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Short communication

Preparation and characterization of carboxymethyl derivatives of yeast mannans in aqueous solutions

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ABSTRACT

Novel carboxymethyl derivatives of yeast mannans of different degrees of substitution (DS) were prepared by optimized reaction of concentrated polysaccharides in alkaline aqueous solution. Mannans from various yeasts differing in size and degree of branching show similar reactivity. Strong alkaline conditions during carboxymethylation caused degradation of the polysaccharides. The degree of substitution (DS) of *Candida albicans* mannan and dextran were proportional to the amount of monochloroacetate added. However, degrees of carboxymethylation of *Candida albicans* mannan (0.30, 0.41, 0.73) were lower than those of dextran (DS = 0.33, 0.6, 1.1) using the same amounts of monochloroacetate. Evidently the resulted polyanionic derivatives have higher hydrodynamic sizes than the original polysaccharides. Non-uniform, variable position of substitutions results to non-proportional change of optical rotation and increase of complexity of NMR spectra. Basic physico-chemical characteristics of novel carboxymethyl mannans obtained by potentiometric titration, FT-IR, UV, HPLC, ^{1}H NMR and optical rotation measurements are presented here.

1. Introduction

Mannans are considered the main polysaccharides on the surface of the yeasts. Different species of yeasts, from pathogenic *Candida albicans* to baker species *Saccharomyces cerevisiae*, contain mannans with different structural features. Yeast mannans consist of α-(1,6)-linked mannose backbone with different frequency of branching. Branches contain α-(1-2; 1-3) and β-(1-2)-linked mannooligomers (Shibata, Kobayashi, & Suzuki, 2012). Mannans are readily soluble in water in contrast to water-insoluble yeast β-glucan, another common polysaccharide that is found under mannoprotein layer on the surface of yeasts. Mannan and β-glucan have interesting biological activities. They are intensively studied as immunoactive components of perspective vaccines (Liu et al., 2012; Pietrella et al., 2005). The polysaccharides are modified in order to enhance or activate their biological properties. The beneficial properties of carboxymethylated β-glucan (which is soluble in water) were already found (Šandula, Machová, & Hríbalová, 1995; Šandula, Kogan, Kačuráková, & Machová, 1999). With novel CM-mannans we can expect alternation of antioxidant, antitumor and antimutagen activities known from original mannans (Chorvatovičová, Machová, Šandula, & Kogan, 1999; Kogan et al., 2008).

The carboxymethylation is broadly used to improve the solubility of polysaccharides in water. The reaction medium generally used for etherification of polysaccharide is water/organic solvent mixture. The most common is the method where the water-insoluble material such as cellulose, starch (Heinze & Koschella, 2005; Heinze, Pfeiffer, Liebert, & Heinze, 1999) or chitin (Kurita, 2001; Trujillo, 1968) are emulsified in solvent mixture. Further, the water-soluble polysaccharides, e.g. dextran (Huynh, Chaubert, & Jozefonvicz, 1998) and hyaluronan (Mőller et al., 2012), were carboxymethylated in alcohol/water mixture. The functionalization of yeast mannans by carboxymethylation has not been studied yet.

We made effort to prepare carboxymethyl mannans (CM-mannans) only in water and tried to elucidate their properties. The carboxymethyl dextrans (CM-dextrans) were prepared for comparison. The influence of reaction conditions as well as the basic structural and molecular characteristics were studied by potentiometric titration, FT-IR, ^{1}H NMR spectroscopy, optical rotation and HPLC.

2. Materials and methods

Yeast strains *Candida albicans* CCY 29-3-32, *C. tropicalis* CCY 29-7-6, *C. dubliniensis* CCY 29-177-1, *C. glabrata* CCY 26-20-1 and

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Saccharomyces cerevisiae CCY 9-84-1 were from the Culture Collection of Yeasts (CCY, Institute of Chemistry of Slovak Academy of Sciences, Bratislava, Slovakia). Dextran from *Leuconostoc* spp. (Mr ~70,000) was purchased from Sigma Aldrich (Bratislava, Slovakia) where it was characterized as a branched glucan composed of linear α-(1,6)-linked glucose units and α-(1,3)-link initiated branches.

2.1. Carboxymethylation of polysaccharides

Mannans isolated from the yeast strains mentioned above (Ližičárová, Matulová, Machová, & Capek, 2007) were carboxymethylated in aqueous or DMSO systems.

Briefly: 100 mg of mannan was dissolved in 1 mL of 6 M sodium hydroxide in deionized water and reaction mixture was stirred at room temperature for 30 min. Subsequently, the various amounts of sodium monochloroacetate (MCA, 150 mg, 300 mg and 600 mg, respectively) was slowly added and the reaction proceeded at 70 °C for 5 h. The reaction mixture was neutralized by acetic acid to pH ~ 7.0 and dialyzed against distilled water (Visking Dialysis Membranes, MWCO 12–14 kDa). CM-dextran was prepared by the same procedure.

Carboxymethylation in non-aqueous DMSO system via induced phase separation at high temperature was accomplished according to Heinze, Liebert, Heinze, & Schwikal, 2004.

