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Short communication

Yeast mannans protect liposomes against peroxidation but do not scavenge free radicals

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ABSTRACT

The mannans from *Candida albicans* serotype A, *Candida dubliniensis*, *Candida tropicalis* and *Saccharomyces cerevisiae* protected liposomes against peroxidation by OH• radicals in a concentration-dependent manner. The most efficacious antioxidant was mannan from *C. albicans* serotype A with antioxidant activity ~49% comparable with known antioxidant carboxymethylated glucan (~46%). The natural antioxidant α-tocopherol exhibited 90% protection of liposomes. The mannans and glucans scavenged negligible amount of free radicals in the common 2,2-diphenyl-1-picrylhydrazyl test. It seems likely that protection of liposomes against OH• radicals by polysaccharides is not due to their scavenging properties, but may be caused by a sterical hindrance. The polysaccharides containing flexible β-linkages are more effective protectors of liposome peroxidation than these polysaccharides that contain α-linkages only.

1. Introduction

Nowadays, the considerable interest is given to polysaccharides as natural antioxidants isolated from various sources, e.g. plants (Capek, Machová, & Turjan, 2009; Kardošová & Machová, 2006; Xu et al., 2009; Yuan et al., 2010; Zha et al., 2009), fungi (Babincová et al., 1999; Ding et al., 2010; Ferracini-Santos & Sato, 2010; Križková et al., 2006), algae (Chen, You, Huang, Yu, & Chen, 2010; Ye, Wang, Zhou, Liu, & Zeng, 2008) and bacteria (Asker, Ahmed, & Ramadan, 2009; Asker & Shawky, 2010; Kodali & Sen, 2008). Despite of that, mechanism of antioxidant polysaccharides' properties has not been elucidated. Mostly studied (1,3)-β-D-glucan prepared from the cell walls of *Saccharomyces cerevisiae* enhances the immune system systemically. This enhancement results in antitumor, antibacterial, antiviral, anticoagulatory and wound healing activities (Bohn & BeMiller, 1995). Patchen, D'Alesandro, Brook, Blakely, and MacVittie (1987) described the radioprotective effect of *S. cerevisiae* glucan by enhancing macrophage function in mice. Its water-soluble derivative, carboxymethyl (1,3)-β-D-glucan (CM-glucan), protected phosphatidylcholine liposomes against peroxidation induced by OH• radicals (Babincová, Bačová, Machová, & Kogan, 2002; Babincová et al., 1999). Also, the radical-scavenging properties of CM-glucan studied by electron paramagnetic resonance spectroscopy were described (Kogan et al., 2005). However, failure of CM-glucan to inhibit oxidative DNA damage induced by OH• radicals was found (Gábelová & Plešková, 2000).

Only little information concerning the antioxidant activities of the second major surface yeast polysaccharide, mannan, was published so far. Križková et al. (2006) described antioxidant activity of neoglycoconjugate prepared from mannan of *S. cerevisiae* and human serum albumin, Ferracini-Santos and Sato (2010) studied mannan-protein complex isolated from the cell walls of *S. cerevisiae*. Both papers deal with mannan as a complex of polysaccharide and protein.

Here we focus on the study of antioxidant properties of the surface mannans obtained from *Candida albicans* serotype A, *Candida dubliniensis*, *Candida tropicalis* and *S. cerevisiae* using liposomes as a model for lipid oxidation. This assay includes the peroxidation of phosphatidylcholine liposomes caused by OH• radicals produced via Fenton's reaction (Babincová et al., 1999) in the presence of mannans. For comparison, glucan polysaccharides, CM-glucan from *S. cerevisiae* and pullulan (α-1,4-; α-1,6-glucan) were monitored. According to current concepts, the antioxidant properties of polysaccharides are the consequence of quenching of OH• radicals. To verify this theory, the radical scavenging activities of selected polysaccharides (*C. albicans* serotype A and *S. cerevisiae* mannans as well as CM-glucan and pullulan) were investigated by commonly used 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay and compared with standard antioxidants (ascorbic acid, α-tocopherol and glutathione).

