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Enzymatic Method To Measure β-1,3-β-1,6-Glucan Content in Extracts and Formulated Products (GEM Assay)

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An enzymatic method to measure β-glucan content (GEM assay) is applicable in a variety of matrices. The method is composed of swelling the sample with KOH and initial digestion with a lyticase, which is followed by treatment with a mixture of exo-1,3-β-D-glucanase and β-glucosidase that converts the β-glucan to glucose. The glucose generated by the enzymatic hydrolysis is measured by another enzymatic method. The method is shown to be accurate and precise. The method is selective and applicable to both highly branched and unbranched β-1,3-glucans.

KEYWORDS: β-1,3-β-1,6-glucan; β-glucan; lyticase; exo-1,3-β-D-glucanse; β-glucosidase

INTRODUCTION

β-1,3-β-1,6-Glucans, branched to various degrees at the 6-position, have been isolated from a variety of sources, including yeasts, fungi, plants, algae (*1*), and lichens (*2*). The β-(1,3)-main chain of β-glucan provides the basic structural motif, but significant variation in chemical structure affects chemical analysis and biological activity (*3*, *4*). The cereal-based β-glucans have a basic linear structure of a β-(1,3/1,4) chain (*1*). These β-glucans are found in oats, barley, and rye. Fungal β-glucans are isolated primarily from mushroom and yeast sources. The mushroom β-glucans are varied, but typically are composed of a β-(1,3)-D-glucan main chain that is highly branched (>30%) with short (one glucose unit) side chains linked β-(1,6) to the main chain (*1*). Yeast β-glucan also has a β-(1,3)-D-glucan main chain with lesser branching (<10%) and with longer β-(1,3)-glucan side chains linked β-(1,6) to the main chain (*1*). Yeast β-glucan also contains variable amounts of a branched β-(1,6)-D-glucan main chain with β-(1,6)-D-glucan side chains linked β-(1,3) to the main chain (*5*).

There have been a number of health benefits attributed to the consumption of β-glucans, including immunomodulatory effects (*6*). Published research has shown that certain β-glucans may enhance the ability of white blood cells to attack tumor cells through a defined mechanism of action (*7*), reduce the symptoms of respiratory tract infections (*8*−*10*), and protect mammals against infectious disease (*11*). The influence of the source and structure on biological activity was demonstrated in a study that compared the therapeutic efficacy of various sources of β-glucans (*12*).

To assess the quality of β-glucan-containing products, it is important to be able to analyze quantitatively the β-glucan content either in a purified powder extract ingredient or in a formulated product as an active ingredient. Several methods to quantify β-glucan content have been previously described. Some are based on acid hydrolysis followed by determination of the total glucose released (*13*). The shortcoming of this approach is that other glucose polymers present in the sample matrix may also release glucose, resulting in an inaccurate measurement. Another method employs solubilization of the glucan in DMSO and measurement against an internal standard by ^{1}H NMR spectroscopy to give β-glucan content on a dry weight basis (*14*). This method requires that the sample is freely soluble in DMSO and does not contain any interfering substances. An enzymatic method has been described that employs acidic hydrolysis of the β-glucan followed by enzymatic digestion to glucose (*15*). A commercially available kit (Megazyme, Wicklow, Ireland) employs this principle. However, acid hydrolysis releases glucose from any other glucose-containing polysaccharide present, in particular glycogen, which then requires a separate glycogen determination by an amylase digestion arm so that the glycogen content can be subtracted from the amount of total glucose. A modification of this approach, the Glucan Enzymatic Method (GEM), is described. This method employs two enzymatic digestions to specifically convert β-glucan to glucose, which is then measured by a colorimetric enzymatic assay.

In the GEM assay, a sample containing β-glucan is first gelatinized in aqueous KOH. The sample is then diluted with buffer and treated with a commercial lyticase. Lyticase is an endoglucanase that partially breaks down the β-glucan and solubilizes it. The lyticase digest is treated with a mixture of commercially available exo-1,3-β-D-glucanase and β-glucosidase that completely converts the solubilized β-glucan oligosaccharides to glucose. There are a variety of commercially available enzymatic assays that can be used to determine glucose for the β-glucan analysis, for example, the hexokinase assay. However, the glucose oxidase/peroxidase (GOPOD) system is preferable for its sensitivity.

