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GLUCOMANNAN

ASSAY PROCEDURE

K-GLUM 12/20

(50 Assays per Kit)

INTRODUCTION:

Glucomannan is a linear polysaccharide comprising 1,4-β-linked D-glucosyl and D-mannosyl residues, where the glucose:mannose (Glc:Man) ratio varies considerably. Konjac (*Amorphophallus konjac*) glucomannan has a Glc:Man ratio of approx. 2:3, *Aloe vera* glucomannan a ratio of 1:2, and salep (*Orchis mureo*) glucomannan has a ratio of approximately 1:4. All of the polysaccharides are acetylated, which imparts water solubility. Glucomannans are major cell-wall reserve carbohydrates in tubers of several plant genera. At certain stages in the development of the plants, the glucomannan is degraded and serves as an energy source. The only industrially exploited glucomannan is konjac. Like carob galactomannan, this polysaccharide interacts with agar, carrageenan and xanthan to form solutions of increased viscosity or gels. This interaction is exploited in the pet-food industry in canned dog food. Recently, glucomannan has also been used in jelly sweets. In this application, the glucomannan has been found to be dangerous, leading to choking deaths of some consumers. This has led to a ban of these types of sweets in Europe and America, and a need for an accurate analytical procedure for glucomannan analysis.

PRINCIPLE:

The quantification of glucomannan requires several enzyme reactions and a treatment at high pH to remove the acetyl-groups from the polysaccharide. The first enzymic reaction involves depolymerisation of acetylated-glucomannan (Ac-GlcMan) by *endo*-β-mannanase to produce acetylated glucomanno-oligosaccharides (Ac-GlcManol) (1). For some samples (those with a high glucomannan content), this is performed before deacetylation, while for other samples (lower concentration of glucomannan), it is more convenient to perform the deacetylation step (2) first.

$$\text{(1)}\quad \text{Ac-GlcMan} + H_2O \xrightarrow{(\beta\text{-mannanase})} \text{Ac-GlcManol}$$

After depolymerisation into acetylated glucomanno-oligosaccharides of degree of polymerisation (DP) 2-6, the oligosaccharides are deacetylated by increasing the pH to 12.5 (2).

$$\text{(2)}\quad \text{Ac-GlcManol} \xrightarrow{(\text{pH } 12.5)} \text{GlcManol} + \text{acetate}$$

For samples containing a high concentration of glucomannan (e.g. konjac flour) it is important to depolymerise with β-mannanase before deacetylation as deacetylation of the polysaccharide leads to polymer self-association and thus resistance to enzymic hydrolysis.

After the acetyl-groups have been removed, the glucomanno-oligosaccharides are quantitatively hydrolysed to D-glucose and D-mannose by the combined action of β-glucosidase (β-Gos) and β-mannosidase (β-Mos) (3).

$$\text{(3)}\quad \text{GlcManol} + H_2O \xrightarrow{(\beta\text{-Gos} + \beta\text{-Mos})} \text{D-glucose} + \text{D-mannose}$$

D-Glucose and D-mannose are phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) (4), (5) to glucose-6-phosphate (G-6-P) and mannose-6-phosphate (M-6-P), respectively, with the simultaneous formation of adenosine-5'-diphosphate (ADP).

$$\text{(4)}\quad \text{D-Glucose} + \text{ATP} \xrightarrow{(\text{HK})} \text{G-6-P} + \text{ADP}$$

$$\text{(5)}\quad \text{D-Mannose} + \text{ATP} \xrightarrow{(\text{HK})} \text{M-6-P} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (6).

$$\text{(6)}\quad \text{G-6-P} + NADP^+ \xrightarrow{(\text{G6P-DH})} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose. It is the NADPH which is measured by the increase in absorbance at 340 nm.

On completion of reaction (6), M-6-P is converted to fructose-6-phosphate (F-6-P) and then to G-6-P by the sequential action of phosphomannose isomerase (PMI) and phosphoglucose isomerase (PGI) (7).

$$\text{(7)}\quad \text{M-6-P} \xrightarrow{(\text{PMI})} \text{F-6-P} \xrightarrow{(\text{PGI})} \text{G-6-P}$$

G-6-P reacts in turn with $NADP^+$ forming gluconate-6-phosphate and NADPH, leading to a further rise in absorbance that is stoichiometric with the amount of D-mannose.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for glucomannan and its breakdown products. Free D-glucose, D-mannose or D-fructose in the extract are removed by aqueous ethanol washing or borohydride reduction in the sample preparation steps. Additionally, residual free D-glucose, D-mannose or D-fructose in the sample extract are measured by analysing the sample solution before hydrolysis of the glucomanno-oligosaccharides with the β-glucosidase plus β-mannosidase mixture.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. For a powdered sample, this corresponds to 0.368 g/100 g of sample. The detection limit is 0.736 g/100 g which is derived from an absorbance difference of 0.020.

