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MUSHROOM
and
YEAST BETA-GLUCAN

ASSAY PROCEDURE

K-YBGL 11/19

(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 medicinal properties of many species of mushroom have been valued and utilised in traditional Chinese medicine for centuries. More recent studies[1-7] have demonstrated that the key active compounds are triterpenoids, ergosterol and most importantly 1,3:1,6-β-glucan. This β-glucan activates the immune system and may even have anti-carcinogenic properties.[1-7] There is concern within the regulatory community regarding health claims relating to nutritional supplements as well as the identity and purity of these products,[8] and this relates particularly to medicinal mushrooms where the key active components have been identified as 1,3:1,6-β-glucan, triterpenoids and ergosterol. The 1,3:1,6-β-glucans of several mushroom species have been studied in considerable detail and the most predominant structural feature has been identified as a 1,3-β-glucan backbone with single D-glucosyl residues linked 1,6-β to every third[9] or fourth D-glucosyl unit in the 1,3-β-glucan backbone. However, much more complex structures have also been reported.[10-14] The structures of mushroom and fungal β-glucans are quite different to the cereal β-glucans (so-called mixed-linkage β-glucans) that are linear polysaccharides in which D-glucosyl residues are linked 1,3-β- and 1,4-β, and the ratio of these linkage types varies with the source of the β-glucan (e.g. oats, barley and wheat). Other β-glucans include cellulose (1,4-β-D-glucan) and curdlan (1,3-β-glucan). A highly specific enzymic procedure has been described for the measurement of cereal 1,3:1,4-β-D-glucans.[15,16] Enzymic procedures have also been described for measurement of 1,3:1,6-β-D-glucans in commercial yeast products,[17,18] however while these procedures are very useful for this particular application, they are less specific than the method that has been developed for the measurement of cereal β-glucan.[15,16] No quantitative enzymic procedure has been described for measurement of β-glucan in mushroom fruiting bodies or mycelium. To date, many of the methods developed for the measurement of β-glucan[19,20] in mushroom are modifications of the Prosky[21,22] dietary fiber procedure.

This booklet describes a method for the specific measurement of 1-3:1-6-β-glucan in mushroom and mycelial products, yeast and fungal preparations.[23] The procedure readily allows the measurement of mushroom products which are highly contaminated with α-glucans such as cereal starches.

PRINCIPLE:

1,3:1,6-β-D-Glucans, 1,3-β-D-glucans and α-glucans are solubilised in ice cold 12 M H_2SO_4 and then hydrolysed to near completion in 2 M H_2SO_4.[24,25] Remaining glucan fragments are then quantitatively hydrolysed to glucose using a mixture of highly purified *exo*-1,3-β-glucanase and β-glucosidase. This gives a measure of total glucan. α-Glucans and sucrose are specifically hydrolysed to D-glucose and D-fructose and glucose is measured with amyloglucosidase and invertase using GOPOD reagent. β-Glucan is determined by difference. Pure mushroom samples contain a much higher β-glucan content than do many commercial mushroom/mycelial products available in capsule form (Tables 1 and 2, page 8 and 10).

In an alternative format for total glucan hydrolysis, the sample is suspended and stirred in 12 M HCl at 30°C for 1 h. The solution is then diluted to 2 M with water and incubated at ~ 100°C in a boiling water bath for 2 h. With most mushroom samples analysed, very similar total glucan was obtained with both acids (Table 2, page 10).[23] However, for a few mushrooms, namely *Ganoderma lucidum*, *Poria cocus* and *Cordyceps militaris*, values with sulphuric acid are significantly higher than with hydrochloric acid.

ACCURACY:

Standard errors of < 5% are achieved routinely (Table 3, page 10).

KITS:

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

Bottle 1: (x2) ***exo*-1,3-β-Glucanase** (100 U/mL) plus **β-Glucosidase** (20 U/mL) ammonium sulphate suspension, 2.0 mL.
Stable for > 4 years at 4°C.

Bottle 2: **Amyloglucosidase** (1,630 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 below -10°C.

Bottle 3: **GOPOD Reagent Buffer.**
Buffer (50 mL, pH 7.4).
p-hydroxybenzoic acid and sodium azide (0.09%).
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, 1.00 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years; store sealed at room temperature.

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

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. To bottle 1, add 9 mL of 200 mM sodium acetate buffer (pH 4.5) (i.e. dilute the contents of one vial to 11 mL). Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use and on ice during use. Once diluted, the reagent is stable for > 2 years below -10°C. Dilute the contents of second vial when required.

2. Use the contents of bottle 2 as supplied.
Stable for ~ 2 years at 4°C or > 4 years below -10°C.

3. Dilute the contents of bottle 3 to 1 L with distilled water. **This is Solution 1.** Use immediately.

NOTE:

1. 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.
2. This buffer contains 0.09% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of bottle 4 in approx. 20 mL of solution 1 and quantitatively transfer to the bottle containing the remainder of solution 1. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is **Glucose Determination Reagent (GOPOD Reagent).**
Stable for ~ 3 months at 2-5°C or > 12 months below -10°C.

