HPLC and ^{13}C-NMR Study of Carboxymethyl-β-(1 → 6)-D-gluco-β-(1 → 3)-D-glucan Derived from *Saccharomyces cerevisiae*

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SYNOPSIS

Sodium salt of carboxymethyl-β-(1 → 6)-D-gluco-β-(1 → 3)-D-glucan (CMG-Na) was prepared from β-D-glucan isolated from baker's yeast (*Saccharomyces cerevisiae*). Three samples, Fractions I, II, and III, were further separated from the crude CMG-Na derivative. For the physicochemical characterization of the separated fractions, the methods of high-performance liquid chromatography (HPLC) in the size-exclusion mode and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy were applied. The HPLC method revealed that the molecular weights, M_n, M_w, and M_z averages, of Fraction II were 9.71 $\times 10^4$, 2.27 $\times 10^5$, and 3.59 $\times 10^5$ Da, respectively, whereas those of Fraction III were 1.52 $\times 10^4$, 2.13 $\times 10^4$, and 3.57 $\times 10^4$ Da, respectively. The ^{13}C-NMR spectra of Fraction II showed a ratio of 3 : 1 for β-(1 → 3)/β-(1 → 6), whereas for Fraction III, the content of β-(1 → 3) units was smaller. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

There have been several reports on the relationship between the biological or pharmacological activity and the chemical structure of polysaccharides, β-glucans, isolated from various microorganisms.[1-7] Most of these glucans are linked by β-1,3-glycosidic bonds with β-1,6-monoglucosyl side chains. The biologically most important glucans, with molecular weights in the order from 1×10^4 to 2×10^6 Da, are usually neutral, not charged, and poorly water-soluble macromolecules.

The polysaccharide lentinan obtained from *Lentinus edodes* (Berk.) Sing., a popular edible mushroom found in Japan, exhibited strong antitumor activity against sarcoma 180 implanted subcutaneously in mice.[8-11] The molecular weight (M_w average) of lentinan in the injection form used clinically was found to be 2.66×10^5 Da.[12] Schizophyllan, isolated from *Schizophyllum commune,* with a molecular weight established to be 6.24×10^5,[12] has also been applied clinically.

Many original and characteristic activities of neutral polysaccharides have been revealed in studies on a yeast cell wall polysaccharide preparation: zymosan.[4] However, the entities involved in these biological activities based on their chemical constituents are still obscure because zymosan is a crude preparation containing glucan, mannan, chitin, protein, lipid, and inorganic constituents. The isolation of "*active agents*" from yeast has been developing progressively for about 20 years. From these compounds, the particulate DiLuzio's water-insoluble yeast β-(1 → 3)-glucan has been stated to be the most promising for host defense in the regulation of inflammatory and immune responses.[5]

In some of our previous works, we focused attention on isolation and purification of the β-D-glucan portion of zymosan or of the cell wall of *Saccharomyces cerevisiae*.[13] To solubilize the isolated glucan, we established a chemical derivatization reaction yielding carboxymethyl sodium salt of glucan (CMG-Na).[14]

The aim of this report was to describe in detail (1) the procedure of CMG-Na crude derivative

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preparation, (2) the isolation of individual samples coded Fractions I, II, and III, (3) the analysis of their molecular weight distribution (M_n, M_w, and M_z averages of the molecular weights) by a rapid HPLC method, and (4) the physicochemical analysis of the samples by ^{13}C-NMR spectroscopy.

EXPERIMENTAL

Materials and Chemicals

Commercial baker's yeast (*Saccharomyces cerevisiae*) was from LIKO, Trenčín, Czechoslovakia. The dialyzing bag with the molecular weight cutoff 10–14 kDa was purchased from Serva Feinbiochemica, Heidelberg, Germany. Pullulan reference compounds P-400, P-200, P-100, P-20, P-10, and P-5 with M_w (M_w/M_n) of 3.80×10^5 (1.12), 1.86×10^5 (1.13), 1.00×10^5 (1.10), 2.37×10^4 (1.07), 1.22×10^4 (1.06), and 5.80×10^3 (1.07), respectively, were the product of "SHODEX®", Macherey-Nagel, Düren, Germany.

