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Antioxidant properties of yeast (1→3)-β-D-glucan studied by electron paramagnetic resonance spectroscopy and its activity in the adjuvant arthritis☆

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Abstract

Radical-scavenging activity of the water-soluble derivative obtained from cell wall of the baker's yeast *Saccharomyces cerevisiae* was investigated using the technique of electron paramagnetic resonance. The experiments involved a study of the scavenging activity of carboxymethyl (1→3)-β-D-glucan (CMG) towards the radicals formed in the thermally initiated decomposition of potassium persulfate, hydrogen peroxide, or 2,2′-azo-bis(2-amidinopropane)-dihydrochloride in aqueous solutions using spin trapping as an indicative technique. In the absence of glucan, high intensity spectra of generated free radicals in the form of their adducts with 5,5-dimethylpyrroline-*N*-oxide were observed.

Addition of CMG resulted in concentration-dependent substantial decrease of spectral intensities of adducts as a result of competition of CMG in the scavenging of reactive radicals formed.

In the in vivo experiments involving administration of CMG to rats with experimentally induced adjuvant arthritis (AA) a substantial decline of the level of plasmatic carbonyls, a parameter indicating oxidative tissue damage during the progress of arthritic diseases, was observed. We assume that radical-scavenging properties of CMG can be responsible for its antioxidant activity in the AA model, suggesting possible application of the yeast glucan derivatives in the treatment of arthritis.

Keywords: β-D-glucan; Electron paramagnetic resonance spectroscopy; Spin trapping; Radical scavenging; Antioxidant; Adjuvant arthritis

1. Introduction

In the last decade, much support has been obtained for the assumption that many pathogenic processes including rheumatoid arthritis and osteoarthritis, neoplastic and mitochondrial diseases, as well as ageing and death of cells and complete tissues may be initiated by damaging action of free radical species, especially reactive oxygen species (ROS) (Basu, Temple, & Garg, 1999; Halliwell & Gutteridge, 1989). For this reason, growing attention of researchers and physicians has been paid to the group of natural substances, known as antioxidants, which are able to protect living organisms from the attack of reactive radical species, and in this way to decrease the risk of several diseases (Babincová & Sourivong, 2001; Wang et al., 1996).

Although most often applied and investigated natural antioxidants are ascorbic acid (vitamin C) and α-tocopherol (vitamin E), recently increased attention has been paid to the antioxidants of polysaccharide origin (Bobek, Ozdin, & Kuniak, 1997; Xie, Xu, & Liu, 2001). Among the representatives of this class of antioxidants are (1→3)-β-D-linked glucose polymers that occur as a primary

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component in the cell walls of fungi and several bacteria or they are secreted extracellularly by various fungi (Bohn & BeMiller, 1995; Kogan, 2000). Due to their ability to modulate the immune system of the host, glucans belong to the group of substances known as biological response modifiers. (1→3)-β-D-glucans have been reported to have stimulating effects on the defense mechanisms of the living organisms by enhancing the host resistance to viral, bacterial, fungal, and parasitic infections, as well as to neoplastic and other pathogenic conditions (Tzianabos, Kasper, Cisneros, Smith, & Onderdonk, 1995; Williams, 1997; Williams, Mueller, & Browder, 1996). Some indications have been obtained that protective and immunomodulating properties of (1→3)-β-D-glucan may in part be ascribed to its ability to exert free radical scavenging properties. It has been demonstrated that (1→3)-β-D-glucans prepared from yeast or mushrooms are able to protect blood macrophages from X- or γ-ray irradiation, to restore bone marrow production, to increase hemopoiesis, and to protect animals from the lethal effect of the γ-ray irradiation (Hofer, Pospíšil, Boháček, Pipalová, & Šandula, 1995; Patchen, D'Alessandro, Brook, Blakely, & MacVittie, 1987; Patchen & MacVittie, 1983).

Using the prepared water-soluble carboxymethylated (1→3)-β-D-glucan (CMG), Chorvatovičová (1991) described its inhibitory effect on the micronucleus frequencies induced in mice by γ-ray irradiation and later demonstrated the ability of this compound to suppress the mutagenic and toxic activity of cyclophosphamide in mice (Chorvatovičová, Machová, & Šandula, 1996). It was suggested that the observed protective activity of CMG as well as the described protective effect of sulfoethylated (1→3)-β-D-glucan (SEG) against mutagenic action of hexavalent chromium (Chorvatovičová, Kovačíková, Šandula, & Navarová, 1993) could be attributed to the free radical scavenging activity of the used glucan derivatives. Recently, using the same compounds, Križková et al. (2003) described pronounced antioxidant effect of CMG and SEG in the Trolox equivalent antioxidant capacity (TEAC) assay and demonstrated that, due to the observed antioxidant activity, these two glucan derivatives were able to inhibit significantly *Euglena gracilis* chloroplast DNA damaging action of ofloxacin, which is known to be associated with the generation of reactive radical species.

Previously, we have studied the ability of CMG prepared from the cell walls of baker's yeast *Saccharomyces cerevisiae* to inhibit lipid peroxidation in liposomes induced by hydroxyl radicals produced using Fenton's reagent (H_2O_2/Fe^{2+}) or using microwave radiation. The liposome model is an ideal approximation to the lipid bilayers in the cell membranes and therefore the protective efficiency of glucan in such a model can imply its possible application as antioxidant in protection of living cells. Our results showed that CMG exerted a potent protective effect against lipid peroxidation induced in both experimental models, comparable to that of the well-known antioxidants, α-tocopherol and D-mannitol (Babincová, Bačová, Machová, & Kogan, 2002; Babincová, Machová, & Kogan, 1999). More recently, we demonstrated a protective effect of CMG and another water-soluble derivative, sulfoethyl glucan, against oxidative DNA lesions in hamster lung cells, using comet assay. On the basis of the observations made we suggested that the glucans exhibited their protective effect against oxidative DNA damage as a consequence of scavenging both $^{\cdot}OH$ radicals and singlet oxygen (Slameňová et al., 2003).

