Megazyme

MUSHROOM and YEAST BETA-GLUCAN

ASSAY PROCEDURE

K-YBGL 07/11

(100 Assays per Kit)

INTRODUCTION:

(1-3)-β-Glucans are widely distributed in Nature, especially in algae, fungi and yeast, but also in higher plants. They serve a variety of biological functions. They form the major structural components of cell walls, they act as storage carbohydrates and they sometimes play a protective role by forming at specific sites in response to particular stimuli such as wounding.[1] The significance of (1-3)-β-glucans in the wine making industry is related to the presence in wine of a particular high molecular weight (~ 800 Kd) β-glucan, produced by the fungus, *Botrytis cinerea* (grey rot). This glucan is composed of a (1-3)-β-linked D-glucan backbone to which single D-glucosyl residues are attached β-(1-6) to every third main-chain residue. The β-glucan passes into the must and, as the alcohol levels rise in the latter stages of wine making, aggregation occurs leading to clarification and filtration problems. β-Glucan levels as low as 10 mg/L can cause serious filtration problems[2]. These problems can only be removed by the addition of specific enzyme preparations active on this polysaccharide. At present the only method for detecting this β-glucan in wine is the use of a non-specific alcohol precipitation test. β-Glucans also have medicinal implications and literature indicates that the potent anti-tumour properties of polysaccharide fractions extracted from certain strains of mushrooms, in particular *Grifola frondosa* (also known as Maitake), can be attributed to linear 6-branched, 1,3-β-glucan.[3-5] It has been claimed that yeast β-glucan substantially enhances the function of the immune system by activating macrophages, one of the primary defences of the immune system.

This booklet describes a method for the measurement of (1-3) (1-6)-β-glucan in yeast and mushrooms. Work is continuing on extraction and decolourisation procedures for the analysis of β-glucan in wine.

PRINCIPLE:

1,3:1,6-β-D-Glucan, 1,3-β-D-glucans and α-glucans are solubilised in concentrated (37 %; 10 N) hydrochloric acid and then extensively hydrolysed by 1.3 N HCl at 100°C for 2 h. Hydrolysis to D-glucose is completed by incubation with a mixture of highly purified *exo*-1,3-β-glucanase and β-glucosidase. While some β-glucans are readily soluble in hot water or hot KOH, these solvents are not effective in solubilising the β-glucans from yeast or mushrooms. Analysis of these glucans requires prior partial acid hydrolysis to remove gel-forming properties and covalent links to other polysaccharides (e.g. chitin) or proteins.

ACCURACY:

Standard errors of approximately < 5 % are achieved routinely.

KITS:

Kits suitable for carrying out 100 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: *exo*-1,3-β-Glucanase (100 U/mL) plus β-Glucosidase (20 U/mL) suspension, 2.0 mL.
Stable for > 4 years at 4°C.

Bottle 2: Amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) solution in 50 % v/v glycerol, 20 mL.
Stable for ~ 2 years at 4°C or > 4 years at -20°C.

Bottle 3: **GOPOD Reagent Buffer.** Buffer (48 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.4 % 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 at -20°C.

Bottle 5: D-Glucose standard solution (5 mL, 1.00 mg/mL) in 0.2 % w/v benzoic acid.
Stable for > 5 years at room temperature.

Bottle 6: Control yeast β-glucan preparation (~ 2 g, β-glucan content stated on the bottle label).
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Add 8 mL of 200 mM sodium acetate buffer (pH 5.0) to bottle 1 (i.e. dilute the contents of the vial to 10 mL). Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Once diluted, the reagent is stable for > 2 years at -20°C.

2. Use the contents of bottle 2 as supplied.
Stable for ~ 2 years at 4°C or > 4 years at -20°C.

3. Dilute the contents of bottle 3 to 1.0 L with distilled or deionised water. Stable for > 2 years at 4°C.

4. Dissolve the contents of bottle 4 in the diluted contents of bottle 3 (see the preceding reagent). Divide this reagent mixture **(GOPOD reagent)** into aliquots of desired volume for storage. Stable for 2-3 months at 4°C in a dark bottle, or for > 12 months at -20°C.

