-Amylase from human saliva, lyophilized Powder, Sigma Prod. No. A-0521)[2]

I. α-Amylase Solution (PAYS)
(Immediately before use, prepare a solution containing 40 unit/ml of α-Amylase in cold deionized water using α-Amylase from procine pancreas, saline suspension, Sigma Prod. No. A-4268.)[2]

J. α-Amylase Inhibitor Solution Against α-Amylase from Human Saliva (INH-HAYS)
(Immediately before use, prepare a solution in cold deionized water using α-Amylase from Triticum Aestivum at 1 mg / ml. Store on ice. Immediately prior to adding to pre-incubation mixture, dilute to 50 μg Protein / ml in cold deionized water.)

K. α-Amylase Inhibitor Solution Against α-Amylase from procine pancreas(INH-PAYS)
(Immediately before use, prepare a solution in cold deionized water using α-Amylase from Triticum Aestivum at 1 mg / ml. Store on ice. Immediately prior to adding to pre-incubation mixture, dilute to 200 μg Protein / ml in cold deionized water.)

PROCERDURE:

A. Trial Enzymatic Assay of Amylase:

Preincubation-Step-1:

Pipette (in milliliters) the following reagents into suitable containers:

	Uninhib Test-1	Uninhib. Test-2
Reagent G (Buffer)	2.00	2.00
Reagent H (HAYS)	0.10	----
Reagent I (PAYS)	----	0.10
MilliQ-deionized Water	0.04	0.04
Reagent J (INH-HAYS)	----	----
Reagent K (INH-PAYS)	----	----

Mix by swirling and equilibrate to 25◦C for 30 minutes. Then proceed to Enzymatic-Step-2:

Enzymatic-Step-2(Blanks):

	Uninhib Test-1-BLANK	Uninhib. Test-2-BLANK
Reagent B (Starch)	1.0	1.0
Deionized Water	0.5	0.5

Mix by swirling and equilibrate to 20◦C. Then add:

Reagent E (Clr Rgt Soln)	1.0	1.0
Preincubation-Step-1	0.5	0.5

Cap and place in a boiling water bath for exactly 15 minutes,then cool to room temperature and add:

Deionized water	9.0	9.0	9.0	9.0

Mix by inversion and record the A_{540nm} for both the Uninhibited-Test-Blank and the Test-Blank using a suitable spectrophotometer.

Enzymatic-Step-2(Uninhibited TEST-Amylase):

	Uninhib. Test-1	Uninhib. Test-2
Reagent B (Starch)	1.0	1.0
Deionized Water	0.5	0.5

Mix by swirling and equilibrate to 20◦C. Then add:

Preincubation-Step-1	0.5	0.5

Mix by swirling and incubate for exactly 3.0 minutes at 20◦C. Then add:

Reagent E	1.0	1.0

Cap and place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and add:

Deionized water	9.0	9.0

Mix by inversion and record the A_{540nm} for both the Uninhibited-Test and Test using a suitable spectrophotometer. Determine the Amylase Units per Uninhibited Reaction. Adjust enzyme concentration and prepare a fresh enzyme solution to obtain 2.0 Units per Uninhibited Reaction. Proceed with Amylase Inhibitor Assay.

B. Enzymatic Assay of Amylase Inhibitor:

Preincubation-Step-1:

Pipette (in milliliters) the following reagents into suitable containers:

	Uninhib Test-1	Uninhib. Test-2	Test-1[2]	Test-2[2]
Reagent G (Buffer)	2.00	2.00	2.00	2.00
Reagent H (HAYS)	0.10	----	0.10	----
Reagent I (PAYS)	----	0.10	----	0.10
MilliQ-deionized Water	0.04	0.04	----	----
Reagent J (INH-HAYS)	----	----	0.04	----
Reagent K (INH-PAYS)	----	----	----	0.04

Mix by swirling and equilibrate to 25◦C for 30 minutes. Then proceed to Enzymatic-Step-2:

Enzymatic-Step-2(Blanks):

	Uninhib Test-1-BLANK	Uninhib. Test-2-BLANK	Test-1-BLANK	Test-2-BLANK
Reagent B (Starch)	1.0	1.0	1.0	1.0
Deionized Water	0.5	0.5	0.5	0.5
Mix by swirling and equilibrate to 20°C. Then add:				
Reagent E (Clr Rgt Soln)	1.0	1.0	1.0	1.0
Preincubation-Step-1	0.5	0.5	0.5	0.5

Cap and place in a boiling water bath for exactly 15 minutes, then cool to room temperature and add:

Deionized water	9.0	9.0	9.0	9.0

Mix by inversion and record the A_{540nm} for both the Uninhibited-Test-Blank and the Test-Blank using a suitable spectrophotometer.

