Carbohydrate Research, 253 (1994) 293–298
Elsevier Science B.V., Amsterdam

Note

Molecular weight analysis of a water-insoluble, yeast-derived (1 → 3)-β-D-glucan by organic-phase size-exclusion chromatography

David L. Williams [a,*], Henry A. Pretus [b], Harry E. Ensley [c] and I. William Browder [a]

[a] *Glucan Research Laboratory, Department of Surgery, James H. Quillen College of Medicine, East Tennesse State University, Johnson City, TN 37614-0575 (USA)*
[b] *Department of Physiology, Tulane University School of Medicine, New Orleans, LA 70112 (USA)*
[c] *Department of Chemistry, Tulane University, New Orleans, LA 70118 (USA)*

(Received for publication April 22th, 1993; accepted July 7th, 1993)

We have extensively investigated (1 → 3)-β-D-glucan immune stimulants that are isolated from the inner cell wall of *Saccharomyces cerevisiae*[1–4]. Natural product (1 → 3)-β-D-glucan homopolysaccharides, which stimulate immunity, belong to the class of drugs known as biological response modifiers (BRMs). Upon initial isolation from the yeast cell wall, (1 → 3)-β-D-glucans are water-insoluble microparticulates[1,5,6]. A major obstacle to the clinical development of (1 → 3)-β-D-glucan BRMs is their relative lack of solubility in aqueous media[1,5,6]. Recently, we have reported on a methodology for the conversion of water-insoluble, yeast (1 → 3)-β-D-glucan to water-soluble pharmaceutical-grade preparations[1]. In addition, we have described the methodology for the physicochemical characterization of water-soluble (1 → 3)-β-D-glucan pharmaceuticals[1,5–8]. Water-insoluble (1 → 3)-β-D-glucan serves as the raw material for the production of water-soluble pharmaceutical-grade (1 → 3)-β-D-glucan BRMs[1]. While it is possible to partially characterize water-insoluble yeast (1 → 3)-β-D-glucan by NMR spectroscopy and elemental analysis[1,5,6], establishing molecular weight moments and other physicochemical parameters has proven exceedingly difficult. In order to enhance our knowledge of (1 → 3)-β-D-glucans and to further their development as pharmaceutical agents, it is essential to develop a methodology which will allow precise molecular weight and structural characterization of the water-insoluble form. Herein, we describe a

* Corresponding author.

0008-6215/94/$07.00 © 1994 – Elsevier Science B.V. All rights reserved
SSDI 0008-6215(93)E0265-3

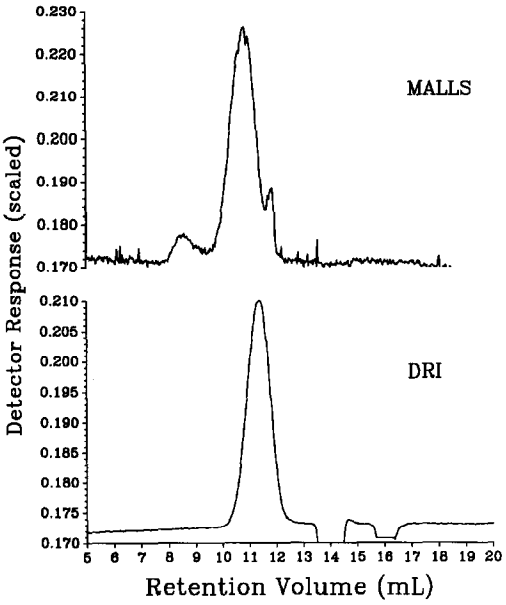

Fig. 1. Size-exclusion chromatogram of water-insoluble (1 → 3)-β-D-glucan as determined by HPSEC/MALLS. Two polymer peaks were resolved. Peak 1, the high molecular weight peak, represents < 1% of total polymers. Peak 2 contains ~ 99% of total polymers. The MALLS data represents the 90° light scattering angle (detector 11)

methodology for establishing molecular weight averages, polymer distribution, rms radius, polydispersity (I), and intrinsic viscosity $[\eta]$ of a water-insoluble, microparticulate (1 → 3)-β-D-glucan.

