Carboxymethylated Glucan Inhibits Lipid Peroxidation in Liposomes

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Protective capabilities were studied of carboxymethylated (1→3)-β-D-glucan from *Saccharomyces cerevisiae* cell wall against lipid peroxidation in phosphatidylcholine liposomes induced by $OH^{\cdot}$ radicals produced with Fenton's reagent (H_2O_2/Fe^{2+}) and also by microwave radiation using absorption UV-VIS spectrophotometry. A significant decrease in the conjugated diene production, quantified as Klein oxidation index, was observed in the presence of a moderate amount of added glucan. Increase of the oxidation index was accompanied with enhanced carboxyfluorescein leakage as a result of liposome membrane destabilization. This process was markedly suppressed with glucan present in the liposome suspension. Therefore, glucan may be considered as a potent protector against microwave radiation-induced cell damage.

Introduction

Glucan, a (1→3)-β-linked polyglucopyranose biological response modifier isolated from baker's yeast *Saccharomyces cerevisiae,* is effective in the therapy of experimental neoplasia, immunosuppressive states and infectious diseases of bacterial, fungal, and viral origin (Bohn and BeMiller, 1995; Williams *et al.*, 1996, Patchen *et al.*, 1989; Baker *et al.*, 1992; Chorvatovičová *et al.*, 1996; Mork *et al.*, 1998; Kogan *et al.*, 1997; Chorvatovičová *et al.*, 1998). Currently, soluble derivatives of glucan are undergoing phase I clinical trials.

β-Glucan revealed also free radical scavenger properties. It is able to protect blood macrophages from the free radical action during and after β-irradiation allowing these cells to continue their important functions in the irradiated body and to release factors required for the restoration of normal bone marrow production (Patchen *et al.*, 1987; Santiago and Mori, 1993; Bobek *et al.*, 1997). Recently, there is an increased interest to investigate biological effects of non-ionizing electromagnetic fields on the living body, and specifically on its cellular systems (Lin, 1994; Kaiser, 1996). The main concern has been given to a possible effect on health which may be associated with exposure to such fields. Evidence for non-thermal effects occurring at the cellular level has been found in many experiments. In the studies of the interaction of microwave radiation with liposomes, an increased permeability of their lipid bilayers has been observed (Saalman *et al.*, 1991). That was ascribed to the degradation of lipid fatty acid hydrocarbon chains induced by peroxidation (Babincová, 1993; Babincová, 1994), which is analogous to the effects observed in the interaction of liposomes with ionizing radiation (Chatterjee and Agrawal, 1988). Successful protection of the cellular systems against even lethal doses of radiation (Patchen *et al.*, 1987) exerted by (1→3)-β-D-glucan stimulated the present study of microwave-induced lipid peroxidation in the presence of this compound.

Materials and Methods

Glucan preparation and characterization

Water-insoluble (1→3)-β-D-glucan was obtained by extraction of commercial baker's yeast (Slovlik, Trenčín, Slovak Republic) with 6% NaOH at 60 °C (Kogan *et al.*, 1988). Carboxymethylation of the insoluble glucan was performed using a procedure described by Machová *et al.*, 1995. Determination of the degree of substitution (DS) of car-

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boxymethylated (1→3)-β-D-glucan was performed by potentiometric titration.

Preparation of ultrasonically treated glucan derivatives

To produce a well soluble preparation, carboxymethylated (1→3)-β-D-glucan (M_w = 3.46 × 10^5, DS = 0.71) was suspended in distilled water and sonicated (20 kHz, 100 W) 10 min in an ice-bath using a horn-type ultrasound generator (Ultragen, Nitra, Slovak Republic). Gel filtration of the ultrasonicated glucan was performed on a glass column (150 × 1.5 cm) packed with Sepharose CL-6B (Pharmacia Uppsala, Sweden). The separation process was monitored by a differential refractometric detector (RIDK 102; Laboratorní přístroje, Prague).The pooled fractions were dialyzed against distilled water and freeze-dried.