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2. Materials and methods

Mannans of *C. albicans* serotype A (CCY 39-3-32), *C. dubliniensis* (CCY 29-177-1), *C. tropicalis* (29-7-6), *S. cerevisiae* (21-4-13) and CM-glucan from *S. cerevisiae* were prepared previously in our laboratory. Pullulan was purchased from Serva; $FeCl_2{\cdot}4H_2O$, H_2O_2, chloroform and ethanol for spectroscopy from Merck; α-tocopherol, ascorbic acid, glutathione, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), Coomassie Brilliant Blue G-250, human serum albumin (HSA) and L-α-phosphatidylcholine from Sigma–Aldrich; Bio-Gel P2 from Bio-Rad.

2.1. Preparation of polysaccharides

All yeast strains used for preparation of mannans were from Culture Collection of Yeasts (Institute of Chemistry, Slovak Academy of Sciences). Firstly, mannoproteins were extracted from freeze-dried yeast cells by autoclaving for 1 h at 120 °C three times with 0.2 M NaCl. The supernatants were combined and mannoproteins were precipitated with ethanol, dissolved in distilled water and dialyzed. The freeze-dried mannoproteins were suspended in 2% KOH and heated for 1 h at 100 °C. Insoluble residues were separated by centrifugation, and mannans were precipitated from supernatants using Fehling reagent (Jones & Stoodley, 1965). CM-glucan was prepared from *S. cerevisiae* according to Machová, Kogan, Alfoldi, and Šoltés (1995).

2.2. Monosaccharide composition

Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C, released monosaccharides were reduced to alditols by sodium borohydride. Quantitative determination of neutral sugars was carried out in the form of their alditol-trifluoroacetates by gas chromatography on Hewlett-Packard Model 5890 Series II chromatograph equipped with PAS-1701 column (0.32 mm × 25 m), a temperature program of 110–125 °C (2 °C/min) to 165 °C (20 °C/min) and flow rate of hydrogen 20 mL/min.

2.3. Protein determination

The content of protein was determined by Bradford method using Coomassie Brilliant Blue G-250 with human serum albumin as a reference compound (Sedmak & Grossberg, 1977).

2.4. Molecular weight determination

Molecular weights of polysaccharides were determined by a high performance liquid chromatography (HPLC) system at ambient temperature with two columns (250 mm × 8 mm) packed with Biospher GM 300 and GM 1000 (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. A set of pullulans (Gearing Scientific, Polymer Lab. Ltd., UK) was used for the calibration of the HPLC system.

2.5. Liposome preparation and determination of oxidation index

Multilamellar liposomes were prepared by hydration of phospholipid film (Babincová et al., 1999). 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 (negative control) or with aqueous solutions of the tested oligo- and polysaccharides. As a positive control, α-tocopherol was used. It was dissolved in chloroform–methanol mixture along with L-α-phosphatidylcholine and was embedded into the liposome bilayer. Lipid peroxidation of liposomes 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 isolated radical induced conjugated dienes in liposomes were recorded in the wavelength range of 200–270 nm using UV-VIS spectrophotometer 1240 (Shimadzu, Japan). 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 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 and the tested compound, respectively. α-Tocopherol (positive control) was used as reference inhibitor of peroxidation. The value of IC_{25} represents the concentration of polysaccharides or α-tocopherol that provides 25% protection of liposomes against peroxidation with OH• radicals.

2.6. DPPH• radical scavenging assay

For study of radical scavenging activity of the selected saccharides, the DPPH• assay was chosen.

DPPH• radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized. Free radical scavenging properties of the selected polysaccharides were determined spectrophotometrically at 517 nm using UV-VIS spectrophotometer 1240 (Shimadzu, Japan) (Molyneux, 2004). To 1 mL of polysaccharide samples (0.68 mg/mL; 1.37 mg/mL; 2.74 mg/mL) 1 mL of freshly prepared methanolic solution of DPPH• (0.069 mM) was added and incubated at room temperature for 1 h. Analyses were performed at triplicates. Scavenging ability (%) = $[(A_0 - A_{sample})/A_0] \times 100$, where A_0 was the absorbance of control (without sample). Ascorbic acid (0.003; 0.007; 0.013 mg/mL), α-tocopherol (0.009; 0.028; 0.046 mg/mL) and glutathione (0.006; 0.029; 0.057 mg/mL) were used as reference scavengers of DPPH• radicals.