Despite all the indications that CMG acts as free radical scavenger and antioxidant, no direct evidence of trapping of free radicals by glucan has ever been produced. Thus, in this paper we attempted to demonstrate directly the free $^{\cdot}OH$ radical scavenging activity of CMG by using the EPR technique.

Another goal of our work was to investigate whether the antioxidant activity of CMG could lead to beneficial effects in animals with induced adjuvant arthritis, an experimental model of human rheumatoid arthritis (RA), a severe disease of joints, in which pathogenesis an important role is played by reactive oxygen species and various nitrogen oxides (Bauerová & Bezek, 1999; Jaswal, Mehta, Sood, & Kaur, 2003; Ostrakhovitch & Afanas'ev, 2001).

2. Materials and methods

2.1. Reagents

All chemicals used were of analytical purity grade and distilled water was used throughout all procedures. 5,5-Dimethylpyrroline-*N*-oxide (DMPO) was purchased from Aldrich Chemical Company (Milwaukee, WI); $K_2S_2O_8$ and D-mannitol were from Merck GmbH (Darmstadt, Germany); H_2O_2 from Lachema (Brno, Czech Republic); 2,2′-azo-bis(2-amidinopropane)-dihydrochloride (AAPH) from Polysciencies, Inc. (Warrington, PA). Cyclosporin A (CsA) (Consupren®, Ivax-CR, Opava, Czech Republic) was used in the biological experiments as a reference antiarthritic preparation.

2.2. Preparation and characterization of glucan derivative

Water-insoluble (1→3)-β-D-glucan was obtained by extraction of commercial baker's yeast (Slovlik, Trenčín, Slovakia) with 6% aqueous NaOH at 60 °C as previously described (Kogan, Alföldi, & Masler, 1988). Carboxymethylation of the glucan was performed using monochloroacetic acid in an isopropyl alcohol dispersion according to the previously described procedure (Machová, Kogan, Alföldi, Šoltés, & Šandula, 1995). Degree of carboxymethylation of the synthesized derivative was equal to 0.8 as determined by potentiometric titration with aqueous KOH. The molecular mass was established to be 250 kDa by using

an HPLC procedure under the previously described conditions (Babincová et al., 1999).

2.3. EPR experiments

EPR spin trapping studies were carried out using the field-modulated X-band EPR CW Bruker EMX spectrometer (Bruker, Rheinstetten, Germany). DMPO served as a spin-trapping agent, and free radicals were generated in thermal decomposition of potassium persulfate ($K_2S_2O_8$), and also in photochemically initiated splitting of hydrogen peroxide (H_2O_2) or AAPH.

All series of experiments started with a reference sample being prepared by adding to 200 μl of distilled water 25 μl aqueous solutions of the following radical initiators: 3% H_2O_2, or 0.1 M AAPH, or 0.01 M $K_2S_2O_8$; further, 25 μl of 1.0 M phosphate buffer, and finally 25 μl of aqueous 0.2 M DMPO spin trap. The mixture was purged for 1 min with argon and then transferred into a flat EPR cell, and inserted in the cavity of the spectrometer. Decomposition of H_2O_2 and AAPH at room temperature was initiated photochemically using an HPA 400/330S lamp (Philips, The Netherlands)—a medium-pressure metal halide source with iron and cobalt additives emitting ozone-free radiation mainly between 300 and 400 nm. Decomposition of the $K_2S_2O_8$ was initiated thermally at 60 °C, directly in the cavity of EPR spectrometer. At testing the radical scavenging activity, the initial 200 μl of distilled water, used in the reference sample, were replaced with solutions of the individual antioxidants in variable concentrations as specified in Section 3. The reproducibility of EPR measurements from the repeatedly prepared samples was within ±5%.

2.4. Induction of adjuvant arthritis and design of the biological experiments

AA was experimentally induced in Lewis type male rats (150 g body mass) by means of a single intradermal administration of *Mycobacterium butyricum* in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). The effect of the administered CMG was evaluated and compared with that of the known immunosupressant CsA, which is used as a reference standard for antirheumatic efficacy testing.

Rats (breeding farm Dobrá Voda, Slovakia) with induced AA were daily administered with either CsA (2.5 mg/kg body mass, orally) or CMG (5 mg/kg body mass, intraperitoneally). A control group and a group with induced arthritis without any treatment were used as references. Each group contained eight animals. The major physiological markers for the evaluation of arthritis progression—(a) swelling of hind paws (changes in hind paw volume) and (b) changes in animal body mass—were monitored on the experimental days 7, 14, 21, and 28 after induction of AA. The biochemical marker for protein oxidative damage—amount of plasmatic carbonyls—was assessed at the end of the experiment, namely on day 28. The arthritis parameters were expressed according to the mode applied by Tanaka et al. (1998). Protein carbonyl determination in plasma was performed according to the method described by Levine et al. (1990) and modified in agreement with the previously applied experimental conditions (Bauerová, Neradová, Žitňanová, Horáková, & Štefek, 2002). The biochemical characterization was completed by determination of a marker of lysosomal activity—the plasmatic activity of lysosomal enzyme *N*-acetyl-β-D-glucosaminidase (NAGA) according to the method described by Barrett and Heath (1977).

2.5. Statistical analysis of the data

The experimental data obtained were expressed as mean and standard error (SEM). The program GraphPad InStat® was used for calculations. Statistical analysis was performed using unpaired *t*-test with two-tailed *P*-value. The group of arthritic animals treated neither with CMG nor with CsA was compared to control animals (+). The effect of treatment in the arthritic groups was compared with the arthritic animals that did not receive any treatment (*). $P<0.0001$ was considered extremely significant (+++ or ***); $P<0.001$ was considered very significant (++ or **); $P<0.01$ was considered significant (+ or *).