The assay is linear over the range of 4 to 80 µg of glucomannan per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur, this corresponds to a glucomannan concentration of approx. 0.184-0.368 g/100 g of sample. As in sample preparation, the sample is weighed, e.g. 0.10 g/250 mL, a difference of 0.001-0.002 g/0.10 g can be expected.

To confirm that the glucomanno-oligosaccharides are completely hydrolysed by the β-glucosidase plus β-mannosidase mixture, perform the incubation for the recommended time and for twice the recommended time. The final determined values for glucomannan should be the same.

INTERFERENCE:

If the hydrolysis of glucomannan has been completed within the times specified in the various steps in the assay, it can be generally concluded that no interference has occurred. Quantitative measurement of D-glucose and D-mannose can be checked by adding D-glucose and D-mannose (about 20 µg of each in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance indicates that no interference has occurred.

Interfering substances in the sample being analysed can be identified by including an internal standard (e.g. glucomanno-oligosaccharides). Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding glucomannan or glucomanno-oligosaccharides to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (25 mL, pH 4.5).
Stable for > 2 years at 4°C.

Bottle 2: Buffer (25 mL, pH 7.6) plus magnesium chloride and sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 3: $NADP^+$ plus ATP.
Stable for > 5 years below -10°C.

Bottle 4: β-Mannanase suspension (1.2 mL).
Stable for > 2 years at 4°C.

Bottle 5: β-Glucosidase plus β-mannosidase suspension, 1.1 mL.
Stable for > 2 years at 4°C.

Bottle 6: Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.2 mL.
Stable for > 2 years at 4°C.

Bottle 7: Phosphoglucose isomerase plus phosphomannose isomerase suspension, 2.2 mL.
Stable for > 2 years at 4°C.

Bottle 8: D-Glucose plus D-mannose standard solution (0.2 mg/mL of each).
Stable for > 2 years; store sealed at room temperature.

PREPARATION OF REAGENT SOLUTIONS/ SUSPENSIONS:

1. Dilute the contents of bottle 1 to 450 mL with distilled water. Check pH and, if necessary, adjust to pH 4.5 using either 1 M HCl or 1 M NaOH. Adjust the volume to 500 mL and store in a well-sealed Duran® bottle. Store at 4°C between use. Stable for 12 months at 4°C if 2 drops of toluene are added to prevent microbial contamination, or for 2 months at 4°C if toluene is not added. Preferably, store the diluted buffer in appropriately sized aliquots in polypropylene containers below -10°C, under which conditions it is stable for > 2 years.

2. Use the contents of bottle 2 as supplied.
Stable for > 2 years at 4°C.

3. Dissolve the contents of bottle 3 in 12 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

4-7. Use the contents of bottles 4, 5, 6 and 7 as supplied. Before opening for the first time, shake the bottles to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position. Stable for > 2 years at 4°C.

8. Use the contents of bottle 8 as supplied.
Stable for > 2 years; store sealed at room temperature.

NOTE: 0.1 mL of standard solution should be used in the cuvette test and water volume should be adjusted accordingly.
The standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample.

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL and 100 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of buffer 2 and 0.1 mL aliquots of $NADP^+$/ATP solution).
   - with 25.0 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Thermostated hot-block heater set at 35°C.
9. Stop clock.
10. Whatman GF/A glass fibre (9 cm) filter papers.
11. Hot-plate magnetic stirrer.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: preferably 35°C in a dry hot-block heater, but otherwise ~ 25°C
Final volume: 2.86 mL
Sample solution: 4-80 µg of D-glucose + D-mannose per cuvette (in 0.50 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 35°C) sample solution suspension 5 (β-Gos/β-Mos)	0.02 mL 0.50 mL -	- 0.50 mL 0.02 mL
Mix*, then cap the cuvettes and incubate at 35°C for 20 min. **Then add:**		
distilled water (at ~ 35°C) solution 2 (buffer) solution 3 ($NADP^+$/ATP)	2.00 mL 0.20 mL 0.10 mL	2.00 mL 0.20 mL 0.10 mL
Mix**, incubate at ~ 25-30°C, read absorbances of the solutions after approx. 3 min (A_1) and start the reactions by addition of:		
suspension 6 (HK/G6P-DH)	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min. **Then add:**		
suspension 7 (PGI/PMI)	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_3) at the end of the reaction (approx. 20 min). If the reaction has not stopped after 20 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min.		

