Enzymatic and Ultrasonic Depolymerization of Carboxymethylated β-1,3-D-Glucans Derived from *Saccharomyces cerevisiae*

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SYNOPSIS

Enzymatic and ultrasonic degradations of the carboxymethyl derivative of the β-1,3-D-glucan, isolated from the cell walls of baker's yeast *Saccharomyces cerevisiae*, are described. The samples obtained upon partial depolymerization of the two carboxymethylated glucans with the substitution degree of 0.56 and 0.91 were characterized by the distributions of their molecular weights and ^{13}C-NMR spectra. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Fungal β-1,3-D-glucans are known to have potent immunostimulating and antitumor properties.[1-3] A skeletal β-1,3-D-glucan from the cell walls of *Saccharomyces cerevisiae* belongs to the class of substances known as biological response modifiers. By stimulation of the host immune system, it exerts a beneficial effect on a variety of experimental disease states of bacterial, viral, fungal, and parasitic origin. Antitumor and radioprotective properties of such glucans have been described as well.[4,5] A successful clinical use of β-1,3-D-glucan derived from the baker's yeast cell wall is, however, greatly limited by its insolubility in aqueous media.

In order to improve the solubility of β-1,3-D-glucans, several derivatization procedures have been developed.[6-9] Carboxymethylation of glucans, along with their solubility, was reported to improve also the antitumor activity against Sarcoma 180 in mice[9,10] as well as to enhance the protective antibacterial properties of their derivatives.[11]

In the present study, we describe the depolymerization of the *S. cerevisiae* cell wall β-1,3-D-glucan (the carboxymethylated derivatives) with β-1,3-D-glucanase as well as by means of the ultrasonication carried out in order to obtain biopolymer samples with lower molecular weights. The native and degraded products were characterized by the distributions of their molecular weights and ^{13}C-NMR spectra.

EXPERIMENTAL

Materials

The water-insoluble β-1,3-D-glucan was obtained by means of the extraction of *S. cerevisiae* cells with 6% NaOH at 60°C followed by 4% phosphoric acid at room temperature according to the procedure outlined by Masler et al.[12] The source of commercial baker's yeast was the Slovlik, Trenčín, Slovak Republic.

Endo-β-1,3-D-glucanase, from the extracellular production of *Trichoderma reesei*, was a generous gift of Dr. Farkaš from the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic. It was purified by the liquid chromatography by using Bio-Gel P-100 and Ostsorb DEAE columns. The activity of the enzyme preparation was 2.0 U mg^{-1} (1 U catalyzes the liberation, from laminarin, of 1 μmol of reducing sugar (as glucose equivalent) per minute at 30°C, pH 5.0).

A set of pullulans P-100, P-200, P-400, and P-

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800 (Shodex Standard P-82, Macherey-Nagel GmbH + Co KG, Düren, Germany) was used for the calibration of the high-performance gel permeation chromatography system. Therefore the molecular weight parameters presented herein are relative to the pullulan reference material.

Glucan O-Carboxymethylation

Derivatization of the β-1,3-D-glucan was performed using the modified procedure described by Sasaki et al.[9] Briefly, 10 g of the glucan was suspended in a mixture of 12.4 mL of aqueous NaOH (300 g L^{-1}) and 125 mL of isopropanol. The suspension was vigorously stirred at 10°C for 1 h. Subsequently, sodium salt of monochloroacetic acid was added [8.8 g for achievement of the substitution degree (DS) of 0.56; 14.3 g for DS = 0.91] in 14 mL of water, and the mixture was stirred at 70°C for 2 h. The NaOH excess was neutralized and the salts were removed by dialysis. The nondialyzable portion was dried, dissolved in water, centrifugated and lyophilized. Determination of the substitution degree of the carboxymethylated glucan preparations was performed by means of potentiometric titration with 0.05 mol L^{-1} KOH solution.[13]

Enzymatic Degradation

Water solutions of the carboxymethylated β-1,3-D-glucans ($\bar{M}_w$ = 3.46 × 10^5 Da, DS = 0.56; $\bar{M}_w$ = 1.9 × 10^5 Da, DS = 0.91), 5.0 mg mL^{-1}, were kept at 28°C for 20 min. Enzymatic degradation was performed using a partially purified endo-β-1,3-glucanase from *T. reesei* (0.3 U) with 30 mg of carboxymethylated β-1,3-D-glucan in 0.05 mol L^{-1} acetate buffer (pH 5.0) at 28°C. The digestion was allowed to proceed for 6, 24, 48, 72, 168, and 192 h and was terminated by heating the vessel in a boiling water bath for 10 min. The samples were cooled to a room temperature and centrifuged at 10,000*g* for 3 min to separate the precipitated proteins.