High-performance liquid chromatography

HPLC experiments were performed at ambient temperature with a system that included a high-pressure pump (LCP 3001; Laboratorní přístroje, Prague, Czech Republic), an eight-port switching valve equipped with two 100-μL loops (Model PK 1; Vývojové dílny, Czechoslovak Academy of Sciences, Prague), and two in series connected stainless-steel HPLC columns (250 × 8 mm) packed with Biospher GM 300 and Biospher GM 1000 sorbents (mean particle size = 10 μm; Labio, a.s. Prague). The separation process was monitored with a differential refractometric detector. The mobile phase used was 0.1 M aqueous $NaNO_3$ solution. The flow rate was 0.4 mL/min. A set of pullulans P-5, P-100, P-200, P-400, and P-800 (Shodex Standard P-82; Macherey-Nagel, Düren, Germany) was used for the calibration of the HPLC system. The M_w and M_n molecular-weight averages of the carboxymethylated (1→3)-β-D-glucan (before and after ultrasonication) were calculated using the computer program described by Šoltés *et al.* (1993). Taking into account that the HPLC system was calibrated using pullulan standards as the reference materials, the molecular-weight characteristics, weight-average and number-average molecular weight values (M_w and M_n, respectively) of glucan samples should be regarded as relative values.

Liposome preparation

Lipid soy-bean phosphatidylcholine (Sigma, St. Louis, USA) was dissolved in organic solvents (a mixture of chloroform and methanol 2:1 v/v). The lipid solution was evaporated in vacuum by a rotary evaporator. After evaporation of the solvent, Tris (tris(hydroxymethyl)aminomethane) buffer (pH 7.4) and carboxymethylated (1→3)-β-D-glucan with ultrasonically decreased molecular weight (M_w = 1.36 × 10^5, DS = 0.71) and with desired concentration were added into the glass vessel with lipid film, and solution was shaken up mechanically. The suspension was then sonicated with Brown Labsonic 2000 sonicator at 80 W for 15 min under nitrogen in an ice-bath to obtain clear suspension of liposomes. For the microwave experiments, the lipid film was hydrated with the Tris buffer (pH 7.4), containing 100 mM 6-carboxyfluorescein (Eastman Kodak Company, Rochester, USA) and sonicated liposomes were separated from free 6-carboxyfluorescein by centrifugation and resuspended in Tris buffer containing a desired concentration of glucan. The final lipid concentration was 5 mg/mL in all experiments.

Induction of lipid peroxidation

For the study of lipid peroxidation of liposomes we have used OH· radicals produced via Fenton's reaction initiated by addition of H_2O_2 and $FeCl_2$ with a final concentration of 100 mM and 2 mM, respectively, to the liposome suspension containing varying concentration of glucan.

Determination of oxidation index

Absorption spectra of conjugated dienes were recorded in the wavelength range 215–320 nm using a SPECORD M-20 (Zeiss, Jena, Germany) UV-VIS spectrophotometer. The increase of the absorption at 233 nm was considered as an evidence of the formation of conjugated dienes, and the oxidation index was calculted from the ratio of the absorbances (A_{233}/A_{215}) (Chatterjee and Agrawal, 1988; Klein, 1970).

Microwave irradiation

Microwave radiation was generated by a continuous wave klystron (20 SR 51, Tesla, Czech Republic) operating at 2.45 GHz. The klystron was

coupled to a waveguide which was terminated with a horn antenna with an aperture with the dimensions of 157 mm × 135 mm in the E and H planes, respectively. All irradiations were performed at a distance of 30 cm. The sample container was made from teflon and was placed inside a polymethylmetacrylate cell through which water circulated from a thermostated bath situated outside the radiation field. Stirring of the sample was achieved by a slow flow of air bubbles via a small plastic tube protruding through the lid of the sample container. The temperature was monitored continuously by a nonabsorbing Vitek thermistor probe placed into the sample. Irradiation was performed at 20 °C, temperature fluctuation being less than 2 °C . Absorbed power of the microwave radiation was determined using the following procedure. The sample was exposed to radiation for about 5–15 min without sample thermostating and the temperature of the sample was recorded as a function of exposure time. Specific absorbed power (SAP) was calculated using the expression $SAP=C_p\ dT/dt$, where C_p is heat capacity (we have used C_p = 4200 J/kg) and dT/dt is initial slope of the temperature *vs* time dependence. The specific absorbed power in our experiments was SAP=500±25 mW/g.

