POLYGALACTURONIC ACID (from Citrus Pectin) (Lot 200801)

CAT. NO: **P-PGACIT** **08/20**
CAS: **9049-37-0**
Source: **Citrus pectin**

STRUCTURE

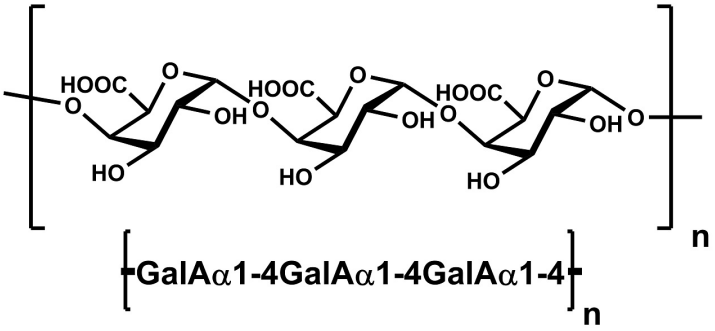

Figure 1. Schematic representation of polygalacturonic acid.

DESCRIPTION

Polygalacturonic acid is a linear homopolymer comprised of α-1,4-linked galacturonic acid residues. Note that this product contains ~ 5% methylation and is the **recommended substrate for the assay of endo-polygalacturonanase** using either the Nelson-Somogyi or DNS assay procedures. Details of these procedures are outlined in this document.

PREPARATION

Polygalacturonic acid is prepared from citrus pectin by demethylation, neutralisation, filtration and recovery. Typically, the polysaccharide is > 85% pure, the additional weight being sodium. Uronic acid content is ~ 94-96% w/w of the polysaccharide.

PROPERTIES

Purity: > 96%
Sugar Composition:

Galacturonic acid	94
Galactose	1.0
Arabinose	0.2
Rhamnose	1.0
Xylose	1.0
Glucose	0.3
Protein	1.0%
Ash:	0.3%
Moisture:	8.0%

STORAGE CONDITIONS

Store dry at room temperature in a well-sealed container. Under these conditions, the product is stable for several years.

COMPARISON OF ACTIVITY DATA OBTAINED FROM NELSON/SOMOGYI AND DNS METHODS:

When using the Nelson-Somogyi (NS) and DNS methods for the measurement of *endo*-polygalacturonanase activity, the DNS method gives an activity approximately twice that obtained with the NS method. For example, with *endo*-polygalacturonanase from *Aspergillus aculeatus* (Megazyme cat. no. **E-PGALUSP**), activity measured with the NS procedure (as detailed below) is 1,351 U/mL at pH 5.5 and 40°C. When assayed with the DNS procedure under the same incubation conditions (also detailed below), an activity value of 2,330 U/mL is obtained.

To understand the reason for this difference in determined activities using the NS and DNS procedures, the colour responses of equimolar concentrations of galacturonic acid, digalacturonic acid and trigalacturonic acid were determined with the NS and DNS procedures and are shown below. It is evident that essentially the same colour response is obtained with equimolar amounts on mono-, di- and tri-galacturonic acid with the NS procedure, but that with the DNS procedure, a higher colour response is obtained with the di- and tri-galacturonic acids than with mono-galacturonic acid. In other words, the NS procedure gives a true measure of glycosidic bonds cleaved by *endo*-polygalacturonanase whereas, the DNS method overestimates the number of glycosidic bonds cleaved. This overestimation may vary between different *endo*-polygalacturonanases based on the particular action pattern of the specific enzyme.