Ultrasonic Degradation

Carboxymethylated glucan ($\bar{M}_w$ = 3.46 × 10^5 Da, DS = 0.56; $\bar{M}_w$ = 3.66 × 10^5 Da, DS = 0.91), 20 mg, was suspended in 20 mL of distilled water and sonicated (20 kHz, 150 W) at room temperature using a UGA 204 52 ultrasound generator (Tesla, Vráble, Slovak Republic). At definite time intervals the samples were withdrawn and analyzed by high-performance gel permeation chromatography (HPGPC) and ^{13}C-NMR spectroscopy.

High-Performance Gel Permeation Chromatography

HPGPC was performed using a Laboratorní přístroje instrument (Prague, Czech Republic) equipped with two Tessek Separon HEMA-BIO 1000 columns (8 × 250 mm) with a 100-μL sample loop. The mobile phase used was a 0.1 mol L^{-1} aqueous $NaNO_3$ and the flow rate was 0.4 mL min^{-1}. All experiments were carried out at room temperature. The carbohydrate content in the effluent was monitored with a differential refractometer (RIDK-101 Laboratorní přístroje, Prague, Czech Republic). In order to convert the HPGPC data into the sample molecular weight distribution, a computing procedure based on a linear effective calibration curve has been applied.[14]

NMR spectroscopy

Proton-decoupled ^{13}C-NMR spectra were recorded with a Bruker AM-300 Fourier transform spectrometer at 75.468 MHz field frequency with methanol as the internal standard (50.15 ppm relative to tetramethylsilane). The solutions in D_2O (80 mg mL^{-1}) were used and the measurements were carried out at 25°C.

RESULTS AND DISCUSSION

For the carboxymethylated glucan with the substitution degree of 0.56 enzymatic depolymerization proved to be more effective (Fig. 1, curve 1). After

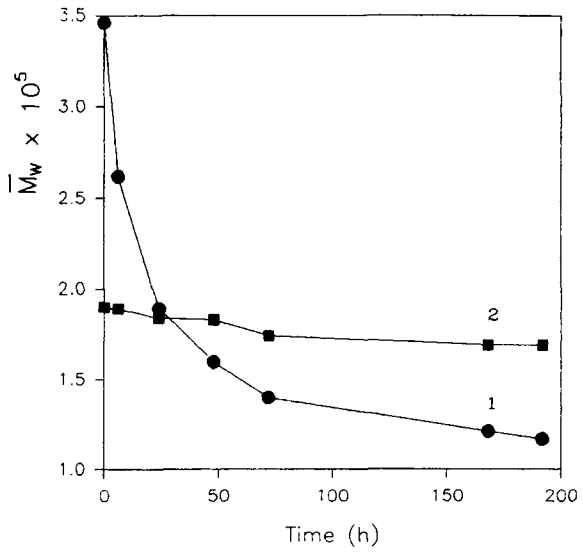

Figure 1 Enzymatic depolymerization of the carboxymethylated glucan, derived from *S. cerevisiae*, with DS = 0.56 (curve 1) and DS = 0.91 (curve 2).

72 h digestion, its weight-average molecular weight ($\bar{M}_w$) was lowered by 60%. With digestion prolongation the depolymerization process slowed down significantly. In the following 96 h, the $\bar{M}_w$ value decreased only by a further 14%. The resulting $\bar{M}_w$ value, after the termination of the enzymatic degradation (192 h), was 1.17×10^5 Da, corresponding to 66% decrease of the sample initial $\bar{M}_w$ value, which was 3.46×10^5 Da.

The other, higher substituted carboxymethylated glucan (DS = 0.91) was found to be an inappropriate substrate for the β-1,3-D-glucanase used. After 72 h digestion its $\bar{M}_w$ decreased only by 10.5%, and in the following 72 h it remained almost unchanged (Fig. 1, curve 2).

Better accessibility of the hydrolyzing enzyme to the carbohydrate chain with a lower substitution degree was already observed by Parfondry and Perlin[15] in their study of the cellulase action on the carboxymethylated celluloses with DS = 0.5, 0.7, or 1.2. This finding along with our results are in accord with the previous assumptions[16-18] that the scission of the cellulose ether chains by a cellulase takes place preferentially at residues that do not bear the O-alkyl substituent. Thus, enzymatic scission of all accessible β-1,3- linkages in the carboxymethylated glucan with DS = 0.91 corresponded to a 10.5% decrease in its molecular weight (M_w – value).
– value).