5. Use the contents of bottle 5 as supplied.
Stable for > 4 years at room temperature.

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

REQUIRED REAGENTS (not supplied):

1. **Sodium acetate buffer (200 mM, pH 5.0).**
Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water and adjust to pH 5.0 using 4 M (16 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 litre.
Stable for ~ 1 year at 4°C.

2. **Sodium acetate buffer (1.2 M, pH 3.8).**
Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 litre with distilled water.
Stable for > 2 years at room temperature.

3. **Potassium Hydroxide** (**2 M**).
Add 112 g of KOH to 800 mL of distilled water and dissolve by stirring. Adjust the volume to 1 litre.
Stable for > 2 years at room temperature.

4. **Hydrochloric acid** (**37 % v/v; ~10 M**).
Merck No. 100317-2500.
Stable for > 10 years at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed, 16 x 100 mm, 14 mL capacity).
2. Corning Culture Tubes
   - Screw cap tubes, 20 x 125 mm (Fisher Scientific Cat No. FB59563) plus caps (Cat. No. FB51355).
   - Screw cap tubes, 16 x 125 mm (Fisher Scientific Cat No. TKV-173-030B) plus caps (Cat. No. TKV-178-020V). Fisher Scientific, interact@fisher.co.uk
3. Boiling water bath (deep-fry cooker filled with water).
4. Micro-pipettors, 100 µ litre (e.g. Gilson Pipetman® or Rainin EDP-2® motorised dispenser).
5. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of hydrolysed sample solution and 0.2 mL of enzymes).
   - with 25 mL Combitip® (to dispense 1.5 mL aliquots of 37 % HCl and 3.0 mL of GOPOD Reagent).
6. Magnetic stirrer plus stirrer bars (5 x 15 mm).
7. Analytical balance.

8. Spectrophotometer set at 510 nm.
9. Vortex mixer.
10. Thermostated water bath set at 40°C.
11. Bench centrifuge (required speed 3,000 rpm; i.e. approx. 1,500*g*), with tube holders to accommodate 20 x 125 mm tubes.

NOTE:
The tube holders routinely supplied with bench centrifuges accommodate tubes of up to 16 mm diameter. In the current method, the optimal tube diameter is 20 mm (to accommodate a sample size of 100 mg). If a tube holder which can hold 20 mm diameter tubes is not available, then use Corning Culture Tubes, 16 x 125 mm, as described above. In this case, reduce the sample size and all volumes to 50 % of that described in the method.

CONTROLS AND PRECAUTIONS:

1. Safety goggles, gloves and laboratory coat must be worn at all times. When incubating the tube in the boiling water bath, the tube (with cap loosened), should be placed into the boiling water bath for 5 min to allow the contents to heat. The cap should then be tightened. This prevents excess pressure accumulation in the tube and removes the possibility that tubes might explode.
2. Concentrated hydrochloric acid (37 % v/v) is a very strong acid. Extreme caution must be exercised when handling this solution. These operations should be performed in a well ventilated fume cupboard.

MEASUREMENT OF 1,3:1,6-β-GLUCAN IN YEAST AND MUSHROOM PREPARATIONS:

A. MEASUREMENT OF TOTAL GLUCAN (α-glucan + β-glucan) plus D-Glucose in Oligosaccharides, Sucrose and free D-Glucose

a. Solubilisation and partial hydrolysis of total glucan (α-glucan + β-glucan) plus D-glucose in oligosaccharides, sucrose and free D-glucose.

1. Mill yeast or mushroom sample to pass a 0.5 mm screen using a Retsch centrifugal mill, or similar.
2. Add milled sample (approximately 100 mg, weighed accurately) to a 20 x 125 mm Fisher Brand culture tube. Tap the tube to ensure that all of the sample falls to the bottom of the tube.