D_2O, deuterated dimethylsulfoxide (DMSO-d_6), hydrochloric acid, NaOH, NaCl, $NaNO_3$, methanol, acetone, isopropyl alcohol, diethyl ether, and sodium salt of monochloroacetic acid were all of analytical-reagent grade (mostly from Lachema, Brno, Czechoslovakia). Water was of redistilled quality grade.

Procedure

Glucan was isolated from the cell walls of baker's yeast (*Saccharomyces cerevisiae*) according to the procedure developed by Masler et al.[15]: 1 kg of pressed baker's yeast (28% of dry weight) was dispersed in 1.5 L of 6% aqueous NaOH and stirred at 60°C for 4 h. One liter of water was then added to the dispersion and the insoluble part was collected by centrifugation at 1200 g for 20 min. The sediment was suspended in 1.5 L of 3% aqueous NaOH and heated at 90°C for 2 h and centrifuged. The insoluble material was washed three times with 200 mL of water and then extracted twice with 200 mL of 4% HCl at room temperature for 2 h and centrifuged. The residue was decanted with water until neutral reaction and the sediment achieved was freeze-dried. The yield was 14.5 g.

The residual water-insoluble glucan, containing β-(1 → 3)-glycosically linked D-glucopyranosyl units with a small amount of β-(1 → 6) linkages,[16] was further solubilized by carboxymethylation[14] as follows: 100 g of the solid glucan was mixed with 216 mL of aqueous NaOH (300 g/L) and 2.5 L of isopropyl alcohol for 1 h at 10°C. Then, 115.8 g of sodium salt of monochloroacetic acid in 140 mL of water was added to the reaction vessel. The content was stirred at 75°C for 3 h. After the reaction, the excess of NaOH was neutralized with concentrated hydrochloric acid and the low molecular weight salts were removed by dialysis. The yield of the sodium salt of carboxymethylglucan (CMG-Na) derivative, with the substitution degree (DS) of 0.91, was 95%.

An aliquot of the obtained crude CMG-Na derivative was fractionated by stepwise precipitation with acetone as follows[17]: 220 mL of acetone was added to 1 g of the crude CMG-Na sample dissolved in 250 mL of 0.5% NaCl solution. The yield of Fraction I was 10.2%. That of the main Fraction II, isolated by subsequent polymer precipitation with further 60 mL of acetone, was 72.3%. The yield of Fraction III, obtained by addition of 200 mL acetone excess, was 3.2%.

All three fractions were reprecipitated, thoroughly washed with acetone and diethyl ether, and dried at laboratory temperature. Whereas Fractions II and III were fully soluble in water (D_2O) and 0.1*M* aqueous $NaNO_3$, yielding clear transparent solutions, on dissolving Fraction I, having a small insoluble polymer content, the resulting solution was opalescent and turbid. (Microscopic insoluble particles in Fraction I might be originated from the insufficient derivatization of the cell wall bud scars containing chitin, which are more compact than are other parts of the cell wall, and/or they were formed after dissolution of the freeze-dried substance.) That is why only the two samples of Fractions II and III were characterized by HPLC.

High-Performance Liquid Chromatography

The HPLC experiments were performed with a high-pressure pump (HPP 5001, Laboratorní přístroje, Prague, Czechoslovakia), an eight-port switching valve equipped with two 100 μL loops (Model PK 1, Vývojové dílny, Czechoslovak Academy of Sciences, Prague), and two in a series connected stainless-steel columns (250 × 8 mm i.d.) packed with Separon HEMA-BIO 1000 for Fraction II analysis or Separon HEMA-BIO 100 at characterizing Fraction III. The mean particle size of the sorbent was 10 μm (Tessek Ltd., Prague). Chromatograms were recorded by a differential refractometric detector (RIDK 101, Laboratorní přístroje, Prague). The mobile phase was 0.1*M* aqueous $NaNO_3$ solution. Its flow rate was set constant at 0.4 mL/min. The concentration of the injected sample was 0.2%. All

