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Carbohydrate Research 299 (1997) 203–208

CARBOHYDRATE RESEARCH

The application of various protic acids in the extraction of (1 → 3)-β-D-glucan from *Saccharomyces cerevisiae*

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Received 3 May 1996; accepted 15 November 1996

Abstract

Glucans are (1 → 3)-β-linked glucose polymers which have immune-stimulating capability. The extraction of water-insoluble (1 → 3)-β-D-glucan form *Saccharomyces cerevisiae* employs hydrochloric acid. Hydrochloric acid is difficult to employ in the large-scale pharmaceutical extraction of glucans due to its corrosive nature and toxicity. To address these concerns, we determined whether acetic, formic or phosphoric acid can be substituted for hydrochloric acid in the process for the isolation of (1 → 3)-β-D-glucan. The resulting microparticulate glucans were employed as the starting material for the production of (1 → 3)-β-D-glucan phosphate. ^{13}C NMR analysis of the glucan phosphates derived from the acetic, formic or phosphoric acid-extracted microparticulate glucan show excellent correspondence to hydrochloric acid extracted glucan and laminarin, a (1 → 3)-β-D-glucan standard, indicating that the primary structure is not altered by the acid used for extraction. Glucan phosphate prepared from hydrochloric acid had a M_w of 7.2×10^4 g/mol, rms_z of 17.7 nm, of 1.50 and (η) of 49.0 mL/g. Glucan phosphate prepared from acetic acid had a primary polymer peak with a M_w of 1.4×10^6 g/mol, rms_z of 23.6 nm, I of 1.93 and (η) of 62.4 mL/g. Glucan phosphate prepared from formic acid had a main polymer peak with a M_w of 1.2×10^6 g/mol, rms_z 27.1 nm, I of 1.56 and (η) of 89.0 mL/g. Glucan phosphate prepared from phosphoric acid had a primary polymer peak with a M_w of 6.6×10^5 g/mol, rms_z of

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PII S0008-6215(97)00004-9

32.3 nm, I of 2.70 and (η) of 91.3 mL/g. These data indicate that the molecular mass, size, polydispersity and intrinsic viscosity of the glucan phosphate obtained is influenced by the pK_a of protic acid employed to extract the microparticulate glucan. However, the primary structure and side-chain branching are not substantially altered regardless of the acid employed. © 1997 Elsevier Science Ltd.

Keywords: Glucan; Polymer; Carbohydrate; Acid; Extraction

1. Introduction

Glucans are naturally occurring (1 → 3)-β-linked glucose polymers that belong to the class of drugs known as biological response modifiers (BRMs) [1–4]. Numerous studies have demonstrated that (1 → 3)-β-D-glucans exhibit considerable immunomodulatory activity [4]. Prophylaxis or therapy with (1 → 3)-β-D-glucan based immunopharmaceuticals may be beneficial in the treatment of wounds [5], sepsis [1,4] and inflammatory disease [6].

We have extensively studied (1 → 3)-β-D-glucans isolated from the inner cell wall of *Saccharomyces cerevisiae* [2,7,8]. These glucans are isolated by an alkaline–acid hydrolysis method, originally developed by Hassid [9] and later refined by Di Luzio [10], Williams [2,8] and colleagues. Upon initial isolation from yeast, (1 → 3)-β-D-glucans are water-insoluble microparticulates. We have shown that microparticulate yeast (1 → 3)-β-D-glucans extracted by this method are chemically pure, i.e. they contained no other carbohydrates, proteins or residual lipids [11,12]. The microparticulate (1 → 3)-β-D-glucan is employed as the starting material for the production of water soluble pharmaceutical grade (1 → 3)-β-D-glucans, such as glucan phosphate [8] and glucan sulfate [2,13]. The current methodology employs hydrochloric acid for the extraction of microparticulate (1 → 3)-β-D-glucan [2,8–10]. Hydrochloric acid is a strong protic acid which would be difficult to use in the extraction of pharmaceutical grade glucans due, in part, to its corrosive nature, toxicity, and waste management.

