Megazyme

MUSHROOM and YEAST BETA-GLUCAN

ASSAY PROCEDURE

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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 of detecting this β-glucan in wine is by 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 boolet 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 and 1,3-β-D-glucans and α-glucans are solubilised in 60% sulphuric acid and then hydrolysed to near completion in 2 N HCl. Any remaining glucan fragments are then quantitatively hydrolysed to glucose using a mixture of highly purified *exo*-1,3-β-glucanase and β-glucosidase. While some β-glucans are readily solubile 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 solubilisation in 60% sulphuric acid and extensive 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:

1. *exo*-1,3-β-Glucanase plus β-Glucosidase.
2. Amyloglucosidase
3. Glucose Determination Reagent
4. Glucose Reagent Buffer (concentrate)
5. Glucose Standard Solution
6. Control Yeast β-Glucan preparation.

ENCLOSED ENZYMES:

(A) *exo*-1,3-β-Glucanase (100 U/ml) plus β-Glucosidase (20 U/ml)
2 ml in 3.2 M ammonium sulphate).

Before use, add 8 ml of 100 mM sodium acetate buffer (pH 4.5) (i.e. dilute the contents of the vial to 10 ml).
Store frozen between use in polypropylene tubes.

(B) Amyloglucosidase (10 ml, 3200 U/ml, in 50% glycerol).

Use as supplied.

ENCLOSED REAGENTS:

(A) Glucose Determination Reagent (for 1 Litre). Store at -20°C.

Reagent concentrations after dissolution in buffer:

Glucose oxidase	>12,000 U/litre
Peroxidase	>650 U/litre
4-Aminoantipyrine	0.4 mM

(B) Glucose Reagent Buffer (concentrate) (50 ml). Store at 4°C (up to 1 year) or at -20°C (for periods >1 year).

Dilute the entire contents to 1 litre with distilled water and use to dissolve the **Glucose Determination Reagent.** Divide this reagent **(GOPOD reagent)** into aliquots of desired volume for storage.
Stability: 2-3 months at 4°C; 12 months at -20°C.

ENCLOSED STANDARDS:

(A) Glucose standard solution (100 µg/0.1 ml in 0.2% benzoic acid). Store at room temperature.

(B) Yeast β-Glucan preparation (2 g) (level of β-glucan specified on vial label). Store dry at room temperature.

REAGENTS:

1. **Sodium acetate buffer** (100 mM, pH 4.5).

   Add glacial acetic acid (5.7 ml, 1.05 g/ml) to 900 ml of distilled water. Adjust the pH to pH 4.5 by the addition of 2 M (8 g/100 ml) sodium hydroxide solution. Approximately 70 ml is required. Store at 4°C.

   Adjust the volume to 1 litre and store the buffer at 4°C.

2. **Sodium acetate buffer** (1.2 M, pH 3.8).
   Add 69.6 mL of glacial acetic acid 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. Store 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. Store at room temperature.

4. **Sulphuric acid** (60% v/v; ~11 M).
   Carefully add 240 ml of concentrated acid (98%, sp. gr. 1.835) to 120 ml of distilled water. Mix well.
   Store at room temperature.

5. **Aqueous ethanol or IMS** (~ 80% v/v).
   Dilute 800 mL of 99% ethanol or Industrial Methylated Spirits to 1 L with H_2O. Store in a well sealed bottle 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 μlitre (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 extracts and buffered enzyme solutions),

   - with 50 ml Combitip® (to dispense 2.0 ml aliquots of 2 N KOH and 2 ml of 11 N H_2SO_4 and 3.0 ml of GOPOD Reagent).

6. Bench centrifuge (required speed 3,000 rpm; i.e. approx.1,500g), with tube holders to accommodate 20 x 125 mm tubes.
7. Magnetic stirrer plus stirrer bars (5 x 15 mm).
8. Analytical balance.
9. Spectrophotometer set at 510 nm.
10. Vortex mixer.
11. Thermostatted water bath set at 40.0°C.

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. When cooking tubes in the boiling water bath, safety goggles, gloves and laboratory coat must be worn at all times. 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. Sulphuric acid (60%) is a very strong acid. Extreme caution must be exercised when preparing and handling this solution. These operations should be performed in a well ventilated fume cupboard.
3. The enzyme preparation solutions should not be cross-contaminated.

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

A. Measurement of Total Glucan (α-glucan + β-glucan)

a. Removal of low degree of polymerisation (DP) sugars.

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.

NOTE: For samples not containing reducing sugars (e.g. commercial yeast β-glucan preparations that are already washed), the alcohol washing steps below are not required. In this case, simply "wet" the sample with 0.2 ml of aqueous ethanol before proceeding with "b".