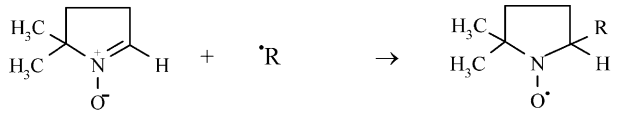
3. Results and discussion

3.1. EPR experiments

Previously, we have demonstrated antioxidant properties of CMG in the lipid peroxidation model induced by $^{\cdot}OH$ radicals produced by Fenton's reagent and microwave irradiation (Babincová et al., 2002, 1999). In these experiments, CMG proved to be a potent antioxidant preventing oxidative damage of liposomes. Its antioxidant activity revealed at relatively low concentration (30 μM) was intermediate between that of α-tocopherol and D-mannitol, which were however used at much higher concentrations (800 and 500 μM, respectively). In the present study, we have expanded our experiments in an effort to demonstrate directly free radical scavenging properties of CMG, which can be defined as the ability to reduce the concentration of reactive radical species, generated in several radical-producing systems. To our knowledge, this is the first report providing direct evidence of radical-scavenging properties of (1→3)-β-D-glucan derivative by means of EPR technique.

We have used three different free radical initiators (H_2O_2, $K_2S_2O_8$, and AAPH). As the generated free radicals ($^{\cdot}OH$, $SO_4^{\cdot-}$, and carbon-centered, respectively) are very unstable, their stationary concentrations are very low, and therefore spin trapping technique has to be applied to prove their presence. Consequently, we used DMPO spin trap

which, according to the scheme below, can react with free radicals (˙R) to form more stable and thus EPR-detectable radicals (spin adducts ˙DMPO-R).



DMPO spin trap Free radical ˙R ˙DMPO-R adducts

The antioxidants under investigation compete in free radical scavenging with DMPO spin trap. Consequently, the action of antioxidants is indicated with a decreasing yield of ˙DMPO-R adducts. This resulted in a decrease of EPR lines intensities of ˙DMPO-R adducts as it is demonstrated in more detail below on the example of AAPH initiator. In this way, the antioxidant activities of the investigated substrates were characterized and compared.

Herein, we present the results of application of the described EPR technique in order to assay the free radical scavenging activity of the prepared water-soluble carboxymethyl derivative of yeast (1→3)-β-D-glucan using the three free radical initiators and to compare this activity with the properties of the standard water-soluble antioxidant, D-mannitol.

3.1.1. AAPH initiator

Azocompounds of the general formula R_1–N=N–R_2 thermally or photochemically decompose to form reactive carbon centered radicals ˙R (Eq. (1)). These are, in the presence of oxygen, easily converted to the unstable peroxyl radicals ROO˙ (Eq. (2)). Peroxyl radicals quickly rearrange via tetroxides most likely to alkoxy radicals RO˙ (Eq. (3)) or react with the antioxidant HA rendering hydroperoxides ROOH (Eq. (4)), which can subsequently decompose (e.g. under photochemical activation '*hν*') to yield alkoxy RO˙ and hydroxy ˙OH radicals (Eq. (5)). The radicals generated in this way (˙R, ROO˙, RO˙, and ˙OH) can be trapped to form ˙DMPO-adducts (Eq. (6))

$$\mathrm{R{-}N{=}N{-}R \rightarrow R^{\cdot} + N_2 + {}^{\cdot}R} \quad (1)$$

$$\mathrm{R^{\cdot} + O_2 \rightarrow ROO^{\cdot}} \quad (2)$$

$$\mathrm{2ROO^{\cdot} \rightarrow 2RO^{\cdot} + O_2} \quad (3)$$

$$\mathrm{ROO^{\cdot} + HA \rightarrow ROOH + {}^{\cdot}A} \quad (4)$$

$$\mathrm{ROOH + h\nu \rightarrow RO^{\cdot} + {}^{\cdot}OH} \quad (5)$$

$$\mathrm{DMPO + ({}^{\cdot}R, ROO^{\cdot}, RO^{\cdot}, {}^{\cdot}OH) \rightarrow {}^{\cdot}DMPO(\text{-}R, \text{-}OOR, \text{-}OR, \text{-}OH)} \quad (6)$$

Time evolution of EPR spectra of ˙DMPO-adducts observed in 0.02 M DMPO and 1 mM AAPH solutions with progressing irradiation time in the presence of various initial CMG concentrations is presented in Fig. 1. The highest adduct concentration was found in the reference sample (Fig. 1a), when no CMG was added. In the experiments illustrated in Fig. 1b–g, with growing initial concentrations of glucan, the spectral intensities, i.e. the concentration of radicals trapped, were decreasing as a result of a competitive action of CMG, demonstrating in this way its radical scavenging activity. The evaluation of these experiments is shown in Fig. 2, where the limiting relative EPR intensities (intensities after 10 min of photochemical AAPH decomposition) are quoted in respect to varying initial CMG concentrations. The scavenging action of CMG is unambiguously evident from these data. The inset in Fig. 2 shows a typical EPR spectrum for these series of experiments. It is well simulated with splitting constants $a_N = 1.46$ mT, $a_H = 1.482$ mT and g-value = 2.0058, characteristic for oxygen-centered adducts (RO˙ and ˙OH) (Li, Cummings, Roething, Buettner, & Chignell, 1988). Tsiapali et al. (2001) have also used AAPH as a source of free radicals and reported that water-soluble derivatives of (1→3)-β-D-glucan carrying negatively charged substituents (similar to CMG) exerted concentration-dependent radical-scavenging activity that was however significantly lower than that of lipopolysaccharide or water-soluble antioxidant pyrrolidine dithiocarbamate.