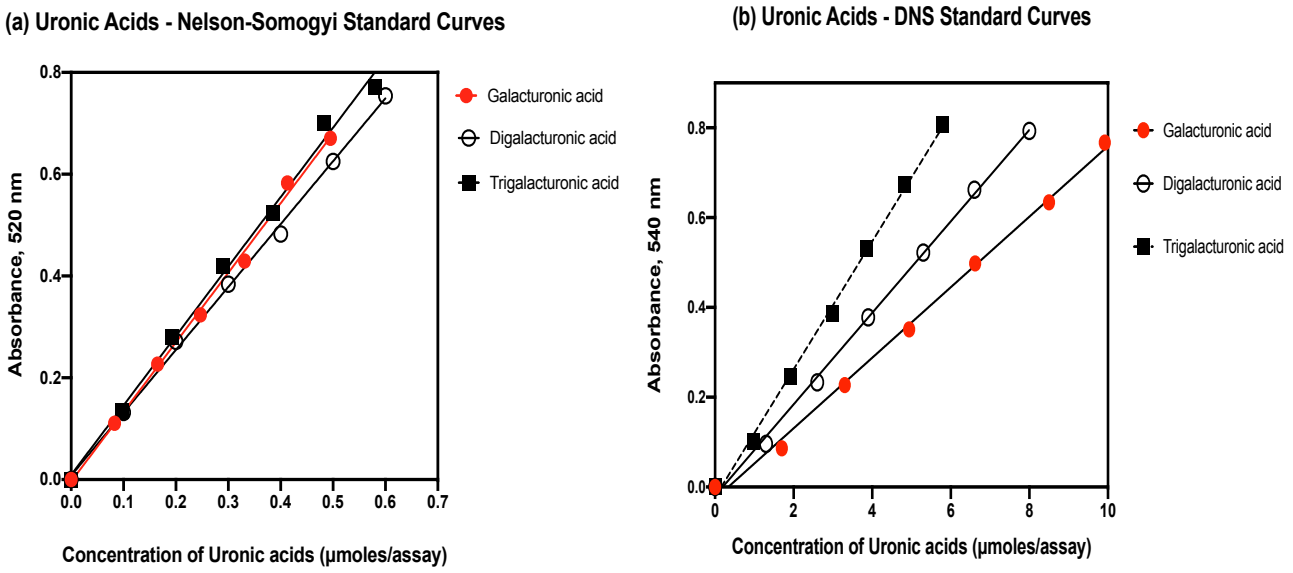

Figure 2. Standard curves for galacturonic acid, digalacturonic acid and trigalacturonic acid obtained using (a) the NS reducing sugar method and (b) the DNS reducing sugar method.

MEASUREMENT OF *ENDO*-POLYGALACTURONANASE ACTIVITY – NELSON/SOMOGYI METHOD

1. PRINCIPLE

endo-Polygalacturonanase (polygalacturonanase) hydrolyses the α-1,4-glycosidic bond in polygalacturonic acid with an increase in free galacturonic acid reducing-sugar end-groups. These reducing galacturonic acid end-groups react with alkaline copper tartrate, reducing the copper from the cupric to the cuprous state, forming cuprous oxide. Cuprous oxide then reduces molybdic acid to molybdenum blue. This blue colour is measured in a colorimeter and compared with standards.

2. UNIT DEFINITION

One Unit of *endo*-polygalacturonanase activity is defined as the amount of enzyme that produces one mmole of D-galacturonic acid reducing sugar equivalents per minute under the conditions specified in this assay.

3. PROCEDURES

3.1. Safety

Perform all operations in a well-ventilated fume cupboard. Safety glasses and disposable gloves must be worn. Chemicals used in this assay, copper sulphate, ammonium molybdate, sodium arsenate and concentrated sulphuric acid, extremely hazardous. Read safety data sheets before performing the assay.

3.2. Purpose

To determine the activity of *endo*-polygalacturonanase using the Nelson Somogyi Method.

3.3. Equipment required

3.3.1. Water bath set at 40°C + 1.0°C.
3.3.2. Stop clock.
3.3.3. Visible range spectrophotometer.
3.3.4. Boiling water bath.
3.3.5. Thick walled test tubes (15 mL, 16 x 120 mm) suitable for centrifugation at ~ 3,000 rpm.
3.3.6. Bench centrifuge capable of 3,000 rpm.
3.3.7. P200 Gilson Dispensor.
3.3.8. Eppendorf Multipette dispenser.
3.3.9. Test Tube Stirrer.

3.4. Reagents

3.4.1. Sodium acetate buffer (100 mM, pH 4.5):

Add 5.72 mL of glacial acetic acid to 900 mL of deionised water in a 2 L beaker on a magnetic stirrer. Adjust the pH to 4.5 or 5.5 ± 0.05 by careful addition of 2 M NaOH. Adjust the volume to 1 L.

