β-GLUCAN
(Yeast and Mushroom)

ASSAY PROTOCOL

K-YBGL

08/23

(100 Assays per Kit)

NOTE: PLEASE READ THIS BOOKLET CAREFULLY AS CHANGES TO THE PROTOCOL HAVE OCCURRED

INTRODUCTION:

IMPORTANT INFORMATION:

This procedure has been updated as of AUGUST 2023 (protocol 08/23) to include trehalase suspension (Bottle 7). Inclusion of this enzyme in Section B (Measurement of α-glucan) will result in an increased α-glucan measurement for samples containing trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside). As a result, the calculated β-glucan value will be reduced. The updated procedure should be followed for all sample types.

(1-3)-β-Glucans are widely distributed in nature, especially in algae, fungi and yeast, but also in higher plants. β-Glucans serve a variety of biological functions; they form the major structural components of cell walls, act as storage carbohydrates and sometimes play a protective role by forming at specific sites in response to stimuli such as wounding.[1]

The medicinal properties of many species of mushrooms 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-D-glucan). A highly specific enzymic procedure has been described for the measurement of cereal 1,3:1,4-β-D-glucans.[15,16] An assay kit based on this method is available to purchase from Neogen: β-Glucan Assay Kit (Mixed Linkage) (**K-BGLU**).

Enzymic procedures have also been described for measurement of 1,3:1,6-β-D-glucans in commercial yeast products,[17,18] (including Neogen's Enzymatic Yeast β-Glucan Assay Kit (**K-EBHLG**)), however 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 mushrooms are modifications of the Prosky[21,22] dietary fiber procedure.

This booklet describes a method for the indirect measurement of 1,3:1,6-β-D-glucan in mushroom and mycelial products, yeast and fungal preparations.[23] The method

described herein employs amyloglucosidase, invertase and trehalase to hydrolyse α-glucans found in starch, maltodextrins, sucrose and trehalose. If a sample contains α-glucans that are not hydrolysed by the enzymes described above this will result in an overestimation of β-glucan content.
This procedure is not suitable for the analysis of yeast/mushroom β-glucan in the presence of other β-glucans, for example, cellulose (1,4-β-D-glucan).

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. D-glucose released is then measured using **GOPOD reagent.** This gives a measure of total glucan.

α-Glucans found in starch, maltodextrins, sucrose and trehalose are degraded by incubation with amyloglucosidase, invertase and trehalase. Free D-glucose and D-glucose released during hydrolysis are then measured using **GOPOD reagent**.

β-Glucan is calculated indirectly as the difference between total glucan and α-glucan, determined as described here.

Previous assay protocols for this kit utilised an alternative total glucan hydrolysis method with hydrochloric acid. More details on this change are outlined in McCleary *et al*.[23]

ACCURACY:
Standard errors of < 5% are achieved routinely.

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 100 assays are available from Neogen. The kits contain the full assay method plus:

Bottle 1: (x2) ***exo*-1,3-β-Glucanase** plus **β-Glucosidase** ammonium sulphate suspension, 2.0 mL.
Store at 4°C. See individual label for expiry date.

Bottle 2: **Amyloglucosidase** plus **invertase** solution in 50% (v/v) glycerol, 20 mL.
Store below -10 °C. See individual label for expiry date. NOTE: If stored at 4°C the expiry of this product will be decreased to ≥ 2 years.

Bottle 3: **GOPOD Reagent Buffer.**
Buffer (50 mL, pH 7.4). *p*-hydroxybenzoic acid and sodium azide (0.09%).
Store at 4°C. See individual label for expiry date.

Bottle 4: **GOPOD Reagent Enzymes**. Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Store below -10°C. See individual label for expiry date.

Bottle 5: **D-Glucose standard solution** (5 mL, 1.00 mg/mL)
in 0.2% (w/v) benzoic acid.
Store sealed at room temperature. See individual label for expiry date.

Bottle 6: **Control yeast β-glucan preparation** (~ 2 g,
β-glucan content stated on the bottle label).
Store sealed at room temperature. See individual label for expiry date.

