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Biophysical properties of carboxymethyl derivatives of mannan and dextran

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ABSTRACT

Mannan from *Candida albicans*, dextran from *Leuconostoc* spp. and their carboxymethyl (CM)-derivatives were tested on antioxidant and thrombolytic activities. As antioxidant tests, protection of liposomes against OH radicals and reducing power assay were used. Dextran and mannan protected liposomes in dose-dependent manner. Carboxymethylation significantly increased antioxidant properties of both CM-derivatives up to concentration of 10 mg/mL, higher concentrations did not change the protection of liposomes. The reducing power of CM-mannan (DS 0.92) was significantly lower ($P < 0.05$) than underivatized mannan. No reductive activity was found for dextran and CM-dextran. All CM-derivatives demonstrated statistically significant increasing activity compared with underivatized polysaccharides. The highest thrombolytic activity was found using CM-mannan (DS 0.92). The clot lysis here amounted to 68.78 ± 6.52% compared with 0.9% NaCl control (18.3 ± 6.3%). Three-dimensional surface profiles of mannan, dextran, and their CM-derivatives were compared by atomic force microscopy.

1. Introduction

At present, the considerable interest is given to polysaccharides from various sources possessing number of positive biological and physico-chemical properties (Yuan et al., 2010; Ding et al., 2010; Machová & Bystrický, 2013). One of them, yeast mannan is located on the *Candida* spp. surface as mannoprotein complex which performs immunoprotective function and induces specific antibody formation. It is easily prepared and manipulated as water soluble neutral polysaccharide. In our previous paper we described the preparation of carboxymethyl derivatives of yeast mannans (CM-mannan) and *Leuconostoc* spp. α-glucan (dextran) with tailored degree of substitution (Machová, Bystrický, Malovíková, & Bystrický, 2014a) and tested their antioxidant properties (Machová, Čížová, & Bystrický, 2014b). Compared to underivatized mannans, CM-derivatives showed stronger antioxidant activities against reactive OH radicals determined by standard salicylate assay. Moreover, accompanied radical degradation was less marked at CM-derivatives compare to parent polysaccharides. On the other hand, scavenging abilities of CM-mannans against s DPPH radical were lower than those of underivatized ones. Further, yeast mannans and CM-β-glucan protected liposomes against peroxidation with OH radicals but the main reason of their antioxidant effect could be the protective polysaccharide shield coating the surface of liposomes (Machová & Bystrický, 2012).

Several studies revealed that antioxidants are related to other bioactivities (Kalaivani, Rajasekaran, & Mathew, 2011). Almost all currently available thrombolytic regimens have some vital shortcomings including limited fibrin specificity, noteworthy bleeding tendency and large dose requirement. To counter this dilemma, there is a tendency that carried out to find other effective resources (Khan et al., 2011).

The aim here was the study of antioxidant properties of *Candida albicans* mannan and dextran from *Leuconostoc* spp. and their CM-derivatives using liposomes exposed to OH radicals as model of experimental biological membrane. Also, the reducing power and thrombolytic activities of above mentioned polysaccharides were tested. The three-dimensional surface profiles of underivatized polysaccharides and their CM-derivatives were compared using atomic force microscopy.

2. Material and methods

2.1. Polysaccharide material

Mannan was isolated as mannan–copper complex (Jones & Stoodley, 1965) from *C. albicans* (CCY 29-3-32 from Culture

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Collection of Yeasts, Institute of Chemistry of Slovak Academy of Sciences, Slovakia). Dextran from *Leuconostoc* spp. (Mr ~70,000) was purchased from Sigma Aldrich (Bratislava, Slovakia). CM-derivatives with various degrees of substitution (DS), CM-mannan (DS 0.3 and 0.92) CM-dextran (DS 0.37 and 1.14), were prepared and characterized according to Machová et al. (2014a).

2.2. Preparation of liposomes and determination of degree of peroxidation

Multilamellar liposomes (MLLs) were prepared by hydration of phospholipid film (Babincová, Šusteková, & Machová, 2000). Briefly: 5 mg of L-α-phosphatidylcholine was dissolved in 1 mL of chloroform–methanol mixture (2:1, v/v), the suspension was evaporated in vacuum. Lipid film was hydrated either with distilled water or with aqueous solutions of the tested polysaccharides–mannan and dextran, as well as their CM-derivatives. Polysaccharides were used in the concentration range of 1–20 mg/mL according to the previous results showing the dose-dependent manner of antioxidant activities in this range (Machová & Bystrický, 2012). Lipid peroxidation of MLL was induced by OH radicals generated by Fenton's reaction [100 mM of H_2O_2 and 2 mM Fe^{2+} ($FeCl_2{\cdot}4H_2O$)]. The absorption spectra of radical induced conjugated dienes in liposomes were recorded in the wavelength range of 200–270 nm. The increase of the absorption at 233 nm was considered as an evidence of the formation of the conjugated dienes. The degree of peroxidation was calculated according to the Klein method (Klein, 1970). The Klein oxidation index was calculated from the ratio of the absorbance values (A_{233}/A_{215}). The antioxidant activity (AOA) was expressed as AOA (%) = $100 \times (I_L - I_C)/I_L$, where I_L and I_C stand for the Klein index of the pure liposome hydrated with distilled water (I_L) and the liposomes hydrated with tested compounds (I_C), respectively. Liposomes exposed to OH radicals without polysaccharides as possible scavenger of radicals represent positive control (AOA = 100%). The value of EC_{40} represents the concentration of polysaccharides that provides 40% protection of liposomes against peroxidation with OH radicals (Table 1).

