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Note

NMR spectral analysis of a water-insoluble (1 → 3)-β-D-glucan isolated from *Saccharomyces cerevisiae*

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A (1 → 3)-β-Linked poly-D-glucose immune stimulant is isolated from the inner cell wall of *Saccharomyces cerevisiae* [1,2] and belongs to the class of drugs known as biological response modifiers (BRMs). This polysaccharide exerts a beneficial effect on a variety of experimentally induced disease states, and its biological effects continue to receive significant attention [3–10].

Upon initial isolation from *S. cerevisiae*, the (1 → 3)-β-glucan is a water-insoluble microparticulate [11]. A major obstacle to the clinical utilization of the β-glucan is its relative lack of solubility in aqueous media. Recently, our laboratory group has succeeded in developing a methodology for the conversion of water-insoluble yeast β-glucan to a nontoxic, water-soluble, immunologically active pharmaceutical form [11,12].

Another impediment to the understanding and development of (1 → 3)-β-glucan based pharmaceuticals is the often incomplete chemical characterization of glucan polysaccharides isolated from natural sources. Since the substitution pattern of the isolated glucan can vary depending on both the strain of *S. cerevisiae* used and on the isolation methodology, careful characterization of the isolated material is necessary in order to ensure consistency. To address this problem we undertook studies to characterize the water-insoluble glucan polysaccharide that is employed as the starting material for the production of pharmaceutical grade (1 → 3)-β-glucan. Herein, we report the NMR spectral characterization and structure determination of glucan isolated from *S. cerevisiae*.

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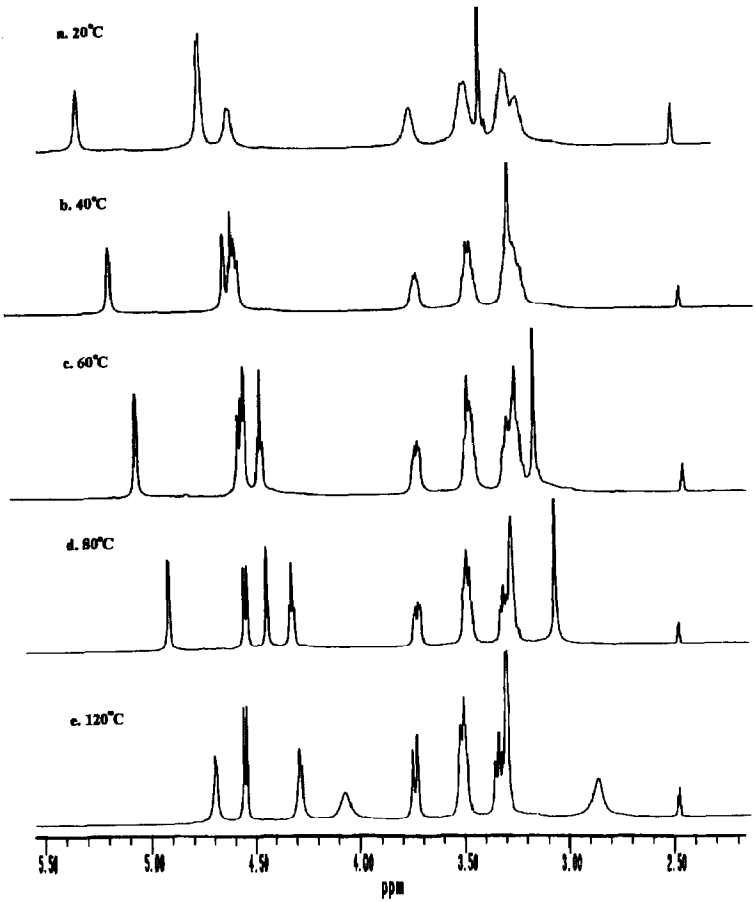

Fig. 1. Variable temperature 500-MHz ^{1}H NMR spectrum of particulate glucan (10 mg/mL, after D_2O exchange) in Me_2SO-d_6 at the temperatures indicated.

^{13}C NMR spectroscopy has proved useful in the determination of the degree of branching in β-glucans. The ^{13}C NMR spectrum of the β-glucan shows six carbon signals at 102.70 (C-1), 85.98 (C-3), 76.14 (C-5), 72.59 (C-2), 68.23 (C-4), and 60.71 (C-6) ppm. These chemical shifts agree well with previously reported shifts of other β-glucan polymers [13], and the assignments were confirmed by the COSY and HETCOR spectra discussed below.

