ENZYMATIC YEAST β-GLUCAN

ASSAY PROTOCOL

K-EBHLG

07/23

(50 Assays per Kit)

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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 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]

Yeast β-glucan substantially enhances the function of the immune system by activating macrophages. Literature indicates that linear 6-branched (1-3)-β-glucan extracted from the mushroom *Grifola frondosa* (also known as Maitake) can be linked to potent anti-tumour properties.[2-4] The use of yeast β-glucan in animal feed, especially broiler, has been demonstrated to be beneficial against some bacterial infections with comparable results to those displayed using antibiotics. Yeast β-glucan is therefore a promising replacement for antibiotics use in animal nutrition.[5]

Some β-glucan forms (e.g. high molecular weight (~ 800 KDa) β-glucan produced by the fungus *Botrytis cinerea* (grey rot) can however pose technological challenges in winemaking and are linked to clarification and filtration problems.[6]

An enzymic method for yeast β-Glucan measurement has been published by Danielson *et al*.[7] which is known as the 'GEM' assay. The method employs an *exo*-1,3-β- glucanase/β-glucosidase (**E-EXBGOS**) from Megazyme and the enzymes lyticase and pustulanase. This method is suitable for yeast β-glucan measurement, but its widespread adoption is limited by the high cost of lyticase and the potential over-estimation due to contaminating enzyme activities in the enzyme mix. In this assay protocol, we describe the **K-EBHLG** method, an alternate enzymic procedure for the measurement of (1-3)(1-6)-β-glucan in commercial extracted yeast β-glucan preparations. The **K-EBHLG** method is significantly simpler to perform than the GEM assay and the recombinant nature of the enzymes present in **K-EBHLG** provides excellent reproducibility while also removing the potential for enzymatic side activities (from impure enzymes) that can artificially inflate the β-glucan content in samples. Indeed, hydrolysis of α-glucans (starch, mutan, glycogen and sucrose) and cellulose by the ***Glucamix*** enzyme in **K-EBHLG** is minor (< 2%, see Table 1, page 7).

The **K-EBHLG** method described in this assay protocol does not give quantitative measurement of β-glucan in mushrooms. For these samples, we recommend the use of Neogen's Megazyme brand 'β-Glucan Assay Kit (Yeast and Mushroom)' (**K-YBGL**) assay kit. This kit is based on an acid hydrolysis/enzymic procedure for the determination of β-glucan in yeast and mushroom.[8] The acid hydrolysis measures the Total Glucan content (α-Glucan + β-Glucan) while the enzymic treatment is specific for α-glucan. The β-Glucan content in this method is determined by difference (i.e. Total Glucan- α-Glucan).

PRINCIPLE:

(1-3)(1-6)-β-D-Glucan, (1-3)(1-4)-β-D-glucan and (1-3)-β-D-glucans are solubilised/hydrated in 2 M potassium hydroxide with stirring and the solution is subsequently adjusted to pH 4.0-4.5 with 1.2 M sodium acetate buffer.[9] The slurry is incubated with the ***Glucamix*** enzyme mixture (highly specific yeast β-glucan hydrolytic enzyme suspension) for 16 h at 40°C. After dilution and centrifugation, an aliquot is

removed for determination of glucose with GOPOD reagent.

ACCURACY:

Standard errors of < 3% are achieved routinely (see Table 2, page 7).

KITS:

Kits suitable for carrying out 50 assays are available. The kits contain the full assay method plus:

Bottle 1: ***Glucamix* preparation** (Yeast β-glucan hydrolytic enzyme suspension), 2.2 mL.
Store at 4°C. See individual label for expiry date.

Bottle 2: **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.09% w/v).
Store at 4°C. See individual label for expiry date.

Bottle 3: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze dried powder.
Store below -10°C. See individual label for expiry date.

Bottle 4: **D-Glucose standard solution** (5 mL, 1.5 mg/mL) in 0.2% w/v benzoic acid. Store sealed at room temperature. See individual label for expiry date.

Bottle 5: Control fungal 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 6: **Control starch preparation** (~ 2 g, 96% starch dwb).
Store sealed at room temperature. See individual label for expiry date.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use bottle 1 as supplied. Swirl the container contents before removing aliquots. Stand the bottle in an upright position between use.

2. Dilute the contents of **GOPOD Reagent Buffer** bottle to 1.0 L with distilled or deionised water. **This is Solution 1. Use immediately.**

NOTE:

1. On storage, salt crystals may form in the concentrated GOPOD Reagent 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 handled accordingly.

3. Dissolve the contents of **GOPOD Reagent Enzyme** bottle in approx. 20 mL of **Solution 1** and quantitatively transfer this 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 ≥ 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.

4, 5 & 6. Use bottles 4, 5 & 6 as supplied.