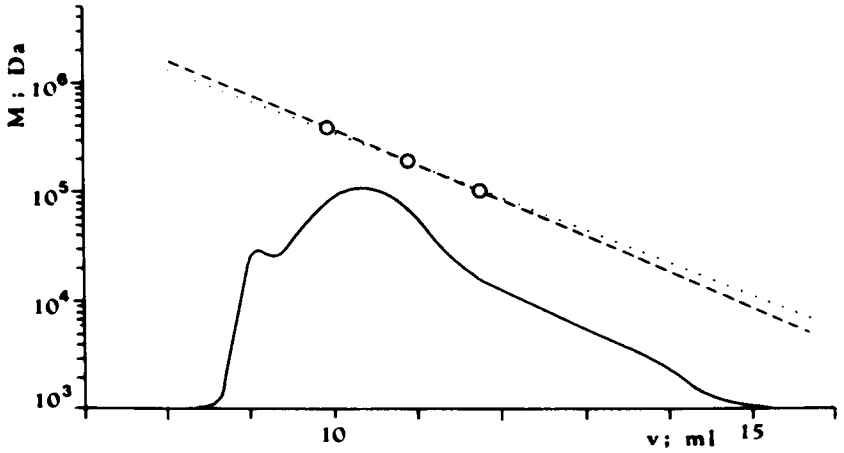

Figure 1 (——) Normalized HPLC record of Fraction II. Calibration curve [$M = f(v)$] of HPLC columns (- - - - -) obtained by using the pullulans P-400, P-200, and P-100 or (· · · · ·) computed applying one single calibrant P-200.

experiments were carried out at laboratory temperature.

NMR Spectrometry

Proton-decoupled ^{13}C-NMR spectra were recorded on an FT–NMR spectrometer Bruker AM-300 at 75.468 MHz in D_2O or DMSO-d_6 solution at 25°C. Methanol was used as the internal standard [50.15 ppm relative to $(CH_3)_4Si$].

RESULTS

Figures 1 and 2 show the normalized chromatographic records of Fractions II and III with corre-

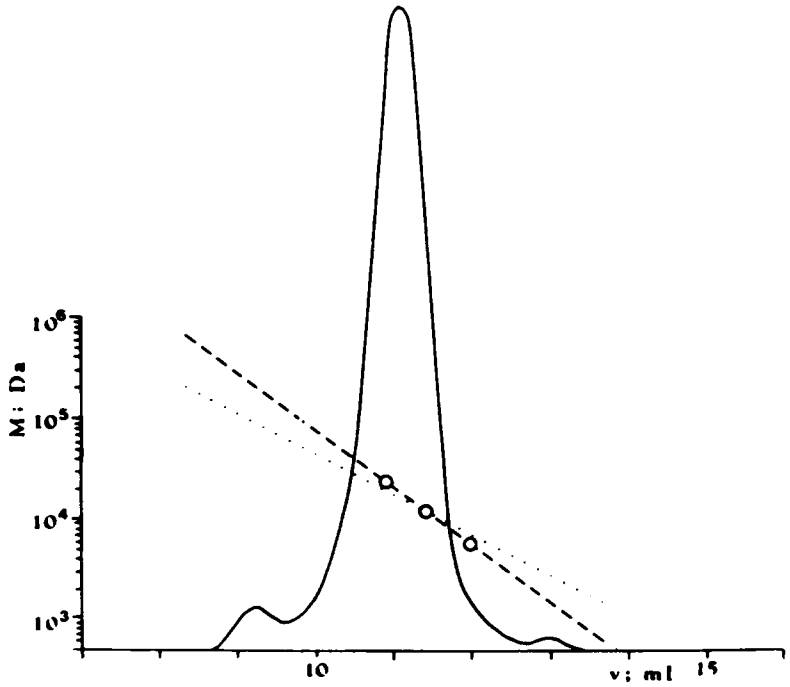

Figure 2 (——) Normalized HPLC record of Fraction III. Calibration curve [$M = f(v)$] of HPLC columns (- - - - -) obtained by using the pullulans P-20, P-10, and P-5 or (· · · · ·) computed applying one single calibrant P-10.