Bottle 7: **Trehalase suspension** (5 mL).
Store at 4°C. See individual label for expiry date.

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). This is **Solution 1.**
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 the second vial only when required.

2. Use the contents of **bottle 2** as supplied.

3. Dilute the contents of the **GOPOD Reagent Buffer** bottle to 1 L with distilled water. **This is Solution 2**. 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 the **GOPOD Reagent Enzymes** bottle in approx. 20 mL of **Solution 2** and quantitatively transfer to the bottle containing the remainder of **Solution 2**. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is **Glucose Determination Reagent** (**GOPOD Reagent**). Stable for ≥ 1 month at 4°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. The solution may develop a stronger pink colour upon storage 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.

6. Use the contents of **bottle 6** as supplied.

7. Use the contents of **bottle 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.

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.

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.

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.

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.

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.

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.
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 3X 50 mL Combitip® (to dispense 2.0 mL aliquots of 12 M H_2SO_4, 6 mL aliquots of 8 M NaOH 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.
13. Centrifugal mill, with 12-tooth rotor and 1.0 mm sieve

CONTROLS AND PRECAUTIONS:

1. This method is **NOT** applicable to the analysis of yeast/mushroom β-glucan in the presence of other β-glucans, for example cellulose (1,4-β-D-glucan).
2. If a sample contains α-glucans that are not hydrolysed by amyloglucosidase, invertase and trehalase an overestimation of β-glucan content will occur.

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 of **bottle 5** (D-glucose standard, 1 mg/mL) + 0.1 mL of sodium acetate buffer (200 mM, pH 4.5) + 3.0 mL **GOPOD Reagent**.

The absorbances of the samples assayed should not exceed that obtained for the D-glucose control samples. If the sample absorbance exceeds the control values dilute the sample further to achieve a suitable absorbance.

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

A. MEASUREMENT OF TOTAL GLUCAN (α-glucan + β-glucan)

a. Solubilisation and partial hydrolysis of total glucan

1. Mill mushroom or yeast sample to pass a 1.0 mm screen using a centrifugal mill.
2. Add the milled sample (approx. 90 mg, weighed accurately) to a 20 x 12 mm Fisher Brand culture tube. Tap the tube to ensure that all 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

incubate for 2 h. Periodically, 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

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 **Solution 1** [*exo*-1,3-β-glucanase plus β-glucosidase] 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.

B. MEASUREMENT OF α-GLUCAN

a. Solubilisation, hydrolysis and measurement of α-glucan

1. Add the milled sample (approx. 100 mg, weighed accurately) to a 20 x 125 mm Fisher Brand culture tube. Tap the tube to ensure that all 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 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 **Bottle 2** [amyloglucosidase plus invertase] **and 0.05 mL of Bottle 7** [Trehalase], mix well and place the tubes in a water bath at 40°C.

4. Incubate the tubes at 40°C for 60 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. **10.35 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 > 75% w/w.[23]

CALCULATIONS:

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

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

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

α-Glucan (% w/w) = $\Delta A \times F \times 1000 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$
(+ sucrose etc) **(or 103.5)**

$= \Delta A \times F/W \times 90$ (final volume 100 mL)
$= \Delta A \times F/W \times$ **9.315** (final volume **10.35** mL)

β-Glucan = Total Glucan (+ sucrose etc.) - α-Glucan (+ sucrose etc.)

where:

ΔA = 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).

103.5 = volume correction factor; for α-glucan (0.1 mL out of 10.35 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.