3. Add 8 ml of aqueous ethanol (80% v/v) to each tube and stir the tubes vigorously on a vortex mixer. Incubate the tubes at ~ 80°C for 15 min. Ensure that the solution does not boil out of the tube. Add another 8 ml of aqueous ethanol and stir the tubes vigorously on a vortex mixer. Cool, and centrifuge at 1,500g for 10 min. Carefully decant and discard the supernatant solution.

4. Resuspend the pellet in 8 ml of aqueous ethanol and stir vigorously. Add another 8 ml of aqueous ethanol and stir. Centrifuge the tubes at 1,500g for 10 min. Carefully decant the supernatant. Invert the tubes on absorbent paper to ensure complete removal of all free liquid.

b. Solubilisation of Total Glucan (α-glucan + β-glucan):

1. Add a magnetic stirrer bar (5 x 15 mm) to each tube and place the tubes in a test-tube rack in an ice water bath over a magnetic stirrer. Add 2 ml of ice-cold, 60% v/v sulphuric acid to each tube with vigorous stirring on the magnetic stirrer (to ensure complete dispersion). Continue stirring for **1 hr**.

2. Add 12 ml of water to each tube and continue stirring for a few minutes.

3. Loosely cap the tubes and place them in a boiling water bath at ~ 100°C. Allow the remaining alcohol to boil off over 5 min, and then tighten the cap and continue the incubation for exactly **2 hr.**

4. Cool the tubes to room temperature and carefully loosen the caps.

5. Quantitatively transfer the contents of each tube to a 100 ml volumetric flask using a water wash bottle and adjust to volume with distilled water. Mix the contents well by inversion.

6. Filter an aliquot of the suspension through Whatman GF/A glass fibre filter paper, or centrifuge at 1,500g for 10 min.

c. Measurement of total glucan:

1. For **mushroom samples**, transfer 0.2 ml aliquots (in duplicate) of filtered or centrifuged extract to the bottom of glass test tubes (16 x 100 mm). For **yeast samples**, transfer 0.1 ml of extract plus 0.1 ml of water to the bottom of the glass test tubes.

2. Add 3.0 ml of Glucose oxidase/peroxidase mixture (GOPOD) to each tube and incubate at 40°C for 20 min.

3. 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 micrograms (in quadruplicate) .

The **reagent blank** consists of 0.2 ml of acetate buffer + 3.0 ml glucose oxidase/peroxidase reagent.

The **glucose standard** consists of 0.1 ml glucose standard + 0.1 ml of acetate buffer (100 μg/0.1 ml) + 3.0 ml glucose oxidase/peroxidase reagent.

NOTE:
In a previous method, hydrolysis was perfomed with trifluoroacetic acid at 120°C, and this was found to be incomplete with the hydrolysate containing 10-15% of gluco-oligosaccharides. Consequently, it was essential to complete the hydrolysis to glucose with a mixture of *exo*-1,3-β-glucanase plus β-glucosidase. In contrast, with the use of sulphuric acid, as described here, the reaction is essentially complete (~ 97%). Consequently, treatment with *exo*-1,3 β-glucanase plus β-glucosidase is generally not required (it leads to insignificant increases in the measured absorbance values). However, this enzyme mixture is still included in the assay kit to allow the analyst to check that hydrolysis with sulphuric acid is complete.

To include the treatment with *exo*-1,3-β-glucanase plus β-glucosidase, the hydrolysate needs to be neutralised. This is done as follows:

From Step b.4.:

i Quantitatively transfer the contents of each tube to a 100 ml volumetric flask using a water wash bottle. Add 20 ml of 2 M KOH and adjust to volume (100 ml) with 100 mM sodium acetate buffer, pH 4.5.

ii. Filter an aliquot of the suspension through Whatman GF/A glass fibre filter paper, or centrifuge at 1,500*g* for 10 min.

iii. Perform step c.1 above.

iv. Add 0.1 ml of a mixture of *exo*-1,3-β-glucanase (20 U/ml) plus β-glucosidase (4 U/ml) in 0.1 M sodium acetate buffer to the bottom of each tube, mix the tube contents on a vortex mixer and incubate at 40°C for 60 min. Proceed from Step c.2, above

v. Since the sample incubation volume has increased to 0.3 ml, also increase the reagent blank and the glucose standard to 0.3 ml (i.e. add an extra 0.1 ml of 0.1 M sodium acetate buffer.

B. MEASUREMENT OF α-GLUCAN (phytoglycogen and starch).

a. Removal of low degree of polymerisation (DP) sugars.

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.

NOTE: For samples not containing reducing sugars (e.g. commercial yeast β-glucan preparations that are already washed), the alcohol washing steps are not required. In this case, simply "wet" the sample with 0.2 ml of aqueous ethanol before proceeding with "b".