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 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. **Potassium Hydroxide** (2 M).
Add 112 g of KOH to 800 mL of distilled water and dissolve by stirring. Adjust the volume to 1 L.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (16 x 100 mm, 14 mL capacity).

2. Glass culture tubes with screw cap (16 x 125 mm).

3. Micro-pipettors, 100 µL (e.g. Gilson Pipetman).

4. Positive displacement pipettor, (e.g. Eppendorf Multipette®).

5. Magnetic stirrer plus stirrer bars (5 x 15 mm).

6. Analytical balance.

7. Spectrophotometer set at 510 nm.

8. Vortex mixer.

9. Thermostated water bath set at 40°C.

10. Bench centrifuge (required speed 3,000 rpm; i.e. approx. 1,500 *g*), with tube holders large enough to fully accommodate tubes.

11. Centrifugal mill, with 12-tooth rotor and 0.5 mm sieve.

NOTE:

With each set of determinations, include at least one control fungal preparation (analyse **bottle 5** as described in the procedure below).

A sample blank is also recommended for each sample in order to account for any free glucose that may be present. Prepare the sample blank by including a duplicate of the sample analysis as outlined in the procedure below. At Step 4 in the procedure, add deionised water in place of ***Glucamix*** enzyme. Subtract the absorbance value for the sample blank from the result obtained for the sample prior to calculation of the β-glucan content.

Also include reagent blanks and glucose standards of 150 µg (in quadruplicate).

The **reagent blank** consists of 0.1 mL of sodium acetate buffer (200 mM, pH 5.0) + 4.0 mL glucose oxidase/peroxidase reagent.

The **D-glucose standard** consists of 0.1 mL D-glucose standard (1.5 mg/mL) + 4.0 mL glucose oxidase/peroxidase reagent.

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

1. Mill yeast sample or other material to pass a 0.5 mm screen using a centrifugal mill.

2. Add milled sample (approx. 20 mg, weighed accurately to the nearest 0.1 mg) to a glass culture tube. Record the weight. Tap the tube to ensure that all of the sample falls to the bottom of the tube.

3. Add 0.4 mL of 2 M KOH and a 5 x 15 mm stirring bar. Stir the contents for 30 min in an ice water bath over a magnetic stirrer (Figure 1, page 6).

4. Add 1.6 mL of 1.2 M sodium acetate buffer (pH 3.8), mix well and then add 40 µL of ***Glucamix*** enzyme mixture and cap the tubes. Continue mixing in the ice water bath for 2 min and then transfer the tubes to a water bath set at 40°C and incubate (without stirring) overnight (~ 16 h).

5. Add 10 mL of distilled water to each tube and mix the contents thoroughly. Centrifuge the tubes at 3,000 rpm for 10 min in a bench centrifuge. As an alternative, the sample can be filtered through the Whatman Type I filter paper (or equivalent).

6. Carefully transfer 0.1 mL aliquots of the sample in duplicate to the bottom of glass test tubes.

7. Add 4 mL of **GOPOD reagent** to each of the reaction tubes, the controls, the standards and reagent blanks, and incubate the tubes for 20 min at 40°C.

8. Read the absorbance at 510 nm of each solution against a reagent blank.

CALCULATIONS:

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).

β-Glucan (% w/w)

$$= \Delta A \times F \times \frac{12.04}{0.1} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180}$$

$$= \Delta A \times F/W \times 10.836.$$

where:

ΔA	=	Absorbance (reaction) - Absorbance (sample blank)
F	=	Conversion from absorbance to µg (150 µg of D-glucose) standard divided by GOPOD absorbance of this 150 µg).
12.04/0.1	=	Volume correction (0.1 mL taken from 12.04 mL).
100/W	=	Factor to present β-glucan as a percentage of sample weight.
1/1000	=	Conversion from µg to mg.
W	=	Weight of sample analysed in mg.
162/180	=	Factor to convert from free D-glucose to anhydro-D-glucose as occurs in β-glucan.

REFERENCES:

1. Stone, B. A. & Clarke, A. E. (1992). "Chemistry and Biology of (1-3)-β-Glucans." La Trobe University Press, Victoria, Australia.

2. Ohno, N., Suzuki, I., Oikawa, S., Sato, K., Miyazaki, T. & Yadonne, T. (1984). Antitumour Activity and Structural Characterisation of Glucans Extracted from Cultured Fruit Bodies of *Grifola frondosa. Chem. Pharm. Bull.,* **32**, 1142-1151.

3. Ohno, N., Lino, K., Takahiro, T., Suzuki, I., Sato, K., Oikawa, S., Miyazaki, T. & Yadonne, T. (1985). Structural Characterisation and Antitumour Activity of the Extracts from Matted Mycelium of Cultured *Grifola frondosa*. *Chem. Pharm. Bull.,* **33**, 3395-3401.