MATERIALS AND METHODS

Materials. β-Glucosidase, exo-1,3-β-D-glucanase, glucose oxidase/peroxidase reagent (GOPOD), and GOPOD reagent buffer were purchased from Megazyme. Lyticase, laminarin, and barley β-glucan were purchased

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from Sigma-Aldrich (St. Louis, MO). β-Glucan from *Saccharomyces cerevisiae* (Wellmune WGP) was obtained from Biothera (Eagan, MN). Oyster glycogen was purchased from TCI America (Portland, OR). Pachyman was purchased from Biosupplies Australia, Pty. Ltd. (Parkville, VIC, Australia). Pustulan from *Umbilicaria papullosa* was purchased from Calbiochem/EMD Chemicals Inc. (Gibbstown, NJ). Scleroglucan (Actigum) was obtained from Cargill, Inc. (Atlanta, GA). Curdlan was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Methods. Samples of β-glucan were weighed (15−20 mg) in triplicate into 16 mm × 100 mm screw-cap culture tubes. During agitation of the tube on a vortex mixer, 400 μL of chilled 2 M aqueous potassium hydroxide was added directly to the powder. Tubes were then capped and cycled between agitation and storage in an ice bath for approximately 20 min. Upon removal from the ice bath, lyticase digestion was performed by adding 1.6 mL of pH 3.8 aqueous 1.2 M sodium acetate followed by 600 μL of a 10 U/μL solution of lyticase containing 0.01 M Tris, 0.001 M EDTA, and 0.02 M sodium chloride and then incubating the mixture at 50 °C for 12−18 h. After the samples had cooled to room temperature, an aliquot of 130 μL of each sample was then removed and further digested by treatment with 650 μL of a solution of 12 U/mL exo-1,3-β-D-glucanase and 2.4 U/mL β-glucosidase in pH 5 aqueous 0.2 M sodium acetate for 1 h at 40 °C. Samples were then cooled to room temperature and centrifuged, and duplicate 50 μL aliquots were removed for glucose testing.

Glucose detection was accomplished by the addition of 3 mL of GOPOD reagent dissolved in GOPOD reagent buffer (a solution of >12 U/mL glucose oxidase, >0.65 U/mL peroxidase, and 0.4 mM 4-aminoantipyrine in pH 7.4 aqueous 0.26 M dibasic potassium phosphate, 0.22 M *p*-hydroxybenzoic acid, and 0.06 M sodium azide) followed by incubation for 20 min at 40 °C. The absorbance of each solution was then read at 510 nm and compared to a similarly treated series of glucose standards to determine the concentration of liberated glucose.

Because the enzyme solutions absorb slightly at 510 nm, an enzyme blank is prepared by combining the various solutions proportionately, and the absorbance of this solution is subtracted from the absorbances of the sample solutions. The percent β-glucan value is determined by dividing the amount of glucose measured in each sample by the amount of the powder. These values can be adjusted by multiplying the total glucose determined by the GOPOD assay by a factor of 0.9, to account for the difference in molecular weight of a glucose monomer bound in a polysaccharide (162) as opposed to free glucose (180). If it is preferred to report the percent β-glucan on a dry weight basis, a separate moisture determination can be performed on the powder, and a corresponding correction can be applied in the calculation.

For linkage analysis by the partially methylated alditol acetate method (*16*), glucan samples were dissolved in dimethyl sulfoxide (DMSO) and treated with NaOH and methyl iodide to methylate all free hydroxyl groups. The methylated polysaccharide was then hydrolyzed with trifluoroacetic acid (TFA), the reducing ends of the resulting sugars were reduced with $NaBD_4$, and then the resulting free hydroxyl groups were acetylated with acetic anhydride. The mixture of partially methylated alditol acetates was analyzed by gas chromatography (GC). Each derivative corresponding to a particular linkage has been identified by a characteristic retention time and mass spectrum (MS) using a mass detector. The relative amount of each derivative was measured by GC with flame ionization detection (FID). The data presented are the average of two determinations; in each the quantity of the various derivatives is calculated as the percent integrated area of each peak multiplied by a response factor (*16*) normalized against the total area under the peaks in the gas chromatogram.