Enzymatic-Step-2(TEST):

	Uninhib. Test-1	Test-2	Uninhib. Test-1	Test-2
Reagent B (Starch)	1.0	1.0	1.0	1.0
Deionized Water	0.5	0.5	0.5	0.5
Mix by swirling and equilibrate to 20°C. Then add:				
Preincubation-Step-1	0.5	0.5	0.5	0.5
Mix by swirling and incubate for exactly 3.0 minutes at 20°C. Then add:				
Reagent E	1.0	1.0	1.0	1.0
Cap and place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and add:				
Deionized water	9.0	9.0	9.0	9.0

Mix by inversion and record the A_{540nm} for both the Uninhibited-Test and Test using a suitable spectrophotometer.

Standard Curve:

A standard curve is made by pipetting (in milliliters) the following reagents into suitable containers:

	Std 1	Std 2	Std 3	Std 4	Std 5	Std-6	Std Blank
Reagent F (Std Soln)	0.05	0.10	0.20	0.50	0.75	1.00	----
Deionized Water	1.95	1.90	1.80	1.50	1.25	1.00	2.00

Reagent E (Clr Rgt Soln)	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Cap and place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and add:

Deionized Water	9.00	9.00	9.00	9.00	9.00	9.00	9.00

Mix by inversion and record the A_{540nm} for the Standards and Standard Blank using a suitable spectrophotometer.

CALCULATIONS:

Standard Curve:

ΔA_{540nm} Standard = A_{540nm} Std - A_{540nm} Std Blank

Plot the ΔA_{540nm} of the Standards vs milligrams of Maltose.

Sample Determination:

Δ_{540nm} Uninhibited-Test-1 = A_{540nm} Uninhibited-Test-1 - A_{540nm} Uninhibited-Test-1-Blank
Δ_{540nm} Uninhibited-Test-2 = A_{540nm} Uninhibited-Test-2 - A_{540nm} Uninhibited-Test-2-Blank
Δ_{540nm} Test-1 = A_{540nm} Test-1 - A_{540nm} Test-1-Blank
Δ_{540nm} Test-2 = A_{540nm} Test-2 - A_{540nm} Test-2-Blank

Determine the milligrams of Maltose liberated using the Standard Curve.

$$\frac{\text{Uninhibited Amylase Units}}{\text{Inhibitor Reaction}} = \frac{\text{(mg of Maltose released)(2.14)}}{0.50}$$

2.14 = Volume (in milliliter) of Total Inhibition/Uninhibited Reaction Mixture
0.50= Volume (in milliliter) of Step-1 Uninhibited Enzyme used in Colorimetric Detection Reaction

$$\%\ \text{Inhibition (with HAYS)}^3 = \frac{[(\text{UIA Units/UIA RXN}) - (\text{UIA Units/IA RXN}] \times 100}{(\text{UIA Units/UIA RXN})}$$

$$\%\ \text{Inhibition (with HAYS)}^3 = \frac{[(\text{UIA Units/UIA RXN}) - (\text{UIA Units/IA RXN}] \times 100}{(\text{UIA Units/UIA RXN})}$$

UIA = Uninhibited Amylase
RXN = Reaction
IA = Inhibited Amylase
CF(50%) = 50%/% Inhibition

Abbreviation:
CF(50%) = Correction Factor for Percent Inhibition at 50% per unit definition
CF(2 Units) = Actual Units of Amylase per Uninhibited Reaction/ 2.00
CF(2 Units) = Correction Factor for 2.00 Units per Uninhibited and Inhibited Reaction Mixture per Unit Definition

Milligrams of Amylase Inhibitor = 0.04 X Conc.(mg Solid/ ml) X Percent Protein
Reaction Mixture

mg = Milligrams
Percent Protein as determined by Protein Biuret Method
0.04 = Volume (in milliliters) of Reagent J or Reagent K

Normalized Inhibitory Units = (Milligrams of Amylase Inhibitor/RXM) X CF(50%)
CF(2 Units)

Actual Inhibitory Units = 2.00/Normalized Inhibitory Units

UNIT DEFINITION:

α-Amylase:
One unit will liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C.

Amylase Inhibitor Units:
One unit will reduce the activity of two units of α-Amylase,Human Saliva(A-0251) by 50% after preincubation at 25°C.

One unit will reduce the activity of two units of α-Amylase,Porcine(A-4268) by 50% after preincubation at 25°C.

FINAL ASSAY CONCENTRATIONS:

In a 2.00 ml reaction mix, the final concentrations are 10 mM sodium phosphate, 0.50% (w/v) starch, 3.4 mM sodium chloride and 1 unit α-amylase.

REFERENCE:

Bernfeld, P. (1955) *Methods in Enzymology* **1**, 149-158
O'Donnell,M. and McGeeney, K.(1976) *Biochim. Biophys. Acta* **422,** 159-169
Silano, V. (1975) *Biochim. Biophys. Acta* **391**, 170

NOTES:

1. α-Amylases, A-4268 and A-0521, must be run with each lot of α-Amylase Inhibitor.
2. The inhibitor can be pipetted at different levels, 10µL, 20µL, 30µL, and 40µL to ensure a percent inhibition in the range of 40 to 65.
3. This assay is only valid with the percent inhibition from 40 to 65.
4. This assay is based on the cited reference.
5. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.