Carbohydrate Research, 219 (1991) 203–213
Elsevier Science Publishers B.V., Amsterdam

A method for the solubilization of a (1→3)-β-D-glucan isolated from *Saccharomyces cerevisiae*

David L. Williams*,†,‡, Rose B. McNamee*,†, Ernest L. Jones*,†, Henry A. Pretus*,†, Harry E. Ensley§, I. William Browder*,†,‡, and Nicholas R. Di Luzio*,†

Glucan Research Laboratory, Departments of Physiology† and Surgery‡, Tulane University School of Medicine, New Orleans, LA 70112 (U.S.A.) and Department of Chemistry§, Tulane University, New Orleans, LA 70118 (U.S.A.)*

(Received December 24th, 1990; accepted for publication April 8th, 1991)

ABSTRACT

This report describes a method for the solubilization of a micro-particulate β-D-glucan. Insoluble glucan is dissolved in methyl sulfoxide and urea (8M) and partially phosphorylated at 100°. The resulting water-soluble product is called glucan phosphate. The conversion rate is 70%, and the preparation is endotoxin free as determined by the *Limulus* lysate procedure. Glucan phosphate is composed of 34.66% C, 6.29% H, 42.83% O, and 2.23% P and has a repeating-unit empirical formula of $(C_6H_{10}O_5)_7 \cdot PO_3H_2$, indicating a phosphate group substitution on every seventh glucose subunit. Molecular-weight averages, polydispersity, and intrinsic viscosity were determined by aqueous high-performance size-exclusion chromatography (s.e.c.) with on-line, multi-angle laser light scattering (m.a.l.l.s.) photometry and differential viscometry (d.v.). Two polymer peaks were resolved. Peak 1 ($M_w = 3.57 \times 10^6$ daltons), represents ~2% of the total polymers, while peak 2 ($M_w = 1.10 \times 10^5$ daltons) comprises ~98% of polymers. ^{13}C- and ^{31}P-n.m.r. spectroscopy confirmed the β-1,3 interchain linkage and the presence of a phosphate group. In solution, glucan phosphate polymers self-associate in a triple-helical arrangement. The ability to prepare a immunologically active, non-toxic, water-soluble β-D-glucan preparation will greatly enhance the clinical utility of this class of compounds.

INTRODUCTION

We have extensively investigated a β-linked glucan immune stimulant that is isolated from the inner cell wall of *Saccharomyces cerevisiae*[1,2]. Glucans belong to the class of drugs known as biological response modifiers (BRMs). The glucan isolated in our laboratory exerts a beneficial effect on a variety of experimental desease states of bacterial[3,4], viral[5,6], fungal[7], and parasitic[8] origin. This glucan has also been shown to modify immune suppression[9] and the course of experimental neoplastic disease[10]. These and other observations have stimulated research on the potential biomedical applications of polymeric β-D-glucan BRMs[11,12]. A major obstacle to the clinical utilization of β-glucan BRMs is their relative lack of solubility in aqueous media. Specifically, the β-D-glucan isolated from *S. cerevisiae* is a water-insoluble, micro-particulate (~1–2 μm), polymer upon initial isolation. While topical or intralesional administration of a micro-particulate glucan induces no toxicity[13,14], systemic (*i.e.*, intravenous) administration of the micro-particulate form is associated with hepatosplenomegaly[15], granulo-

0008-6215/91/$ 03.50

ma formation[10], micro-embolization, and enhanced endotoxin sensitivity[16]. If β-D-glucans are to become clinically applicable, they have to be converted to a biologically effective, water-soluble form that can be safely administered *via* the systemic route. Numerous reports exist which describe isolation methodology and biological effects of water-soluble polymeric carbohydrate BRMs isolated from a variety of plant and microbial sources[12,17]. Specific examples include Lentinan[18,19], Krestin[20], schizophyllan[21], aminated β-D-glucan[22], Grifolan[23], and SSG[17]. While all of these compounds possess immune stimulatory activity to a greater or lesser extent, many still exhibit significant toxicity, including vasodilatation[19], microvascular hemorrhage[19], and circulatory collapse[24]. Clearly, there is a need for development of a process for converting water-insoluble β-D-glucans to safe, effective, water-soluble forms.

Based on the therapeutic potential of glucan, our laboratory undertook studies to develop methodology for the conversion of insoluble yeast β-D-glucan to a non-toxic, immunologically active water-soluble form. This report describes: (*i*) a method for the solubilization of yeast-derived β-D-glucan which results in a water-soluble preparation that is a potent immune stimulant[25,26] and is suitable for parenteral administration to humans[26] and animals[25], and (*ii*) preliminary data on the physiochemical characterization of the water-soluble preparation, which we have termed glucan phosphate.