3.1.2. H_2O_2 initiator

The photochemical decomposition of hydrogen peroxide represents one of the purest sources of hydroxyl radicals (˙OH). The methodology of experiments was similar to that described above for AAPH initiator. Experiments started with a reference sample, then samples with increasing CMG concentrations were UV irradiated at room temperature in the cavity of the EPR spectrometer at a constant initial H_2O_2 concentration (0.08 M). The obtained relative limiting EPR intensities of ˙DMPO-R adduct are quoted in respect to the CMG concentrations in Fig. 3. The trend in the time evolution of EPR spectra was almost identical to that observed at AAPH initiation, where with an increased CMG concentration a gradual decline of spin adducts concentration was observed. Typical EPR spectra recorded in these experiments were very similar to those shown in Fig. 2 and confirmed the presence and scavenging of oxygen centered radicals.

3.1.3. $K_2S_2O_8$ initiator

This initiator is known as a powerful source of free radicals (Harbour & Hair, 1979). Previously, we have systematically investigated thermally initiated $K_2S_2O_8$ decomposition at 60 °C (Staško, Brezová, Liptáková, & Šavel, 2000). It has been shown that in the first step very reactive sulfate radicals ($SO_4^{\cdot -}$) are formed, which may be trapped under the formation of ˙DMPO–SO_4^{-} adducts. However, these adducts are very unstable (half-life time below 21 s (Staško et al., 2000)) and, consequently, were not detectable in our experiments. In the next step, $SO_4^{\cdot -}$ radicals can react with the antioxidant (AH) present, with

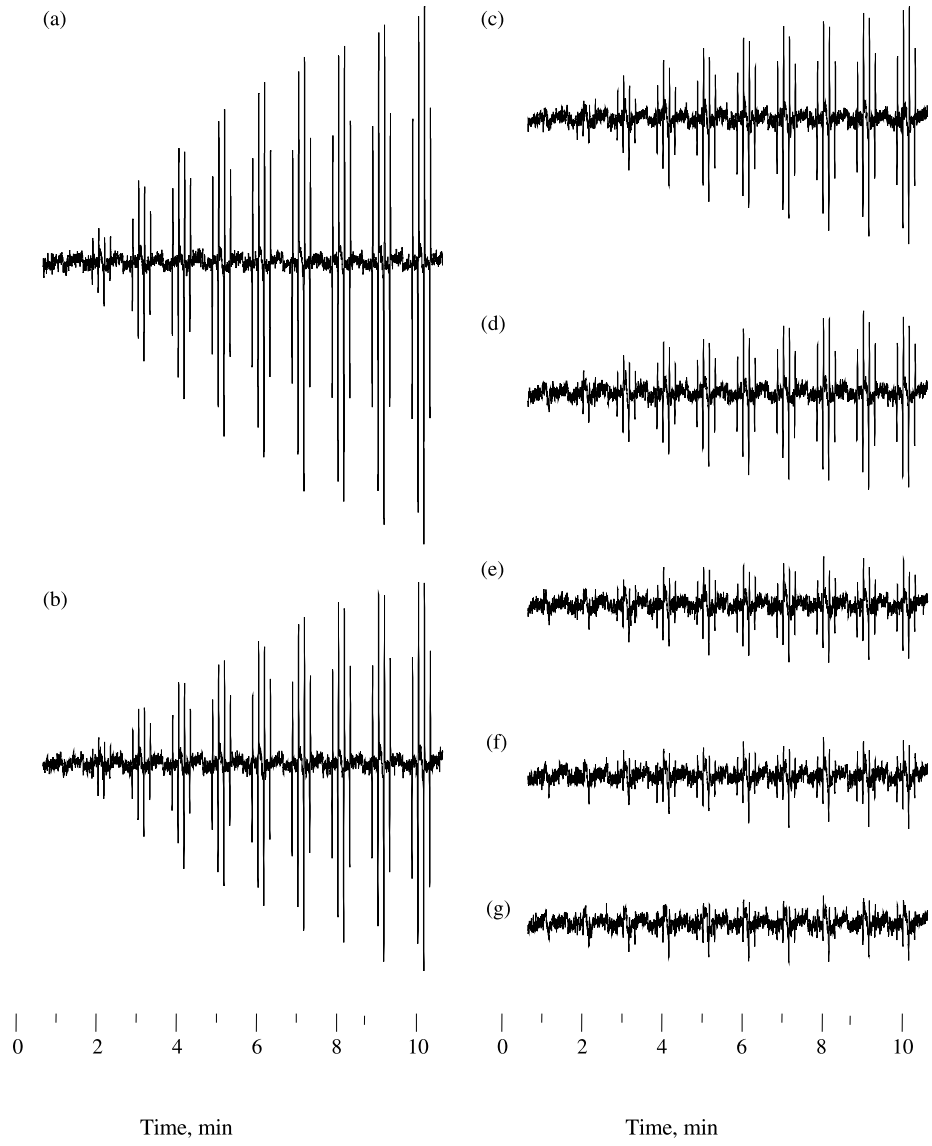


Fig. 1. Time evolution of EPR spectra of ˙DMPO-adducts observed in 0.02 M DMPO solution irradiated during 10 min in the presence of 1 mM AAPH initiator at various initial CMG concentrations (in g/l): (a) 0; (b) 0.48; (c) 0.96; (d) 1.92; (e) 3.85; (f) 5.80; (g) 7.7.