2.7. Statistical analysis

The data concerning peroxidation of liposomes and scavenging of DPPH• radicals are presented as mean ± standard deviation of three determinations.

3. Results

3.1. The antioxidant activities of yeast mannans

The surface mannans prepared from the yeast cell walls of *C. albicans* serotype A, *C. dubliniensis*, *C. tropicalis* and *S. cerevisiae* showed considerable antioxidant activities. They protected the liposomes against peroxidation by OH• radicals in a concentration-dependent manner (Fig. 1A). The antioxidant activities (% AOA) varied from ~20% (*S. cerevisiae* mannan) to ~49% (*C. albicans* serotype A mannan). The negligible changes of AOA were investigated for all mannans at concentrations of above ~25 mg/mL. In consideration of IC25 values, mannan from *C. albicans* serotype A was the most efficacious antioxidant of liposome peroxidation (IC25 = 6.3 mg/mL) while *S. cerevisiae* mannan exhibited weaker antioxidant ability (IC25 = 43.1 mg/mL) (Table 1). Moreover, we monitored two glucan polysaccharides as potential antioxidants using the same liposomal model. CM-glucan protected liposomes against OH• radicals (AOA ~ 46%; IC25 = 6.5 mg/mL) very similar to *C. albicans* serotype A mannan. Pullulan showed weak antioxidant ability (AOA ~ 20%; IC25 > 45 mg/mL) (Fig. 1B, Table 1). The IC25 of α-tocopherol, used as positive control, was 0.06 mg/mL.

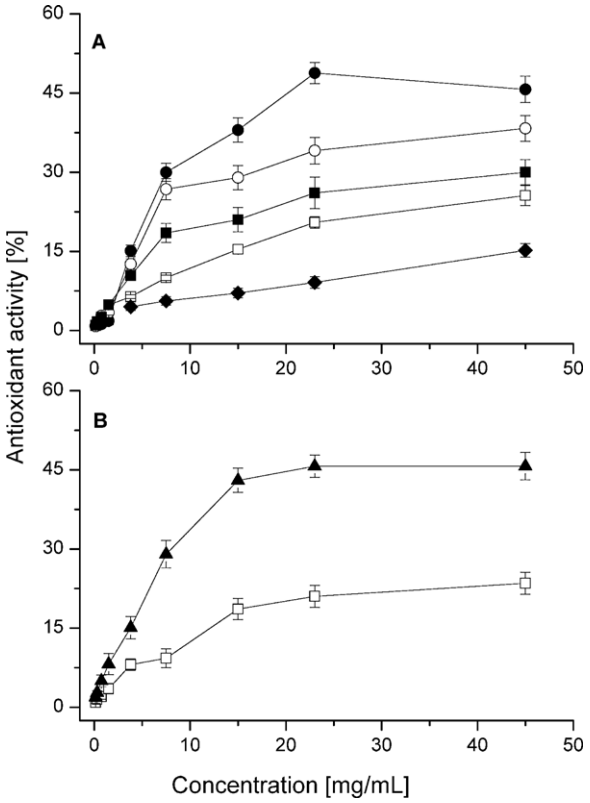

Fig. 1. Antioxidant activities of polysaccharides – concentration dependence: (A) yeast mannans (● – *C. albicans* serotype A; ○ – *C. tropicalis*; ■ – *C. dubliniensis*; □ – *S. cerevisiae*), mannitol (♦) and (B) glucan polysaccharides (▲ – CM-glucan; □ – pullulan).