These data were obtained by employing organic high-performance size-exclusion chromatography (HPSEC) with in-line multi-angle laser light scattering (MALLS) and differential viscometry (DV). The mobile phase was HPLC-grade dimethyl sulfoxide. Water-insoluble (1 → 3)-β-D-glucan exhibits a polymodal distribution. Two polymer peaks were resolved (Fig. 1). The high molecular weight peak (peak 1) had a M_w of 4.57×10^6 g/mol, an rms radius of 47.3 nm and a polydispersity of 1.83 (Table I). Peak 1 accounted for < 1% of total polymers. Peak 1 occurred at such a low concentration that it was not possible to establish the intrinsic viscosity. This may be due to the formation of macromolecular aggregates that are readily detectable by MALLS, yet much less so by DV and differential refractive index (DRI). The lower molecular weight peak (Peak 2) had a M_w of 3.53×10^4 g/mol, an rms radius of 21.8 nm, an I of 1.12, and a $[\eta]$ of 0.366 dL/g. Peak 2 accounted for ~ 99% of total polymers. A small peak was observed by MALLS on the trailing edge of Peak 2. This peak occurred at such a low concentration that it was not possible to establish the M_w. Elemental analysis of lyophilized, water-insoluble (1 → 3)-β-D-glucan revealed a composition (mol%) of 40.03% C, 6.72% H and 49.90% O. N and S were both < 0.5%.

TABLE I

Molecular weight averages, rms radii, polydispersity, and intrinsic viscosity of water-insoluble, yeast derived (1 → 3)-β-D-glucan [a]

Parameter	Peak 1	Peak 2
M_n (number-average MW, g/mol)	2.49×10^6	3.50×10^4
M_w (weight-average MW, g/mol)	4.57×10^6	3.53×10^4
M_z (z-average MW, g/mol)	11.31×10^6	7.62×10^4
r rms radius (nm)	47.3	21.8
I (polydispersity)	1.83	1.12
$[\eta]$ (intrinsic viscosity dL/g)	–	0.366
% of total polymers	< 1.0	99.0

[a] Water-insoluble (1 → 3)-β-D-glucan was dissolved in Me_2SO at a concentration of 2 mg/mL with heating (5–10 min at 40°C). A 200-μL injection volume was used for all analyses.

Previously, we have employed aqueous HPSEC/MALLS/DV for the characterization of water-soluble pharmaceutical-grade (1 → 3)-β-D-glucans[1,5,6,8]. Further, we have reported the development and characterization of a water-soluble phosphorylated (1 → 3)-β-D-glucan BRM, which we have termed glucan phosphate[1]. Glucan phosphate is prepared from the water-insoluble yeast-derived (1 → 3)-β-D-glucan described in this report[1]. The process for the conversion of water-insoluble (1 → 3)-β-D-glucan to water-soluble pharmaceutial-grade glucan phosphate employs dimethyl sulfoxide to initially solubilize the water-insoluble glucan[1]. It was this observation that suggested dimethyl sulfoxide as a mobile phase for the characterization of water-insoluble glucan. In addition, once in solution the glucan–dimethyl sulfoxide solution is stable at ambient temperature. During our preliminary studies, we examined several potential mobile phases. Specifically, we examined *N*,*N*-dimethylformamide, *N*,*N*-dimethylacetamide, *N*,*N*-dimethyacetamide–lithium chloride, guanidine hydrochloride, methanol, hydrazine, and ethanolamine. Of these organic solvents only dimethyl sulfoxide provided acceptable results.

