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ORIGINAL PAPER

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Optimised quantification method for yeast-derived 1,3-β-D-glucan and α-D-mannan

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Abstract The polysaccharides 1,3-β-D-glucan and α-D-mannan show numerous beneficial effects for the health of humans and animals. For several years, an increasing number of glucan- and mannan-containing products intended for food and feed applications are commercially available. For the determination of glucan and mannan contents, however, widely accepted methods have not yet been established. We have developed a practicable and reliable quantification method characterised by acidic hydrolysis with trifluoroacetic acid and subsequent determination of released D-glucose and D-mannose. The unavoidable loss of the monosaccharides due to the acidic conditions was minimised by optimisation of the hydrolysis parameters. The best conditions found were compared with literature methods in order to demonstrate the suitability. Finally, glucan and mannan contents of various commercial products were determined and compared to the specifications given by the manufacturers.

Keywords 1,3-β-D-Glucan · α-D-Mannan · Acidic hydrolysis · Quantification · MOS

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Introduction

1,3-β-D-Glucan and α-D-mannan comprise two classes of polysaccharides which occur in numerous bacteria, fungi, mushrooms, algae and higher plants. In the last decades, both compounds have been described to show many benefits for the health of humans and animals [1, 2, 3, 4, 5, 6]. Accordingly, the number of commercial products containing 1,3-β-D-glucan—usually named 'β-glucan' or 'glucan'—or α-D-Mannan ('mannan' will be used in the following text) have increased in the last years. Glucan is primarily obtained from bacteria (curdlan [7]), baker's and brewer's yeast [8], fungi (scleroglucan [9]), and edible mushrooms (lentinan [10], shizophyllan [11]). It is used as a health food ingredient or in tablets and capsules for supporting the immune system, and curdlan has found use as a gelling agent and thickener in puddings, jellies and salad dressings. Mannan is often named MOS meaning 'mannan oligosaccharides'. MOS products are derived from common yeast and are used in the feed industry to prevent the colonisation of pathogens in the intestinal tract of farm animals [12, 13].

The marketing of glucan and MOS is often supported by certificates of analysis which are intended to prove a high content or purity of glucan and mannan. However, widely accepted quantification methods have not yet been introduced. One great obstacle concerns the more or less distinctive insolubility of many glucans, particularly of yeast glucans. Yeast glucans with a molecular weight of more than 10^5 kDa show a low solubility in dimethyl sulfoxide and are insoluble in other organic solvents and in water [14]. Therefore, direct analytical methods have predominantly been reported for well-soluble glucans. In the case of water-soluble β-1,3–1,4-mixed glucans occurring in cereals, complexes with fluorescent dyes like calcofluor white [15] and congo red [16] are used for quantification [17]. Recently described methods by means of NMR [18] or of an enzyme-linked immunosorbent assay [19] also require a solubility in dimethyl sulfoxide or in water. This is sometimes difficult or impossible if the glucan—as for example in yeast cell walls—is as-

sembled with other carbohydrates, proteins or lipids resulting in a strongly decreased solubility.

In contrast to glucans, mannans are usually soluble in water and in dimethyl sulfoxide actually facilitating analytical procedures. Nevertheless, determination methods have hardly been described. NMR spectra of yeast mannans have been published and may serve for confirming the identity [20, 21, 22]. Quantification by NMR seems, however, to be very difficult since the ^{1}H NMR spectrum does not show representative signals. Obviously, mannan yields are mostly determined by weight, and mannan purities are then equated with 100%.

Due to the problems with direct determination methods, quantification of glucan and mannan after cleavage into the monomeric units is the most applied way for evaluating their purity. For glucans, this can be achieved enzymatically by treatment with 1,3-glucanases [23, 24] provided that the completeness of the hydrolysis to D-glucose is not hampered by the presence of other biopolymers limiting the access of the enzyme. In the case of β-1,3–1,4-mixed glucans, however, an analytical method using the enzyme lichenase has been established as the standard method [25, 26].