* pipette sample solution and suspension 5 into the bottom of the cuvette and mix by gentle swirling.

** for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATIONS:

Determine the absorbance differences (A_2-A_1) and (A_3-A_2) for both blank and sample, and calculate values of $\Delta A_{D\text{-glucose}}$, $\Delta A_{D\text{-mannose}}$ and $\Delta A_{glucomannan}$ as described below:

Determination of D-glucose (from glucomannan):

$\Delta A_{D\text{-glucose}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$.

Determination of D-mannose (from glucomannan):

$\Delta A_{D\text{-mannose}} = (A_3\text{-}A_2)_{sample} - (A_3\text{-}A_2)_{blank}$.

Determination of glucomannan:

$\Delta A_{glucomannan} = (A_3\text{-}A_1)_{sample} - (A_3\text{-}A_1)_{blank}$.

The value of $\Delta A_{glucomannan}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The content of glucomannan as % (w/w) can be calculated as follows:

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{V}{1000} \times MW \times \frac{FEV}{v} \times \frac{100}{w} \times F$$

where:

$\Delta A_{glucomannan} = (A_3\text{-}A_1)_{sample} - (A_3\text{-}A_1)_{blank}$

6300 = extinction coefficient of NADPH at 340 nm [l x mol^{-1} x cm^{-1}]

$\Delta A_{glucomannan/6300}$ = factor to determine molarity of NADPH

V = final volume in assay cuvette [mL]

V/1000 = factor to convert molarity to moles of NADPH (i.e. to moles of D-glucose plus D-mannose in the assay cuvette)

MW = molecular weight of anhydro-D-glucose/D-mannose (162.14) as occurs in glucomannan polysaccharide

FEV = final extraction volume (e.g. 250 mL for konjac powder; 100 mL or 50 mL for jelly sweets)

v = volume of sample added to the cuvette

FEV/v = factor to determine the number of grams of glucomannan in the final extract

w = weight of sample extracted (grams)

100/w = factor to express glucomannan content as a percentage of the sample (w/w)

F = dilution factor (after extraction, some samples are diluted in sample treatment, e.g. jelly sweets in sample extraction format (d) where 2 mL of sample after treatment is adjusted to 4 mL, i.e. F=2)

Thus, for KONJAC POWDER when extracted according to sample preparation example (a), page 10,

Glucomannan (g/100 g):

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{V}{1000} \times MW \times \frac{FEV}{v} \times \frac{100}{w} \times F$$

where:

$\Delta A_{glucomannan} = (A_3\text{-}A_1)_{sample} - (A_3\text{-}A_1)_{blank}$
V = 2.86 mL
MW = 162.14
FEV = 250 mL
v = 0.5 mL
w = 0.100 g
F = 1 (i.e. no dilution)

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{2.86}{1000} \times 162.14 \times \frac{250}{0.5} \times \frac{100}{0.100} \times 1$$

$= \Delta A_{glucomannan} \times 36.8$ [g/100 g]

For JELLY SWEETS, when extracted according to sample preparation examples (b) and (c), page 11,

Glucomannan (g/100 g):

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{V}{1000} \times MW \times \frac{FEV}{v} \times \frac{100}{w}$$

where:

$\Delta A_{glucomannan}$ = $(A_3\text{-}A_1)_{sample} - (A_3\text{-}A_1)_{blank}$
V = 2.86 mL
MW = 162.14
FEV = 100 mL
v = volume of sample analysed (0.5 mL)
w = weight of gel (approx. 12-18 g)

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{2.86}{1000} \times 162.14 \times \frac{100}{0.5} \times \frac{100}{w}$$

$$= \Delta A_{glucomannan} \times 1.472/w \quad [g/100\ g]$$

For JELLY SWEETS, when extracted according to sample preparation example (d), page 12,

Glucomannan (g/100 g):

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{V}{1000} \times MW \times \frac{FEV}{v} \times \frac{100}{w} \times F$$

where:

$\Delta A_{glucomannan}$ = $(A_3\text{-}A_1)_{sample} - (A_3\text{-}A_1)_{blank}$
V = 2.86 mL
MW = 162.14
FEV = 50 mL
v = volume of sample analysed (0.5 mL)
w = weight of gel (approx. 12-18 g)
F = 2 (2-fold dilution)

$$= \frac{\Delta A_{glucomannan}}{6300} \times \frac{2.86}{1000} \times 162.14 \times \frac{50}{0.5} \times \frac{100}{w} \times 2$$

$$= \Delta A_{glucomannan} \times 1.472/w \quad [g/100\ g]$$

In hydrolysates containing just D-mannose, the quantity of D-mannan polysaccharide can be determined using the same equation.

Ratio of D-glucose : D-mannose:

$= \Delta A_{D\text{-}glucose} : \Delta A_{D\text{-}mannose}$

SAMPLE PREPARATION EXAMPLES:

(a) Determination of glucomannan in *Amorphophallus konjac* (konjac) powder.

Accurately weigh approx. 0.100 g of milled sample (to pass a 0.5 mm screen) into glass, round-bottomed test tubes (16 x 120 mm). Tap the tube to ensure all of the sample falls to the bottom of the tube. Add 5 mL of 80% (v/v) aqueous ethanol to each tube, stir on a test-tube stirrer and incubate at 85-90°C for 5 min. Add a further 5 mL of aqueous ethanol, cover the tube with Parafilm® and carefully stir on a vortex mixer. Centrifuge at 1,500 *g* for 10 min. Carefully decant and discard the supernatant solution. Resuspend the pellet in 5 mL of 80% (v/v) aqueous ethanol, stir the tube and add another 5 mL of 80% (v/v) aqueous ethanol. Stir the tube and centrifuge at 1,500 *g* for 10 min. Carefully decant and discard the supernatant solution and allow excess liquid to drain onto absorbent paper.

NOTE: These washing steps are performed to remove low molecular weight sugars (such as D-glucose and D-fructose).

Resuspend the pellet in 8 mL of solution 1 (pH 4.5) and stir the tube vigorously on a vortex mixer to effect complete dispersion. Immediately place the tube in a boiling water bath and incubate for 30 sec. Remove the tube and stir it vigorously on a vortex mixer. Repeat this heating and stirring step. Finally, incubate the tube in the boiling water bath for 4 min to ensure full hydration of the glucomannan. Remove the tube, stir vigorously on a vortex mixer and place in a water bath at 40°C. After 5 min, add 20 µL of β-mannanase (suspension 4) and stir the tube vigorously on a vortex mixer for 30 sec. Incubate the tubes at 40°C for 60 min with intermittent vigorous stirring (about 2-3 times) on a vortex mixer. Alternatively, stir the tube contents continuously with a magnetic stirrer using an arrangement similar to the Megazyme Multistir Incubation Bath (www.megazyme.com). Quantitatively transfer the solution to a 200 mL beaker and increase the volume to approx. 70 mL. Adjust the pH of the solution to approx. 12.5 by dropwise addition of 1 M NaOH and leave at room temperature for 10 min to effect complete deacetylation of the glucomanno-oligosaccharides. Add 20 mL of 200 mM sodium phosphate buffer (pH 6.5) (not supplied) and adjust the pH to 6.5 by dropwise addition of 1 M HCl. Quantitatively transfer the solution

to a 250 mL volumetric flask, adjust to volume using distilled water and mix well. *Typically, for milled, dried konjac, a sample volume of 0.5 mL is satisfactory and for pure konjac glucomannan, a dilution factor of 2.5 (using a 0.5 mL sample volume) is required for analysis.*

(b) Determination of glucomannan in jelly sweets (Sugar Removal Procedure 1: Dialysis).