3.2. Scavenging of DPPH• radicals

The radical scavenging properties of *C. albicans* serotype A and *S. cerevisiae* mannans as well as CM-glucan and pullulan against DPPH• radicals were investigated. Despite of wide concentration range of polysaccharides, their scavenging properties did not exceed 4.2% (2.7 mg/mL of *C. albicans* serotype A mannan) resp. 3.4% (2.7 mg/mL of CM-glucan). Natural antioxidants used as positive controls quenched at optimal concentrations 93% of DPPH• radicals (ascorbic acid), 92.6% (α-tocopherol) and 88.5% (glutathione) (Fig. 2).

4. Discussion

We studied the antioxidant activities of yeast mannans and glucan polysaccharides using phosphatidylcholine multilamellar liposomes as model scavengers of OH• radicals induced by Fenton's reagent (Babincová et al., 1999). This method uses UV absorption spectra of phosphatidylcholine for investigation of antioxidant properties of polysaccharides. Liposomes are artificially prepared vesicles made of lipid bilayer. Nonconjugated double bonds in natural phospholipids have an absorption peak at 200–210 nm. OH• radicals induce oxidation of phospholipids what is demonstrated by the change of isolated double bonds to conjugated dienes in oxidated lipids. The second peak at 233 nm appears and Klein peroxidation index (A_{233}/A_{215}) can be calculated.

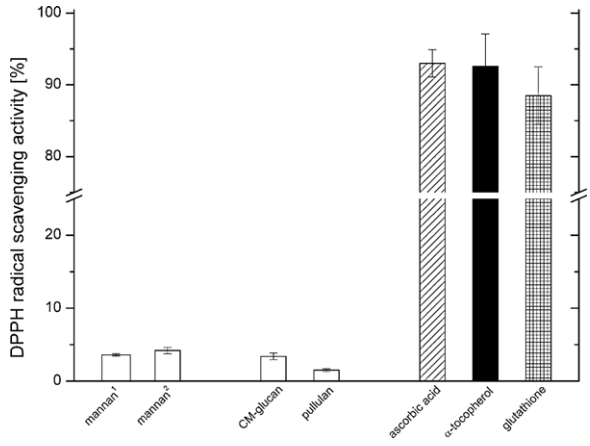

Fig. 2. DPPH radical scavenging activities of *S. cerevisiae* mannan (mannan[1]), *C. albicans* serotype A mannan (mannan[2]), CM-glucan, pullulan, and natural antioxidants – ascorbic acid, α-tocopherol and glutathione using DPPH• assay.

It is known that yeast mannans are branched homopolymers differing in structure and size. They are composed from mannopyranose units containing the residual protein and small trace of N-acetylglucosamine from chitobiose bridges. Mannans from *Candida sp*. used in our experiments have similar structure comprising α-1,6-linked backbone highly branched with side chains containing α-1,2-; α-1,3-; and β-1,2-linked mannopyranose units (Kobayashi et al., 1994; Ližičárová, Matulová, Capek, & Machová, 2007; Shibata, Suzuki, Kobayashi, & Okawa, 2007). Structurally different mannan from *S. cerevisiae* includes only α-1,2- and α-1,3-linked mannopyranose units in side chains, no β-linkage is present here (Kobayashi et al., 1992). Prepared mannans considerably differed in molecular size (*S. cerevisiae* mannan – $M_p \sim 34$ kDa; *C. tropicalis* mannan – $M_p \sim 73$ kDa; *C. dubliniensis* mannan – $M_p \sim 75$ kDa and *C. albicans* serotype A mannan – $M_p \sim 193$ kDa) and the protein content (0.6–4.9%). All mannans contained traces of D-glucose (1–5.9%) and D-galactose (0.7–8.7%) (Table 1).

The results revealed the considerable effect of all studied mannans in the protection of liposomes against OH• radicals (Fig. 1A). Mannans protected the peroxidation of liposomes in a concentration-dependent manner. It seems that the percent of AOA grew with increasing concentrations as well as the molecular weights of mannans (Fig. 1A, Table 1). The largest mannan prepared

Table 1
The characterization of mannan and glucan polysaccharides and their IC25 values.