3.4.2. Nelson-Somogyi reagents

3.4.2.A. Nelson-Somogyi Solution A:
i) Sodium carbonate anhydrous (> 99%); 25 g
ii) Potassium sodium tartrate tetrahydrate (> 99%); 25 g
iii) Sodium sulphate anhydrous (> 99%); 200 g

Dissolve the above in 800 mL of distilled water with stirring. Dilute to 1 L and filter if necessary. Store in a Duran bottle at room temperature.

3.4.2.B. Nelson-Somogyi Solution B:
i) Copper sulphate pentahydrate (> 98%); 30 g
ii) Concentrated sulphuric acid (> 98%)

Dissolve the copper sulphate in 200 mL of distilled water containing 4 drops of concentrated sulphuric acid. This step should be performed in fume hood with gloves and safety glasses.

3.4.2.C. Nelson-Somogyi Solution C:
i) Ammonium molybdate tetrahydrate (> 99%); 50 g
ii) Concentrated sulphuric acid (> 98%); 42 mL
iii) Sodium arsenate heptahydrate (> 98%); 6 g

Dissolve the ammonium molybdate in 900 mL of distilled water and add the concentrated sulphuric acid. Dissolve the sodium arsenate heptahydrate separately in 50 mL of distilled water and add to the above solution. Dilute to 1 L. Perform this step in a fume hood using gloves and safety glasses.

NOTE: If necessary, warm the solution to 55°C to dissolve completely.

3.4.2.D. Nelson-Somogyi Solution D:

Add 1 mL of Solution B to 25 mL of Solution A

3.4.2.E. Nelson-Somogyi Solution E:

Dilute solution C 5-fold; i.e. dilute 50 mL to 250 mL with distilled water just before use (stable at 4°C for approx. 1 week).

3.4.3. Polygalacturonic acid (0.5 % w/v):
Add 0.5 g of polygalacturonic acid (PGA; Megazyme cat. no. **P-PGACIT**) to a 200 mL beaker and wet with 2 mL of ethanol. Add 90 mL of deionised water and stir at room temperature for approximately 10 min. Adjust the pH to the pH of the assay (usually 4.5 or 5.5) with 1 M sodium hydroxide or 1 M HCl. Adjust the volume to 100 mL and store in a 100 mL Duran® bottle at room temperature. Add 2 drops of toluene to prevent microbial infection. For the *endo*-polygalacturonanase (from *Aspergillus aculeatus*) employed in the current evaluation, the substrate was adjusted to pH 5.5.

3.4.4. D-galacturonic acid standard solution
Weigh 1.06 g of D-galacturonic acid (this is equivalent to 0.97 g dry weight D-galacturonic acid. This carbohydrate cannot be dried, it exists as $C_6H_{10}O_7.H_2O$) into a 250 mL beaker and add 150 mL of deionised water and dissolve the galacturonic acid with stirring at room temperature. Transfer the solution to a 200 mL volumetric flask and adjust to volume to give a solution of 2.5 mM. Transfer this solution to a 250 mL Duran® bottle and add 2 drops of toluene to prevent microbial infection. Store at 4°C. Stable for ~ 1 year at 4°C. Transfer 100 mL of this solution to a 250 mL Duran® bottle and dilute with an equal volume of water to give a solution of (1.25 mM). Add 2 drops of toluene to prevent microbial infection. Stable for ~ 1 year at 4°C.

3.5. Preparation of enzyme dilutions

3.5.1 Dilutions are prepared in 100 mM sodium acetate buffer (pH 4.5 or 5.5) (3.4.1) as described below:

3.5.2 **For powder samples:** In a well-ventilated flow booth, accurately weigh 1.00 g of powder enzyme preparation into a 200 mL beaker. Add a magnetic stirrer bar and 100 mL of 100 mM sodium acetate buffer (pH 4.5 or 5.5) and stir the beaker contents for approx. 10 min until the powder is completely dissolved or dispersed. Further dilute the enzyme preparation to obtain a concentration suitable for assay (as described above). If necessary, clarify the solution by centrifuging 2 mL of the preparation in a microfuge at 13,000 rpm for 3 min.