EXPERIMENTAL

Preparation of particulate glucan. — Particulate glucan was isolated from *S. cerevisiae* by a modification of the methods of Hassid *et al.*[1] and Di Luzio *et al.*[2]. The flow diagram in Fig. 1 describes the step-by-step preparation of water-insoluble, micro-particulate β-glucan from *Saccharomyces cerevisiae*.

Preparation of glucan phosphate. — Soluble glucan phosphate was prepared as outlined in Fig. 2. Micro-particulate glucan (4 g) was dissolved in 200 mL of methyl sulfoxide (Me_2SO) containing 8M urea. Forty (40) mL of 85% phosphoric acid was added dropwise immediately prior to heating. The solution was heated to 100° in a water bath, and the reaction was carried out for 6 h. A crystalline precipitate (presumed ammonium phosphate) formed at 90 min. Following heating, the solution was cooled to ambient temperature and diluted in 4 L of ultra-pure, pyrogen-free, deionized water obtained from a water purification system (Millipore, Bedford, MA). The solution was passed through a 1-μm pre-filter to remove unreacted micro-particulate glucan. The glucan phosphate preparation was dialyzed with a Pellicon tangential flow dialyzer (Millipore, Bedford, MA) against 100 L of ultra-pure, pyrogen-free water, concentrated to 500 mL, shell-frozen, and lyophilized to dryness (Virtis, Gardiner, NJ). The yield was 70%, and the lyophilized product was shown to be endotoxin free as determined by the *Limulus* lysate procedure. Solubility of glucan phosphate is $\geq$ 50mg/mL in aqueous media. The ultra-pure water employed in the dilution and dialyzing of glucan phosphate was demonstrated to be endotoxin free by the *Limulus* lysate procedure (Sigma Chemical Co., St. Louis, MO). All chemicals were analytical reagent grade.

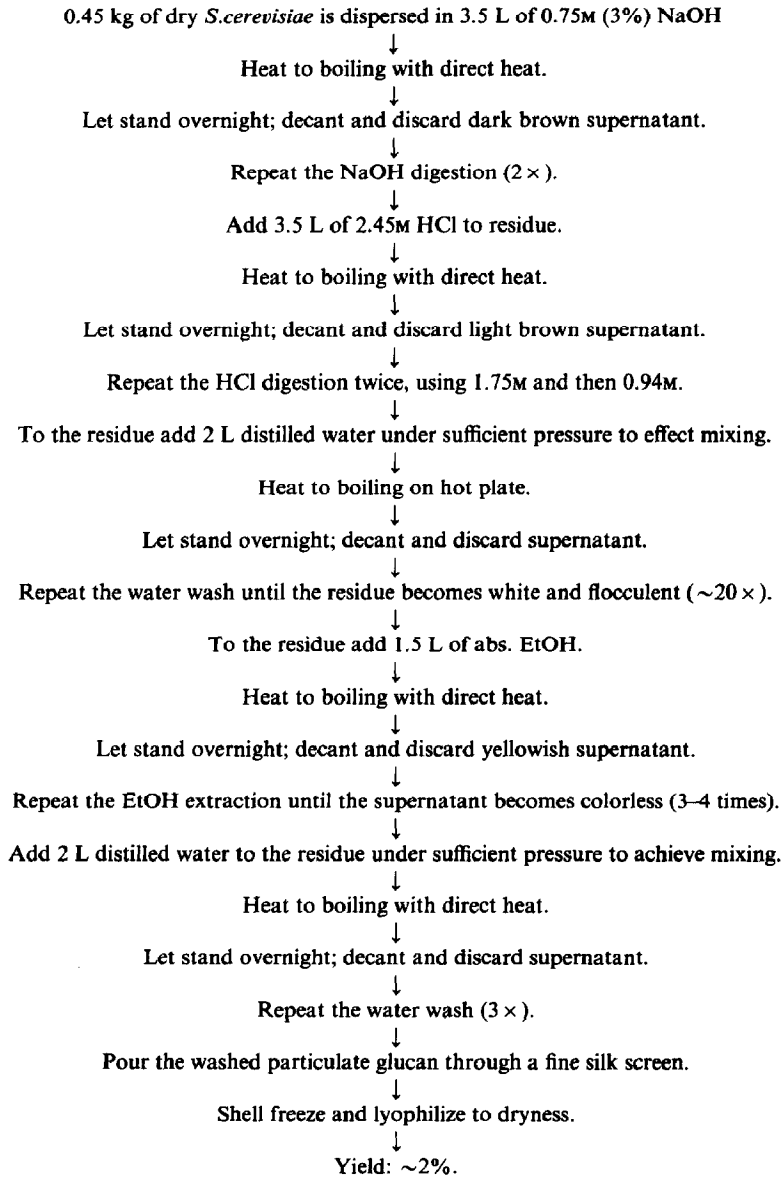

Fig. 1. Flow-chart describing the procedure for extraction of water-insoluble, micro-particulate β-glucan from *Saccharomyces cerevisiae*.