If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots. Do not freeze/thaw more than once.

When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be less than 0.05 when read against distilled water.

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

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

REQUIRED REAGENTS (not supplied):

1. **Sodium Acetate Buffer (200 mM, pH 4.5)**
Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water and adjust to pH 4.5 using 4 M (16 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 L.
Stable for ~ 1 year at 4°C.

2. **Sodium Acetate Buffer (1.2 M, pH 3.8)**
Add 68.6 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 L with distilled water.
Stable for > 2 years at room temperature.

3. **Sodium Hydroxide (8.0 M)**
In a well ventilated fume cupboard, add 320 g of NaOH to 700 mL of distilled water and dissolve by stirring. Allow the solution to cool to room temperature and then adjust the volume to 1 L.
Stable for > 2 years at room temperature.

4. **Sodium Hydroxide** (**1.7 M**)
Add 68 g of NaOH to 800 mL of distilled water and dissolve by stirring. Adjust the volume to 1 L.
Stable for > 2 years at room temperature.

5. **Sulphuric Acid** (**12 M, 72% w/w**)
In a well ventilated fume cupboard, carefully add 640 mL of concentrated acid (98%, sp. gr. 1.835) to 300 mL of distilled water. Dilute to 1 L and mix well.
Stable at room temperature for > 4 years.

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. FB59559) plus caps (Cat. no. FB51354).
3. Boiling water bath (deep-fry cooker filled with water).
4. Micro-pipettors, 100 µL (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 0.1 mL of enzymes).

- with 25 mL Combitip® (to dispense 2.0 mL aliquots of 12 M H_2SO_4, 6 mL aliquots of 8 M KOH and 3.0 mL of GOPOD Reagent).

6. Magnetic stirrer plus stirrer bars (5 x 15 mm).
7. Analytical balance.
8. Microfuge centrifuge - capable of 13,000 rpm.
9. Disposable 2.0 mL polypropylene microfuge tubes.
10. Spectrophotometer set at 510 nm.
11. Thermostated water bath set at 40°C.
12. Vortex mixer.

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 sulphuric acid 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 mushroom or yeast sample to pass a 1.0 mm screen using a Retsch centrifugal mill, or similar.
2. Add milled sample [approx. 90 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 2.0 mL of ice cold 12 M sulphuric acid to each tube, cap the tubes and stir them vigorously on a vortex mixer. Place the tubes in an ice-water bath and leave them there for 2 h.

Over this period of time, vigorously stir the tube contents (for 10-15 sec) several times on a vortex mixer (to ensure complete dissolution of the β-glucan).

4. Add 4 mL of water to each tube, cap the tubes and vigorously stir the contents on a vortex mixer for 10 sec. Then add 6 mL of water, cap the tubes and stir the contents for a further 10 sec.
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 h.
6. Cool the tubes to room temperature and carefully loosen the caps.
7. Quantitatively transfer the contents of each tube to a 100 mL volumetric flask using a wash bottle containing 200 mM sodium acetate buffer (pH 4.5).
8. Add 6 mL of 8.0 M NaOH solution to the volumetric flask and adjust to volume with 200 mM sodium acetate buffer (pH 4.5). Mix the contents well by inversion and collect an aliquot of the sample in a microfuge tube.
9. Centrifuge an aliquot of the solution at 13,000 rpm for 5 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 4.5) 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 GOPOD Reagent 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). Run these through the entire incubation procedure with GOPOD Reagent

The **reagent blank** consists of 0.2 mL of sodium acetate buffer (200 mM, pH 4.5) + 3.0 mL GOPOD 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 4.5) + 3.0 mL GOPOD Reagent.

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

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

1. Add milled sample (approx. 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 1.7 M NaOH to each tube and suspend the pellets (and dissolve the phytoglycogen/starch) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer.
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 (1,630 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 13,000 rpm for 10 min or filter through Whatman No. 1 filter paper (9 cm).
6. ***For samples containing < 10% α-glucan content***; transfer 2 mL of solution to a microfuge tube and centrifuge at 13,000 rpm for 5 min. For such samples the final volume in the tube is approx. 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 4.5) 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:
Mushroom and yeast 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).

Table 1. Total glucan, α-glucan and β-glucan contents of a range of encapsulated mushroom and mycelium based products.