water, or with spin trap DMPO. In the reaction with the antioxidant AH (Eq. (7)), radicals A˙ are formed, which are usually not observed in EPR spectra. In the reaction with water, ˙OH radicals and consecutively ˙DMPO-OH adducts are formed (Eqs. (8) and (9)). Anion-radicals $SO_4^{\cdot -}$ can also oxidize spin trap to its cation (Eq. (10)), which is subsequently hydrolyzed under formation of ˙DMPO-OH adducts (Eq. (11))

$$SO_4^{\cdot -} + AH \rightarrow HSO_4^- + A^{\cdot} \quad (7)$$

$$SO_4^{\cdot -} + H_2O \rightarrow SO_4^{2-} + H^+ + {}^{\cdot}OH \quad (8)$$

$$DMPO + {}^{\cdot}OH \rightarrow {}^{\cdot}DMPO\text{-}OH \quad (9)$$

$$SO_4^{\cdot -} + DMPO \rightarrow SO_4^{2-} + DMPO^{\cdot +} \quad (10)$$

$$DMPO^{\cdot +} + H_2O \rightarrow {}^{\cdot}DMPO - OH + H^+ \quad (11)$$

Using $K_2S_2O_8$, an additional experiment was also carried out in comparison with that performed with the above-described initiators AAPH and H_2O_2. In the experimental set-up, in a 0.02 M DMPO solution the $K_2S_2O_8$ concentrations were increased (a) without CMG and (b) at a constant CMG concentration (0.9 g/l). The decomposition of $K_2S_2O_8$ was thermally initiated at 60 °C. As the predominant paramagnetic species, ˙DMPO-OH adducts were observed. Table 1 summarizes the observed dependence of relative limiting EPR intensities of ˙DMPO-OH adducts on the increased concentrations of $K_2S_2O_8$ from both experiments (a) and (b). A considerable decrease of limiting EPR intensity was observed in the presence of CMG, confirming again its strong scavenging action at various concentrations of radical initiator.

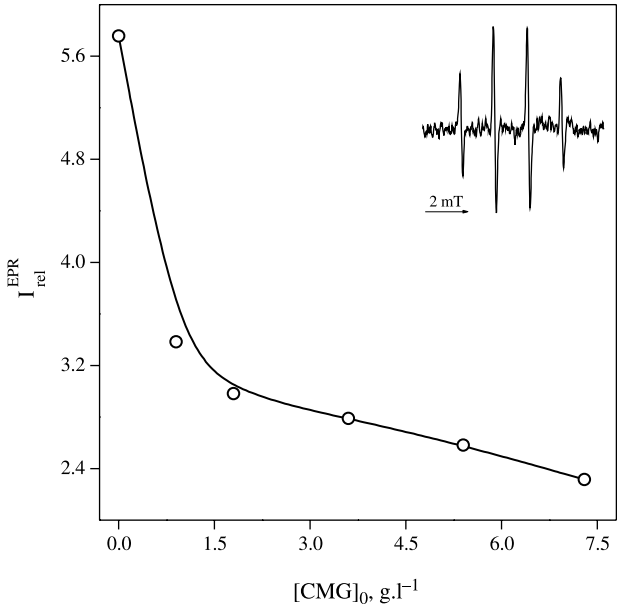


Fig. 2. Limiting relative EPR intensities of ˙DMPO-adducts at various initial CMG concentrations evaluated from Fig. 1 for 10 min irradiation time. Inset represents characteristic EPR spectrum of ˙DMPO-adducts observed.

As can be seen from the presented data, addition of CMG to the radical-producing systems involving different types of free radical initiators (APPH, H_2O_2, and $K_2S_2O_8$) led to a significant concentration-dependent decrease of spectral amplitudes of ˙DMPO-adducts as a result of competition of CMG in the scavenging of generated reactive free radicals. To our knowledge, this is the first direct proof of a high

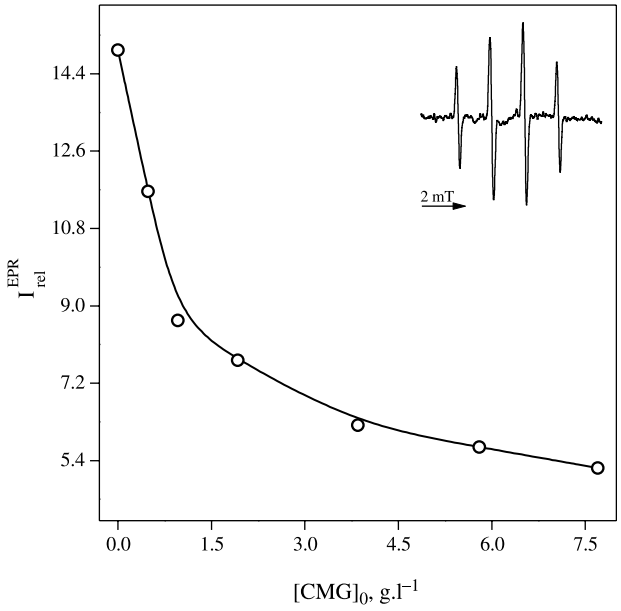


Fig. 3. Limiting relative EPR intensities of ˙DMPO-adducts found after 10 min irradiation, in 0.02 M DMPO, 0.08 M H_2O_2 at various initial CMG concentrations. Inset represents characteristic EPR spectrum of adducts observed.

scavenging or antioxidant activity of the (1→3)-β-D-glucan derivative.

