

Megazyme

ASSAY OF
RHAMNOGALACTURONAN
HYDROLASE
AND
RHAMNOGALACTURONAN
LYASE
USING
RHAMNOZYME TABLETS

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INTRODUCTION

Rhamnogalacturonan (RG) polysaccharides are heteropolysaccharides that constitute the so-called "hairy regions" of pectin. The backbone of RG polysaccharide is composed of α -1,4- and α -1,2-linked D-galacturonosyl- and L-rhamnopyranosyl residues respectively, mainly in alternating sequence. Side chains composed of arabinose and/or galactose are glycosidically linked to backbone rhamnose residues. Various types of enzymes that catalyse *endo*-depolymerisation of the rhamnogalacturonan backbone have been identified, namely rhamnogalacturonan hydrolase (RG-hydrolase) and rhamnogalacturonan lyase (RG-lyase). RG-hydrolase hydrolyses the 'galacturonic acid α -1,2-rhamnose' linkage to release oligosaccharides with rhamnose at the non-reducing end, whereas RG-lyase cleaves the 'rhamnose α -1,4-galacturonic acid' linkage to release oligomers that have an unsaturated galacturonic acid at the non-reducing end. Both enzymes are inhibited by the presence of acetyl- or methyl-substituents on the RG backbone.

Various procedures have been developed for the assay of RG-hydrolase and RG-lyase, none of which have been entirely satisfactory. This problem is now resolved with the development of Rhamnozime tablets by Megazyme.

SUBSTRATE:

The substrate employed is Azurine-crosslinked-rhamnogalacturonan (AZCL-Rhamnogalacturonan). This is prepared by treating highly purified pectic galactan from potato fibre with a mixture of *endo*-galactanase, *endo*-arabinanase, α -L-arabinofuranosidase and polygalacturonanase to remove most of the enzyme-susceptible galactan and arabinan fragments in the polysaccharide and remove regions susceptible to polygalacturonanase. The resultant polysaccharide still contains significant levels of galactose and arabinose, but it is highly resistant to *endo*-depolymerisation by *endo*-galactanase and *endo*-arabinanase. The polysaccharide is then dyed and crosslinked. AZCL-rhamnogalacturonan is a highly sensitive and very selective substrate for the assay of RG-hydrolase (at pH 4.0) and RG-lyase (at pH 8.0) and can be used to selectively measure these activities in the presence of large excesses of other pectin degrading enzymes and in the presence of sugars and other stabilisers present in industrial powder and liquid pectinase enzyme preparations. This substrate is supplied commercially in a ready-to-use form as Rhamnozime tablets.

EXTRACTION/DILUTION BUFFERS:

A. Sodium acetate buffer (50 mM, pH 4.5) containing sodium azide (0.02%)(for RG-hydrolase).

Add 3.05 g of glacial acetic acid (1.05 g/ml) to 900 ml of distilled water. Adjust the pH to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution. Approximately 12 ml is required. Add 0.2 g of sodium azide and adjust the volume to 1 litre. Stable at 4°C for > 6 months.

NOTE: Sodium azide is a toxic chemical and should be treated accordingly.

B. Tris/HCl buffer (50 mM, pH 8.0) containing sodium azide (0.02%)(for RG-lyase).

Add 6.05 g of Trizma Base(Sigma T 1503) to 900 ml of distilled water. Adjust the pH to 8.0 by the addition of 1 M hydrochloric acid. Add 0.2 g of sodium azide and adjust the volume to 1 litre. Stable at 4°C for > 6 months.

STOPPING SOLUTION:

Add 20 g of tri-sodium phosphate to 900 ml of distilled water and adjust the pH to 11.0 with 1 M HCl. Adjust the volume to 1 litre. Stable at room temperature for 6 months.

ENZYME EXTRACTION AND DILUTION:

Liquid enzyme preparation (1.0 ml) is added (using a positive displacement dispenser) to either sodium acetate buffer (buffer A) or Tris/HCl buffer (buffer B)(19.0 ml) and mixed thoroughly. This is termed the **Original Extract**. This extract is then further diluted by addition of 1.0 ml of extract to 9.0 ml of either buffer A or B. This process is repeated until a concentration of enzyme suitable for assay is obtained.

With **powder samples**, enzyme preparation (1.0 g) is added to 20 ml of either buffer A or B and mixed until either completely dissolved or dispersed. This solution is clarified by centrifugation (1,000 g, 10 min) or filtration through Whatman No.1 filter circles and is termed the Original Extract. This extract is then further diluted as for the liquid samples.

Reaction absorbance values should be in the **range 0.2 to 1.4** after subtraction of the reaction blank. The **reaction blank** is approximately 0.2 absorbance units.