3.5.3 **For liquid samples:** Accurately transfer 1.00 mL of enzyme preparation using a positive displacement dispenser into 99 mL of 100 mM sodium acetate buffer (pH 4.5 or 5.5) in a 200 mL beaker and stir for 5 min. Further dilute the enzyme preparation to obtain a concentration suitable for assay (as described above). If necessary, clarify the solution by centrifuging 2 mL of the preparation in a microfuge at 13,000 rpm for 3 min. In the current evaluation, *endo*-polygalacturonanase from *Aspergillus aculeatus* was employed and this is diluted in 100 mM sodium acetate buffer at pH 5.5.

3.6. Standard enzyme assay procedure

3.6.1 Dispense 0.5 mL of polygalacturonic acid substrate solution (3.4.3; 0.5 mg/mL) into 16 test tubes.

3.6.2 To 2 tubes as above add 0.2 mL of sodium acetate buffer (Reagent blank). To other tubes (in duplicate) above add 0.2 mL of D-galacturonic acid (3.4.4) at 2.5 mM (0.5 μM) or 1.25 mM (0.25 μM) (Standards). To other tubes (in duplicate) add 0.5 mL of N/S Solution D and mix thoroughly. Then add 0.2 mL of diluted enzyme solution as assayed (3.5.3) and mix thoroughly (Zero Time Incubation).

3.6.3 Pre-equilibrate the remaining 8 tubes at 40°C for 5 min.

3.6.4 To initiate the enzyme reaction, add 0.2 mL of suitable diluted enzyme solution (3.5.3) to four tubes containing 0.5 mL of pre-equilibrated substrate as above.

3.6.5 Incubate the tubes at 40°C and terminate the reaction in two tubes after 3 min, 6 min, 9 min and 12 min by adding 0.5 mL of Solution D (3.4.2.D) and stirring vigorously.

3.6.6 Add 0.5 mL of N/S Solution D (3.4.2.D) to the Reagent Blank and Standard tubes (3.8.2) and mix thoroughly.

3.7. Colour development with Nelson-Somogyi reagents

3.7.1 Heat all test tubes (Reagent and sample blanks, Standards and Samples) in a boiling water bath for 20 min.

Note: The timing is not critical but all tubes for a particular run must be boiled together and for exactly the same time.

3.7.2 Cool the tubes to room temperature (5 min) and stir them for 10 sec (until carbon dioxide is completely released).

3.7.3 Add 3.0 ml of N/S Solution E (3.4.2.E) and stir well (10 sec. on vortex mixer),

3.7.4 Allow the tubes to stand for 10 min at room temperature and mix again.

3.7.5 Centrifuge all tubes at 3,000 rpm for 10 min to remove insoluble polygalacturonic acid.

3.7.6 Measure the absorbance of all tubes at 520 nm against the Reagent Blank.

4. CALCULATIONS:

4.1. Plot absorbance at 520 nm (y axis) versus incubation time in minutes (x axis) to determine the initial rate of reaction of the enzyme.

4.2. Calculate the enzyme activity in IU/mL using the following equation:

$$\Delta A \times 1/\text{Incubation time} \times 5 \times F \times D$$

where:
ΔA = absorbance of the reaction solution read against zero time absorbance.

Incubation time = time of incubation of the enzyme with the substrate in minutes.

5 = Conversion of 0.2 mL of enzyme solution as analysed back to 1 mL (1/0.2).

F = a factor to convert absorbance to μM of D-galacturonic acid

$$= \frac{0.25 \text{ or } 0.50 \text{ (}\mu\text{M of D-galacturonic acid)}}{\text{absorbance for 0.25 or 0.50 }\mu\text{M of reducing sugar}}$$

D = Dilution of the original enzyme preparation.