4. Lino, K., Ohno, N., Suzuki, I., Miyazaki, T. & Yadomae, T. (1985). Structural Characterisation of a Neutral Antitumour β-D-Glucan Extracted with Hot Sodium Hydroxide from Cultured Fruit Bodies of *Grifola frondosa. Carbohydrate*

Research, **141**, 111-119.

5. Ott, C. P., Omara, I. I., Persia, M. E. & Dalloul, R. A. (2018). The Impact of Beta-Glucans on Performance and Response of Broiler Chickens During a Coccidiosis challenge, *Poultry Sci*, **97**, 2713-2721.
6. Villettaz, J. C., Steiner, D. & Trogus, H. (1984). The use of a Beta Glucanase as an Enzyme in Wine Clarification and Filtration. *Am. J. Enol. Vitic.,* **35**, 253-256.
7. Danielson, M. E., Dauth, R., Elmasry, N. A., Langeslay, R. R., Magee, A. S. & Will, P. M. (2010). Enzymatic method to measure β-1,3-β-1,6- glucan content in extracts and formulated products (GEM assay). *J. Agric. Food Chem.,* **58**, 10305-10308.
8. McCleary, B. V. & Draga, A. (2016). Measurement of beta-Glucan in Mushroom and Mycelial Products. *J. AOAC International*, **99**, 364-373.
9. McCleary, B. V. & Monaghan, D. A. (2002). Measurement of resistant starch. *J. AOAC International*, **85**, 665-675.

APPENDICES:

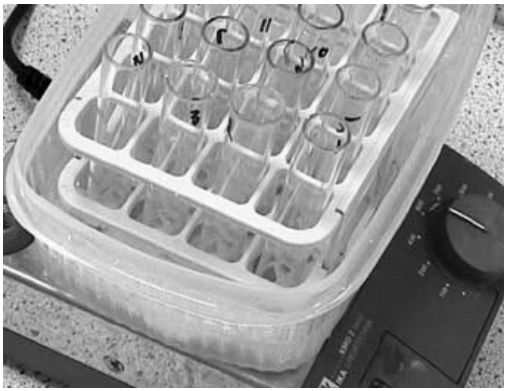

Figure 1. Arrangement of ice-water bath over a magnetic stirrer for dissolution/hydration of β-glucan in KOH.

Table 1. β-glucan content is measured using the enzymic procedure, **K-EBHLG**, for a range of standards and enzyme contamination screenings. The undesired hydrolysis of α-glucans and oligosaccharides (mutan, starch, glycogen and sucrose) and cellulose by the ***Glucamix*** enzyme mixture is minor (< 2 %) which is indicative of the purity of the enzyme components.

Sample Description	**Percentage hydrolysis, reported as β-glucan (%, *w/w,* as-is)**
Fungal β-glucan control (K-EBHLG, Lot 211101a)	34.0
USP Yeast β-glucan, (Ref cat 1048288)	65.6*
Alpha-Cellulose (Avicel, FMC)	2.1
Soluble starch (Sigma, S9765)	0.1
Glycogen (Sigma, G8751)	1.1
Mutan (P-AGLU13, Lot 210401)	1.0
Sucrose (Sigma, S7903)	1.7
* The content on the label states ~ 78% glucose on a dried weight basis (dwb). The K-EBHLG analysis is expressed as % w/w of β-glucan (as-is basis). When the reference sample moisture and the conversion factor between β-glucan and glucose are taken into account, the result obtained with K-EBHLG and is 80% glucose (dwb).	

Table 2. The repeatability of the assay for yeast samples was determined by analysis of 10 yeast samples over a period of 3 days, with 6 separate extractions of β-glucan, analysed in duplicate as per the standard procedure.

	β-Glucan (% w/w)						
	Day 1	**Day 2**	**Day 3**	**Average**	**St. Dev**	**n**	**%CV**
Sample 1	68.28	67.53	67.80	67.87	0.38	6	0.57
Sample 2	67.99	67.14	67.96	67.70	0.48	6	0.71
Sample 3	21.16	20.58	20.67	20.80	0.31	6	1.50
Sample 4	16.17	15.95	15.82	15.98	0.17	6	1.09
Sample 5	66.70	65.09	65.03	65.61	0.94	6	1.44
Sample 6	13.67	13.37	13.00	13.35	0.33	6	2.48
Sample 7	22.20	21.71	21.85	21.92	0.26	6	1.17
Sample 8	31.70	31.58	31.75	31.68	0.09	6	0.27
Sample 9	24.96	24.83	24.79	24.86	0.09	6	0.36
Sample 10	23.68	23.49	22.78	23.31	0.47	6	2.02

Contact us for more information: neogen.com/contact

Without guarantee

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