ASSAY PROCEDURE:

1. Pre-equilibrate 0.5 ml aliquots of suitably diluted enzyme in acetate buffer (50mM,pH 4.5)(RG-hydrolase) or tris/HCl buffer (50 mM, pH 8.0)(RG-lyase) at 40°C for 5 min.
2. Initiate reaction by adding a Rhamnozyme tablet. (The tablet hydrates rapidly). Do not stir the suspension.
3. Terminate the reaction after exactly 10 min at 40°C by adding 10 ml of tri-sodium phosphate solution (2% w/v, pH 11.0).
4. Allow the tubes to stand at room temperature for about 5 min, stir the slurry again and then filter through a Whatman No.1 (9 cm) filter circle.
5. Measure the absorbance of the reaction solutions at 590 nm against the reaction blank.

A **substrate/enzyme (reaction) blank** is prepared by adding tri-sodium phosphate solution (2%,10 ml) to the enzyme solution (0.5 ml) before the addition of the Rhamnozyme tablet.

A **single blank** is required for each set of determinations and this is used to zero the spectrophotometer.

STANDARDISATION:

A standard curve relating the activity of purified RG--hydrolase on borohydride-reduced rhamnogalacturonan and Rhamnozyme tablets (Lot 20601) at pH 4.5 is shown in Figure 1. The activity of highly purified RG-hydrolase on rhamnogalacturonan (7 mg/ml,pH 4.5) was determined using the Nelson/Somogyi reducing sugar procedure with galacturonic acid as standard. One Unit of activity is that amount of enzyme required to release one micromole of galacturonic acid reducing-sugar equivalents per minute under the defined assay conditions.

A standard curve relating the activity of purified RG-lyase on rhamnogalacturonan and Rhamnozyme tablets (Lot 20601) at pH 8.0 is shown in Figure 2. The activity of highly purified RG-lyase on borohydride-reduced rhamnogalacturonan (4 mg/ml, pH 8.0) was determined using a recording spectrophotometer set at 235 nm. One Unit of activity is that amount of enzyme required to release one micromole of unsaturated galacturonic acid equivalent per minute under the defined assay conditions.

STANDARDISATION OF RHAMNOGALACTURONAN LYASE ACTIVITY ON RHAMNOGALACTURONAN:

Dilute rhamnogalacturonan lyase in 50 mM tris/HCl buffer (pH 8.0).

Dissolve borohydride-reduced rhamnogalacturonan (prepared from potato pectic galactan) to a concentration of 10 mg/ml in 50 mM tris/HCl buffer (pH 8.0).

Assay Procedure:

To a 1 cm lightpath quartz cuvette in the heating chamber of a recording spectrophotometer;

Add: 1.0 mL of borohydride-reduced rhamnogalacturonan (10 mg/ml) solution in Tris/HCl buffer, plus 1.0 mL of Tris/HCl buffer (50 mM, pH 8.0). Mix well and allow the solution to equilibrate to 40°C over 5 min.

Add: 0.5 mL of suitably diluted enzyme solution.

Mix the solution well and measure the absorbance increase at 235 nm over a period of approximately 20 min.

Prepare enzyme and substrate blanks, by replacing these components with an equal volume of Tris/HCl buffer, and run the reactions concurrently with the enzyme-substrate reaction.

Measure the initial rate of reaction in the linear range of the kinetic reaction curve.

Calculation of Rhamnogalacturonan Lyase Activity:

Activity, Units/mL of original solution:

$$= \frac{A}{T} \times \frac{1}{4600} \times \frac{0.5}{2.5} \times 10^6 \times \text{Dilution.}$$

where:

- A/T = rate of increase in absorbance at 235 nm.
- 4600 = molar extinction coefficient of the unsaturated bond at the 4-5 position of the uronic acid residue (i.e. $\epsilon_{235} = 4600 \text{ M}^{-1} \text{ cm}^{-1}$).
- 0.5 = the volume of enzyme used in the assay.
- 2.5 = the total volume of the reaction mixture.
- 10⁶ = conversion from moles to micromoles
- Dilution = dilution of the original enzyme preparation.

Calculation of RG-Hydrolase Activity Using Rhamnozyme Tablets:

RG-Hydrolase activity in the sample being assayed is determined by reference to the standard curve for RG-hydrolase on Rhamnozyme at pH 4.5 (Figure 1). This allows the conversion of absorbance values at 590 nm to milli-Units per assay (ie. per 0.5 ml).

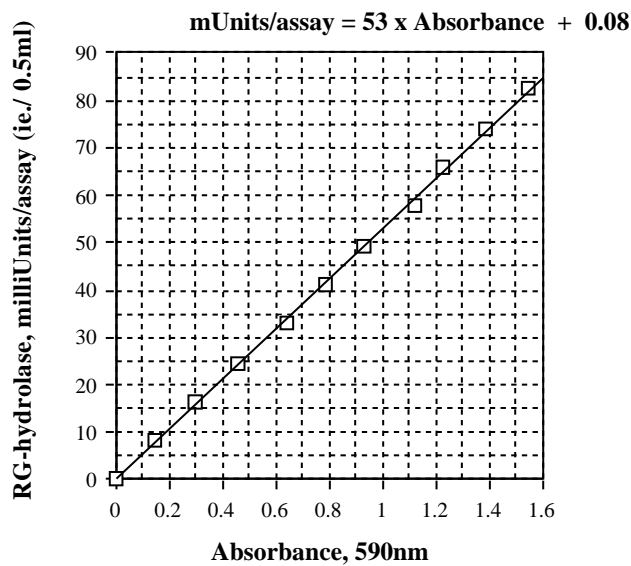


Figure 1. Rhamnogalacturonan hydrolase standard curve on Rhamnozyme tablets (Lot 20601).