Measurement of carboxyfluorescein release

Liposome leakage was assessed by determination of the fluorescence increase due to the release and subsequent dissolution of the liposome-entrapped, self-quenching probe 6-carboxyfluorescein. At high concentrations (in our experiments, 100 mM), 6-carboxyfluorescein reveals only a very weak fluorescence due to self-quenching, probably as the result of the intermolecular interactions (dimerization). For this reason, 6-carboxyfluorescein which was entrapped inside liposomes at high concentration produced no increase in fluorescence, even when the liposome suspension was diluted, since the concentration of the entrapped liposomal contents remained unchanged. On the other hand, 6-carboxyfluorescein which has leaked out of the liposomes into the extraliposomal medium led to the increase of fluorescence upon dilution of the suspension. Fluorescence intensity (I) was measured at 520 nm (excitation at 490 nm) using a Perkin-Elmer MPF-3L fluorimeter. Percent increase of dye leakage was determined as $[(I-I_{min})/(I_{max}-I_{min})] \times 100$ (%), where I_{min} is the minimum fluorescence intensity given by a nonexposed sample and I_{max} is maximum fluorescence intensity revealed by a sample after total disintegration of the liposome structure by Triton X-100 (10% v/v).

Results and Discussion

Natural phospholipids contain only non-conjugated double bonds, and therefore have a UV absorbance peak at a very short wavelength (200–205 nm). Removal of a hydrogen atom from a methylene group located between two double bonds spreads the unsaturation over five carbon atoms and results in the formation of a conjugated diene which is energetically more favourable than the two double bonds in isolation. As a result, second absorbance maximum at 233 nm appears. This phenomenon allows to study radical degradation of the natural phospholipids, that results in extraction of hydrogen atoms from the fatty acid chains, using UV-VIS spectrophotometry.

First, we have studied lipid peroxidation induced by OH· radicals produced via Fenton's reac-

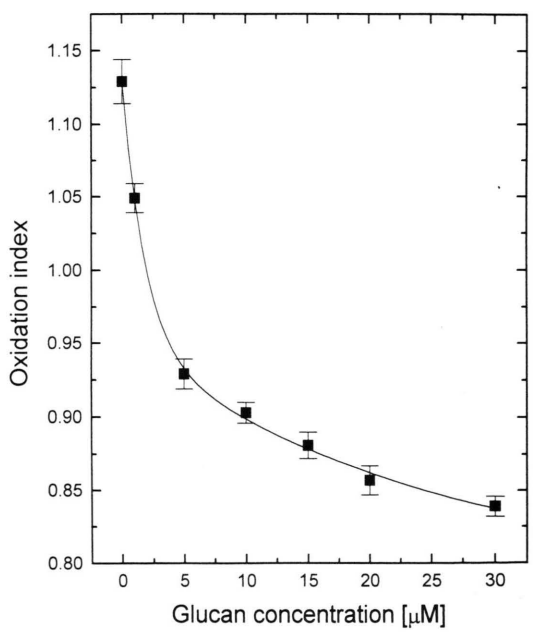

Fig. 1. Dependence of Klein oxidation index on the carboxymethylated (1→3)-β-D-glucan concentration. Lipid peroxidation was initiated with Fenton's reagent producing OH· radicals.

tion, which is a standard method in the investigation of the capabilities of OH· radical scavengers. The concentration dependence of the Klein peroxidation index is shown in Fig. 1. As can be seen, glucan scavenging effect is most pronounced in the concentration range 5–20 μM. Therefore, in the study of the influence of glucan on microwave-induced lipid peroxidation we have used 7.5 μM carboxymethylated (1→3)-β-D-glucan with ultrasonically decreased molecular weight ($M_w = 1.36 \times 10^5$, DS = 0.71).

