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RAFFINOSE/ SUCROSE/ GLUCOSE

ASSAY PROCEDURE

K-RAFGL 04/18

(120 Assays per Kit)

Megazyme

INTRODUCTION:

Grain legumes are an important component of both human and livestock diets. Galactosyl-sucrose oligosaccharides (raffinose, stachyose and verbascose) are major components in many food legumes,[1] and the anti-nutritional activity of grain legumes is frequently associated with the presence of these oligosaccharides.[2] Galactosyl-sucrose oligosaccharides are not hydrolysed in the upper gut due to the absence of α-galactosidase. In the lower intestine they are metabolised by bacterial action, producing methane, hydrogen and carbon dioxide, which lead to flatulence and diarrhoea. Galactosyl-sucrose oligosaccharides are thus a factor limiting the use of grain legumes in monogastric diets.[3]

Several solvents have been employed for the extraction of galactosyl-sucrose oligosaccharides from legume-seed flours. These are generally water/alcohol mixtures. Before (or concurrent with) extraction, it is vital that endogenous α-galactosidase and invertase are inactivated. This can be achieved by refluxing the flour in ethanol or in an aqueous ethanol mixture before the flour is subjected to aqueous extraction.

Identification and quantification of the extracted galactosyl-sucrose oligosaccharides have been achieved using an array of chromatographic procedures, however many of these methods are, at best, semi-quantitative. Chromatographic procedures employing high performance liquid chromatography and low pressure liquid chromatography (using Bio-Gel P2) are quantitative, but can be time consuming, particularly in the area of sample preparation.

It is well known that raffinose, stachyose and verbascose are hydrolysed by α-galactosidase to D-galactose and sucrose. Biochemical kits for the measurement of raffinose are commercially available. The α-galactosidase used in these kits (from green coffee beans) rapidly hydrolyses raffinose, but acts quite slowly on stachyose and verbascose, and thus does not give complete hydrolysis of these oligosaccharides under the incubation conditions recommended. In contrast, the enzyme used in the current procedure (from *Aspergillus niger*) readily and rapidly catalyses complete hydrolysis of raffinose, stachyose and verbascose to D-galactose and sucrose.

PRINCIPLE:

Galactosyl-sucrose oligosaccharides are hydrolysed to D-galactose, D-glucose and D-fructose using α-galactosidase and invertase. The D-glucose is then determined using glucose oxidase/peroxidase reagent. The method does not distinguish between raffinose, stachyose and verbascose, but rather measures these as a group. Since one mole of each of the raffinose-series oligosaccharides contains one mole of D-glucose, the concentrations are presented on a molar basis. Free sucrose and D-glucose in sample extracts are determined concurrently.

KITS:

Kits suitable for performing 120 assays of D-glucose, sucrose and raffinose-series oligosaccharides are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: α-Galactosidase suspension (*A. niger*; 2 mL) in ammonium sulphate.
Stable for > 5 years at 4°C.

Bottle 2: Invertase solution (yeast; 6 mL) containing sodium azide (0.02%) as a preservative.
Stable for > 5 years at 4°C.

Bottle 3: **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.095% w/v).
Stable for > 4 years at 4°C.

Bottle 4: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Stable for > 5 years below -10°C.

Bottle 5: D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.

Bottle 6: Soy-Flour Reference Sample (containing glucose, sucrose and galactosyl-sucrose oligosaccharides).
Stable for > 5 years stored dry at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Add the entire contents of Bottle 1 to 21 mL of Buffer 1 (see page 3). Then add 2 mL of the contents of Bottle 2 (invertase) and mix the contents thoroughly by careful inversion. Divide the solution into aliquots of suitable volume (approx. 5 mL) and store in polypropylene tubes below -10°C.
   Stable for > 5 years below -10°C.

2. Dilute 1.0 mL of the contents of Bottle 2 to 12 mL with Buffer 1 [50 mM sodium acetate buffer, (pH 4.5)].
   Stable for > 2 years below -10°C.

3. Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water (this is solution 3). Use immediately.

NOTE: On storage, salt crystals may form in the concentrated buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.

NOTE: This buffer contains 0.095% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of bottle 4 with 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is Glucose Determination Reagent **(GOPOD Reagent)**. Stable for ~ 3 months when stored at 2-5°C or > 12 months below -10°C.

5 & 6. Use the contents of bottles 5 and 6 as supplied. Stable for > 5 years at room temperature.

BUFFER 1 (NOT SUPPLIED):

Sodium acetate buffer (50 mM, pH 4.5).
Add 2.9 mL of glacial acetic acid to 900 mL of distilled water. Adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 L. Store at 4°C. Sodium azide (0.2 g) can be added as a preservative. Stable for > 3 months at 4°C (no sodium azide).
Stable for > 2 years at 4°C (with sodium azide).

NOTE: Sodium azide is a toxic chemical and should be treated accordingly. It is added to buffers (after pH adjustment) solely as a preservative. It can be deleted from buffer recipes but buffers should then be stored at 4°C.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 120 mm and 18 x 150 mm).
2. Micro-pipettors, e.g. Gilson Pipetman 100/200 µL.
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of diluted invertase and invertase/α-galactosidase mixtures).
   - with 12.5 mL Combitip® (to dispense 1.0 mL aliquots of invertase).
4. Analytical balance.
5. Spectrophotometer (set at 510 nm).
6. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
7. Thermostated water bath (set at 50°C).
8. Boiling water bath (set at 84-88°C).
9. Bench centrifuge (capable of 1,000 *g*).
10. Stop clock.