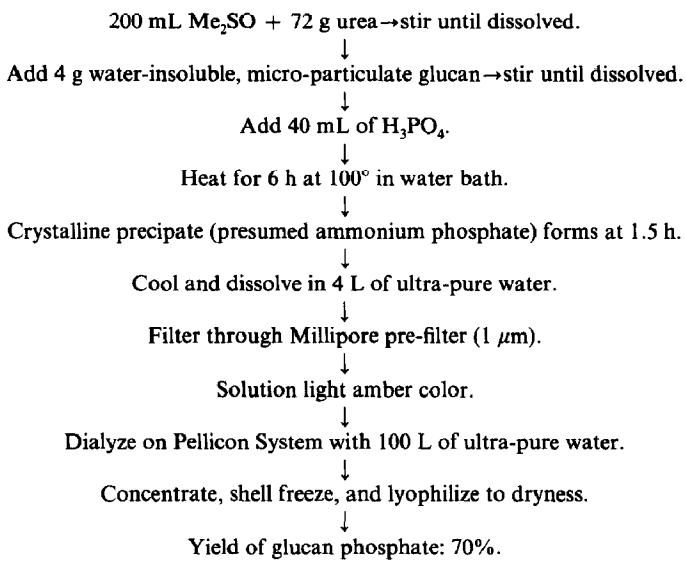

Fig. 2. Flow-chart describing the preparation of glucan phosphate from insoluble β-glucan (U.S. Patent No. 4 739 046).

Elemental analysis of glucan phosphate. — Elemental analysis of carbon, hydrogen, oxygen, phosphorous, nitrogen, and sulfur were conducted by a commercial laboratory (Galbraith Laboratories, Inc. Knoxville, TN).

High-Performance size-exclusion chromatography of glucan phosphate. — To evaluate polymer distribution, glucan phosphate was analyzed by aqueous, high-performance, size-exclusion chromatography (h.p.s.e.c.). The basic h.p.s.e.c. system consisted of a Waters 600E solvent delivery system, a U6K manual injector, and a column heating chamber (Waters Chromatography Division, Millipore Corp., Milford, MA). The mobile phase, 0.05M sodium nitrite, was stored in a sterile reservoir (Kontes, Vineland, NJ) and was thoroughly degassed by sparging and blanketing with helium prior to use. The mobile phase was delivered at a flow rate of 0.5 mL/min. Three Ultrahydrogel (Waters Chromatography Division, Milford, MA) aqueous s.e.c. columns having exclusion limits of 2×10^6, 5×10^5, and 1.2×10^5 daltons were connected in series along with an Ultrahydrogel guard column. The columns were maintained at 30°. Flow rate, column temperature, and pump operating conditions were controlled by Maxima 820 GPC software (Dynamic Solutions, Ventura, CA). The system was calibrated using narrow-band pullulan standards (Showdex P-82 series, J. R. Science, NY) and broad-band dextran standards (Pharmacia, Piscataway, NJ). For analysis, glucan phosphate was dissolved in the mobile phase at a concentration of 2–3 mg/mL by gentle rocking until completely hydrated (~2–3 h). A 200-μL injection volume was used for all analyses. Mass-balance studies demonstrated that all of the injected material eluted.

Determination of molecular weight, polydispersity, and root-mean-square radius of glucan phosphate by multi-angle laser light scattering photometry. — To determine absolute molecular weight (M_w), glucan phosphate was analyzed by h.p.s.e.c. with on-line, multi-angle laser light scattering (m.a.l.l.s.) photometry employing a Dawn F m.a.l.l.s. photometer fitted with a K5 flow cell (Wyatt Technology Corp, Santa Barbara, CA). Absolute M_w distribution, number-average M_w, Z-average M_w, weight-average M_w, peak M_w, polydispersity, and root-mean-square (r.m.s.) radius in nm was established with ASTRA software (v. 2.0). A differential index of refraction (dn/dc) of 0.146 cm^3/g was employed[27]. Pullulan and dextran standards were employed to establish that column calibration showed good agreement with m.a.l.l.s. values.