Several reports indicate that (1 → 3)-β-D-glucans exist as stable triple helices[9–12]. The triple-helical conformation is thought to be of importance with regard to immunobiological activity[12]. Specifically, Maeda et al.[12] have reported that the immune stimulatory and antitumor activity of lentinan, a highly branched, triple-helical (1 → 3)-β-D-glucan, are intimately linked to the triple-helical conformation. The triple-helical conformation of (1 → 3)-β-D-glucans can be disrupted by dissolution of the glucan in dimethyl sulfoxide or sodium hydroxide solution (pH > 11)[10,11]. Norisuye and colleagues[10] have reported that following dissolution in dimethyl sulfoxide the triple helical structure of the (1 → 3)-β-D-glucan, schizophyllan, melts, and the individual glucan polymer strands assume a random coil conformation. These investigators reported that the molecular weight moments obtained for schizophyllan dissolved in dimethyl sulfoxide reflected only one-third of the native molecular weight due to the disruption of the triple helix[10]. Whether the triple helix of schizophyllan and water-insoluble, yeast-derived glucan react identically to

dissolution in dimethyl sulfoxide is not known. However, based on these observations we speculate that the native weight average M_w of the predominant polymer peak (Peak 2) of water insoluble (1 → 3)-β-D-glucan is $\sim 1.06 \times 10^5$ g/mol or three times the M_w obtained in the present study (3.53×10^4 g/mol).

Recently, we have reported on the development and characterization of a water-soluble phosphorylated (1 → 3)-β-D-glucan pharmaceutical, which we have termed glucan phosphate[1]. Glucan phosphate, prepared from water-insoluble (1 → 3)-β-D-glucan, has two polymer peaks and exists as a stable triple helix[1]. The predominant polymer peak of glucan phosphate (Peak 2, which contains 98% of polymers), has a M_w of 1.10×10^5 g/mol[1]. Comparison of the estimated native weight-average molecular weight for water-insoluble (1.06×10^5 g/mol) and water-soluble phosphorylated (1 → 3)-β-D-glucan (1.10×10^5 g/mol), suggests that the phosphorylation process developed in our laboratory for the conversion of water-insoluble (1 → 3)-β-D-glucan to water-soluble pharmaceutical-grade glucan phosphate does not substantially degrade the native water-insoluble molecule. The minor differences in M_w between water-insoluble glucan (1.06×10^5 g/mol) and water-soluble glucan phosphate (1.10×10^5 g/mol) fall within the experimental error of the technique (~ 3–5%).

While the present data enhance our basic knowledge of (1 → 3)-β-D-glucans, they are also of practical significance. Acquiring precise molecular weight data on water-insoluble (1 → 3)-β-D-glucans will greatly enhance our ability to isolate and characterize these natural product carbohydrate polymers as well as provide quality control for the derivatization of this novel class of pharmaceutical compounds.

EXPERIMENTAL

Preparation of water-insoluble (1 → 3)-β-D-*glucan*.—Microparticulate glucan was isolated from *S. cerevisiae* by modifications of the methods of Hassid et al.[13] and Di Luzio et al.[14] as reported by Williams and co-workers[1].

Elemental analysis of water-insoluble (1 → 3)-β-D-*glucan*.—Elemental analyses of (1 → 3)-β-D-glucan for carbon, hydrogen, oxygen, phosphorus, nitrogen, and sulfur were conducted by a commercial laboratory (Galbraith Laboratories, Inc., Knoxville, TN).

High-performance size-exclusion chromatography (HPSEC) of water-insoluble (1 → 3)-β-D-*glucan*.—To evaluate the polymer distribution, (1 → 3)-β-D-glucan was analyzed by high-performance size-exclusion chromatography (HPSEC). The basic HPSEC system consisted of a Waters 6000A solvent delivery system, a U6K manual injector, and a column heating chamber (Waters Chromatography Division, Millipore Corp., Milford, MA). Two in-line pulse dampers were employed. The mobile phase was HPLC grade dimethyl sulfoxide (Me_2SO) delivered at a flow rate of 0.5 mL/min. A single linear Ultrasytragel column (Waters Chromatography Division, Milford, MA) was connected in series with a guard column (Waters

Chromatography Division, Milford, MA). The column was maintained at 30°C. Flow rate, column temperature, and pump operating conditions were controlled by Maxima 820 HPSEC software (Dynamic Solutions, Ventura, CA). The system was calibrated using narrow and broad-band dextran standards (Pharmacia, Gaithersburg, MD). For analysis, (1 → 3)-β-D-glucan was dissolved in the mobile phase at a concentration of 2 mg/mL, heated for 5–10 min at ~ 40°C, followed by gentle rocking until completely dissolved (~ 1 h) and filtered (0.22 μm). A 200-μL injection volume was used for all analyses.