Table I Molecular Weight Averages of Fractions II and III

Fraction	M_n	M_w $\times 10^{-5}$ Da	M_z	Pullulan Calibrant(s)[a]
II	0.971	2.27	3.59	P-200
II	*0.895*	*2.41*	*4.00*	*P-400 P-200 P-100*
III	0.152	0.213	0.357	P-10
III	*0.154*	*0.312*	*0.931*	*P-10 P-10 P-5*

[a] The values obtained by using three pullulan calibrants correspond to the uncorrected M_n, M_w, and M_z averages, whereas the values calculated by using one single calibrant, P-200 or P-10, are equivalent to the corrected M_n, M_w, and M_z parameters.

sponding calibration curves used for molecular weight distribution analysis. The calculated molecular weight averages (M_n, M_w, and M_z) for Fractions II and III, on applying two different approaches to calibrate the HPLC apparatus used, are listed in Table I.

Figure 3 represents the proton-decoupled ^{13}C-NMR spectrum of water-insoluble β-(1 → 3) glucan with β-(1 → 6)-linked side chains in DMSO-d_6.

Figure 4 shows the ^{13}C-NMR spectra of Fractions II and III in D_2O. The chemical shifts of the fractions studied were taken from the proton-decoupled ^{13}C-NMR spectra, using the WALTZ sequence.

DISCUSSION

The β-D-glucan isolated from the cell walls of baker's yeast (*Saccharomyces cerevisiae*) is a water-insoluble branched polysaccharide with β-1,3- and a small amount of β-1,6-glycosidic linkages.[16] The heterogeneous etherification of the particulate β-D-glucan with monochloracetic acid in alkaline medium yielded the sodium salt of the water-soluble derivative carboxymethyl-β-(1 → 6)-D-gluco-β-(1 → 3)-D-glucan (CMG-Na).

The crude CMG-Na polymer was fractionated and characterized by combined methods of gel permeation chromatography, light scattering, and capillary viscometry.[18] Systematic high-performance liquid chromatographic studies in this field have not yet been reported. CMG-Na with the DS up to 0.6 is poorly soluble in water, whereas that one with DS over 1.0 is biologically less active.

The glucans presented here with the DS = 0.91 were tested for their immunomodulatory activity. They were found to enhance phagocytosis,[19,20] potentiate the antibacterial effect of antibiotics,[13] and exert a radioprotective effect,[21] and they may be used as a macromolecular antileukemic drug carrier.[22]

We established and used a routine procedure for the determination and calculation of the corrected molecular weight averages of the polymers,[12,18,23–27] applying the approach originally proposed by Tung.[28] For this procedure, both the dependence of