The variable-temperature ^{1}H NMR spectra were recorded at 20, 40, 60, 80, and 120°C (±0.1°C) (Fig. 1). No decomposition was evident at temperatures up to 100°C. Heating at 120°C caused the solution to darken, but after 3 h at this temperature the spectrum was unchanged, and lowering the temperature produced spectra identical to those previously observed. The ^{1}H NMR of the β-glucan at 20°C (Fig. 1a) shows only a series of ill-defined peaks at 5.3 (1 H), 4.72 (2 H), 4.6 (1 H), 3.7 (1 H), 3.46 (2 H), 3.26 (2 H), and 3.22 (1 H) ppm. The residual water peak

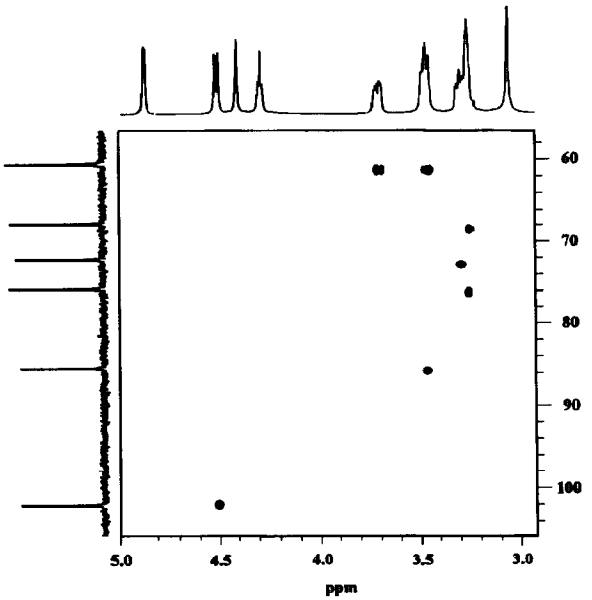

Fig. 2. HETCOR of particulate glucan (20 mg/mL) in Me_2SO-d_6 at 80°C. The ^{13}C NMR spectrum is displayed on the vertical axis and the ^{1}H NMR spectrum on the horizontal axis.

was observed at 3.4 ppm. On warming the solution, all of the peaks for protons attached to oxygen shifted to higher field, while the protons bonded to carbon remained essentially unchanged, and the observed coupling patterns become increasingly clear. Since maximal spectral resolution for protons was achieved at 80°C, the other NMR experiments were conducted at that temperature. As the temperature of the solution was increased to 120°C, internal exchange of the protons bonded to oxygen became rapid, and the HOCH coupling disappeared (Fig. 1e). The 80°C ^{1}H NMR (Fig. 1d) spectrum, in conjunction with the 80°C HETCOR (Fig. 2) and COSY (Fig. 3), allows assignment of all of the observed signals.

In Fig. 1d, the proton which appears as a doublet at 4.52 ppm (J 8 Hz) is clearly attached to C-1 (HETCOR, Fig. 2) and is coupled to H-2 (COSY, Fig. 3) which appears at 3.28 ppm (a triplet in Fig. 1e). The H-2 signal is also coupled to two other protons; HO-2 at 4.87 ppm (J 3.5 Hz), and H-3, which is part of the two-proton signal at 3.46 ppm. The hydroxyl signal at 4.29 ppm can be assigned as HO-6 since it appears as a triplet (J 6 Hz) and is coupled to the two different C-6 protons. One of the C-6 protons appears as a doublet of doublets (J_1 6, J_2 11 Hz) at 3.7 ppm, and the other as part of the two-proton signal at 3.46 ppm. The remaining hydroxyl signal at 4.4 ppm can be assigned as HO-4 and is coupled to H-4, which is part of a two-proton signal at 3.25 ppm that includes the H-5 signal. The coincidence of H-4 and H-5 at 3.25 ppm, and of H-3 and one of the C-6 protons at 3.46 ppm can clearly be seen in the HETCOR spectrum (Fig. 2).