Determination of intrinsic viscosity by differential viscometry. — Intrinsic viscosity ([η]) of glucan phosphate was determined by on-line differential viscometry (d.v.). For determination of [η], the column eluent was analyzed by on-line differential viscometry employing a Viscotek Model 200 differential refractometer/viscometer (Viscotek, Porter, TX). The differential refractive index (d.r.i.) signal was employed for m.a.l.l.s. and d.v. calculations. Molecular weight determinations of standards using this technique showed good agreement with m.a.l.l.s. data. Intrinsic viscosity of pullulan standards was in agreement with previous data[28].

^{13}C- and ^{31}P-nuclear magnetic resonance spectroscopy. — To investigate the type of interchain linkages and to elucidate the polymer backbone, micro-particulate glucan and glucan phosphate were in dissolved in Me_2SO-d_6 and analyzed by ^{13}C-n.m.r. spectroscopy[20]. Analyses were performed on a Bruker 200 MHz spectrometer (Bruker Instruments, Inc., Billerica, MA) operating in the pulsed Fourier-transform mode. All samples were prepared at 50 mg/mL. Laminarin in Me_2SO-d_6 was employed as a β-1,3-linked triple-helical glucopyranose standard[29]. ^{13}C-N.m.r. chemical shifts were expressed in p.p.m. downfield from the central carbon peak of Me_2SO-d_6, which was observed at 39.5 p.p.m. To confirm the presence of a phosphate group, glucan phosphate dissolved in Me_2SO-d_6 was analyzed by ^{31}P-n.m.r. spectroscopy. Conditions under which the ^{13}C-spectra were obtained are as follows: field strength, 50 MHz; relaxation delay, 1 s; pulse window, 15°–20°. Approximately 15 668 scans were collected for samples in Me_2SO-d_6. All spectra were obtained with broadband proton decoupling.

Helix–coil transition analysis. — The conformational structure of glucan phosphate in solution was established by helix–coil transition analysis according to a modification of the Ogawa procedure[30]. Briefly, Congo red (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.001M NaOH to a final concentration of 88μM. Glucan phosphate was dissolved in 0.001M NaOH. Laminarin was employed as a β-1,3-linked triple-helical control[29]. Dextran (40 000 daltons) was employed as a random-coil control. All carbohydrates were prepared at 10 mg/mL in 0.001M NaOH. Glucan phosphate or polysaccharide standards (250 μL) were added to 10-mm microcuvettes containing 750 μL of either Congo red–NaOH or water–NaOH. Absorbance (λ_{max}) was determined using an LKB Ultrospec II spectrophotometer (LKB Instruments, Gaithersburg, MD). Polysaccharides existing in an ordered conformation form a complex with Congo red in dilute aqueous NaOH solution as denoted by a shift in λ_{max}. To assess

the order–disorder transition, the λ_{max} for solutions of Congo red–β-D-glucan phosphate or Congo red–polysaccharide standards were determined at NaOH concentrations ranging from 0.001M to 1.0M.

RESULTS AND DISCUSSION

Herein we describe a method for the solubilization of β-D-glucan from *S. cerevisiae*. The resulting water-soluble preparation (glucan phosphate) is a biological response modifier that can be safely administered to humans[26] and animals[25] *via* the intravenous route. Solubilization is achieved by partially phosphorylating the β-glucan polymer in the presence of Me_2SO and a chaotropic agent (urea). Elemental analysis of lyophilized glucan phosphate revealed a chemical composition (mol.%) of 34.66% carbon, 6.29% hydrogen, 42.83% oxygen, and 2.23% phosphorus, respectively. Nitrogen and sulfur were both <0.03%. Based on the elemental analysis, the repeating-unit empirical formula for glucan phosphate is $(C_6H_{10}O_5)_7 \cdot H_2PO_3$, suggesting that, on the average, a phosphate group is substituted on every seventh glucose subunit along the polymer.