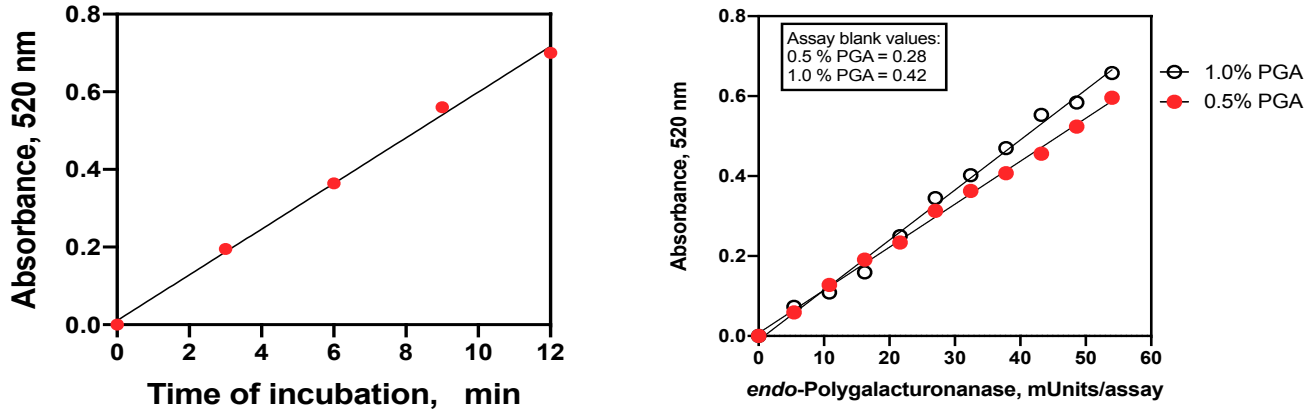

Figure 3. (a) Time course of hydrolysis of polygalacturonic acid (Megazyme cat. no. **P-PGACIT**) by *Aspergillus aculeatus endo*-polygalacturonanase (54 U/assay; Megazyme cat. no. **E-PGALUSP**) in 100 mM sodium acetate buffer (pH 5.5) plus BSA (0.5 mg/mL) at 40°C. Degree of hydrolysis was detemined using the Nelson-Somogyi reducing sugar procedure.
(b) Standard curve showing the activity of *Aspergillus aculeatus endo*-polygalacturonanase (0-54 mU assay) on polygalacturonic acid (Megazyme cat. no. **P-PGACIT**) plotted against the absorbance increase at 520 nm. Incubations were performed in 100 mM sodium acetate (pH 5.5) plus BSA (0.5 mg mL) at 40°C for 10 min, and assays were performed with the DNS procedure.

MEASUREMENT OF *ENDO*-POLYGALACTURONANASE ACTIVITY – DNS METHOD

1. PRINCIPLE

endo-Polygalacturonanase (polygalacturonanase) hydrolyses the α-1,4-glycosidic bond in polygalacturonic acid with an increase in free galacturonic acid reducing-sugar end-groups. This reducing end-group reacts with dinitrosalicylic acid (DNS). The colour produced is proportional to the amount of reducing end-groups and thus, the number of glycosidic bonds cleaved by the enzyme. The absorbance change is measured at 540 nm and is used to calculate the activity of the enzyme in International Units of activity (i.e. μmoles of reaction produced per minute under the defined assay conditions of temperature and pH.

2. UNIT DEFINITION

One Unit of *endo*-polygalacturonanase activity is defined as the amount of enzyme that produces one μmole of D-galacturonic acid reducing sugar equivalents per minute under the conditions specified in this assay.

3. PROCEDURES

3.1. Safety

Perform all operations in a well-ventilated fume cupboard. Safety glasses and disposable gloves must be worn. DNS is an extremely hazardous chemical.

3.2. Purpose

To determine the activity of *endo*-polygalacturonanase using the Dinitrosalicylic (DNS) Method.

3.3. Equipment required

3.3.1. Water bath set at 40°C + 1.0°C.
3.3.2. Stop clock.
3.3.3. Visible range spectrophotometer.
3.3.4. Boiling water bath.
3.3.5. Cooling water bath.
3.3.6. Corning culture tubes – screw cap tubes; 20x 125 mm (Fisher Scientific cat. no. B59563) plus caps (cat. no. FB1355).
3.3.7. Thick walled test tubes (15mL, 16 x 120 mm) suitable for centrifugation at ~ 3,000 rpm.
3.3.8. Bench centrifuge capable of 3,000 rpm.