Results of the enzymatic degradation of the carboxymethylated β-1,3-D-glucan derived from *S. cerevisiae* (DS = 0.56) are represented in Figure 2. As evident, the chromatographic curve of the original carboxymethylated glucan revealed two peaks. The β-1,3-D-glucanase treatment, however, depolymerized both biopolymer components leading to samples with broader distribution of molecular weights.

The assignment of the signals in the ^{13}C-NMR spectra of the carboxymethylated glucans was performed on the basis of the known chemical shifts of the signals of nonderivatized glucan,[14] as well as taking in account the substitution effect on the chemical shift values caused by carboxymethylation.[15] It is known that the baker's yeast glucan contains β-1,3- and β-1,6-linked D-glucosyl units, the former component being the predominant one.[14] Therefore, ^{13}C-NMR spectra of the carboxymethylated glucans contain the signals corresponding to the carbon atoms of both types of units, as well as the signals of the carbon atoms of the units bearing carboxymethylated substituents (Table I).

Comparison of the ^{13}C-NMR spectra of the original carboxymethylated glucan (DS = 0.91) and its ultrasonically degraded preparation shows changed intensity of the C-3 in the spectrum of the latter, which is due to splitting of β-1,3- glucosidic linkages (Fig. 3). The presence of an anomeric signal at 97.8 ppm implies that some low-molecular-weight fragments had formed during the degradation.

The ^{13}C-NMR spectra of the carboxymethylated

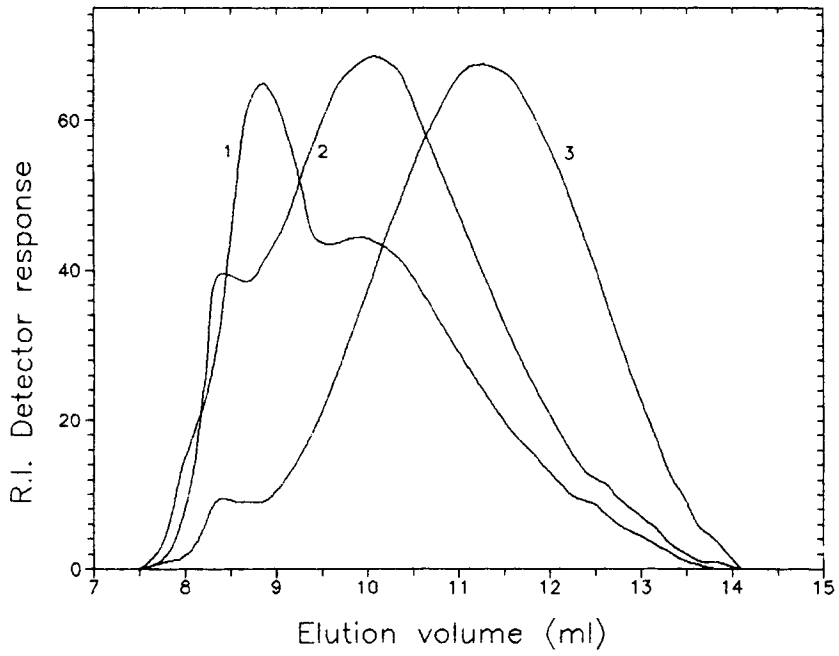

Figure 2 Chromatograms of the original carboxymethylated glucan with DS = 0.56 (curve 1) and its products obtained after enzymatic treatment 24 h (curve 2) and 192 h (curve 3).