The molecular-weight averages, polydispersity, and intrinsic viscosity of glucan phosphate are presented in Table I. A size-exclusion chromatogram of glucan phosphate showing analysis of the column eluent by m.a.l.l.s. and d.r.i. detectors is presented in Fig. 3. Two polymer peaks were resolved by m.a.l.l.s. photometry. Peak 1, which represents ~2% of the total polymer mass, has a weight-average M_w of 3.57×10^6 daltons, r.m.s. radius of 31.7 nm, and a polydispersity (I) of 3.2. Peak 2, which comprises ~98% of the polymers, has a weight-average M_w of 1.10×10^5 daltons, r.m.s. radius of 25.4 nm, and a polydispersity (I) of 6.2. The average $[\eta]$ was 0.29 g/dL. The high M_w peak (peak 1) comprises such a small amount of the total polymers that detection was not possible with differential refractive index or viscometry detectors. The m.a.l.l.s. detec-

TABLE I

Molecular-weight averages, r.m.s. values, polydispersity, and intrinsic viscosity of glucan phosphate[a]

Parameter	*Peak 1*	*Peak 2*
M_n (number-average mol. wt.)	1.28×10^6	0.25×10^5
M_w (weight-average mol. wt.)	3.57×10^6	1.10×10^5
M_z (Z-average mol. wt.)	12.23×10^6	3.04×10^5
M_w r.m.s. radius (nm)	31.7	25.4
I (polydispersity)	3.2	6.2
$[\eta]$ (intrinsic viscosity)	—	0.29 g/dl
% of total polymers	~2%	~98%

[a] The weight-average mol. wt. (M_w), expressed in daltons, represents the average M_w of the polymers in each peak. The number-average M_w (M_n) is indicative of the proportion of low M_w polymers. Z-average M_w (M_z) reflects the proportion of high molecular weight polymers. The polydispersity number (I) reflects polymer homogeneity.

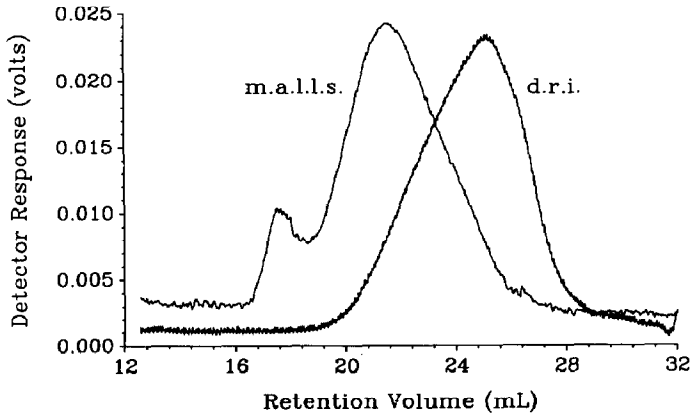

Fig. 3. Size-exclusion chromatogram of glucan phosphate as determined by multi-angle laser light scattering (m.a.l.l.s.) photometry and differential refractive index (d.r.i.) detectors. Two glucan phosphate polymer peaks were observed by m.a.l.l.s.. The m.a.l.l.s. data represents the 90° light scattering angle.

tor, which measures absolute molecular mass (*i.e.*, M_w) of the polymers in the column eluent, is the only technology available which provides the sensitivity required for such critical polymer analysis.

To confirm the type of interchain linkages associated with glucan phosphate, samples were analyzed by ^{13}C-n.m.r. spectroscopy in deuterated methyl sulfoxide (Me_2SO-d_6). This allows elucidation of the polymer backbone[23] and can also be employed to evaluate the type of side-chain branching, if any, along the backbone[23]. The ^{13}C-n.m.r. spectrum of water-insoluble, micro-particulate β-D-glucan (*a*) isolated from *S. cerevisiae* and water-soluble glucan phosphate (*b*) prepared from the insoluble material are presented in Fig. 4. Laminarin (*c*), in Me_2SO-d_6 served as the β-1,3-linked triple-helical control[31]. Comparison of the insoluble, micro-particulate glucan and glucan phosphate peaks shows excellent correspondence with laminarin. In addition, the ^{13}C-n.m.r. spectrum of laminarin reported by Saito *et al.*[29] agrees well with the present laminarin spectrum. ^{13}C-n.m.r. chemical shifts in p.p.m. for insoluble glucan, glucan phosphate, and laminarin are presented in Table II. Comparison of the chemical shifts of insoluble glucan and glucan phosphate with laminarin confirms the β-1,3 assignment. These data also indicate that the solubilization procedure does not substantially alter the basic molecule. The small peaks at 71.32, 73.87, 76.92, 86.72, and 103.87 p.p.m. in the laminarin spectrum can be attributed to the presence of C-6 glucosyl side-chains which occur, on average, every 11th subunit along the polymer[31]. The three other small peaks in the laminarin spectrum at 63.86, 69.95, and 70.18 p.p.m. are unassigned. The small peaks observed in the water-insoluble micro-particulate glucan spectrum (Fig. 4A) correspond to the C-6 glucosyl side-chains peaks observed in the laminarin spectrum (Fig. 4C). Comparison of the small peaks in the micro-particulate glucan with those observed in laminarin suggests that the side-chain branching frequency of micro-particulate glucan is approximately every 22nd glucose subunit.