3.4. Reagents

3.4.1. DNSA

- 3,5-dinitrosalycylic acid (98%) (DNSA)
- Sodium hydroxide (> 97%)
- Potassium Sodium Tartrate tetrahydrate (> 99%)

Cover a 2 L beaker with aluminium foil to protect the contents from light. Add 700mL of deionized water and place the beaker on an IKA magnetic stirrer. Add 10 g of 3,5-dinitrosalycylic acid and dissolve with vigorous stirring. Gradually add 300 g of potassium sodium tartrate tetrahydrate to the beaker with continual stirring. Then add 16 g of NaOH pellets to the beaker with stirring. Continue stirring the contents until the DNSA is completely dissolved. Transfer the solution to a 1 L volumetric flask and adjust to volume with deionised water. Store the solution in a sealed Duran bottle at room temperature (stable for ~ 2 months).

3.4.2. Sodium acetate buffer (100 mM, pH 4.5)

Add 5.72 mL of glacial acetic acid to 900 mL of deionised water in a 2 L beaker on a magnetic stirrer. Adjust the pH to 4.5 ± 0.05 by careful addition of 2 M NaOH. Adjust the volume to 1 L.

3.4.3. Polygalacturonic acid (1.0 % w/v)

Add 1.0 g of polygalacturonic acid (PGA; Megazyme cat. no. **P-PGACIT**) to a 200 mL beaker and wet with 2 mL of ethanol. Add 90 mL of deionised water and stir at room temperature for approx. 10 min. Adjust to the pH of the assay (usually 4.5 or 5.5) with 1 M sodium hydroxide or 1 M HCl. Adjust the volume to 100 mL and store in a 100 mL Duran® bottle at room temperature. Add 2 drops of toluene to prevent microbial infection. For the *endo*-polygalacturonanase (from *Aspergillus aculeatus*) employed in the current evaluation, the substrate was adjusted to pH 5.5.

3.4.4. Stock D-galacturonic acid (Sigma cat. No. 48280) (10 mM)

Weigh 2.12 g of D-galacturonic acid (this is equivalent to 1.94 g dry weight D-galacturonic acid. This carbohydrate cannot be dried, it exists as $C_6H_{10}O_7.H_2O$) into a 100 mL beaker and add 80 mL of deionised water. Transfer the solution to a volumetric flask and adjust the volume to 100 mL to give a solution of 10 mM. Transfer to a 100 mL Duran bottle and add 2 drops of toluene to prevent microbial infection. Store at 4°C. Stable for ~ 1 year at 4°C.

3.5. Preparation of Standard Graph

3.5.1 Prepare the following dilutions from stock D-galacturonic acid (3.4.4).

Required D-galacturonic acid concentration (mM)	Volume of stock 10 mM D-galacturonic acid (mL)	Volume of deionised water (mL)
0	0	10
0.2	0.2	9.8
0.4	0.4	9.6
0.6	0.6	9.4
0.8	0.8	9.2
1.0	1.0	9.0

Note: the absorbance of the reaction solutions should fall in the range of 0.15 to 0.8 for accurate results. This is the linear range of the assay and thus reproducibility is at its peak. Enzyme samples must be altered so that the absorbance values obtained fall within this range.

A standard curve must be run with each set of analyses.

3.5.2 Transfer duplicate 2 mL aliquots of each of the D-galacturonic acid standard solutions (3.5.1) into 16 x 200 mm test-tubes.

3.5.3 Incubate these tubes at 40°C for 10 min, along with the enzyme assay tubes.

3.5.4 After 10 min, add 2 mL of DNS solution to each tube, cover the tubes and place them together with the enzyme assay tubes in a boiling water bath for exactly 10 min.

3.5.5 Rapidly cool the tubes by placing them in an ice water bath and add 10 mL of deionised water to each tube. Mix the contents thoroughly.

3.5.6 Measure the absorbance at 540 nm against the reagent blank. The reagent blank is the tube that contains no D-galacturonic acid.

3.5.7 Plot the absorbance values against the concentration of D-galacturonic acid (mM).

3.5.8 Determine the conversion Factor (F) as μM of D-galacturonic acid divided by the absorbance value over the linear range of the standard curve (see Figure 2b).

3.6. Standard enzyme assay procedure

3.6.1 Dispense 1.0 mL of polygalacturonic acid substrate solution (1.0 % w/v) into a set of 20 x 120 mm test tubes.

3.6.2 Pre-equilibrate all tubes plus the enzyme to be assayed at 40°C for 5 min.

3.6.3 To initiate the enzyme reaction, add 1.0 mL of suitably diluted enzyme solution in duplicate to 1.0 mL of pre-equilibrated substrate solution.