To address these concerns, we determined whether phosphoric, acetic, or formic acid can be substituted for hydrochloric acid in this process for the isolation of microparticulate (1 → 3)-β-D-glucan. The resulting microparticulate glucans were employed as the starting material for the production of (1 → 3)-β-D-glucan phosphate. To compare and contrast the glucans, we investigated the physicochemical features of the glucan phosphates obtained, i.e. primary structure, polymer mass, size and distribution.

2. Results

^{13}C nuclear magnetic resonance analysis.—Table 1 shows the ^{13}C NMR chemical shifts of microparticulate glucans in Me_2SO-d_6 after isolation with the different acids. Table 1 also shows the ^{13}C NMR

Table 1
^{13}C NMR chemical shifts of water-insoluble, microparticulate (1 → 3)-β-D-glucan extracted from *Saccharomyces cerevisiae* with various protic acids and the water-soluble (1 → 3)-β-D-glucan phosphates derived from the corresponding microparticulate (1 → 3)-β-D-glucan [a]

Protic acid	Carbon atom					
	C-1	C-2	C-3	C-4	C-5	C-6
Water-insoluble (1 → 3)-β-D-glucans						
Hydrochloric	103.01	72.83	86.22	68.41	76.33	60.87
Acetic	102.93	72.74	86.17	68.33	76.27	60.80
Formic	102.91	72.73	86.16	68.33	76.26	60.80
Phosphoric	102.94	72.76	86.16	68.35	76.28	60.82
Water-soluble (1 → 3)-β-D-glucan phosphates						
Hydrochloric	103.00	72.76	86.21	68.36	76.29	60.83
Acetic	102.87	72.82	86.13	68.28	76.22	60.75
Formic	102.91	72.70	86.15	68.31	76.24	60.78
Phosphoric	102.90	72.70	86.15	68.31	76.24	60.78
Laminarin [b]	103.12	72.93	86.26	68.49	76.41	60.96

[a] The glucans were dissolved in Me_2SO-d_6 and the chemical shifts are in ppm.
[b] Laminarin was employed as a (1 → 3)-β-D-glucan standard.

chemical shifts of the glucan phosphates derived from the corresponding microparticulate glucan. Comparison of the chemical shifts of the glucans extracted with acetic, formic and phosphoric acid show excellent correspondence to hydrochloric acid extracted glucan and laminarin. All water-insoluble and water-soluble preparations possess the characteristic pattern of a (1 → 3)-β-D-glucan with clearly identifiable peaks for the six carbon atoms of the sugar ring at characteristic chemical shifts [12]. This confirms the (1 → 3)-β assignment and indicates that with regard to primary structure there are no significant differences between any of (1 → 3)-β-D-glucans examined (Table 1). Glucans extracted with hydrochloric acid are typically non-branched or branched at a very low frequency, i.e. one (1 → 6)-β-glycosidic branch occurs every 20 backbone residues [8]. Glucans extracted with phosphoric, acetic or formic acid showed branching similar to that observed with hydrochloric acid extraction.

Polymer mass, size and distribution.—Table 2 shows the refractive index increment (dn/dc), the weight-average molecular mass moments, the Z-average rms radius, the polydispersity and intrinsic viscosity of water soluble glucan phosphates prepared from microparticulate glucan extracted with the different protic acids. Glucan phosphate (hydrochloric acid) had a polymer peak with a M_w of 7.2×10^4 g/mol, a rms_z radius of 17.7 nm and polydispersity (I) of 1.50 [14]. The intrinsic viscosity was 49.0 mL/g [14]. Glucan phosphate (acetic acid) had a broad molecular mass distribution with three separate polymer peaks. The primary peak (49% of the total mass) had a M_w of 1.4×106 g/mol, a rms_z of 23.6 nm and (I) of 1.93. The second polymer peak accounted for 24% of the total mass with a M_w of 3.9×10^4 g/mol, followed by a third small molecular mass peak (27% of the total mass) with a M_w of 1.1×10^4 g/mol. The intrinsic viscosity over the whole polymer distribution was 62.4 mL/g. Glucan phosphate (formic acid) also showed a broad molecular mass distribution with two separate polymer peaks. The determination of molecular mass and size across the primary polymer peak (69% of the total mass) yielded a M_w of 1.2×10^6 g/mol, a rms_z of 27.1 nm and (I) of 1.56. The second polymer peak (31% of the total mass) possessed a M_w of 6.8×10^4 g/mol. The intrinsic viscosity was found to be 89 mL/g. Glucan phosphate (phosphoric acid) also exhibited a very broad molecular mass distribution. The calculation of molecular mass across the main polymer peak (64% of the total mass) yielded a M_w of 6.6×10^5 g/mol, a rms_z of 32.3 nm and (I) of 2.70. The second polymer peak (36% of the total mass) had a M_w of 2.2×10^4 g/mol. The intrinsic viscosity was determined to be 91.3 mL/g.