3.1.4. Comparison of CMG with D-mannitol

Finally, in the last series of experiments, we compared the antioxidant activity of CMG with that of D-mannitol, frequently considered as a reference for carbohydrate-type antioxidants (Tsiapali et al., 2001; Tsou & Yang, 1996). For these experiments we chose photochemically activated H_2O_2 initiator, known as a simple and reliable source of ˙OH radicals. The results obtained are summarized in Fig. 4, where the relative limiting EPR intensities (intensities after 10 min irradiation) are quoted upon equal concentrations of D-mannitol and CMG, while maintaining a constant H_2O_2 concentration in the experiments (0.08 M). The observed decrease of limiting intensity of ˙DMPO-OH adducts upon increased D-mannitol concentrations was very moderate, on the other hand, the effect revealed by the (1→3)-β-D-glucan derivative was very pronounced, documenting the strong radical scavenging efficiency of CMG. These observations are in agreement with the findings of Tsiapali et al. (2001) who have suggested that increased antioxidant activity of polysaccharides in comparison with monosaccharides can be explained by the fact that polysaccharides contain multiple anomeric hydrogen atoms which are primarily abstracted by the active free radicals, while monosaccharides possess only one such anomeric hydrogen. D-mannitol does not have anomeric hydrogen and its antioxidant status can be perhaps explained by some non-specific interaction with free radicals, probably via donation of hydrogen atom from one of the hydroxyl groups, or by chelation of the transition metal that participates in the redox system initiating free radical production (Panda, Chattopadhyay, Ghosh, Chattopadhyay, & Chatterjee, 1999; Tsou & Yang, 1996). Although D-mannitol is used as a reference antioxidant, it was described that in many experimental systems D-mannitol revealed only minor antioxidant activity (Bhat, Sridhar, & Madyastha, 2001; Weiss, Pahernik, Scheruebl, Jauch, & Thasler, 2003; Zhu, Antholine, & Frei, 2002).

Our results clearly demonstrate for the first time in a direct measurement of free radical scavenging activity by means of EPR technique that the prepared water-soluble carboxymethyl derivative of the yeast (1→3)-β-D-glucan possesses strong antioxidant properties that are by far higher than those of D-mannitol. These promising results as well as previously obtained proof on the antioxidant activity of CMG in vitro led us to test whether it would elicit protective effect in the experimental model of adjuvant arthritis induced in rats.

3.2. Activity of CMG in the model of adjuvant arthritis

It has been proven that free radicals play a deleterious role in damaging cartilage tissues and causing a severe pathological state known as rheumatoid arthritis (RA),

Table 1
Dependence of limiting relative EPR intensities (I_{rel}^{EPR}) of DMPO-adducts at various initial concentrations c of $K_2S_2O_8$ thermally decomposed for 10 min at 60 °C in the absence and in the presence of CMG

$c(K_2S_2O_8)$ (mM)		0	0.05	0.1	0.2	0.3	0.4	0.5
I_{rel}^{EPR}	c(CMG) = 0	0	10.5	17	26	32	41	45.3
	c(CMG) = 0.9 g/l	0	3.2	4.6	8.5	9.8	16.5	22.4

an autoimmune inflammatory disease of unknown origin (Brown, 1988; Henrotin, Bruckner, & Pujol, 2003; Yamazaki et al., 2003). It is the most common inflammatory arthritis affecting approximately 1–2% of the general population worldwide (Harris, 1994). The disease incidence increases with age, with women being affected three times more than men (Krane & Simon, 1986). Since an efficient cure for this disease of unclear etiology is not yet established, there is an urgent need for a therapeutic agent capable of preventing disease progression or reversing joint destruction. In this respect, much attention has been recently attracted to the application of antioxidants in the therapy of RA because of the involvement of free radicals in the disease (Jaswal et al., 2003).

An experimental model of adjuvant arthritis (AA) induced in animals by means of intradermal injection of killed bacteria *M. butyricum* has been accepted as having many histological and clinical features common with human rheumatoid arthritis (Brahn, 1991). It was previously shown that administration of antioxidants, such as triterpene Celastrol (Allison, Cacabelos, Lombardi, Alvarez, & Vigo, 2001), vitamin E (Çinar et al., 1998), and liposome-immobilized superoxide dismutase (Corvo et al., 1999) exerted beneficial effect in experimental arthritic rats. However, there are only few reports available on the application of carbohydrates for treatment of arthritis. In comparison to the above-mentioned antioxidants, yeast polysaccharides are much easier to prepare, modify, purify, and administer, moreover, they are completely devoid of any unfavorable side-effects and products of their enzymatic degradation in human organism comprise exclusively harmless and natural monosaccharides.

Methyl-α-D-mannopyranoside, mannooligosaccharides obtained upon acetolysis of yeast mannans, and mannans isolated from the cell wall of the pathogenic yeast *Candida albicans*, as well as from the non-pathogenic *S. cerevisiae* were assessed in the model of adjuvant arthritis (Prokopová et al., 1993). This was the first paper to describe a therapeutic effect of simple saccharides on the development of adjuvant arthritis in rats. The highest efficiency was revealed by methyl-α-D-mannopyranoside.

In the present work, we evaluated a derivative of a microbial polysaccharide—water-soluble carboxymethyl glucan—prepared from cell wall glucan isolated from baker's yeast *S. cerevisiae*. The choice of this polysaccharide was not accidental, but was rather based on available experimental proof of its biological activity in vitro and in vivo including our previous observations.

Di Luzio (1985) described the ability of β-D-glucans to boost host defense mechanisms against bacterial, viral, fungal, and parasitic infections, as well as their inhibitory effects against neoplastic and metastatic processes. Glucan from *S. cerevisiae* is a high molecular mass homopolymer of β-D-glucopyranosyl units linked predominantly with (1→3) glycosidic bonds (Kogan et al., 1988). Since native β-D-glucan isolated from *S. cerevisiae* is insoluble in water, procedures for preparation of several water-soluble derivatives have been developed, among them that of the carboxymethylated one (Machová et al., 1995).