The most common procedure for depolymerising glucan and mannan is the treatment with strong acids. Reaction time, temperature and acid concentration have to be chosen under which the polysaccharides are completely cleaved but which ensure a low degradation of D-glucose and D-mannose sugars released since they are sensitive to acidic degradation at elevated temperatures. The loss of monosaccharides due to degradation has to be compensated by using a reproducible correction factor. Hydrochloric, sulfuric and trifluoroacetic acid are the most commonly used reagents for cleaving glucan and mannan polymers but the conditions described in the literature differ considerably. We decided to use trifluoroacetic acid (TFA) for the hydrolysis of yeast glucan and mannan, and it was our aim to find optimal conditions for the release of D-glucose and D-mannose in terms of a minimal loss of the monosaccharides resulting in a preferably low correction factor. The optimised hydrolysis conditions were compared to published procedures and finally applied to commercial samples in order to evaluate the efficiency.

Materials and methods

Materials

D-Glucose, D-mannose and trifluoroacetic acid were purchased from Fluka (Buchs, Switzerland), and purities of >99% are specified. 1,3-β-D-Glucan from yeast was obtained by a procedure described in ref. 20. After additional washings with water and acetone, the elemental analysis gave C 43.89%, H 6.25%, N 0.36%. Curdlan (C-7821) was purchased from Sigma-Aldrich (Steinheim, Germany). After removal of moisture, a purity of 99% is specified. α-D-Mannan from *Saccharomyces cerevisiae* (M-7504) was purchased from Sigma-Aldrich (Steinheim, Germany). No statement concerning the purity was available. From the NMR spectra showing some impurities, a mannan content of 97% was determined. Examined commercial products were: Aloverose, lot no. 2003-10-12 (Rainbow Naturprodukte GmbH, Hamburg, Germany); Auxoferm YGT Plus, lot no. 237-1 (Deutsche Hefewerke, Hamburg, Germany); βeta-1,3D Glucan, bottle 0336407 12/06 (Transfer Point, L.L.C., Columbia, USA); Bio-Mannan-Mos-500, lot no. 40201310 (Ultra Bio-Logics, Inc., Rigaud, Canada); Biomos, batch no. 27220 (Alltech, Inc., Nicholasville, USA); Glucanocare, ch. 13 (ABAC R&D AG, Schlieren, Switzerland); Glucanosom, ch. 12 (ABAC R&D AG, Schlieren, Switzerland); Glucaferm 70 (Fibona Health Products GmbH, Wiesbaden, Germany); Glucasan (Fibona Health Products GmbH, Wiesbaden, Germany); Mos-Glucan, lot L (Lallemand, Inc., Montreal, Canada); NBG Cos, lot no. 082-3 (Biotec Pharmacon ASA, Tromsø, Norway); NovaGlucan 30 (Trinova Handel & Marketing AG, Wangen, Switzerland); WGP Beta Glucan, lot no. 20020102C (Biopolymer Engineering, Inc., Eagan, USA).

Acidic hydrolysis—general procedure

Samples were dried for 24 h at 70 °C under vacuum for removal of moisture and subsequently weighted (20 mg) into a glass tube (Pyrex SVL, 14×100, VWR International AG, Dietikon, Switzerland). After addition of 3 ml of aqueous acid (concentration percentages mean w/w) and sealing of the tube, the mixture was stirred vigorously at room temperature until a homogenous suspension or solution was achieved. For hydrolyses starting with 72% (w/w) H_2SO_4 [27, 28], the amount of acid was chosen to give a final volume of 3–4 ml after dilution. The tube was placed in a heated oil bath and stirred magnetically. After the end of the hydrolysis time, the mixture was cooled to room temperature and completely transferred to a suitable container in which it was neutralised by addition of 1 N NaOH. The whole mixture was transferred to a graduated flask and adjusted to a volume of 100 ml. Subsequently, the mixture was filtered through paper and an aliquot of the filtrate was taken for the determination of the monosaccharides.

Examination of commercial samples

Samples (20 mg) were treated with 3 ml of aqueous TFA (72.5% w/w for 1,3-β-D-glucan determination; 60.0% w/w for α-D-mannan determination) and worked-up as described in the general procedure. Hydrolyses were carried out for 3 h at 92.5 °C or 1.5 h at 100 °C (1,3-β-D-glucan determination) and for 4 h at 90 °C or 1.75 h at 100 °C (α-D-mannan determination). They were carried out in parallel with curdlan and α-D-mannan from Sigma-Aldrich whose releases of D-glucose and D-mannose were taken for calculation of correction factors.