Transfer the contents of one "Jelly cup" to a 100 mL pre-weighed Duran® bottle and weigh accurately (usually 12-18 g). Add 20 mL of distilled water and heat the bottle plus contents in a microwave oven at maximum power for approx. 45 sec (to increase temperature to 80-90°C). With some samples, this will result in a homogenous (although not necessarily clear) solution. With samples that are heterogenous suspensions, homogenise with an Ultra-turrax® or Polytron® homogeniser (or equivalent) for 20 sec to effect complete disintegration of jelly particles. Wash the homogeniser shaft with a minimum volume of distilled water (approx. 5 mL). Add 3.0 mL of 2 M NaOH solution (to raise the pH to approx. 12.5) and mix thoroughly. Leave the solutions at room temperature for 10 min (to effect complete deacetylation of the glucomanno-oligosaccharides). Add 10.0 mL of 2 M acetic acid and quantitatively transfer to dialysis tubing (2.5 cm flat diameter x approx. 40 cm length). Seal the tubing and dialyse against flowing tap water overnight. Transfer the contents of the dialysis tube to a 100 mL Duran® bottle, rinse the dialysis tube with approx 10 mL of distilled water and add this to the Duran® bottle. Heat the solution to approx. 80°C in a microwave oven and cool to room temperature. Add 1 mL of 2 M sodium acetate buffer, pH 4.5 and 20 µL of β-mannanase (suspension 4) and incubate at 40°C for 30 min. Adjust the pH to 6.5 with 2 M NaOH and adjust the volume to 100 mL with 200 mM sodium phosphate buffer (pH 6.5). Mix thoroughly and filter an aliquot of the solution through Whatman No. 1 filter paper. *Typically, a dilution factor of 1 to 5 using a sample volume of 0.5 mL is required for analysis.*

(c) Determination of glucomannan in jelly sweets (Sugar Removal Procedure 2: Borohydride Reduction).

Transfer the contents of one "Jelly cup" to a 200 mL pre-weighed Pyrex® beaker and weigh accurately (usually 12-18 g). Add 40 mL of distilled water, cover the beaker and heat in a microwave oven at maximum power for approx. 45 sec (to increase temperature to 80-90°C). With some samples, this will result in a homogenous (although not necessarily clear) solution. With samples that are heterogenous suspensions, homogenise with an Ultra-turrax® or Polytron® homogeniser (or equivalent) for 20 sec to effect complete disintegration of jelly particles. Wash the homogeniser shaft with a minimum volume of distilled water (approx. 5 mL). Add 2.0 mL of 2 M NaOH solution and 1 g of sodium borohydride, mix thoroughly and

allow the reaction to proceed for 2 h at room temperature in a well ventilated fume hood (to effect conversion of reducing sugars to sugar alcohols).

NOTE: During this reaction, and in the subsequent neutralisation with acetic acid, hydrogen gas is produced. It is thus **essential** that these operations are performed in a well ventilated fume hood.

Carefully add 20 mL of 2 M acetic acid, in aliquots, to the vigorously stirring solution to degrade remaining sodium borohydride (avoid loss of solution through "foaming" from beaker). Adjust the pH of the solution to 4.5 with 2 M HCl. Add 20 µL of β-mannanase, mix thoroughly and incubate at 40°C for 30 min. Adjust the pH to 6.5 with 2 M NaOH, transfer the solution to a 100 mL volumetric flask and adjust to volume with 200 mM sodium phosphate buffer (pH 6.5). Mix thoroughly and filter an aliquot of the solution through Whatman No. 1 filter paper. *Typically, a dilution factor of 1 to 5 using a sample volume of 0.5 mL is required for analysis.*

(d) Determination of glucomannan in jelly sweets (Sugar Removal Procedure 3: Alcohol Precipitation).

Transfer the contents of one "Jelly cup" to a 100 mL beaker and weigh accurately (usually 12-18 g). Add 20 mL of distilled water and heat the beaker plus contents in a microwave oven at maximum power for approx. 45 sec (until the solution begins to boil). With some samples, this will result in a homogenous (although not necessarily clear) solution. With such samples, quantitatively transfer the solution to a 50 mL volumetric flask, adjust to volume with distilled water and mix well. Store the solution in a well-sealed Duran® bottle awaiting analysis. With other samples, a heterogenous suspension is obtained on heating in the microwave oven. Homogenise these suspensions with an Ultra-turrax® or Polytron® homogeniser (or equivalent) for 20 sec to effect complete disintegration of jelly particles. Wash the homogeniser shaft with a minimum volume of distilled water and quantitatively transfer the solution to a 50 mL volumetric flask and adjust to volume with distilled water.