In our previous papers, we have described the structure and biological activity of CMG evaluated in several models (Kogan, Machová, & Šandula, 1997; Šandula, Kogan, Kačuráková, & Machová, 1999). The protective action against bacterial infection caused by *Klebsiella pneumoniae* was established in the murine model (Kogan, Masler, Šandula, Navarová, & Trnovec, 1989). CMG enhanced hemopoiesis and led to prolonged survival of mice exposed to γ-irradiation (Pospíšil, Šandula, Pipalová, Hofer, & Viklická, 1991). A pronounced synergistic effect was observed on combined application of CMG with prostaglandin production inhibitor diclofenac to γ-irradiated mice (Pospíšil et al., 1992). The established antimutagenic

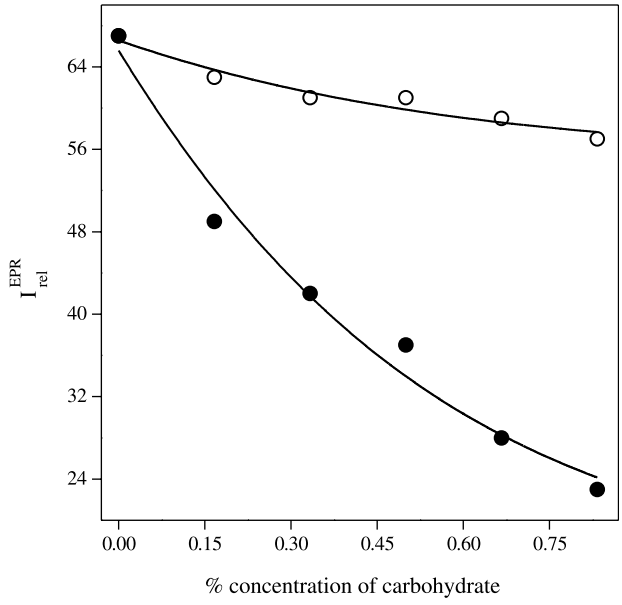


Fig. 4. Limiting relative EPR intensities of ˙DMPO-OH adducts observed after 10 min irradiation of 0.02 M DMPO, 0.08 M H_2O_2 at various initial concentrations of D-mannitol (○) and CMG (●).

activity of CMG (Chorvatovičová et al., 1996) spoke in favor of the possibility to use it as a chemotherapeutic agent. CMG also revealed inhibitory action on the development of Lewis lung carcinoma and suppressed the spread of metastases (Falameeva et al., 2001; Kogan et al., 2002).

Since it is known that pathological processes initiated by free radicals cause certain neoplastic phenomena, the antioxidant activity of CMG was investigated using the model of lipid peroxidation in phosphatidyl liposomes. CMG acted protectively against peroxidation and its antioxidant activity was comparable with that of α-tocopherol and other known antioxidants (Babincová et al., 2002, 1999). CMG also exerted protective effect against oxidative DNA lesions in hamster lung cells (Slameňová et al., 2003).

In the present work we evaluated the potential anti-arthritic effect of CMG in the model of adjuvant arthritis induced in Lewis rats. The experiments involved intraperitoneal administration of CMG and oral administration of CsA—a standard for antirheumatic efficacy testing, and a comparison of the effect of both preparations on the certain arthritic parameters.

As for the major physiological parameters, change in animal body mass and change in hind paws volume, the differences between the arthritic untreated animals and healthy control animals were negligible up to the 14th day of the experiment. Starting from the 21st day, the body mass of the arthritic animals began to decrease dramatically in comparison with that of healthy control animals—the differences between the two groups of animals were assessed as extremely significant (Fig. 5). On evaluating swelling of hind paws, a significant increase of hind paws volume was observed in the arthritic animals already after the 14th experimental day, while the largest increase (over 50%) was established on the 21st day of the experiment with significance $P < 0.0001$ (Fig. 6).

In arthritic animals, which received CMG, no beneficiary effect of this polysaccharide was observed on change in body mass. The CMG effect was even detrimental, leading to additional decrease of the evaluated parameter (Fig. 5). Similarly, from the comparison of changes in hind paws volume in arthritic animals treated with CMG with the

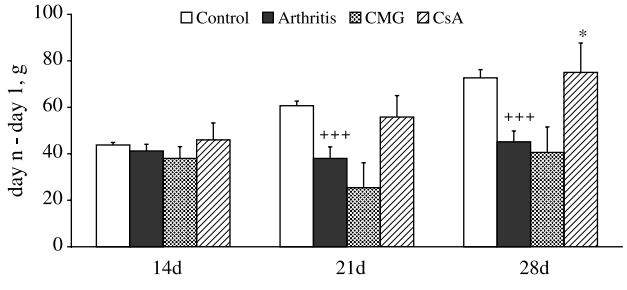


Fig. 5. Increase of total body mass: comparison of a control group (□) and arthritic rats (■) administered with CMG (▩) or CsA (▨). (For statistical symbols, (*, +) see Section 2).

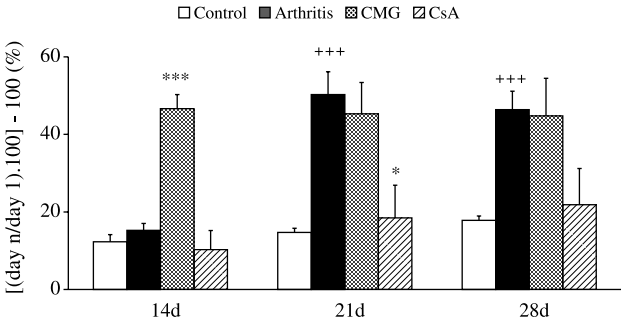


Fig. 6. Increase of hind paw volume: comparison of a control group (□) and arthritic rats (■) administered with CMG (▩) or CsA (▨). (For statistical symbols, (*, +) see Section 2).

group of arthritic untreated animals, it is evident that on the 14th and the following experimental days, no protective effect of CMG was observed, whereas on the 14th day application of CMG resulted in a significant increase of swelling (Fig. 6). Such action of CMG could be ascribed to its known immunomodulatory properties and its ability to activate pro-inflammatory mediators (Kogan, 2000; Williams, 1997) including stimulation of free radical production by macrophages (Tsiapali et al., 2001) and endothelial cells (Iwamoto, Yoshioka, Nitta, & Ito, 1998). On the other hand, administration of CsA resulted in a significant protective effect observed on both parameters by the 14th day—an experimental period, in which the pathological arthritic processes are already well developed in the model of AA (Figs. 5 and 6).