Miscellaneous

For determination of D-glucose, the Gluco-quant Glucose/HK assay from Roche Diagnostics (Mannheim, Germany) was used. The quantitative determination of NADPH formed was performed at 340 nm with a Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Kyoto, Japan). D-Mannose was determined by HPAEC-PAD on a BioLC system (Dionex Corp., Sunnyvale, USA). Elution and quantification was done as described elsewhere [29]. Graphs were generated using MS-Excel XP (Microsoft, Redmond, USA). Datapoints are connected by smoothed lines.

Results and discussion

For a very detailed examination of the influence of hydrolysis time, acid concentration and temperature during the TFA treatment of glucan and mannan, a lot of measurements would be necessary. In order to shorten the number of experiments, we used the results of preliminary

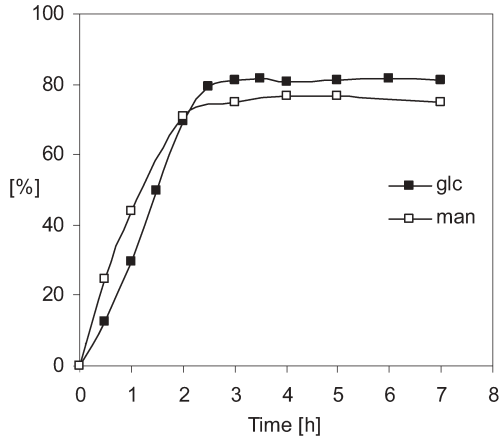

Fig. 1 Time-dependent release of D-glucose and D-mannose after TFA treatment of glucan (75% TFA) and mannan (70% TFA) at 90 °C. Recovery percentages refer to the sample weights of glucan and mannan as 100%

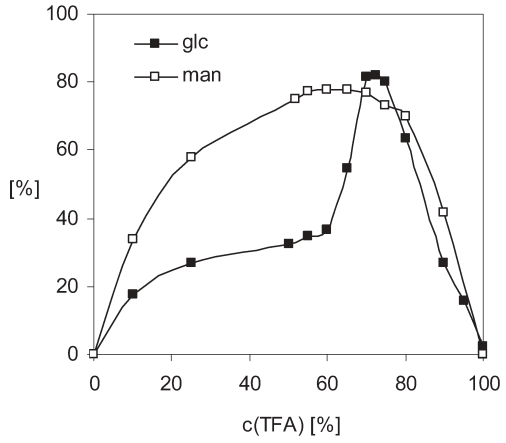

Fig. 2 Release of D-glucose and D-mannose after treatment of glucan (3 h) and mannan (4 h) at different TFA concentrations at 90 °C. Recovery percentages refer to the sample weights of glucan and mannan as 100%

hydrolyses. Thus, TFA concentrations of 70–75% at 90 °C and 3–4 h hydrolysis time proved to be advantageous conditions for a complete depolymerisation. They were used as the starting point and were subsequently varied for finding the maximum amounts of released monosaccharides.

Since the desired quantification method is predominantly intended for the analysis of yeast-derived products, we used a purified yeast glucan obtained according to a published process [20] and a commercial mannan derived from *Saccharomyces cerevisiae*. Both compounds showed purities of more than 90%. The results with yeast glucan were finally verified by comparison with curdlan, a very pure commercial glucan which, however, differs from yeast glucan by the lack of β-1,6-branches and a lower molecular weight. Hydrolyses were accomplished in thick-walled glass tubes. The work-up procedure consisted of few steps: neutralisation with NaOH, adjustment to an appropriate volume and filtration for removal of insoluble particles. The aqueous filtrates containing the monosaccharides were analysed directly for D-glucose and D-mannose which worked without problems despite the presence of sodium salts due to neutralisation with NaOH.

Figures 1, 2, 3 show the influences of hydrolysis time, acid concentration and temperature on the release of D-glucose and D-mannose after TFA treatment of glucan and mannan. Both monosaccharides reached an optimal yield after 3 and 4 h, respectively. Glucan yielded the largest amounts of D-glucose in a narrow region at TFA concentrations between 70 and 75%. TFA concentrations below 65% did not lead to a dissolution of glucan, it remained heterogeneously suspended suggesting a hampered effect of the acid on the undissolved particles. The water-soluble mannan showed a broad maximum between 55 and 70% TFA, a range in which the amount of D-mannose released differs by less than 2%. Optimal temperatures were 90–95 °C for glucan and 90 °C for mannan.