RESULTS AND DISCUSSION

In the GEM assay, a powder sample containing β-glucan is first gelatinized in aqueous KOH for 20 min. An amount of sample corresponding to 15−20 mg of β-glucan is preferred because this is the minimum amount of powder that can be weighed out accurately due to the static electricity that some of these powders acquire. The gelatinization step was originally envisioned to swell insoluble samples to make them more accessible to the enzymes. Although in some cases this step may not be necessary, in other samples, particularly those in which the glucan is formulated with additional ingredients, this treatment provides superior recovery of glucan by partially solubilizing the sample to make it more accessible for reaction with the enzymes. Longer reaction times in alkali do not appear to enhance glucan recovery (data not shown). In fact, care must be taken to minimize destruction of the β-glucan in this step, which is why the sample is held in an ice bath (*17*). The sample is then diluted with buffer and treated with a commercial lyticase. Lyticase is an endoglucanase that partially breaks down the β-glucan and solubilizes it. Different grades of lyticase have different activities, and so the conditions for digestion by each grade need to be optimized for glucose liberation in the assay. Some grades may not be suitable for the assay at all. It was concluded that for the lyticase grade chosen, digestion is complete with 600 μL of a 10 unit/μL solution because addition of more enzyme or a second aliquot of the lyticase solution at this step did not increase the glucan recovery. A $^1/_{20}$ aliquot of this digest is carried into the next step to minimize the consumption of the more expensive enzymes used in the second digestion. Use of a $^1/_{50}$ aliquot gave less precise results. The $^1/_{20}$ aliquot is treated with a mixture of commercially available exo-1,3-β-D-glucanase and β-glucosidase that completely converts the solubilized β-glucan oligosaccharides to glucose. Treatment with a higher quantity of the exo-1,3-β-D-glucanase and β-glucosidase mixture did not increase recovery of glucan (data not shown). A variety of commercially available enzymatic assays are suitable to determine glucose for the β-glucan analysis. The glucose oxidase/peroxidase (GOPOD) system was chosen because the standard curve gives a relatively steep slope.

Other glucans that may be present in a sample, such as glycogen from yeast or microcrystalline cellulose from a tablet formulation, do not react with these enzymes and so do not interfere in the determination. If a given sample is believed to contain free glucose, for example, a formulation containing dextrose, and the β-glucan is insoluble, the glucose can be removed by washing with water before the analysis. Alternatively, a no-enzyme control can be analyzed by the GOPOD method and the value for free glucose subtracted from the results for the samples.

Table 1. Replicate Analysis of Three Preparations of Yeast β-Glucan

		β-glucan content (%)		
sample		trial 1	trial 2	trial 3
1	av	67.4	67.3	67.4
	%RSD[a]	2.7	1.7	0.8
2	av	68.7	68.8	69.7
	%RSD	0.5	3.0	2.6
3	av	68. 8	70.6	71.2
	%RSD	1.2	3.0	0.8

[a] Percent relative standard deviation.

Results are presented in **Table 1** for analyses of percent β-glucan for three different preparations of a commercially available β-glucan (Wellmune WGP) derived from the cell wall of the yeast *S. cerevisiae*. The assay is reported as the average of three determinations. These values are adjusted by multiplying the total glucose determined with the GOPOD assay by a factor of 0.9, to account for the difference in molecular weight of a glucose monomer bound in a polysaccharide (162) as opposed to free glucose (180), and are uncorrected for moisture content. It can be seen that the method is precise, with percent relative standard deviations (%RSD) ranging from 1.9 to 3.9%. As shown in **Table 2**, the values obtained from the GEM method are in good agreement with the total hexose content as measured by the colorimetric anthrone assay (*16*).