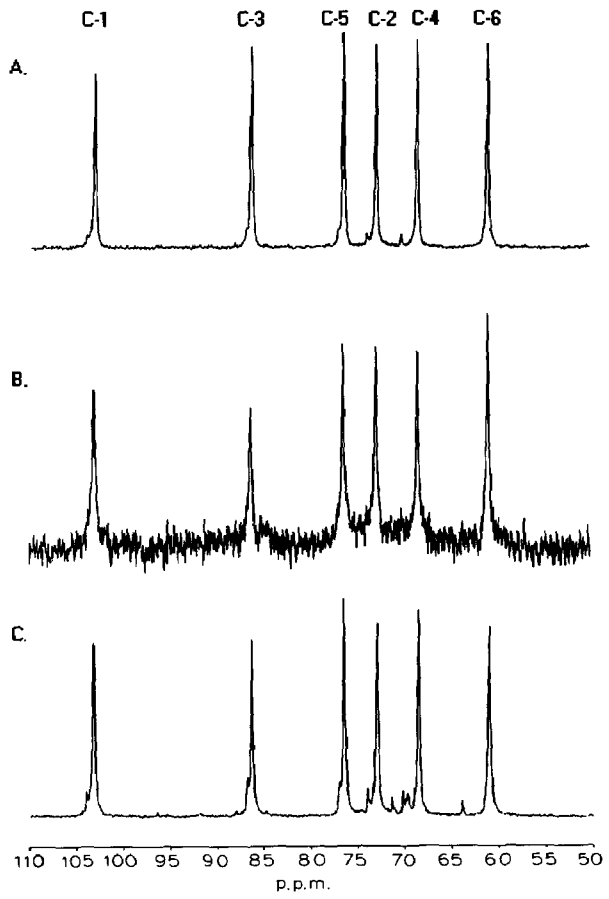

Fig. 4. ^{13}C-N.m.r. spectra of (A) insoluble β-glucan (15 433 scans) and (B) glucan phosphate (15 694 scans). Laminarin (C) served as the β-1,3-linked triple-helial polyglucose control (15 685 scans). All samples were dissolved in Me_2SO-d_6 at 50 mg/mL. Spectra were obtained at 50 MHz.

TABLE II

^{13}C-N.m.r. chemical shifts of insoluble, micro-particulate glucan, glucan phosphate, and laminarin in Me_2SO-d_6[a]

C-atom	*Insoluble glucan*	*Glucan phosphate*	*Laminarin*	*Laminarin*[b]
C-1	103.01	103.00	103.12	103.7
C-2	72.83	72.76	72.93	74.5
C-3	86.22	86.21	86.26	85.5
C-4	68.41	68.36	68.49	69.3
C-5	76.33	76.29	76.41	76.8
C-6	60.87	60.83	60.96	61.9

[a] Chemical shifts in p.p.m. [b] Chemical shifts of laminarin expressed in p.p.m. downfield from external tetramethylsilane as reported by Saito *et al.*[28]

^{31}P-N.m.r. spectral analysis of glucan phosphate in Me_2SO-d_6 was undertaken to confirm the presence of the phosphate group. A single phosphorus signal was observed at 5.41 p.p.m. We speculate that the phoshpate group is substituted at the C-6 position and that it extends away from the glucopyranose backbone of glucan phosphate. We conclude that glucan phosphate is composed of a β-linked glucose backbone with a phosphate group substitution observed on the average every seventh glucose subunit.

Previous reports indicate that the immunologic and antitumor activity of certain β-1,3-D-glucan BRMs is related to the higher structure of the polymer[12,19,32]. Maeda *et. al.*[19] have reported that the denaturation of Lentinan, a triple-helical β-linked glucan BRM, decreases antitumor activity. Renaturation of the polymer restored antitumor activity[19]. These data suggest that the higher structure, specifically the solution conformation, may be critically important with regard to induction of immunobiological activity. The solution conformation of glucan phosphate was determined by the technique of Ogawa and colleagues[30]. Glucan phosphate exhibits a triple-helical conformation as denoted by a shift in the absorption maxima between 0.2 and 0.4M NaOH (Fig. 5). Laminarin, which served as the triple-helical control, exhibited a shift in absorption maxima between 0.1 and 0.2M NaOH. Examination of a 40 000-dalton dextran, which served as the random coil control, revealed no shift in absorption maxima. Congo red in NaOH served as the negative control. The possibility exists that shifts in absorption maxima observed for glucan phosphate may be attributable to chain-ionization effects. However, laminarin a β-1,3-linked water-soluble, triple-helical polyglucose showed a shift in absorption maxima similar to that observed with glucan phosphate. In addition, we have studied a branched, water soluble β-1,3-linked polyglucose derived from *Sclerotium glucanicum* that has no charged groups[33]. The shift in absorption maximum