3. Add 1.5 mL of concentrated hydrochloric acid (37 % v/v) to each tube, cap the tubes and stir them vigorously on a vortex mixer. Place the tubes in a water bath at 30°C for 45 min and stir them on a vortex mixer every 15 min (to ensure complete dissolution of the β-glucan).
4. Add 10 mL of water to each tube, cap the tubes and stir the contents on a vortex mixer.
5. Loosen the caps on the tubes and place them in a boiling water bath (~ 100°C). After 5 min tighten the caps and continue the incubation for **2 hr.**
6. Cool the tubes to room temperature, carefully loosen the caps and add 10 ml of 2 N KOH.
7. Quantitatively transfer the contents of each tube to a 100 mL volumetric flask using 200 mM sodium acetate buffer (pH 5.0) to wash the tube, and to adjust the volume. Mix thoroughly by inversion.
8. Filter an aliquot of each suspension through Whatman GF/A glass fibre filter paper, or centrifuge at 1,500 *g* for 10 min.

b. Measurement of total glucan plus D-glucose in sucrose and free D-glucose.

1. Transfer 0.1 mL aliquots (in duplicate) of filtered or centrifuged extract to the bottom of glass test tubes (16 x 100 mm).
2. Add 0.1 ml of a mixture of *exo*-1,3-β-glucanase (20 U/mL) plus β-glucosidase (4 U/ml) in 200 mM sodium acetate buffer (pH 5.0) to the bottom of each tube, mix the tube contents on a vortex mixer and incubate at 40°C for 60 min.
3. Add 3.0 mL of glucose oxidase/peroxidase mixture (GOPOD) to each tube and incubate at 40°C for 20 min.
4. Measure the absorbance of all solutions at 510 nm against the reagent blank.

NOTE:

With each set of determinations, include at least one control yeast or mushroom preparation. Also include reagent blanks and glucose standards of 100 µg (in quadruplicate).

The **reagent blank** consists of 0.2 mL of sodium acetate buffer (200 mM, pH 5.0) + 3.0 mL glucose oxidase/peroxidase reagent.

The **D-glucose standard** consists of 0.1 mL D-glucose standard (1 mg/mL) + 0.1 mL of sodium acetate buffer (200 mM, pH 5.0) + 3.0 mL glucose oxidase/peroxidase reagent.

B. MEASUREMENT OF α-GLUCAN (phytoglycogen and starch) plus D-glucose in sucrose and free D-glucose.

Solubilisation, hydrolysis and measurement of α-glucan, D-glucose from sucrose and free D-glucose.

1. Add milled sample (approximately 100 mg, weighed accurately) to a 20 x 125 mm Fisher Brand culture tube. Tap the tube to ensure that all of the sample falls to the bottom of the tube.
2. Add a magnetic stirrer bar (5 x 15 mm) followed by 2 mL of 2 M KOH to each tube and suspend the pellets (and dissolve the phytoglycogen/starch) by stirring for approximately 20 min in an ice/water bath over a magnetic stirrer **(see Figure 1).**
3. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring. Immediately add 0.2 mL of amyloglucosidase (1630 U/ml) plus invertase (500 U/mL), mix well and place the tubes in a water bath at 40°C.
4. Incubate the tubes at 40°C for 30 min with intermittent mixing on a vortex stirrer.
5. ***For samples containing > 10% α-glucan content***; quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle) and adjust to volume with water. **Mix well**. Centrifuge an aliquot of the solution at 1,500 *g* for 10 min, or filter through Whatman No. 1 filter paper (9 cm).
6. ***For samples containing < 10% α-glucan content***; directly centrifuge the tubes at 1,500 *g* for 10 min (no dilution). For such samples the final volume in the tube is approximately 10.3 mL (however, this volume may vary slightly with the type of sample being analysed). In some cases, an appropriate allowance for volume should be made in the calculations.