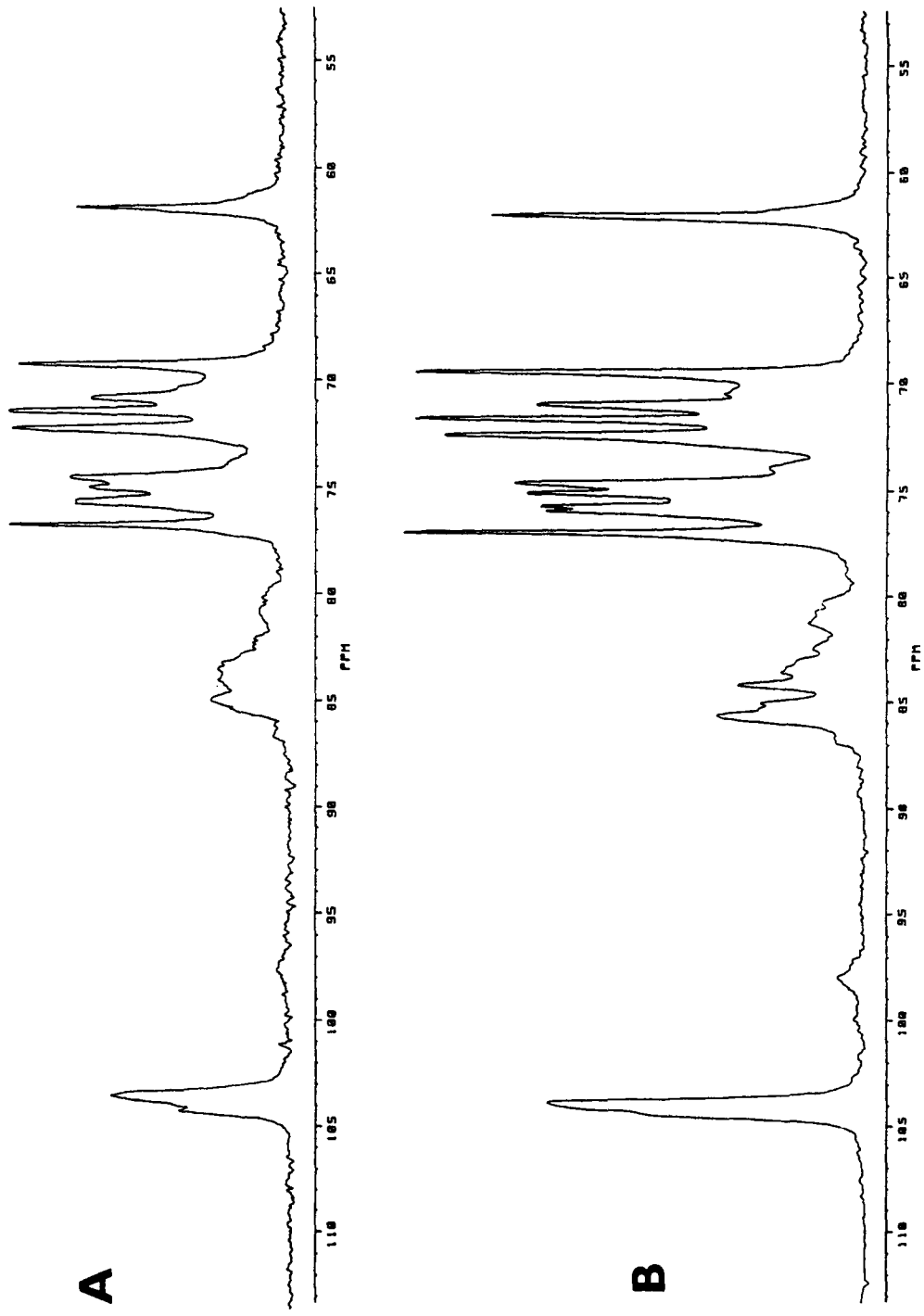

Figure 3 ^{13}C-NMR spectra of the original carboxymethylated glucan with (A) DS = 0.91 and (B) its products obtained after 20 min ultrasonic depolymerization.

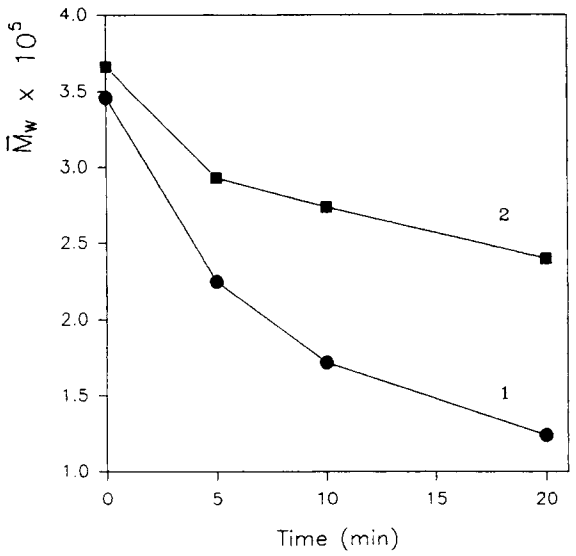

Figure 4 Ultrasonic depolymerization of the carboxymethylated β-1,3-D-glucan, derived from *S. cerevisiae*, with DS = 0.56 (curve 1) and DS = 0.91 (curve 2).

glucan (DS = 0.56) after the ultrasonic treatment were practically identical with the spectra of the original, nondegraded carboxymethylated glucans, and similar results were obtained with the enzymatically degraded samples. However, all these ^{13}C-NMR spectra show no signal from the range corresponding to anomers.