	Sample Details	Total Glucan (g/100 g)	α-Glucan (g/100 g)	β-Glucan (g/100 g)
1	*Ganoderma lucidum*	74.3	29.2	45.1
2	16 *Basidiomycete* species blend	69.5	66.4	3.2
3	7 *Basidiomycete* species blend	73.7	72.5	1.3
4	*Ganoderma lucidum*	44.6	22.6	22.0
5	*Ganoderma lucidum*	87.7	83.2	4.3
6	*Ganoderma lucidum* / *Lentinula edodes*	59.9	41.9	18.0
7	*Cordyceps* sp. (ascomycete)	64.8	53.9	10.9
8	*Cordyceps* sp. (ascomycete)	65.5	64.0	1.5
9	*Ganoderma lucidum*	52.5	45.2	7.3
10	*Cordyceps sinensis* (ascomycete)	13.9	3.0	10.9
11	*Cordyceps sinensis* (ascomycete)	29.3	24.1	5.2
12	*Inonotus obliquua*	69.8	70.0	~ 0.0
13	Control (*A. niger* mycelium) 49 % β-glucan	51.9	1.0	50.9

CALCULATIONS:

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

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

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

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

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

$$\beta\text{-Glucan} = \text{Total Glucan (+ sucrose etc.)} - \alpha\text{-Glucan (+ sucrose etc.)}$$

where:

ΔE = reaction absorbance – blank absorbance.

F = a factor to convert absorbance to μg of D-glucose.
= 100 (μg of the D-glucose standard)
GOPOD absorbance for 100 μg 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).

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 2. A comparison of HCl and H_2SO_4 procedures in the measurement of β-glucan and α-glucan contents of a range of pure mushroom samples.

Mushroom sample	α-Glucan + Free Glucose (g/100g, dwb)	β-Glucan H_2SO_4 Procedure (g/100g, dwb)	β-Glucan HCl Procedure (g/100g, dwb)
Polyporus umbellatus	0.6	50.5	52.8
Trametes versicolor	0.2	47.1	53.3
Inonotus obliqus	0.2	7.9	8.5
Ganoderma lucidum (sample 1)	0.2	54.0	23.6
Agaricus blazei	3.4	13.1	8.9
Grifola frondosa	1.3	35.1	32.1
Ganoderma lucidum (sample 2)	0.6	54.8	26.2
Poria cocus (powder)	0.8	73.9	66.9
Lentinula edodes (powder)	3.2	36.2	37.4
Cordyceps militaris	2.2	34.3	28.6
Hericium erinaceus	3.2	33.9	35.3
Agaricus bisporus (button)	1.3	6.0	7.5
Pleurotus ostreatus	0.4	32.3	33.3
Tremella fuciformis	1.2	14.9	15.6
Grifola frondosa	1.8	31.5	32.5
Lentinula edodes	0.9	23.5	27.4
Pleurotus eryngii	0.4	37.1	39.4
Flammulina velutipes	0.7	20.0	21.0
Agaricus bisporus (portobella)	4.1	5.7	7.2
Aspergillus niger mycelium (control 49%)	0.7	50.6	50.6

Table 3. Repeatability of H_2SO_4 acid hydrolysis procedure for the measurement of total glucan in mushroom products.

Sample	Total glucan, % (w/w)[a], mean[b] ±2 SD (CV[c], %)				
	Day 1	Day 2	Day 3	Day 4	Interday mean ±2 SD (CV, %)
Trametes versicolor	45.5 ± 2.8	45.8 ± 3.2	48.3 ± 4.2	49.1 ± 4.4	47.2 ±4.3
	(3.0%)	(3.4%)	(4.4%)	(4.5%)	(4.6%)
Ganoderma lucidum	52.4 ± 2.6	51.8 ± 2.4	52.7 ± 0.2	53.1 ± 0.5	52.5 ±1.7
	(2.5%)	(2.3%)	(0.2%)	(0.4%)	(1.6%)
Agaricus blazei	16.2 ± 1.4	16.2 ± 0.9	16.9 ± 0.6	16.7 ± 0.2	16.5 ±0.9
	(4.4%)	(2.8%)	(1.6%)	(0.6%)	(2.9%)
Grifola fondosa	35.6 ± 1.3	35.1 ± 1.6	37.8 ± 0.9	37.1 ± 0.5	36.4 ±2.5
	(1.8%)	(2.2%)	(1.1%)	(0.7%)	(3.4%)
Cordyceps militaris	36.8 ± 1.7	36.8 ± 1.8	37.4 ± 0.3	37.3 ± 0.2	37.1 ±1.1
	(2.2%)	(2.5%)	(0.4%)	(0.2%)	(1.5%)
Purified yeast β-glucan (Megazyme Lot n. 20301)	75 ± 5.7	75.5 ± 3.3	77 ± 0.1	77.4 ± 0.1	76.2 ±3.3
	(3.8%)	(2.2%)	(0.1%)	(0.04%)	(2.2%)
Aspergillus niger mycelium (49% β-glucan Megazyme Lot no. 130905a)	53.7 ± 0.4	53.3 ± 1	54 ± 0.6	54.8 ± 0.9	54 ±1.2
	(0.4%)	(0.9%)	(0.5%)	(0.8%)	(1.1%)

[a] All results are presented as total glucan as a percentage of dry weight. α-Glucan and glucose contents of these samples are low and can be seen in Table 3.

[b] On each day, samples of each material were analysed in duplicate.

[c] CV = coefficient of variation.

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