Exposure of liposomal suspension to microwave radiation caused free-radical mediated lipid peroxidation as estimated by measurement of the Klein oxidation index (Fig. 2). Linear dependence on irradiation time was observed in all three cases. To comparatively evaluate the free-radical scavenging capabilities of (1→3)-β-D-glucan we have also studied the influence of 10 mM ascorbic acid, a well-known free-radical scavenger. As can be seen from this Figure, antioxidant properties of (1→3)-β-D-glucan are comparable to those of ascorbic acid.

Peroxidation of unsaturated fatty acids in the lipid bilayer leads to elimination of double bonds or disruption of the fatty acid chains, thus producing physical changes in a membrane leading to enhanced bilayer permeability (Leibowitz and Johnson, 1991). As shown in Fig. 3, the increase of oxidation was accompanied with enhanced carboxyfluorescein leakage, and this process was substantially suppressed by the presence of both antioxidants.

The results of our study revealed the important role of soluble (1→3)-β-D-glucan in the protection of experimental biological membranes against adverse effects of microwave radiation. Such protective effect was most probably mediated through the free-radical scavenging. Since it is now known that free radicals play a detrimental role in the processes of ageing and impairment of the human cells, as well as in some neurodegenerative and neoplastic diseases (Halliwell and Gutteridge, 1989), implementation of preparations based on (1→3)-β-D-glucans may be advantageous in the therapy of diseases involving free radicals.

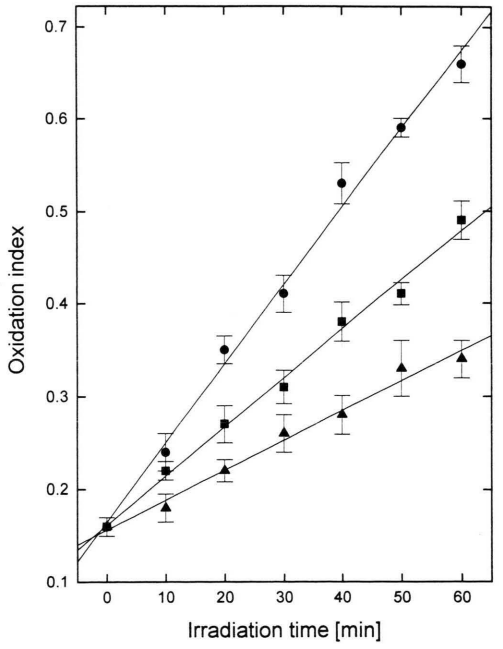

Fig. 2. Dependence of Klein oxidation index on the microwave irradiation time (● – pure liposomes; ■ – liposomes in the presence of 7.5 μM carboxymethylated (1→3)-β-D-glucan; ▲ – liposomes in the presence of 10 mM ascorbic acid).

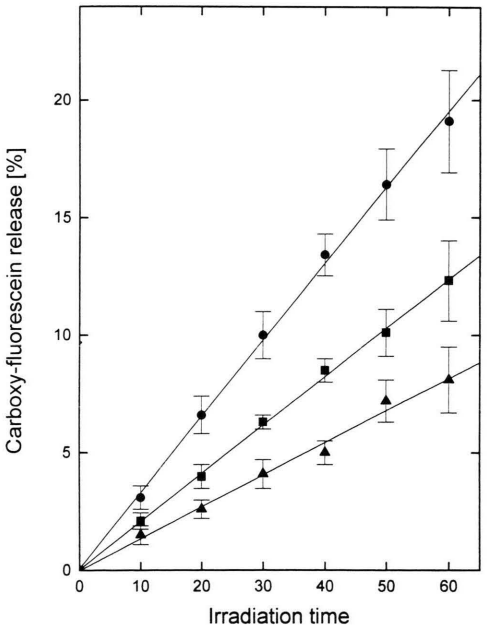

Fig. 3. Dependence of carboxyfluorescein release on the microwave irradiation time. (● – pure liposomes; ■ – liposomes in the presence of 7.5 μM carboxymethylated (1→3)-β-D-glucan; ▲ – liposomes in the presence of 10 mM ascorbic acid).

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