Pre-equilibrate RG-hydrolase (0.5 ml, 0-90 milli-Units) in 50 mM sodium acetate buffer (pH 4.5) containing sodium azide (0.02%) in a glass test-tube (16 x 120 mm) at 40°C for 5 min. Initiate the reaction by the adding a Rhamnozyme tablet without stirring. Terminate the reaction after 10 min by the adding 10 ml of tri-sodium phosphate solution (2% w/v, pH 11.0) with vigorous stirring. Allow the tubes to stand at room temperature for about 5 min, then stir them again. Filter the solutions through Whatman No. 1 (9 cm) filter circles, and measure the absorbance of the filtrate against an enzyme/substrate blank solution at 590 nm.

NOTE: The blank values for this test are quite high (in the range of 0.27 - 0.29 absorbance units at 590 nm).

Rhamnogalacturonan hydrolase activity per mL or gram of original preparation:

$$= Y \times \frac{1}{1000} \times 2 \times 20 \times \text{Dilution}$$

where:

Y = rhamnogalacturonan hydrolase activity (in milli-Units/assay) as obtained from the standard curve.

1/1000 = conversion from milliUnits to units.

2 = conversion from 0.5 ml to 1.0 ml.

20 = Original Extraction volume (i.e. 1 ml of enzyme concentrate or 1 gram of enzyme powder in extraction volume of 20 ml).

Dilution = the further dilution of the original enzyme extract.

One **Unit of activity** is defined as the amount of enzyme required to release one micromole of galacturonic acid reducing-sugar equivalents from rhamnogalacturonan per minute under the defined assay conditions.

- RG-Lyase activity** in the sample being assayed is determined by reference to the standard curve for RG-lyase on Rhamnozyme at pH 8.0 (Figure 2). This allows the conversion of absorbance values at 590 nm to mill-Units per assay (ie. per 0.5 ml of enzyme).

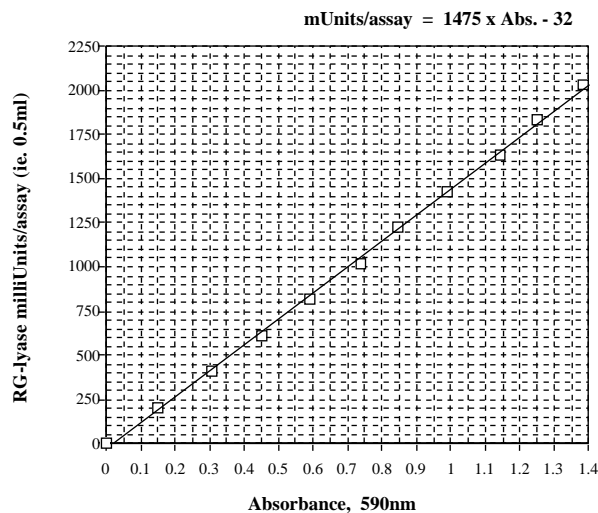


Figure 2. Rhamnogalacturonan lyase standard curve on Rhamnozyme tablets (Lot 20601).

Pre-equilibrate RG-lyase (0.5 ml, 0-2000 milli-Units) in 50 mM tris/HCl buffer (pH 8.0) containing sodium azide (0.02%) in a glass test-tube (16 x 120 mm) at 40°C for 5 min. Initiate the reaction by adding a Rhamnozyme tablet without stirring. Terminate the reaction after 10 min by adding 10 ml of tri-sodium phosphate solution (2% w/v, pH 11.0) with vigorous stirring. Allow the tubes to stand at room temperature for about 5 min, then stir them again. Filter the solutions through Whatman No. 1 (9 cm) filter circles, and measure the absorbance of the filtrate against an enzyme/substrate blank solution at 590 nm.

Rhamnogalacturonan lyase activity per mL or gram of original preparation:

$$= Y \times \frac{1}{1000} \times 2 \times 20 \times \text{Dilution}$$

where:

Y = rhamnogalacturonan lyase activity (in milli-Units/assay) as obtained from the standard curve.

1/1000 = conversion from milli-Units to Units.

2 = conversion from 0.5 ml to 1.0 ml.

20 = Original Extraction volume (i.e. 1 ml of enzyme concentrate or 1 gram of enzyme powder in extraction volume of 20 ml).

Dilution = the further dilution of the original enzyme extract.

One **Unit of activity** is defined as the amount of enzyme required to produce one micromole of unsaturated galacturonic acid equivalents from rhamnogalacturonan per minute under the defined assay conditions.



**Megazyme International Ireland Ltd.,
Bray Business Park, Bray,
Co. Wicklow,
IRELAND**

**Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com**

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