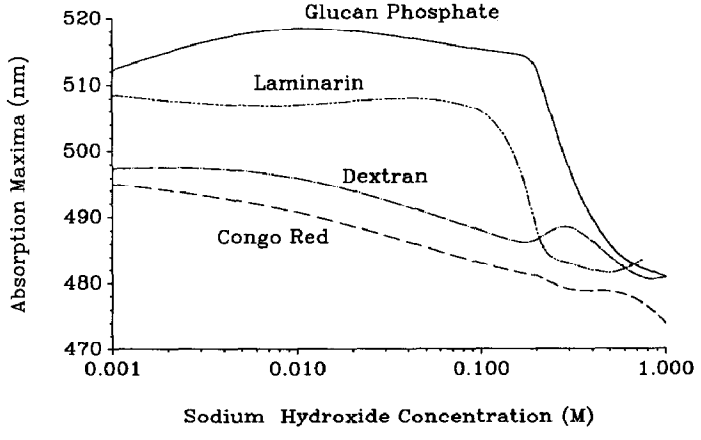

Fig. 5. Helix–coil transition of glucan phosphate in the presence of Congo red and varying concentrations of NaOH. Glucan phosphate exhibits a shift to a lower λ_{max} between 0.2 and 0.4M NaOH, indicating disruption of the ordered (triple helical) conformation. Laminarin served as the β-1,3-linked triple-helical control. Dextran (40 000 dalton) served as the random coil control. Congo red in NaOH served as the negative control.

observed with *S. glucanicum* derived glucan is similar to that observed with glucan phosphate and laminarin. These observations tend to argue against chain-ionization effects in glucan phosphate polymers being solely responsible for the observed shifts in absorption maxima.

Just as the higher structure of β-D-glucan BRMs has been linked to biological activity[12,19], the structure has also been linked to the severe side-effects observed following systemic administration of carbohydrate BRMs such as Lentinan[19]. In striking contrast to the toxicity observed with Lentinan at doses between 0.5 and 8 mg/kg/day[34,35], we have demonstrated that triple-helical β-D-glucan BRMs derived from *S. cerevisiae* can be safely and effectively administered to humans[26] and animals[25] over a wide dose range. We speculate that the difference in toxicity between β-D-glucans prepared in our laboratory and those reported by others may relate to isolation and derivatization methodology.

CONCLUSIONS

The results presented describe a method for the solubilization of a yeast-derived β-1,3-linked glucan BRM. The water-soluble product, glucan phosphate, is a triple-helical β-1,3-D-glucan BRM. The ability to prepare an immunologically active, non-toxic, water-soluble β-1,3-glucan will greatly enhance the clinical application of this class of biological response modifiers.

ACKNOWLEDGMENTS

This work was supported, in part, by the Glucan Research Fund. Funding for instrumentation was provided by the Center for Bioenvironmental Research, Tulane University School of Medicine. The expert technical assistance of Ms. Bridget Molizone in the preparation of this manuscript is also gratefully acknowledged.

REFERENCES

1 W. Z. Hassid, M. A. Joslyn, and R. M. McCready, *J. Am. Chem. Soc.*, 63 (1941) 295–298.
2 N. R. Di Luzio, D. L. Williams, R. B. McNamee, B. F. Edwards, and A. Kitahama, *Int. J. Cancer*, 24 (1979) 773–779.
3 N. R. Di Luzio and D. L. Williams, *Infec. Immun.*, 20 (1978) 804–810.
4 D. L. Williams, I. W. Browder, and N. R. Di Luzio, *Surgery*, 93 (1983) 448–454.
5 D. L. Williams and N. R. Di Luzio, *Science*, 208 (1980) 67–69.
6 D. L. Williams and N. R. Di Luzio, *EOS J. Immunol. Immunopharmac.*, 5(2) (1985) 78–82.
7 D. L. Williams, J. A. Cook, E. O. Hoffmann, and N. R. Di Luzio, *J. Reticuloendothel. Soc.*, 23 (1978) 479–490.
8 J. A. Cook, T. W. Holbrook, and B. W. Parker, *J. Reticuloendothel. Soc.*, 27 (1980) 567–572.
9 N. R. Di Luzio, D. L. Williams, E. R. Sherwood, and I. W. Browder, *Surv. Immunol. Res.*, 4 (1985) 160–167.
10 D. L. Williams, E. R. Sherwood, R. B. McNamee, E. L. Jones, and N. R. Di Luzio, *Hepatology*, 5 (1985) 198–206.
11 D. L. Williams, E. R. Sherwood, R. B. McNamee, E. L. Jones, I. W. Browder, and N. R. Di Luzio, *Hepatology*, 7 (1987) 1296–1304.