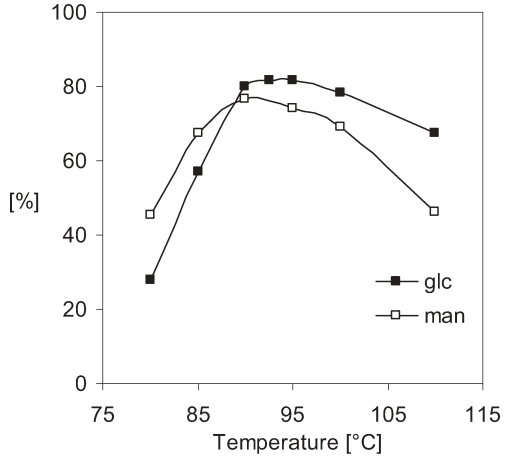

Fig. 3 Release of D-glucose and D-mannose after TFA treatment of glucan (75% TFA, 3 h) and mannan (70% TFA, 4 h) at different temperatures. Recovery percentages refer to the sample weights of glucan and mannan as 100%

The maxima of released monosaccharides shown in Figs. 1, 2, 3 (about 82% for D-glucose, 77% for D-mannose) are in accordance with the expected higher stability of D-glucose. The difference to the desired value close to 100% is due to the partial degradation of the released monosaccharides. A treatment of D-glucose and D-mannose under comparable hydrolysis conditions confirmed a surprisingly fast degradation. After only 30 min, a loss of D-glucose of 7% and of D-mannose of 13% was measured reaching 12.5 and 19%, respectively, after 5 h. Besides the prevailing signals of the monosaccharides, ^{1}H NMR spectra of the residues showed smaller signals of several degradation products from which some could unequivocally be assigned. Thus, D-glucose treated with TFA yielded 1,6-anhydro-β-D-glucose and D-mannose 1,6-anhydro-β-D-mannose [30]. Signal integration gave 4% yield of the anhydro derivative of D-glucose and 8% in the case of D-mannose.

Table 1 Recoveries (%) of glucan, curdlan and mannan under various hydrolytic conditions by determination of the released monosaccharides; *tw* this work

Ref.	Acid	Temperature (°C)	Time (h)	Glucan	Curdlan	Mannan
tw	72.5% TFA	92.5	3.0	82.6	84.0	
tw	72.5% TFA	100	1.5	82.8	83.9	
tw	60% TFA	90	4.0			77.8
tw	60% TFA	100	1.75			78.2
31, 32	2 N TFA	120	1.5	63.5	76.2	78.2
33	2 N TFA	100	2.0			63.8
34	4N TFA	100	4.0	49.3	81.5	77.7
28	2 N HCl	100	2.0	55.8	69.0	84.3
35	6 N HCl	80	2.5	70.9	84.8	
36	1 N H_2SO_4	100	12.0	63.1	77.2	
36	1 N H_2SO_4	100	8.0			83.8
28	1. 72% H_2SO_4	30	3.0	77.5	82.6	77.4
	2. 2 N H_2SO_4	100	4.0			
27	1. 72% H_2SO_4	30	1.0	90.8	94.2	
	2. 3% H_2SO_4	120	1.0			
37	6 N H_2SO_4	100	6.0	18.2	20.4	

We expected that higher temperatures could be combined with shorter hydrolysis times in order to obtain comparable yields of both monosaccharides. Hydrolyses at 100 and 110 °C confirmed that both temperatures can successfully be used. Maximum amounts of released monosaccharides were obtained at 100 °C after 1.5 h for glucan and after 1.75 h for mannan. At 110 °C, the maximal recovery of glucan was achieved after 27 min.

Considering these results, we recommend the following conditions for the quantitative determination of glucans, particularly of insoluble glucans derived from yeast:

T=92.5 °C, t=3.0 h, c=72.5% TFA or T=100 °C, t=1.5 h, c=72.5% TFA.

For mannans, we recommend:

T=90 °C, t=4.0 h, c=60% TFA or T=100 °C, t=1.75 h, c=60% TFA.