3.6.4 Incubate the tubes at 40°C and terminate the reaction after 3, 6, 9 and 12 min by the addition of 2.0 mL of DNS solution with vigorous stirring.

Note: For time 0, the 2 mL of DNSA is added to the substrate solution, before adding the 1 mL of enzyme solution (Reaction blank).

3.6.5 Reagent Blank: prepare this by mixing 1.0 mL of substrate, 1.0 mL of 100 mM sodium acetate buffer (pH 4.5) and 2.0 mL of DNSA.

3.6.6 Standard solutions: These are prepared as described in 3.5.

3.6.7 Heat all test tubes (Reagent and Enzyme blanks, Standards and Samples) in a boiling water bath for exactly 10 min.

Note: All tubes for a particular run must be boiled together and for exactly the same time.

3.6.8 Remove the rack of tubes from the boiling water bath and place them into an ice-water bath. Immediately add 10 mL of deionized water to each tube, cap the tube and mix the contents vigorously.

3.6.9 Transfer the samples to labelled 15 mL centrifuge tubes. Centrifuge for 10 min at 3,000 rpm at room temperature. Take care not to disturb the pellet after centrifugation.

3.6.10 Measure the absorbance of all solutions at 540 nm, using the reagent blank to zero the spectrophotometer.

3.6.11 Plot absorbance at 540 nm (y axis) versus time in minutes (x axis) and determine the initial rate of reaction of the enzyme.

4 Calculation of enzyme activity:

Calculate the enzyme activity in IU/mL using the following equation:

ΔA/Incubation time x F x Dilution

where:

ΔA /incubation time = increase in absorbance at 540 nm/time of incubation

F = a factor to convert absorbance values to μM of D-galacturonic acid:

$$= \frac{\mu M \text{ of D-galacturonic acid}}{\text{absorbance at 540 nm}}$$

Dilution = Dilution of the original enzyme preparation.

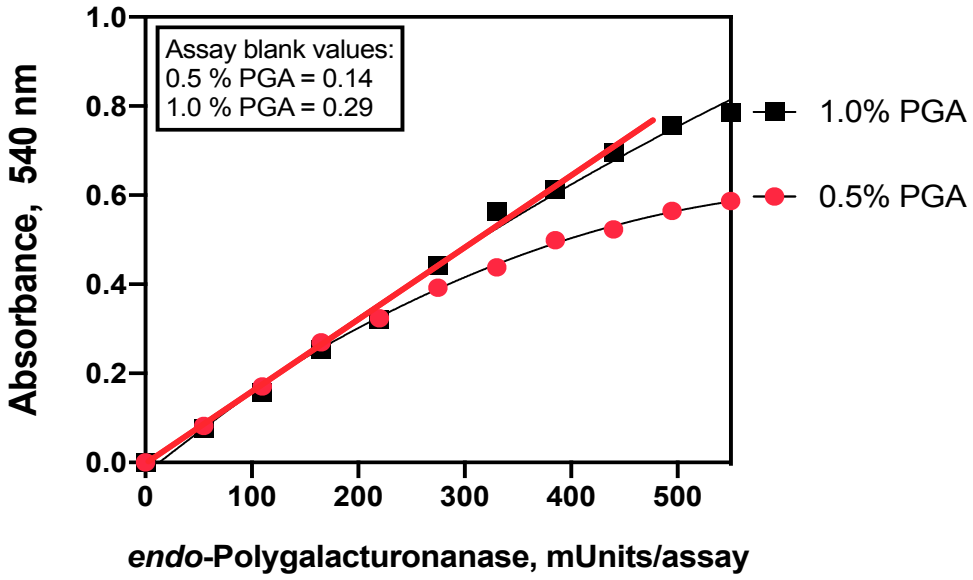

Figure 4. Standard curve showing the activity of *Aspergillus aculeatus endo*-polygalacturonanase (0-550 mU/assay) on polygalacturonic acid (Megazyme cat. no. **P-PGACIT**) plotted against the absorbance increase at 540 nm. Incubations were performed in 100 mM sodium acetate (pH 5.5) plus BSA (0.5 mg/mL) at 40°C for 10 min, and assays were performed with the Nelson-Somogyi procedure.