Determination of molecular weight, polydispersity (I) *and root mean square radius (rms) of water-insoluble* (1 → 3)-β-D-*glucan by multi-angle laser light scattering (MALLS) photometry.*—To determine absolute molecular weight (M_w), the water-insoluble glucan was analyzed by HPSEC with in-line MALLS photometry employing a Dawn F photometer fitted with a K5 flow cell (Wyatt Technology Corp, Santa Barbara, CA). Absolute MW distribution, number-average M_n, z-average M_z, weight average M_w, I, and rms distance in nm were established with Wyatt ASTRA software (v. 2.1). A differential index of refraction (dn/dc) of 0.054 cm^3/g was assumed. Dextran standards were employed to establish that column calibration showed good agreement with MALLS values.

Determination of intrinsic viscosity ([η]) *by differential viscometry (DV).*—Intrinsic viscosity ([η]) of (1 → 3)-β-D-glucan was determined by in-line differential viscometry (DV). For determination of [η], the column eluent was analyzed by in-line differential viscometry employing a Viscotek Model 200 differential refractometer/viscometer (Viscotek, Porter, TX). Intrinsic viscosity and molecular weight moments were established with Viscotek Unical software (v. 4.0). Molecular weight determinations of standards using this technique show good agreement with MALLS data.

REFERENCES

1 D.L. Williams, R.B. McNamee, E.L. Jones, H.A. Pretus, H.E. Ensley, I.W. Browder and N.R. Di Luzio, *Carbohydr. Res.*, 219 (1991) 203–213.
2 D.L. Williams, E.R. Sherwood, I.W. Browder, R.B. McNamee, E.L. Jones and N.R. Di Luzio, *Int. J. Immunopharmacol.*, 10 (1988) 405–411.
3 W. Browder, D. Williams, P. Lucore, H. Pretus, E. Jones and R. McNamee, *Surgery*, 104 (1988) 224–230.
4 W. Browder, D. Williams, H. Pretus, G. Olivero, F. Enrichens, P. Mao and A. Franchello, *Ann. Surg.*, 211 (1990) 605–613.
5 D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley, I.W. Browder and N.R. Di Luzio, *Immunopharmacology*, 22 (1991) 139–156.
6 D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley and I.W. Browder, *Carbohydr. Res.*, 235 (1992) 247–257.
7 D.L. Williams, H.A. Pretus and I.W. Browder, *J. Liquid Chromatogr.*, 15 (1992) 2297–2309.
8 H.A. Pretus, H.E. Ensley, R.D. McNamee, E.L. Jones, I.W. Browder and D.L. Williams, *J. Pharmacol. Exp. Ther.*, 257 (1991) 500–510.
9 D. Lecacheux, Y. Mustiere and R. Panaras, *Carbohydr. Polym.*, 6 (1986) 477–492.
10 T. Norisuye, T. Yanaki and H. Fujita, *J. Polym. Sci.*, 18 (1980) 547–558.

11 T. Yanaki, K. Tabata and T. Kojima, *Carbohydr. Polym.*, 5 (1985) 275–283.
12 Y.Y. Maeda, S.T. Watanabe, C. Chihara and M. Rokutanda, *Cancer Res.*, 48 (1988) 651–675.
13 W.Z. Hassid, M.A. Joslyn and R.M. McCready, *J. Am. Chem. Soc.*, 63 (1941) 295–298.
14 N.R. Di Luzio, D.L. Williams, R.D. McNamee, B.F. Edwards and A. Kitahama, *Intl. J. Cancer*, 24 (1979) 773–779.