Hydrolyses should be accomplished in parallel to reference compounds of high purity whose recoveries should be taken for calculating the correction factors for compensating the loss of released monosaccharides. If for example curdlan and yeast mannan from Sigma-Aldrich (purities of 99 and 97%, respectively) are used, approximate correction factors are 1.18 for D-glucose and 1.25 for D-mannose.

The efficiency of the optimised hydrolysis conditions with respect to high yields of D-glucose and D-mannose was compared to published methods. Table 1 summarises the recoveries of glucan, curdlan and mannan after acidic hydrolysis under various conditions. The data show that most of the procedures gave lower recoveries of curdlan compared to our hydrolysis conditions. More important, almost all methods resulted in significant differences of up to 32% between the recoveries of curdlan and glucan. The reason for this gap is probably due to different dissolution behaviours. If the hydrolytic conditions are too weak, the yeast-derived glucan remains more or less undissolved whereas curdlan goes into solution after a relatively short time. It seems evident that suspended glucan particles are harder to hydrolyse than a dissolved polysaccharide. Surprisingly, this different behaviour during acidic hydrolysis has not been reported before.

Only one hydrolysis method using sulfuric acid [27] resulted in higher yields of released D-glucose compared to our conditions making it an alternative or a complement to our procedure. However, the first step has to be carried out carefully. The examined sample must be completely and homogenously wetted with small amounts of 72% sulfuric acid, which is sometimes difficult if the glucan tends to clump. If the material has partly no contact with the acid, the final recovery will decrease accordingly.

In contrast to glucan and curdlan recoveries, the results of mannan hydrolyses show comparable recoveries with almost all methods described. About 80% was hydrolysed to D-mannose, relatively independent of the kind of acid, the temperature and the time. The water-solubility of mannan allowing an unhampered access of the acids to the mannosidic linkage may be one explanation for the small differences in mannan recovery. Thus, with respect to the time consumption of analyses, there are several hydrolytic conditions that can be alternatively or additionally applied to our method with success [28, 31, 32, 34].

In order to demonstrate the practicability of our determination method, we examined commercial glucan- and mannan-containing products and compared the results with the data of the specification sheets if available. Most products are derived from yeast and are intended for health food applications or as feed ingredients. Glucan contents ranged from 2 up to 92% whereas commercial MOS products usually have mannan contents below 30%. The results in Table 2 confirm a satisfactory efficiency of the method described in this paper. In most cases where specification data were available, the glucan- and mannan contents determined agree quite well. Larger differences may be explained with insufficient determination methods applied by the manufacturers.

As a conclusion, we think that the optimised hydrolytic conditions described in this paper efficiently facilitate the quantification of glucan and mannan derived from yeast. Analyses give reliable and reproducible results for samples containing varying amounts of the polysaccharides. Hydrolyses are easily accomplishable with easily avail-

Table 2 Glucan and mannan contents (%) of commercial products. Data for glucan are averaged from determinations at 92.5 and 100 °C, those for mannan at 90 and 100 °C (as described in Materials and methods) and have a standard deviation of 0.7%. Glucan data are referenced to curdlan with a purity of 99%, mannan data to a reference mannan with an estimated purity of 97%. Data of official specification sheets are given in brackets

Product	Glucan	Product	Glucan	Mannan
Auxoferm YGT Plus	85.3 (80)	NBG Cos	87.4 (89.5±2.2)	
βeta-1,3D Glucan[a]	77.8 (77.2–83.9)	WGP Beta Glucan	77.1 (78)	
Glucanocare	92.4 (>90)	Aloverose[b]	1.8	14.5
Glucanosom	91.1 (>85)	Bio-Mannan-Mos-500	23.8	10.4 (20.7)
Glucaferm 70	72.9 (71)	Biomos	26.1	6.5
Glucasan	18.4 (28)	NovaGlucan 30	23.5 (30)	22.4 (30)
Mos-Glucan	77.0 (63–77)[c]			

[a] Provided as capsules
[b] β-D-Mannan from *Aloe vera*
[c] Personal communication

able laboratory equipment and are completed within few hours. Particularly for yeast glucans, our hydrolytic conditions yield significantly higher recoveries compared to most of the procedures published so far.

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