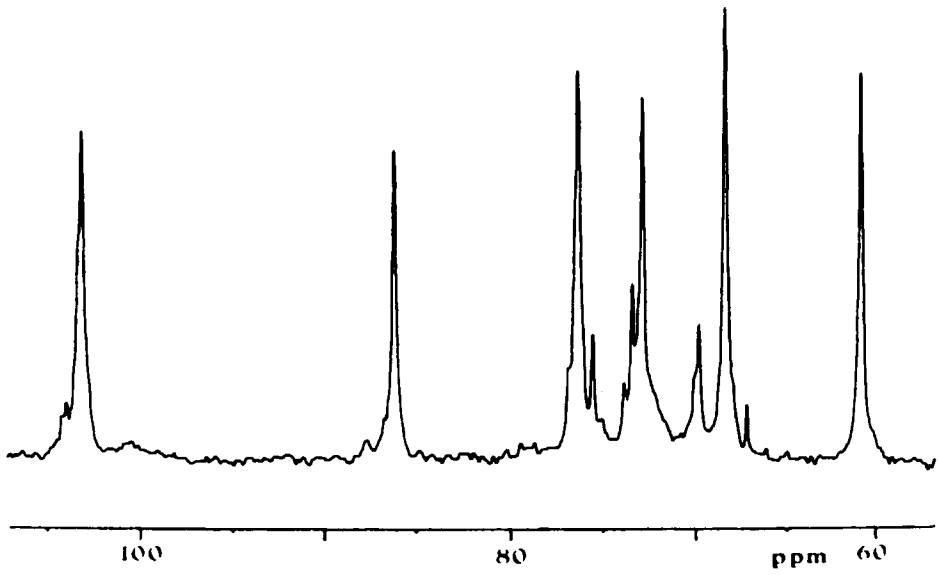

Figure 3 ^{13}C-NMR spectrum of water-insoluble glucan in DMSO-d_6.

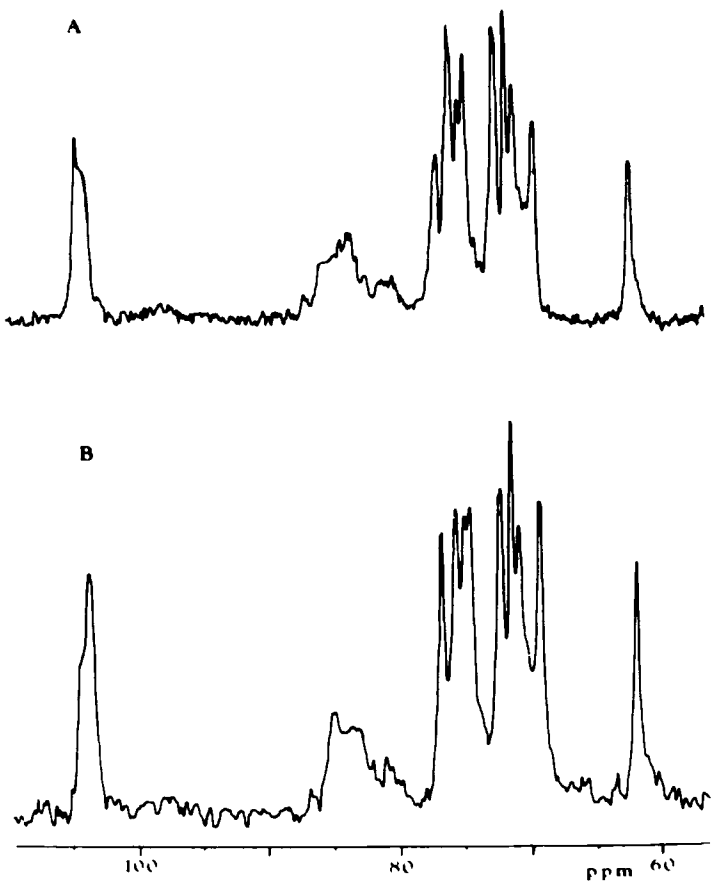

Figure 4 ^{13}C-NMR spectra of (A) Fraction II and (B) Fraction III in D_2O.

the calibrants' elution volumes (v) on their molecular weights (M) and the instrumental spreading parameter have to be calibrated.

Another approach for the correction of the instrumental spreading was proposed by Ishige et al.[29] Their computer program searches for a linear effective calibration curve from calibrant(s) with known average molecular weights, i.e., the effective calibration dependence [$M = f(v)$] should be calculated using either two different calibrants with one known molecular weight parameter (M_n; M_w; or at least the intrinsic viscosity [η]) or one single calibrant with two known molecular weight averages (M_n and M_w).

By using the latter approach, the corrected molecular weight averages of an unknown polymer can be determined by running just this sample and one calibrant. We used this mode and the relevant calibrant was pullulan P-200 or P-10, since their chromatograms peaked at about the same elution volumes as those of Fractions II and III. The obtained corrected molecular weight averages (cf. Table I) are thus valid for our β-1,3-glucans with respect to pullulan calibration.