CONTROLS AND PRECAUTIONS:

1. Time of incubation with GOPOD reagent is not critical but should be at least 20 min. Colour formed should be measured within 60 min.
2. With each set of determinations, reagent blanks and D-glucose controls [0.556 µmoles (i.e. 100 µg) quadruplicate] should be included.
   a. The reagent blank consists of 0.4 mL of 50 mM sodium acetate buffer (Buffer 1) + 3.0 mL GOPOD Reagent.
   b. The glucose control consists of 0.1 mL of D-glucose standard solution (100 µg/0.1 mL) + 0.3 mL of Buffer 1 + 3.0 mL GOPOD Reagent.
3. With each set of determinations a control flour is included.
4. With each new batch of GOPOD Reagent, the time for maximum colour formation with 100 µg (0.556 µmoles) of D-glucose standard should be checked. This is usually about 15 min.

ASSAY PROCEDURE:

Enzyme Inactivation and Sugar Extraction:

1. Accurately weigh 0.50 ± 0.01 g of flour sample into a glass test-tube (18 x 150 mm) and add 5 mL of ethanol (95% v/v).
2. Incubate the tube in a water bath at 84-88°C for 5 min (This treatment inactivates endogenous enzymes).
3. Quantitatively transfer the tube contents to a 50 mL volumetric flask and adjust the volume to the mark with sodium acetate buffer (50 mM, pH 4.5) (Buffer 1). Allow the sample to extract over 15 min and then mix thoroughly.
4. Transfer 5 mL of this solution/slurry to a glass test-tube (16 x 120 mm; suitable for centrifugation at 1,000 *g*).
5. Add 2 mL of chloroform to this solution, mix vigorously on a vortex mixer for 15 sec and centrifuge (1,000 *g*) for 10 min.

NOTE: This treatment removes most of the lipids from the aqueous upper phase. Insoluble plant material tends to concentrate between the phases.

6. Use the upper aqueous solution (Solution A) directly for analysis.

Assay for Glucose, Sucrose and Raffinose-Series Oligosaccharides:

1. Treat 0.20 mL aliquots of Solution A as follows:

   - 0.2 mL of Soln A + 0.2 mL Buffer I [D-Glucose] . . . . . A
   - 0.2 mL of Soln A + 0.2 mL invertase [D-Glucose + Sucrose] . . B
   - 0.2 mL of Soln A + 0.2 mL α-galactosidase + invertase [D-Glucose + Sucrose + Galactosyl-sucrose oligosaccharides] .C

2. Incubate all solutions at 50°C for 20 min.

3. Add 3.0 mL of GOPOD Reagent to solutions A, B and C, as well as to the Reagent Blank and the D-glucose controls, and incubate all at 50°C for 20 min.

4. Read the absorbance of all solutions against the Reagent Blank at 510 nm:

   Absorbances: ΔA = GOPOD absorbance for A
   ΔB = GOPOD absorbance for B
   ΔC = GOPOD absorbance for C

The **reagent blank** consists of 0.4 mL of 50 mM sodium acetate buffer (Buffer 1) plus 3.0 mL of GOPOD Reagent.

The **glucose control** consists of 0.1 mL of D-glucose standard solution (100 µg/0.1 mL) plus 0.3 mL of Buffer 1 and 3.0 mL of GOPOD Reagent.

CALCULATIONS:

D-Glucose, millimoles/100 grams:

$$= \Delta A \times F \times 250 \times 200 \times \frac{1}{1000}$$

$$= \Delta A \times F \times 50$$

Sucrose, millimoles/100 grams:

$$= (\Delta B - \Delta A) \times F \times 250 \times 200 \times \frac{1}{1000}$$

$$= (\Delta B - \Delta A) \times F \times 50$$

Raffinose-series oligosaccharides (RSO), millimoles/100 grams:

$$= (\Delta C - \Delta B) \times F \times 250 \times 200 \times \frac{1}{1000}$$

$$= (\Delta C - \Delta B) \times F \times 50$$

where:

ΔA = GOPOD absorbance for 0.2 mL of samples + acetate buffer.

ΔB = GOPOD absorbance for 0.2 mL of samples + invertase.

ΔC = GOPOD absorbance for 0.2 mL of samples + α-galactosidase and invertase.

F = a factor to convert from absorbance to µmoles of glucose

$$= \frac{0.556 \text{ (µmoles of glucose)}}{\text{GOPOD absorbance for 0.556 µmoles of glucose}}$$

250 = conversion to 50 mL of extract (i.e. to 0.5 g of sample).

200 = conversion from 0.5 to 100 g of sample.

$\frac{1}{1000}$ = conversion from µmoles to millimoles.

The concentrations of D-glucose and sucrose can be represented as millimoles/100 g, or can simply be calculated as g/100 g of flour, as shown below. However, it is not possible to calculate galactosyl-sucrose oligosaccharides as g/100 g of flour because these oligosaccharides are a mixture of raffinose, stachyose and verbascose. If the major component of this mixture for a given seed material is known, then it is possible to use the molecular weight of this compound and calculate an approximate value in grams/100 grams of flour.

D-Glucose (g/100 g flour) = D-Glucose (millimoles)/100 g x 0.1799.

Sucrose (g/100 g flour) = Sucrose (millimoles)/100 g x 0.3425.

Galactosyl-sucrose oligosaccharides (GSO) (g/100 g flour)
= (GSO)/100 g x MW/1000.

where:

0.1799 = the MW of D-glucose (180)/1000 mg of D-glucose.

0.3425 = the MW of sucrose (342)/1000 mg of sucrose.

MW/1000 = the average MW for GSO/1000 mg of RSO.

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