The M_w of the primary polymer peaks of (1 → 3)-β-D-glucan phosphates prepared with acetic, formic

Table 2
Molecular mass moments, Z-average rms radii, polydispersity and intrinsic viscosity of glucan phosphates isolated with different protic acids as determined by SEC/MALLS/DV [a]

Protic acid	Refractive index increment dn/dc (mL/g) [b]	Mass-average molecular mass M_w (g/mol)	Z-average rms radius (nm)	Polydispersity I (M_w/M_n)	Intrinsic viscosity [η] (mL/g) [c]
Hydrochloric [d]	0.158	7.2×10^4	17.7	1.50	49.0
Acetic	0.144				
Peak 1		1.4×10^6	23.6	1.93	62.4
Peak 2		3.9×10^4	n.d. [e]	1.46	
Peak 3		1.1×10^4	n.d.	1.41	
Formic	0.121				
Peak 1		1.2×10^6	27.1	1.56	89.0
Peak 2		6.8×10^4	n.d.	1.10	
Phosphoric	0.157				
Peak 1		6.6×10^5	32.3	2.70	91.3
Peak 2		2.2×10^4	n.d.	1.30	

[a] Carbohydrates were dissolved in 50 mM sodium nitrite, filtered (0.45 μm) and injected.
[b] The dn/dc values were determined across the whole polymer distribution.
[c] The intrinsic viscosities were determined over the whole polymer distribution.
[d] The values for glucan phosphate (hydrochloric acid) were taken from [14].
[e] Not detectable.

or phosphoric acid were one to two orders of magnitude greater than that observed for glucan phosphate prepared with hydrochloric acid extraction. The Z-average rms radii of acetic, formic or phosphoric acid extracted glucans were increased by 33% to 82% when compared to glucan phosphate prepared from hydrochloric acid extracted glucan (Table 2). The intrinsic viscosities of acetic, formic or phosphoric acid extracted glucan phosphates were also higher than for glucan phosphate isolated with hydrochloric acid (Table 2). Another major difference between the glucan phosphates extracted with the various acids and the hydrochloric acid glucan phosphate standard was polydispersity, i.e. with regard to the occurrence of multiple polymer peaks and greater I values than for glucan phosphate isolated with hydrochloric acid. The polymer distributions of the primary polymer peak of acetic, formic or phosphoric acid extracted glucan phosphates were 29%, 4% and 80% greater than that observed in the hydrochloric acid extracted glucan phosphate (Table 2).

Table 3 shows the linear scaling relationship factors α (Mark–Houwink) and ν for the various glucan phosphates. The slope of the linear relationship between log intrinsic viscosity and log molecular mass is known as the Mark–Houwink- or α-value for a polymer system [14]. The slope of the linear relationship between the logarithm of the rms radius of the center of gravity and the logarithm of the molecular mass moment has been termed "ν" [14]. Establishing α and ν for the various glucan phosphates can provide insights into the solution conformation of the polymers [14]. Glucan phosphates prepared from microparticulate glucans extracted with hydrochloric, acetic, formic or phosphoric acid showed α values of 0.649 [14], 0.662, 0.740 and 0.361, respectively. Glucan phosphates prepared from microparticulate glucans extracted with hydrochloric, acetic, formic or phosphoric acid showed ν values of 0.354 [14], 0.793, 1.03 and 0.4, respectively.