Transfer 2 mL of freshly prepared sample (still hot) using a positive displacement dispenser (e.g. Eppendorf Multipette® with 25.0 mL Combitip®) to **pre-weighed** glass test tubes (16 x 120 mm) and immediately add 2 mL of approx. 95% (v/v) ethanol to each tube with vigorous stirring on a vortex mixer. Add another 6 mL of approx. 95% (v/v) ethanol in 2 mL aliquots to each tube with continual stirring. After 2 min, carefully stir the tubes again to remove air bubbles from the polysaccharide precipitates (so that they will

sediment on centrifugation). Centrifuge the tubes in a bench centrifuge at 1,500 *g* for 10 min. Carefully decant the aqueous ethanol taking care not to dislodge the polysaccharide precipitates at the bottom of the tubes. To each tube, add 2 mL of 80% (v/v) aqueous ethanol and stir vigorously on a vortex mixer to obtain complete dispersion of the polysaccharide precipitates. Add a further 8 mL of 80% (v/v) aqueous ethanol to each tube and stir. Centrifuge the tubes at 1,500 *g* for 10 min and decant the supernatant solutions. Repeat this step of re-suspension in 80% (v/v) aqueous ethanol followed by centrifugation one more time. This operation removes essentially all of the free sugars from the sample being analysed.

To each tube, add 2 mL of solution 1 (pH 4.5) and stir vigorously on a vortex mixer. Incubate the tubes in a boiling water bath for 2 min and stir the contents on a vortex mixer to effect complete dissolution. Place the tubes in a water bath at 40°C and allow them to equilibrate over 5 min. Add 20 µL of β-mannanase (suspension 4) and incubate for 10 min. Add 0.1 mL of 2 M NaOH and incubate at room temperature for 10 min (to effect deacetylation of the glucomanno-oligosaccharides). Add 0.1 mL of 0.8 M HCl and 1.8 mL of 200 mM sodium phosphate buffer (pH 6.5) to adjust the pH to approx. 6.5. Weigh the tube to obtain an accurate sample volume (usually 4.00 mL). *Typically, a dilution factor of 1 to 5 using a sample volume of 0.5 mL is required for analysis.*

NOTE: Of the procedures suggested for the removal of sugars from the glucomannan, the recommended format is dialysis. However, for this to work properly, the correct diameter dialysis tubing must be used and dialysis must be performed against flowing tap water for at least 16 h. The other procedures tend to give a 10-20% underestimation of glucomannan content.

NOTE: On hydrolysis of polysaccharides in different jelly sweets with β-mannanase/β-Gos/β-Mos, followed by measurement of D-glucose and D-mannose using the HK/G6P-DH and PGI/PMI enzyme mixtures, either both D-glucose and D-mannose are detected, or just D-mannose. For samples yielding just D-mannose, it can be concluded that this sugar is derived from β-mannanase/β-mannosidase hydrolysis of 1,4-β-D-mannan. However, where both D-glucose and D-mannose are present, these are derived from glucomannan. The source of the glucomannan can usually be deduced from the ratio of D-glucose to D-mannose as measured.

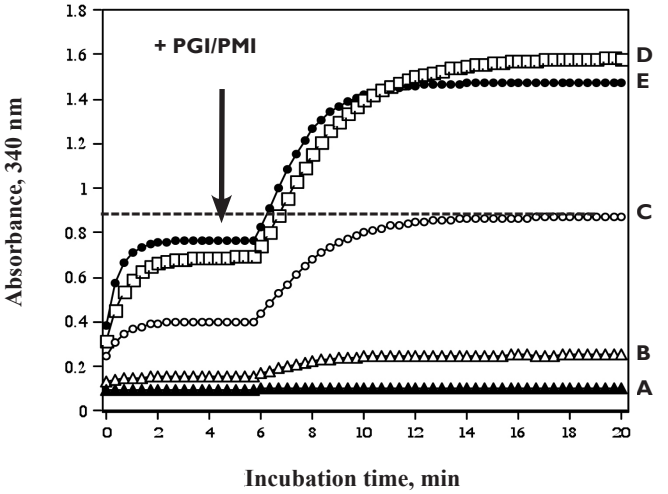

Figure 1. Measurement of D-glucose and D-mannose in a β-mannanase hydrolysate of konjac glucomannan. The reaction was initiated by addition of HK/G6P-DH (to measure D-glucose). At the point shown by the arrow, a PGI/PMI mixture was added to convert M-6-P to F-6-P and then to G-6-P with measurement using the HK/G6P-DH catalysed reaction. Glucomannan in a Jelly sweet was prepared according to procedure (b) and 0.1 mL (B), 0.5 mL (C) or 1.0 mL (D) aliquots were analysed for D-glucose and D-mannose content. Concurrently, a sample blank (A) and a D-glucose + D-mannose control solution (50 µg of each sugar) (E) were analysed.

NOTES:

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