Concerning the biochemical parameters of adjuvant arthritis, we studied changes in the content of plasma carbonyls and in the activity of *N*-acetylglucosaminidase in plasma. Arthritis, similarly to many other diseases, is accompanied by oxidative damage of plasma proteins induced by the action of free radicals. Protein carbonyls (aldehydes and ketones) are produced directly by oxidation or via reactions with other molecules generated by the oxidation process (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003; Punchard & Kelly, 1996) and the assay of protein carbonyls as biomarkers of oxidative stress in various diseases is with advantage used in diagnostics because of the relative early formation and relative stability of carbonylated proteins (Dalle-Donne et al., 2003). The ability of certain compounds to reduce the amount of carbonyls is considered as one of the indirect proofs of their antioxidant activity.

Another biochemical parameter estimated was the activity of NAGA in plasma, which is directly associated with the intensity of systemic inflammation. Changes of its activity are thus a relevant indicator of either antiinflammatory or pro-inflammatory activity modulation of the compound tested.

In the used model of adjuvant arthritis, the content of carbonyls in the arthritic animals increased in comparison with healthy controls from 0.43 to 0.58 nmol/mg proteins

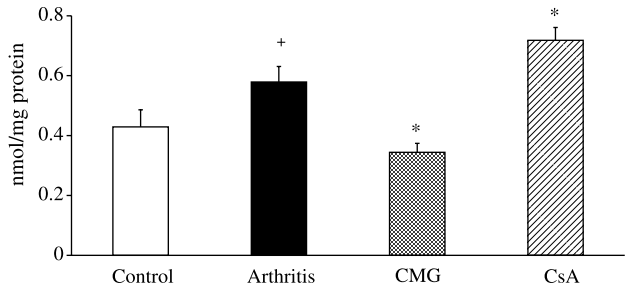


Fig. 7. Comparison of plasma carbonyl levels for a control group (□) and arthritic rats (■) administered with CMG (⊠) or CsA (▨). The data are taken on the 28th experimental day. (For statistical symbols, (*, +) see Section 2).

(Fig. 7). Activity of NAGA per 1 mg of proteins in the plasma of rats with induced adjuvant arthritis was moderately higher than in the group of healthy control animals—0.0648 vs. 0.0597 μg/min ml 4-nitrophenol per 1 mg proteins (Fig. 8). Interestingly, CMG and CsA had a completely opposite effect on the two biochemical parameters assessed. Administration of CsA to arthritic animals resulted in a slight increase of plasma carbonyls, while CMG exerted a significant reducing effect (Fig. 7). On the other hand, on evaluating NAGA plasmatic activity, CsA revealed a protective effect by suppressing NAGA activity in arthritic animals, while administration of CMG led to an enhanced activity of this enzyme (Fig. 8). These findings indicate that application of CsA and CMG implements different mechanisms in affecting the development of adjuvant arthritis. Clearly, the possible antiarthritic action of CMG could reside in its antioxidant properties that resulted in a pronounced inhibition of production of the oxidized (carbonylated) proteins. It has been unequivocally proven that there exists a direct correlation between the carbonyl content and progression of arthritis or other oxidative-impairment related human diseases (Dalle-Donne et al., 2003; Renke, Popadiuk, Korzon, Bugajczyk, & Woźniak, 2000). Thus, an observed antioxidant carbonyl-reducing effect and modulation of pro-inflammatory mediators could be the properties of CMG that should be taken into consideration for its application in

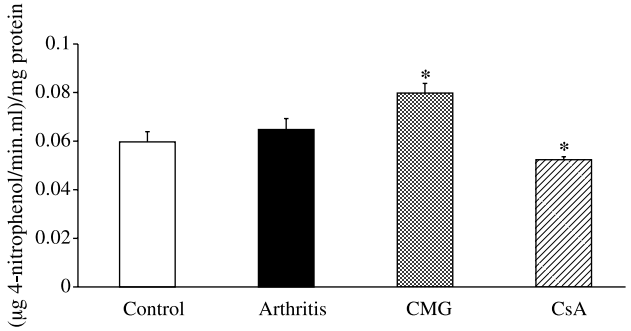


Fig. 8. Comparison of plasmatic NAGA activity for a control group (□) and arthritic rats (■) administered with CMG (⊠) or CsA (▨). The data are taken on the 28th experimental day. (For statistical symbols, (*, +) see Section 2).

arthritis therapy. In the future experiments on adjuvant arthritis, CMG should be thoroughly evaluated for its possible contribution to moderate the arthritic process. More experimental aspects, such as dose-dependency, design of the experiment (treatment vs. pretreatment), experimental period monitored (longer than 28 days), etc., should be also taken into account.

4. Conclusions

By means of EPR spin trapping technique and using several types of free radical initiators, we observed high radical scavenging activity of carboxymethyl (1→3)-β-D-glucan derivative. These properties of yeast polysaccharide have been directly demonstrated for the first time and provide a possible explanation for its antioxidant activity observed in vitro and in vivo in various experimental models. Additional data on antioxidant activity of carboxymethyl (1→3)-β-D-glucan have been obtained in the model of adjuvant arthritis in rats (experimental model of rheumatoid arthritis), where administration of CMG resulted in an evident decrease of plasma carbonyl content—an oxidative parameter associated with the progress of arthritic condition. These observations imply a potential application of (1→3)-β-D-glucan derivatives in treatment of human rheumatoid arthritis and suggest future direction of the research in this area.

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