2.3. Reducing power assay

The reducing power assay of mannan and dextran, as well as their CM-derivatives was quantified following a method described earlier (Yuan, Zhang, Fan, & Yang, 2008). Briefly: 1 mL of samples in different concentrations (0–5 mg/mL) were mixed with 0.2 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1%, w/v). The reaction mixture was incubated at 50 °C for 20 min. Then 1 mL of trichloroacetic acid (10%, w/v) was added to the mixture and centrifuged for 10 min at 3000 rpm. The supernatant (1.5 mL) was mixed with 0.2 mL $FeCl_3$ (1.0%, w/v) and 3 mL of distilled water. The absorbance was measured at 700 nm against blanks. Ascorbic acid was used as the positive control. A higher absorbance of reaction mixture indicates a stronger reducing power of the sample.

2.4. Determination of thrombolytic potential of CM-mannan and CM-dextran

The *in vitro* thrombolytic potential of mannan and dextran and their CM-derivatives was evaluated by modified method of Prasad et al. (2006) using streptokinase from β-hemolytic *Streptococcus* (Lancefield Group, 8750 U/mL, Sigma, Germany) as the standard substance. A total of 10 mL venous blood was drawn from five healthy volunteers (two male and three female) without history of anticoagulant therapy. The study was conducted following ethical principles of research without potential conflicts of interests. Informed consent was obtained by all the participants. Blood from each volunteer was distributed in nine different pre-weighed sterile microcentrifuge tube and incubated at 8 °C. After clot formation, serum was completely aspirated out without disturbing the clot formed and the weight of clot in each tube was measured. To each microcentrifuge tube containing pre-weighed clot, 100 μL aqueous solution of different saccharide materials with the concentration of 20 mg/mL was added separately. Then, 100 μL of streptokinase (streptokinase from β-hemolytic *Streptococcus*, Lancefield Group, 8750 U/mL, Sigma, Germany) and 100 μL of physiological saline were separately added to the control tube as positive and negative controls, respectively. All the tubes were then incubated at 37 °C for 120 min and observed for clot lysis. After incubation, the released fluid was removed and tubes were again weighed to observe the difference after clot disruption. Difference in weight before and after clot lysis was expressed as percentage of clot lysis as shown below: % of clot lysis = (weight of released clot/clot weight) × 100.

Statistical comparison between experimental groups was performed using one-way ANOVA and post-hoc Bonferoni's test. The results were significant if the difference between analyzed groups was equal the 95% confidence level ($P < 0.05$). Statistics were performed with Origin Pro 8 software (OriginLab Corporation, Northampton, USA).

2.5. AFM analysis

Mannan, dextran, CM-mannan (DS 0.92) and CM-dextrans (DS 0.37 and 1.14) films were prepared by casting the polymer solution (50 μg/mL, in DW; 5 μL) on a freshly cleaved mica (Grade V-1, 12 mm diameter, SPI supplies, West Chester, USA). After evaporation of water (room temperature, drying overnight in dessicator) the modified mica disc was fixed on a microscope glass and placed into sample holder. Atomic force microscopy (AFM) measurements were performed using BioScope Catalyst (Bruker, Santa Barbara, USA). Data were acquired using PeakForce QNM mapping technique in air and evaluated by NanoScope Analysis 1.40 (Bruker).

3. Results and discussion

Two structurally different branched polysaccharides, mannan and dextran and their CM-derivatives were examined. *C. albicans* mannan is a highly branched polysaccharide which comprises α-(1,6)-linked mannopyranosyl units in backbone and side chains with α-(1,2)-, α-(1,3)- and β-(1,2)-linked mannopyranosyl units (Shibata, Suzuki, Kobayashi, & Okawa, 2007). Dextran from *Leuconostoc* spp. comprises a straight chain obtaining α-1,6 glycosidic linkages between glucose molecules, while branches begin from α-1,3 linkages. The CM-derivatives of mannan and dextran with various DS were prepared and characterized previously (Machová et al., 2014a).

3.1. Effect of CM-mannans and CM-dextrans on liposomes exposed to OH radicals

Mannan, dextran and their CM-derivatives were tested as possible antioxidants which could protect liposomes against OH radicals in the concentration range of 1–20 mg/mL. Underivatized mannan exhibited protection of liposomal lipid bilayer in dose-dependent manner up to 20 mg/mL (Fig. 1A). At concentration of 20 mg/mL, 42.1% of lipids were protected against peroxidation by OH radicals. Compared with underivatized mannan, carboxymethylation increased antioxidant activities (AOAs) of both CM-mannans (DS 0.3 and 0.92) in dose-dependent manner up to concentration of 10 mg/mL (Fig. 1A). At this concentration, the values of AOA of CM-mannan (DS 0.3) was 51.1% and of CM-mannan (DS 0.92) 44.2%. The two-fold increasing of concentration of both CM-mannans up to 20 mg/mL moderately deteriorated protection of liposomes,