7. Transfer 0.1 mL aliquots (in duplicate) of either the diluted or undiluted supernatants into glass test tubes (16 x 100 mm), add 0.1 mL of sodium acetate buffer (200 mM, pH 5.0) plus 3.0 mL of GOPOD reagent and incubate at 40°C for 20 min.

8. Measure the absorbance of all solutions at 510 nm against the reagent blank.

NOTE:

Yeast and mushroom samples generally contain < 10 % α-glucan. However, some commercial mushroom mycelia are grown on cereal grains, and in this case, the starch content of the recovered product can be as high as 75 % w/w.

This method is **NOT** applicable to the analysis of yeast β-glucan in the presence of cellulose (1,4-β-D-glucan).

CALCULATIONS:

$$\text{Total Glucan (+ oligomers etc.) (\% w/w)} = \Delta E \times F \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta E \times F/W \times 90.$$

$$\text{α-Glucan (+ oligomers etc.) (\% w/w)} = \Delta E \times F \times 1000 \text{ (or 103)} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta E \times F/W \times 90 \text{ (final volume 100 ml)}$$
$$= \Delta E \times F/W \times 9.27 \text{ (final volume 10.3 ml)}$$

$$\text{β-Glucan} = \text{Total Glucan (+ oligomers etc.)} - \text{α-Glucan (+ oligomers etc.)}$$

where:

ΔE = reaction absorbance – blank absorbance.

F = a factor to convert absorbance to μg of D-glucose.

$$= \frac{100 \text{ (μg of the D-glucose standard)}}{\text{GOPOD absorbance for 100μg of D-glucose standard.}}$$

100/0.1 = volume correction factor; for total glucan (yeast), (0.1 mL out of 100 mL was analysed).

103 = volume correction factor; for α-glucan (0.1 mL out of 10.3 mL was analysed).

or

1000 = volume correction factor; for α-glucan (0.1 mL out of 100 mL was analysed).

1/1000 = conversion from µg to milligrams.

100/W = conversion back to 100 mg of sample (i.e. as % w/w).

W = weight of sample analysed.

162/180 = a factor to convert from free D-glucose, as determined, to anhydroglucose, as occurs in β-glucan.

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

Table 1. Total glucan, α-glucan and β-glucan (by difference) contents of a range of commercial mushroom samples.

Sample	Glucan content (dry weight basis)		
	Total	α-Glucan	β-Glucan
1. Dried Black Fungus	48.5	0.2	48.3
2. Dried Black Fungus	42.7	0.3	42.4
3. Dried Maitake Mushrooms	43.4	1.6	42.8
4. Shitake; Clearspring Premium	44.5	5.9	38.6
5. Marel Caps	29.1	8.4	20.7
6. *Boletus edulis*	27.4	3.8	23.8
7. *Morchella conica*	26.8	18.9	7.9
8. Porcini Mushrooms	33.8	4.9	28.9
9. Dried Trompettes	15.5	0.8	14.7
10. Melange Forestier mixture	29.4	1.6	27.8

Sample details:

1. Dried black fungus, Lotus brand, Packed by Du Phong trading company. Hong Kong;
2. Dried black fungus; packed by Fuzhou Golden Banyan Foodstuffs Ltd., China;
3. Yukiguni dried Maitake mushrooms (*Grifola frondosa*), Mitoku Macrobiotic, Japan;
4. Shitake, Clearspring Premium;
5. Choice Morel Caps, Le Gourmet Wholefoods, France;
6. *Boletus edulis* (Porcini 3rd Choice), L'Aquila Importers and Distributors, London; (La Rousse Foods, Ireland).
7. *Morchella conica* (dried morels whole). (La Rousse Foods, Ireland);
8. Porcini mushrooms, Tropical Wholefoods, France;
9. Dried Trompettes, Trompettes de la Mort., La Rousse Foods Ltd., Ireland;
10. Melange Forestier (*Boletus pleurotes*, champignons noirs, cepes).

NOTES:

NOTES:

Figure 1. Arrangement of ice-water bath over a magnetic stirrer for dissolution of sample material in 2 N KOH.

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