Sample	IC_{25} [mg/mL]	M_{peak} ($\times 10^4$)	Protein [%]	Monosaccharide composition [%]			
				Man	Glc	Gal	GlcNAc
Mannan *C. albicans*, ser. A	6.3	19.3	4.9	85.4	5.9	8.7	tr.
Mannan *C. tropicalis*	7.1	7.3	0.6	98	1.1	0.9	tr.
Mannan *C. dubliniensis*	21.3	7.5	0.9	98.3	1	0.7	tr.
Mannan *S. cerevisiae*	43.1	3.4	1.1	96.5	2.6	0.9	tr.
Pullulan	n.d.	4.9	0	0	100	0	0
CM-glucan *S. cerevisiae*	6.5	32.5	3.9	2.5	97.5	0	0

IC_{25} – the concentration of saccharides that provides 25% protection of liposomes against peroxidation with OH• radicals.
n.d. – antioxidant activity did not reached the IC_{25} value up to maximum concentration used.
tr. - traces of GlcNAc from N-acetylchitobiose bridge.

from *C. albicans* serotype A ($M_p \sim 193$ kDa) was the most efficacious antioxidant of liposome peroxidation (Fig. 1A, upper line). The smallest mannan from *S. cerevisiae* ($M_p \sim 34$ kDa) prevented the peroxidation of liposomes less than others mannans (Fig. 1A, lower line). The IC25 values of mannans proportionally increased with their size decreasing (Table 1). The antioxidant activity of CM-glucan ($M_p \sim 325$ kDa; IC25 = 2.1 mg/mL) was similar to that of *C. albicans* serotype A mannan, while pullulan did not reached the IC25 at concentrations used (Fig. 1B, Table 1).

The mechanism of antioxidant activities of polysaccharides has not yet been elucidated. Are the antioxidant activities of polysaccharides caused by scavenging of free radicals from the environment of the liposomes? In our previous paper, a considerable antioxidant activity of the carboxymethyl (1,3)-β-D-glucan from *S. cerevisiae* against OH• radicals was demonstrated (Babincová et al., 1999). Moreover the radical-scavenging activity of the same glucan was observed by means of electron paramagnetic resonance spin trapping technique (Kogan et al., 2005). The question is – why the CM-glucan was completely unable to inhibit oxidative damage of DNA induced by OH• radicals? (Gábelová & Plešková, 2000). To shed light on antioxidant mechanism of polysaccharides the DPPH• free radical scavenging assay was selected. This assay is routinely used for monitoring of the scavenging properties of natural compounds. In comparison with natural antioxidants – ascorbic acid, α-tocopherol and glutathione, polysaccharides proved to have very low scavenging properties of DPPH• radicals. Radical scavenging efficiency of antioxidants against various oxidants such as hydroxyl radicals, DPPH and others used in variety of tests greatly differs. Hydroxyl radical, as it is one of the main biological reactive oxidant has quite strong capacity to rip out hydrogen from carbohydrates. Moreover hydroxyl radicals induce another radical reactions. As we observed, mannitol has low antioxidant reactivity (Schronerová, Babincová, Machová, & Kogan, 2007), its oxidation evokes structural changes.

Different situation is with DPPH. Stable DPPH free-radical is less aggressive. It requires antioxidant scavenger with appropriate electronic structure such as system of double bonds for neutralization. As we have shown, the scavenging ability of polysaccharides against to DPPH radicals is negligibly low (~4%).

5. Conclusion

According to our results we can infer that protection of liposomes against free radicals caused by polysaccharides is not due to their scavenging properties. Polysaccharides are able to coat the surface of liposomes by strong nonspecific binding and such protective polysaccharide shield on liposome surface can build up (Babincová, Machová, Poláková, & Kogan, 2000; Sunamoto, Iwamoto, Kondo, & Shinkai, 1980). We supposed that the main reason of their antioxidant effect could be the protective shield that protects them from attack of OH• radicals produced in their environment. We found that the polysaccharides containing flexible β-linkages are more effective protector of liposomes against peroxidation than the polysaccharides containing α-linkages only. *Candida* mannans and commercial CM-glucans are equally effective against reactive oxygen radicals. The advantage of mannan utilization as antioxidants is that they are water soluble, their preparation is far less laborious and they do not need any carboxymethylation.

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