2.2. Determination of carboxyl groups by potentiometric titration

The degree of substitution (DS) of mannan and dextran derivatives was determined as content of carboxymethyl groups by the potentiometric titration. The solution of CM-polysaccharide was passed through a cation exchanger Dowex 50X2 (H^+-form) and titrated with the solution of KOH (0.106 M) using pH-meter (Mettler Toledo) equipped with the combined electrode. The degree of substitution (DS) defined as the average number of carboxymethyl groups per monomer was calculated according to Rinaudo and Hudry-Clergeon (1967).

2.3. Molecular weight determination

Molecular weights of original and carboxymethylated polysaccharides were measured by a high performance liquid chromatography (HPLC) system at room temperature using two HEMA-BIO 100 and 300 connected columns (8 mm × 250 mm, Tessek, Prague, Czech Republic). 0.1 M aqueous $NaNO_3$ solution was used as a mobile phase at the flow rate of 0.4 mL/min. The separation process was monitored with the differential refractometer (Shodex, Shimadzu, Japan). A set of dextrans (American Polymer Standard Corporation) was used for the calibration of the HPLC system.

2.4. Analytical methods

The specific optical rotation $[\alpha]_D^{20}$ was measured with digital polarimeter Jasco P-2000.

UV spectrophotometric measurement of sample solutions (1 mg/mL) was performed with UV/vis spectrophotometer (Shimadzu 1240).

FT-IR-ATR spectra were measured with Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and controlled by Omnic 8.0 software. The spectra were collected from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, the number of scans was 128. Diamond Smart Orbit ATR accessory was used for measurements in solid state.

^1H NMR spectra were measured in D_2O at 60 °C on Varian 400 MR spectrometer on indirect 5 mm PFG AutoX detection probe after double exchange with D_2O and addition of internal acetone. ^1H NMR spectra were referenced to chemical shift of the acetone (δ 2.217) as the internal standard.

3. Results and discussion

3.1. Carboxymethylation of polysaccharides

Mannans of *Candida* species are accessible surface polysaccharides which are easy isolable from the yeast cell walls. They represent perspective polysaccharide materials with interesting biological activities not yet discovered. The additional functionalization of mannans by carboxymethylation should lead to more reactive products. We tried to use different procedures/media for preparation of derivatized mannans. The products obtained by the conventional method – reaction in water/alcohol system had only low degree of substitution (DS 0.05–0.1). The reaction in non-aqueous DMSO system (slurry) via induced phase separation at high temperature yielded only 40–45% of CM-mannans. These products were not used in further experiments. Finally, the derivatization of mannan in pure water showed promising results. The reaction conditions such as the concentration of mannan, concentration of MCA, the reaction temperature and time were optimized. Consequently, the high molecular CM-mannans with high DS, good reproducibility and high yields were obtained. Moreover, the DS can be directly controlled by the ratio of reactive agents. The same ratio led to similar DS for mannans of different origin (see Table 1). However the DS of carboxymethylation at the same conditions differed when mannans and dextran were compared. The lower DS values of mannans in comparison to dextran probably reflect the different structure of both polysaccharides. It seems that dextran contains a higher number of accessible carbohydrate units for carboxymethylation probably due to its lower branching. The degradation of polysaccharides during the carboxymethylation occured as the hydroxyl groups were activated by the strong alkaline solution (6 M NaOH) at an elevated temperature. It was found that the degradation was quite significant for *Candida* mannans: 16% decrease for *C. albicans* mannan, 24% for *C. tropicalis* mannan, 29% for *C. dubliniensis* mannan, and 19% for *C. glabrata* mannan. The negligible degradation of *S. cerevisiae* mannan (5%) and dextran (7%) could be caused by lower molecular weight and lower degree of branching with shorter side chains (Table 1).

FTIR-ATR spectra of CM-mannans (Fig. 1) showed the new vibration maxima at 1587 cm^{-1} and 1408 cm^{-1} indicating the substitution of COO^-Na^+ group on the mannan chain. These vibrations correspond to stretching vibration of ν_{as} (COO^-) and ν_s (COO^-), respectively. The absorption band at 1322 cm^{-1} could be due to the overlap of —CH_2 and O—H in plane bending (Nguyen, Nguyen, Pham, & Do, 2011). The specific band maxima in the spectral range 800–1200 cm^{-1} are typical for each particular polysaccharide. This region is dominated by ring vibrations overlapped with stretching vibration of ν(C—OH) side groups and the ν(C—O—C) glycosidic bond. The vibration maxima observed for mannan in this region are in accordance with the assignments already published (Galichet, Sockalingum, Belarbi, & Manfait, 2001).

The presence of UV absorbing carboxylate in CM-derivatives prepared was also shown by UV measurement. DS increase of CM-derivatives proportionally increased the absorption at 210 nm (see Table 1).

3.2. NMR spectroscopy

As already mentioned the branched structures of mannans contain numerous differently linked mannosides. As a consequence, ^1H NMR spectra showed complexity with many overlapping peaks (Fig. 2). Due to that complication, the complete assignment of NMR