3. Discussion

In this study, we examined the applicability of various protic acids for the extraction of water insoluble, microparticulate (1 → 3)-β-D-glucan from the saprophytic yeast, *Saccharomyces cerevisiae*. The microparticulate glucans were then employed as the starting material for the production of a water soluble (1 → 3)-β-D-glucan, glucan phosphate. The original method for the isolation of yeast glucans employed hydrochloric acid in the second phase of the alkaline–acid extraction. The use of hydrochloric acid in the large-scale extraction of (1 → 3)-β-D-glucan starting material is problematic due, in part, to the corrosive nature and toxicity of this acid. The ability to obtain a comparable (1 → 3)-β-D-glucan starting material from yeast with a less corrosive acid would be of benefit. The data presented indicate that acetic, formic or phosphoric acid can be employed to extract water insoluble (1 → 3)-β-D-glucan from *Saccharomyces cerevisiae*. Further, these glucans can be used as the starting material for the production of water-soluble glucan phosphate. The primary structure and branching frequency of the glucans extracted with acetic, formic or phosphoric acid were not significantly different from the glucan extracted with hydrochloric acid. However, substantial differences between the glucan phosphates were found with re-

Table 3
Scaling relationships between intrinsic viscosity and molecular mass (α or Mark–Houwink value) and rms radius and molecular mass (ν value) over the corresponding molecular mass ranges for glucan phosphates prepared from microparticulate (1 → 3)-β-D-glucans extracted with various protic acids

Protic acid	α (Mark–Houwink) [a]	ν (rms radius vs. mass) [b]
Hydrochloric	0.65 ($1 \times 10^4 - 5 \times 10^5$ g/mol)	0.35 ($5 \times 10^4 - 4 \times 10^5$ g/mol)
Acetic	0.66 ($8 \times 10^5 - 8 \times 10^6$ g/mol)	0.79 ($9 \times 10^5 - 2 \times 10^6$ g/mol)
Formic	0.74 ($5 \times 10^5 - 5 \times 10^6$ g/mol)	1.03 ($8 \times 10^5 - 2 \times 10^6$ g/mol)
Phosphoric	0.36 ($1 \times 10^5 - 1 \times 10^6$ g/mol)	0.40 ($3 \times 10^5 - 1 \times 10^6$ g/mol)

[a] Mark–Houwink values were determined according to the equation $[\eta] = K_\alpha * M^\alpha$.
[b] ν Values were determined according to the equation $r_z = K_\nu * M^\nu$.

gard to molecular masses, molecular sizes and polydispersities, respectively. The two most striking differences were observed in the molecular mass of the main polymer peak and the polydispersity of the glucans. These data suggest that the weaker the protic acid employed the greater the molecular mass and polydispersity of the glucan phosphate obtained.

We also examined whether the glucan phosphates would behave similarly in aqueous solution. The α (Mark–Houwink) and ν values suggest that glucan phosphates prepared from acetic or formic acid extracted glucan exhibit a somewhat more rigid, rod-like structure than glucan phosphate isolated with hydrochloric acid. We speculate that this may be due to the presence of multiple helical structures in aqueous medium. The phosphoric acid glucan phosphate has a lower α and ν value, suggesting a random coil or spherical coil solution behavior. We speculate that the phosphoric acid glucan phosphate is not behaving as a true random coil but that there may be different types of coiled structures.

There is an important caveat to this study. The present study was undertaken to evaluate whether other protic acids could be substituted for hydrochloric acid in the glucan extraction process. In order to control the experiment, we employed the acid concentrations and extraction times that had previously been defined for HCl extraction [8]. We observed that the weaker the protic acid the greater the molecular mass, size and polydispersity of the glucan phosphate obtained. It is entirely possible and, indeed, probable that if the concentration of the weaker acids were increased and/or the time of hydrolysis increased that the molecular size and distribution of the resulting glucan phosphate obtained could be decreased. Thus, we conclude that the present study has demonstrated the feasibility of using other protic acids to extract microparticulate glucans from yeast which can be used as starting material for the production of glucan phosphate. Additional studies are required to optimize the concentration of acid and the time of hydrolysis necessary to obtain a product which is identical to the hydrochloric acid extracted glucan.