12 K. Tabata, W. Ito, and T. Kojima, *Carbohydr. Res.*, 89 (1981) 121–135.
13 P. W. A. Mansell, H. Ichinose, R. J. Reed, E. T. Krementz, R. McNamee, and N. R. Di Luzio, *J. Natl. Cancer Inst.*, 54(3) (1975) 571–580.
14 W. Browder, D. Williams, P. Lucore, H. Pretus, E. Jones, and R. McNamee, *Surgery*, 104 (1988) 224–230.
15 S. J. Riggi and N. R. Di Luzio, *Nature*, 193 (1962) 1292–1294.
16 G. J. Bowers, M. L. Patchen, T. J. MacVittie, E. F. Hirsch, and M. P. Fink, *Int. J. Immunopharmac.*, 8 (1986) 313–321.
17 N. Ohno and T. Yadomae, *Carbohydr. Res.*, 159 (1987) 293–302.
18 T. Sasaki and N. Takasuka, *Carbohydr. Res.*, 47 (1976) 99–104.
19 Y. Y. Maeda, S. Watanabe, C. Chihara, and M. Rokutanda, *Cancer Res.*, 48 (1988) 671–675.
20 M. Ikuzawa, K. Matsunaga, S. Nishiyama, S. Nakaijma, Y. Kobayashi, T. Andoh, A. Kobayashi, M. Ohara, Y. Ohura, T. Wada, and C. Yoshikumi, *Int. J. Immunopharmac.* 18 (1988) 415–423.
21 M. Belkin, W. G. Hardy, A. Perrault, and H. Sato, *Cancer Res.*, 19 (1959) 1050–1062.
22 R. Seljelid, *Biosci. Rep.*, 6 (1986) 845–851.
23 N. Ohno, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae, *Chem. Pharm. Bull.*, 32 (1984) 1142–1151.
24 R. Seljelid, *Scand. J. Immunol.*, 29 (1989) 181–192.
25 D. L. Williams, E. R. Sherwood, I. W. Browder, R. B. McNamee, E. L. Jones, and N. R. Di Luzio, *Int. J. Immunopharmac.*, 10 (1988) 405–411.
26 W. Browder, D. Williams, H. Pretus, G. Olivero, F. Enrichens. P. Mao, and A. Franchello, *Ann. Surg.*, 211 (1990) 605–613.
27 D. Lecacheux, Y. Mustiere, R. Panaras, and G. Brigand, *Carbohydr. Polym.*, 6 (1986) 477–492.
28 L. Weaver, L. P. Yu, and J. E. Rollings, *J. Appl. Polym. Sci.*, 35 (1988) 1631–1637.
29 H. Saito, T. Ohki, N. Takasuka, and T. Sasaki, *Carbohydr. Res.*, 58 (1977) 293–305.
30 K. Ogawa and M. Hatano, *Carbohydr. Res.*, 67 (1978) 527–535.
31 D. Rolf, J. A. Benner, and G. R. Gray, *Carbohydr. Res.*, 137 (1985) 183–196.
32 Y. Y. Maeda and S. T. Watanabe, *Int. J. Immunopharmac.*, 10 (1988) 87 (Abstract).
33 H. A. Pretus, H. E. Ensley, R. B. McNamee, E. L. Jones, I. W. Browder, and D. L. Williams, *J. Pharmacol. Exptl. Therap.*, 257 (1991) 500–510.
34 H. Chesterman, R. Heywood, T. R. Allen, A. E. Street, N. A. Edmondson, and D. E. Prentice, *Toxicol. Lett.*, 9 (1981) 87–90.
35 R. J. Sortwell, S. Dawa, D. G. Allen, A. E. Street, R. Heywood, N. A. Edmondson, and C. Gopinath, *Toxicol. Lett.*, 9 (1981) 81–85.