REFERENCES:

1. Chihara, G., Hamuro, J., Maeda, Y., Arai, Y. & Fukuoka, F. (1970). *Cancer Res.*, 30, 2776-2781.
2. Ohnu, N., Suzuki, I., Oikawa, S., Sato, K., Miyazaki, T. & Yodomae, T. (1984). *Chem. Pharm. Bull.*, 32, 1142-1151.
3. Ino, K., Ohno, N., Suzuki, I., Miyazaki, T. & Yadomae, T. (1985). *Carbohydr. Res.*, 141, 111-119.
4. Chihara, G. (1990). Lentinan and its related polysaccharides as host defence potentiators. "Immunotherapeutic Properties of infectious diseases", Edited by K. Noel Masihi and W. Lange. Heideelberg: Springer-Verlag.
5. Gunde-Cimerman, N. (1999). *Int. J. Medicinal*, Mushrooms 1, 69-80.
6. Liu, J., Yang, F., Ye, L.-B., Yang, X.-J., Tamani, K. A., Zheng, Y. & Wang, Y.-H. (2004). *J. Ethnopharmacology,* 95, 265-272.
7. Borchers, A. T., Keen, C. L. & Gershwin, M. E. (2004). *Exp. Biol. and Med.,* 229, 393-406.
8. Chilton, J. Redefining medicinal mushrooms, Industry White Paper, http://www.nammex.com/rmm-dps-pdf
9. Stone, B. A. (2009). Chemistry and physico-chemistry. "Chemistry, Biochemistry and Biology of (1-3)-β-glucans and related polysaccharides". Edited by A. Bacic, G. B. Fincher and B. A. Stone, Academic Press, Burlington, MA, USA. p 5-46.
10. Kato, K., Inagaki, T., Shibagaki, H., Yamauchi, R., Okuda, K., Sano, T. & Ueno, Y. (1983). *Carbohydr. Res.*, 123, 259-265.
11. Kato, K., Mutoh, K., Egashira, T., Hiura, M. & Ueno, Y. (1978). *Agric. Biol. Chem.*, 42, 1073-1074.
12. Misaki, A. & Kishida, E. (1995). *Food Rev. Int.*, 11, 219-223.
13. Miyazaki, T., Oikawa, N., Yamada, H. & Yadomae, T. (1978). *Carbohydr. Res.*, 65, 235-243.
14. Ohnu, N., Lino, K., Suzuki, I., Oikawa, S., Sato, K., Miyazaki, T. & Yadomea, T. (1985). *Chem. Pharm. Bull.*, 33, 1181-1186.
15. McCleary, B. V. & Glennie-Holmes, M. (1985). *J. Inst. Brew.*, 91, 285-295.
16. McCleary, B. V. & Codd, R. (1991). *J. Sci. Food Agric.*, 55, 303-312.
17. Danielson, M. E., Dauth, R., Elmasry, N. A., Langeslay, R. R., Magee, A. S. & Will, P. M. (2010). *J. Agric. Food Chem.*, 58, 10305- 10308.
18. Enzymatic yeast beta-glucan assay procedure (**K-EBHLG**). (2020). Megazyme Ltd. (Link to Assay Protocol)
19. Park, Y. K., Ikegaki, M., Alencar, S. M. & Aguiar, C. L. (2003). Cienc. Technol. Aliment., Campinas 23, 312-316.
20. Rhee, S. J., Cho, S. Y., Kim, K. M., Cha, D.-S. & Park, H.-J. (2008).

LWT-Food Sci. Technol., 545-549.

21. Prosky. L, Asp, N.-G., Furda, I., DeVries, J. W., Schweizer, T. F. & Harland, B. F., (1985). *J. AOAC International*, 68, 677-679.
22. Official Methods of Analysis of AOAC International, 19th Ed. 2012, Methods 925.10, 985.29, 991.42, 991.43, 993.19, 994.13, 996.01, 2001.03, 2002.01, 2002.02, 2009.01 and 2011.25. AOAC International, Rockville, Maryland, USA.
23. McCleary, B. V. & Draga, A. (2016). *J. AOAC International*, 99, 364-373.
24. Selvendran, R. P., March, J. F. & Ring, S. G. (1979). *Anal. Biochemistry*, 96, 282-292.
25. Saeman, J. F., Moore, W. E., Mitchell, R. L. & Millett, M. A. (1954). *Tappi*, 37, 336-343.

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