4. Experimental

Isolation of microparticulate water insoluble (1 → 3)-β-D-glucan.—We have previously described the method for the extraction of microparticulate glucans [2,8]. The basic method was modified only with regard to the substitution of different protic acids. Briefly, four batches of *S. cerevisiae* (454 g) were extracted (3 ×) with 0.75 M NaOH at 100 °C. The residue was then sequentially extracted with 2.5 N, 1.75 N and 0.94 N hydrochloric, acetic, formic or phosphoric acid at 100 °C. The residue was then extracted with boiling 1% acidic ethanol (6 ×), followed by boiling 1% alkaline ethanol (1 ×). The acid ethanol was prepared with the corresponding acid. The remaining residue was washed in 18 mΩ water at 100 °C (3 ×), allowed to sediment, harvested and lyophilized to dryness. The yield was 3–5%.

Preparation of water - soluble glucan phosphate.—Water-soluble glucan phosphate was prepared according to the method described by Williams et al. [8]. Our SEC/MALLS/DV data indicate that lyophilization will increase the degree of polymer aggregation present in the glucan phosphate preparations (unpublished observations). To alleviate this problem, the glucan phosphates were not lyophilized following dialysis and concentration (3 to 5 mg/mL) in 18 mΩ water. The glucan phosphates were stored in sealed, depyrogenated glass bottles at 4 °C until assayed. Aliquots for ^{13}C NMR spectroscopy were lyophilized to dryness prior to dissolution in Me_2SO-d_6.

^{13}C NMR.—Analyses were performed on Bruker 200 and 500 MHz instruments (Bruker Instruments Inc., Billerica, MA), operating in the pulsed Fourier-transform mode as previously described [12]. Microparticulate glucans and glucan phosphates were dissolved in Me_2SO-d_6. All samples were prepared at a concentration of 50 mg/mL. Conditions under which the spectra were obtained are as follows: field strength 50 and 125 MHz; relaxation delay 1 s; pulse window 15°–20°. Laminarin, a (1 → 3)-β-D-glucan standard as also examined in Me_2SO-d_6 [8]. Approximately 19,000–61,000 scans were collected for samples. All spectra were obtained with broadband proton decoupling.

Determination of the specific refractive index increment (dn / dc).—The dn/dc values for the glucan phosphate samples were determined with an Optilab 903 interferometric refractometer (Wyatt Technology Corp., Santa Barbara, CA) at 25 °C in 50 mM sodium nitrite mobile phase as previously described [14].

Determination of molecular mass, size, distribution and intrinsic viscosity of the water soluble (1 → 3)-β-D - glucan phosphates by SEC / MALLS / DV.—Molecular mass, size and distribution were established by size- exclusion chromatography (SEC) with in-line multi-angle laser light scattering (MALLS) photometry and differential viscometry (DV) as pre-

viously described [14]. The mobile phase, 0.05 M sodium nitrite, was filtered (0.45 μm), stored in a sterile reservoir and delivered at a flow rate of 0.5 mL/min. The columns were maintained at 30 °C. The system was calibrated using narrow-band pullulan and dextran standards. For analysis, non-lyophilized glucan phosphate samples were adjusted to a concentration of 2–3 mg/mL and then injected with 50 mM sodium nitrite mobile phase. A 200 μL injection volume was used for all analyses.

Acknowledgements

The work was supported, in part, by NIHRO153522 to DLW, a Veterans Administration Merit Review Grant to IWB, an American Heart Association – Tennessee Affiliate grant to DLW, and a postdoctoral fellowship from the German Academic Exchange Service to AM. This work is dedicated to the memory of Ernest L. Jones, friend, colleague and scholar.

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