Table I Assignment of the ^{13}C-NMR Signals in the Spectra of the Carboxymethylated Glucans[a]

Chemical Shift (ppm)	C Atoms	Comments
179.1	C=0	Carboxymethyl group (not shown)
104.1	C—1	β-1,6-linked unit
103.5	C—1	
85.0	C—3	
84.2	C—2	Carboxymethylated
80.5	C—4	Carboxymethylated
76.8	C—5	
75.7	C—3	β-1,6-linked unit
74.9	C—5	β-1,6-linked unit
74.4	C—2	6-O-carboxymethylated unit
72.2	C—2	
71.3	CH_2	Carboxymethyl group
70.7	C—6	Carboxymethylated
69.2	C—4	
61.8	C—6	

[a] Unless specified, the signal refers to the β-1,3-linked units (chemical shift in ppm).

The depolymerization of the carboxymethylated glucan by means of ultrasonication was effective for both the glucan with DS 0.56 (Fig. 4, curve 1) and the sample with DS = 0.91 (Fig. 4, curve 2). Com-

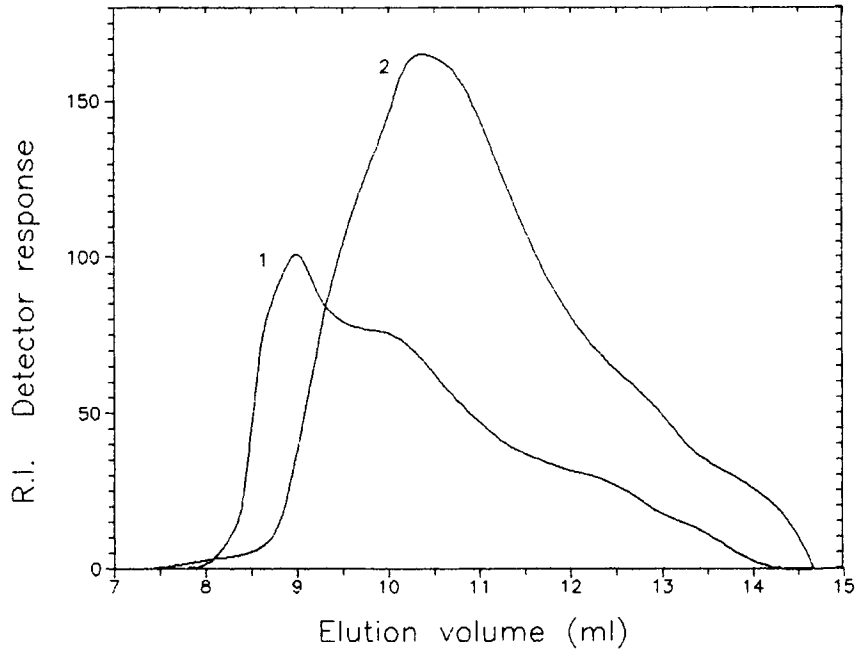

Figure 5 Chromatograms of the original carboxymethylated glucan with DS = 0.91 (curve 1) and its product obtained after ultrasonic treatment lasting 10 min (curve 2).

parison of the $\bar{M}_w$ values of the samples obtained by the ultrasonication of the carboxymethylated glucan with DS = 0.56, lasting 5, 10, and 20 min, shows the degradation to be more effective in the first 5 min when the $\bar{M}_w$ value decreased by 35%. After 20 min ultrasonic treatment $\bar{M}_w$ decreased by 64% and reached the value of 1.24×10^5 Da.

The higher substituted carboxymethylated glucan with DS = 0.91 was also effectively depolymerized. During the first 5 min its $\bar{M}_w$ decreased by 20%. The decrease of $\bar{M}_w$ by 34% was attained after 20 min of ultrasonication and reached $\bar{M}_w = 2.40 \times 10^5$ Da.

The elution profiles of the original carboxymethylated glucan with DS = 0.91 and its sonicated products are represented in Figure 5. Similarly as in Figure 2, the bimodal chromatographic curve indicates that the original carboxymethylated glucan is a bicomponent sample. The ultrasonic treatment resulted in a relatively broad, but unimodal biopolymer.

Our results demonstrated that ultrasonic degradation of the carboxymethylated glucan was more effective for the production of the samples with lower molecular weights than enzymatic hydrolysis. Both the processes of sonication and of enzymatic treatment produced biopolymers with similar molecular weights. Yet ultrasonication avoids the need to separate subsequently the enzyme from products prepared by hydrolytical procedure.[19] We, thus, recommend ultrasonication as a rapid and efficient tool for the preparation of polysaccharides with lower molecular weights.

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