The polymolecularity of the samples, as clearly evident also from Figures 1 and 2, could be classified as relatively broad for Fraction II and as relatively narrow for III. The M_w/M_n parameters for Fractions II and III, found to be 2.34 and 1.40, were comparable to those determined for clinically used injection preparations of lentinan (3.70_5) and schizophyllan (1.30_7).[12]

The comparison of the ^{13}C-NMR spectrum of original water-insoluble glucan (Fig. 3) with the spectra of Fractions II and III (which represent carboxymethyl derivatives after fractionation) evidently shows that in both fractions the characteristic signals for β-(1 → 3)- and β-(1 → 6)-linked glucopyranosyl units are present.[16,30] Thus, Fraction II has the following signals: C1 (103.6 ppm), C2 (72.3 ppm), C3 (85.0 ppm), C4 (69.4 ppm), C5 (76.8 ppm), and C6 (62.0 ppm), which are characteristic for

β-(1 → 3)-linked glucopyranosyl units. Similarly for β-(1 → 6)-linked glucopyranosyl units, the following signals are present: C1 (104.4 ppm), C2 (74.7 ppm), C3 (75.6 ppm), C4 (71.5 ppm), C5 (75.1 ppm), and C6 (70.9 ppm).

The study of *O*-carboxymethylation of glucose showed a strong preference for the OH-2, a lesser one for the OH-6, and the least for the OH-3 group. The carboxymethyl group has a strong α-deshielding effect (8–9 ppm).[31] However, the distribution of the carboxymethyl groups in the glucans depends on their spatial arrangement.[32] The signals at 80.5 ppm in the ^{13}C-NMR spectra of both fractions can be ascribed to the carboxymethylated C-4 atoms. The signal of the carboxymethylated C-2 at 83.6 ppm was observed mainly in the ^{13}C-NMR spectrum of Fraction III, which contained a higher amount of β-(1 → 6)-linked units. Signals at 71.5 and 72.3 ppm in both fractions belong to the $CH_2—COO^-$ groups.

From the preliminarily measured ^{13}C-NMR spectra of Fraction II, using the inversion recovery technique[33] with τ = 0.05–1.0 s, it was not possible to distinguish between the backbone β-(1 → 3)-linked units and the β-(1 → 6)-linked units of the side chains. The carbonyl groups, irregularly distributed, can lead to an interaction that affects the mobility and T1 relaxation time of β-(1 → 6)-linked units. The above-presented ^{13}C-NMR shifts indicate that Fraction II is composed mainly of the β-(1 → 3) glucosyl backbone with branching points in position 6. The ratio for β-(1 → 3) and β-(1 → 6) units was ≈ 3 : 1.

In comparison of the ^{13}C-NMR spectrum of the original insoluble glucan with those of the Fractions II and III, a difference is evident in the intensities of the C-3 signals. Irregular distribution of the carbonyl groups leads to a restricted mobility of the β-(1 → 3)-linked backbone that causes a decrease of the C-3 signal intensity in the ^{13}C-NMR spectra of Fractions II and III. A similarly low-intensity signal was observed for the C-4 atom in the ^{13}C-NMR spectrum of *O*-carboxymethyl cellulose.[31]

Fraction III has a similar ^{13}C-NMR spectrum to that of Fraction II, but there are changes in intensities and chemical shifts that indicate different ratios of β-(1 → 3)- and β-(1 → 6)-linked units. The distribution of carboxymethyl groups is also different in this fraction. The decreasing intensity of the signal at 62.0 ppm [C-6 in β-(1 → 3)-linked units] and the increasing intensity at 70.9 ppm [C-6 in β-(1 → 6)-linked units] indicate that, in comparison with Fraction II, Fraction III contains more β-(1 → 6)-linked units.

CONCLUSIONS

Carboxymethylation of yeast cell wall β-D-glucan and subsequent fractionation by acetone showed its heterogeneous character. Isolated fractions were found to differ in solubility, the distribution of molecular weights, and the ratio of the two types of glycosidic linkages. Carboxymethylation of glucose units showed a strong preference for OH-2, a lesser one for OH-6, and the least for OH-3. The M_w values of the two fractions studied were 2.27×10^5 and 2.13×10^4 Da.

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