The polysaccharide content of these yeast cell wall preparations is principally β-1,3-β-1,6-glucan according to linkage

Table 2. Comparison of β-Glucan Content with Total Hexose

sample	% β-glucan	% total hexose by anthrone
1	67.4	72.9
2	68.7	75.6
3	68.8	76.5

Table 3. Linkage Analysis by the Partially Methylated Alditol Acetate Method

	sample		
linkage	1	2	3
terminal	4.9	6.1	6.3
3-linked	82.1	78.3	77.9
6-linked	4.9	7.3	7.5
4-linked	0.9	1.1	1.2
3,6-linked	4.5	5.4	5.4
other	2.8	2.0	1.8

Table 4. GEM Results from Other Glucans

sample	% β-glucan by GEM assay	% RSD GEM assay	% total hexose by anthrone
microcrystalline cellulose	0.19	6.2	103
glycogen	0.72	6.8	89
pachyman	86	1.2	90
pustulan	78	0.8	89
scleroglucan	83	1.5	87
curdlan	88	2.3	89
laminarin	88	0.4	88
barley β-glucan	84	3.1	88

analysis by the partially methylated alditol acetate method (*16*) as shown in **Table 3**. The yeast β-glucan samples are composed of a relatively pure, lightly branched (5−6%) β-glucan and contain some 6-linked glucan as well as low amounts of 4-linked glucan, presumably α-1,4-linked glycogen, as this material can be removed by treatment with an amyloglucosidase. The "other" residues are a combination of low levels of various derivatives that arise from incomplete methylation of the β-glucan.

To investigate the scope and specificity of the combination of enzymes used in the GEM assay, a variety of other polysaccharides that may be present in β-glucan formulations were subjected to the GEM assay, and the results are presented in **Table 4**. Microcrystalline cellulose is used as an excipient in β-glucan formulations in gel capsules. It can be seen that this material does not interfere in the GEM assay. Whereas the β-glucan samples analyzed above have relatively low α-1,4-linked glycogen content, preparations from other sources have contained up to 20% glycogen (data not shown). In other analysis methods that measure total glucose released from a sample, a separate enzymatic analysis of glycogen content is required to subtract the contribution from the glycogen. It is apparent that a separate glycogen determination is not necessary in this method as very little of the pure glycogen is detected. Also, β-glucan has been accurately measured by the GEM assay in a formulated product that contained maltodextrin as a filler with no interference from the maltodextrin (data not shown).

The GEM assay gives a value in good agreement with total hexose by anthrone for both pachyman and curdlan, which are poorly soluble, unbranched β-glucans. The method performs as well on the more highly branched β-glucans, laminarin (8% branching) and scleroglucan (33% branching). Pustulan, a β-1,6-glucan, gives a high recovery, although lower than β-1,3-glucans. It has been reported that a purified lyticase showed no activity on pustulan (*18*), but the specific batch of exo-1,3-β-D-glucanase used in this study apparently had β-1,6-glucanase activity, as another batch used in subsequent experiments did not digest pustulan and consistently gave lower values of percent β-glucan. Depending on which batch of exo-1,3-β-D-glucanase is used, addition of a pustulanase digestion step after the lyticase digestion may be required to capture the β-1,6-glucan. Barley β-glucan, composed of blocks of β-1,4-glucan oligomers linked by a single β-1,3-glucose, also gives a high recovery, and so this method may be useful for analyzing barley β-glucan as well.

The Glucan Enzymatic Method (GEM) has been shown to provide an accurate and precise measure of β-glucan content in samples of the dry powder ingredient isolated from yeast. The method can be applied to a variety of branched and unbranched β-glucans. The GEM assay is also capable of accurately measuring the β-1,3-β-1,6-glucan content in samples containing other polysaccharides that may be present in a sample such as α-1,4-glucans or microcrystalline cellulose. A β-1,6-glucan and a β-1,4-β-1,3-glucan are also detected by the method.

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