2. Add 8 ml of aqueous ethanol (80% v/v) to each tube and stir the tubes vigorously on a vortex mixer. Incubate the tubes at ~ 80°C for 10 min. Ensure that the solution does not boil out of the tube. Add another 8 ml of aqueous ethanol and stir the tubes vigorously on a vortex mixer. Cool, and centrifuge at 1,500*g* for 10 min. Carefully decant and discard the supernatant solution.

3. Resuspend the pellet in 8 ml of aqueous ethanol and stir vigorously. Add another 8 ml of aqueous ethanol and stir. Centrifuge the tubes at 1,500*g* for 10 min. Carefully decant the supernatant. Invert the tubes on absorbent paper to ensure complete removal of all free liquid.

b. Solubilisation, hydrolysis and measurement of α-glucan.

1. 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).**

2. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring. Immediately add 0.1 mL of AMG (3300 U/ml), mix well and place the tubes in a water bath at 40°C.

3. Incubate the tubes for 30 min with intermittent mixing on a vortex stirrer.

4. ***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, 9 cm filter paper.

5. ***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.

6. Transfer 0.1 mL aliquots (in duplicate) of either the diluted or undiluted supernatants into glass test tubes (16 x 100 mm), treat with 3.0 mL of GOPOD reagent and incubate at 40°C for 20 min.

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

Prepare reagent blank solutions (in duplicate) by mixing 0.1 mL of 0.1 M sodium acetate buffer (pH 4.5) with 3.0 mL of GOPOD reagent.

Prepare glucose standards (in quadruplicate) by mixing 0.1 mL of glucose (1 mg/ml) and 3.0 mL of GOPOD reagent.

After incubation at 40°C for 20 min, measure the absorbance of each solution 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%.

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

CALCULATIONS:

Yeast

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

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

Mushroom

$$\text{Total Glucan (\% w/w)} = \Delta E \times F \times \frac{100}{0.2} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162.}{180}$$

$$= \Delta E \times F/W \times 45.$$

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

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

β-Glucan = Total Glucan - α-Glucan

where:

ΔE = reaction absorbance – blank absorbance;

F = a factor to convert of absorbance to μg of glucose
= 100 (μg glucose)/the GOPOD absorbance for 100 μg Glc;

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

100/0.2 = volume correction factor; for total glucan (mushroom), (0.2 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 = weight of sample analysed;

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

Figure 1. Arrangement of ice-water bath over a magnetic stirrer for treatment of samples with 2 M KOH and dissolution of starch and phytoglycogen.

Table 1. The effect of inclusion of exo-1,3-β-glucanase/β-glucosidase treatment on the determined b-glucan content of a range of samples solubilised in 60% sulphuric acid and then hydrolysed in 2 N acid.

Sample	Determined β-Glucan Content ("as is" basis)	
	Not Treated	Treated
Scleroglucan		
(1 hr at 100°C)	78.3	79.4
(2 hr at 100°C)	76.5	77.3
(3 hr at 100°C)	77.9	77.9
Curdlan	78.6	79.7
Pachyman	78.4	77.6
Yeast β-glucan	72.6	72.3
Immustim	57.5	59.0

Samples were analysed on an "as is" basis (i.e. no allowance was made for moisture content). Scleroglucan has a structure very similar to the major β-glucan in mushrooms. Immustim is a commercial yeast β-glucan product

Table 2. 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	40.44	0.21	40.23
2. Dried Black Fungus	35.01	0.30	34.71
3. Dried Maitake Mushrooms	17.5	1.6	15.8
4. Shitake; Clearspring Premium	31.4	5.9	25.5
5. Marel Caps	23.3	8.4	115.0
6. *Boletus edulis*	12.5	3.8	8.7
7. *Morchella conica*	22.0	18.9	3.1
8. Porcini Mushrooms	12.2	4.9	7.3
9. Dried Trompettes	12.0	0.8	11.2
10. Melange Forestier mixture	45.5	1.6	43.9

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 Gormet 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) containing a mixture of *Boletus granulatus, Pleurotus ostreatus, Auricula judae* and *Boletus edulis*)(La Rousse Foods, Ireland).

Five β-glucan containing materials were analysed in quadruplicate on four separate days. The overall mean β-glucan contents (dry weight basis) and variability (cv %) were as follows:

Non-defatted Immustim:	(mean =59.88 ± 0.75; cv = 1.2 %);
Curdlan:	(mean =86.33 ± 1.30; cv = 1.5 %);
Maitake Tablets:	(mean =17.42 ± 0.45; cv = 2.6 %);
Dried black fungus:	(mean =42.77 ± 1.60; cv = 3.7 %);
Dried Trompettes:	(mean =13.30 ± 0.37; cv = 2.8 %);

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