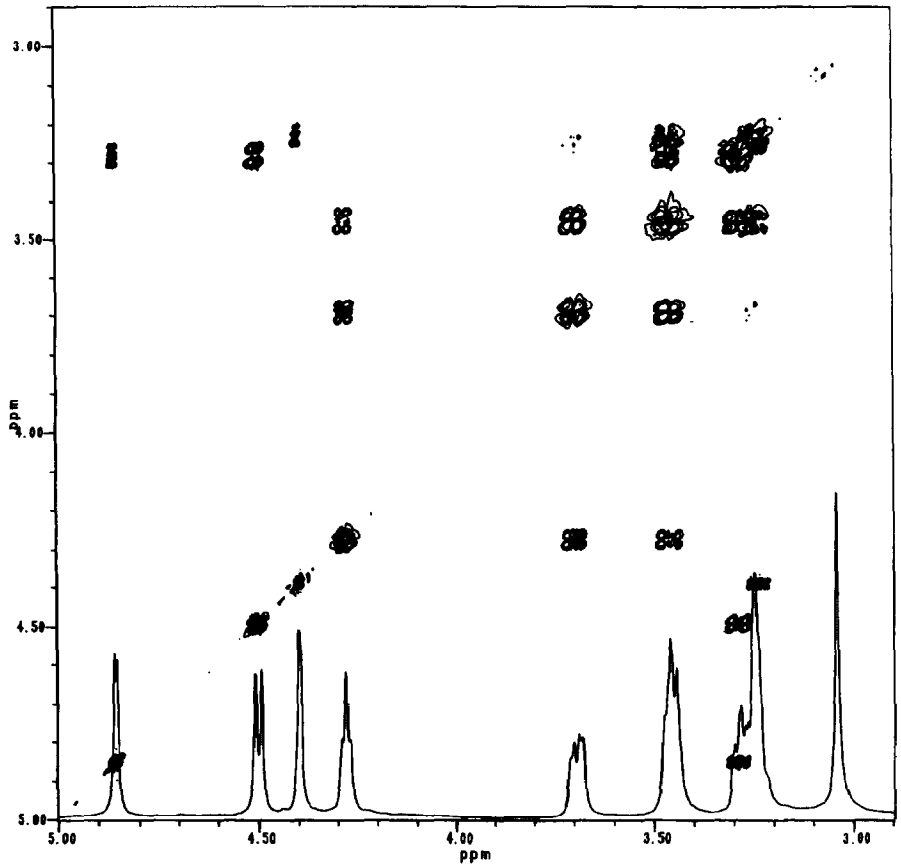

Fig. 3. Phase-sensitive double-quantum-filtered COSY of particulate glucan (10 mg/mL, after D_2O exchange) in Me_2SO-d_6 80°C.

The β-(1 → 3) linkage of the polysaccharide backbone was verified by a HMBC experiment optimized for a three-bond $^3J_{CH}$ (COCH) coupling of 5.5 Hz. This value minimizes signals coming from the $^3J_{CH}$ coupling within the same ring (dihedral angles close to 60° have a $^3J_{CH}$ coupling of ~ 1 Hz [14]). A substantial signal is seen only for the coupling of H-1 and C-3, with a much weaker signal for intraring H-6 and C-4 coupling.

In conclusion, NMR examination of the water-insoluble glucan isolated from *S. cerevisiae* provides conclusive evidence for the assigned β-(1 → 3) linkage and shows that the polysaccharide contains few, if any, side branches. (Note that in some cases the glucan isolated using this procedure has shown up to 4% branching, but usually the branching frequency is much less.) All of the signals in both the ^1H and ^{13}C NMR spectra can be unambiguously assigned.

1. Experimental

Preparation of the water-insoluble, microparticulate glucan.—The water-insoluble, microparticulate (1 → 3)-β-D-glucan was isolated from *S. cerevisiae* as previously described [11,12]. High-performance size-exclusion chromatography (SEC) with on-line multi-angle laser light scattering (MALLS), and differential viscometry (DV), employing an organic mobile phase, show that the polysaccharide has a weight-average molecular weight (M_w) of 3.53×10^4 g/mol, a polydispersity (I) of 1.12, an intrinsic viscosity [η] of 0.366 dL/g, and contains ~ 196 glucose units per chain [15].

NMR spectral studies — Exchangeable protons were removed by suspending the glucan in D_2O (2 mL/25 mg of glucan), stirring for 15 min, freezing and lyophilization. This exchange process was repeated three times. This exchange dramatically reduced the the water signal, but it did not significantly affect the carbohydrate hydroxyl protons. All spectra were recorded in Me_2SO-d_6 (Aldrich Gold Label) on a GE-Omega 500 MHz NMR spectrometer using 10 mg of glucan/mL (^{13}C NMR at 125.75 MHz using 20 mg of glucan/mL). A phase-sensitive double-quantum-filtered COSY experiment was used to track the covalent network [16]. The reverse-detected, heteronuclear correlation sequence (HETCOR) described by Bax and co-workers [17], was used with an assumed $^1J_{C,H}$ coupling of 150 Hz. A reverse-detected long-range heteronuclear correlation sequence (HMBC) [17] optimized for a three-bond $^3J_{C,H}$ coupling of 5.5 Hz confirmed the 1 → 3 linkage.

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