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THE ROLE OF NATURAL BIOPOLYMERS IN GENOTOXICITY OF MUTAGENS/ CARCINOGENS ELIMINATION

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Nowadays naturally occuring compounds with the potential antimutagenic and anticarcinogenic effects are of great importance for their prospective use in cancer chemoprevention and treatment. The new water soluble derivative of microbial polysaccharide β-D-glucan-carboxymethyl glucan (CMG) belongs to such a category of natural substances. CMG isolated from the cell wall of baker's yeast *Saccharomyces cerevisiae* is included into the class of biopolymers known as biological response modifiers (BRMs) with a broad range of activities, above all ones interfering with cancer therapy. It was demonstrated on four experimental model systems that biological and consequential medicinal importance of CMG is based on the combined application with another active compound. In the *Saccharomyces cerevisiae* antimutagenicity assay CMG significantly reduced ofloxacin-induced mutagenicity in the yeast strain D7. CMG exerted bioprotective (anti-toxic and antimutagenic) effect after its simultaneos application with methyl methanesulphonate on the repair-deficient strain *uvs10* of the unicellular green alga *Chlamydomonas reinhardtii.* In the *Vicia sativa* simultaneous phytotoxicity and anticlastogenicity assay CMG exerted statistically significant anticlastogenic efect against maleic hydrazide-induced clastogenicity in *Vicia sativa* L. Only in the *Salmonella*/microsome assay CMG did not exert statistically significant antigenotoxic effect, despite of the fact that it reduced 9-aminoacridine-induced mutagenicity in *S. typhimurium* TA97*,* but his^+ revertants decreasing was statistically significant only at the highest CMG concentration used. The data presented unambiguously documented that even biopolysaccharides (e.g., derivatives of β-glucan) belonging to the most abundant class of natural biopolymers may contribute to cancer prevention and therapy.

INTRODUCTION

A rational use of chemopreventive agents is based not only on the assessment of their efficacy and safety but also on understanding of their mechanisms of action. A detailed classification is proposed which covers variety of mechanisms interfering with different phases of mutagenesis and carcinogenesis. Several mechanisms, such as inhibition of genotoxic effects, antioxidant activity and scavenging of free radicals, inhibition of cell proliferation and signal transduction modulation can be involved[1–3]. To such compounds also belong microbial skeletal polysaccharides which possess marked immunological properties ranging from non-specific stimulation of host immune system, resulting in antitumor, antiviral and anti-infective effects, to antioxidant, antimutagenic or hematopoetic activity[4–7]. In the recent years much evidence has been collected indicating that microbial polysaccharides play a role of signalling molecules for innate immune system[8] where they are recognized by genetically predetermined pattern recognition receptors (PRRs) located on the surface of immunocompetent and also other type of cells (e.g., epitelial)[9–11]. Among the yeast polysaccharides, specific PRRs have been idenfied and described for (1→3)-β-D-glucans[9, 12].

β-Glucans isolated from fungi, bacteria and lichens belong to the class of substances known as biological response modifiers (BRMs), which modify the host's biological response by stimulation of the immune system[13].

Recet decades have brought increased attention to the research of BRMs. Introduction of regulatory peptides and biomodulators in combination with chemotherapy (biotherapy) was a significant contribution to antineoplastic therapy. Among the microbial polysaccharides mainly derivatives of β-glucans isolated from the cell walls of yeast and fungi have been studied regarding their anticancer activities. The efficiency of chemotherapy of Lewis lung carcinoma with cyclophosphamide was affected by administration of the yeast carboxymethyl glucan – a well-known macrophage simulator[14]. Soluble β-glucan enhanced killing of retinal carcinoma micrometastases[15].

As some polysaccharides isolated from the yeast cell walls belong to the class of substances known as BRMs with a broad range of activity, and the principal strategy of new anticancer drug modulators evaluation involves also antimutagenicity/anticarcinogenicity studies, the main concern of the present study has been given to examination of the carboxymethyl glucan (CMG) eligibility to exert antigenotoxic effect after its application on four genetic model organisms.

MATERIAL AND METHODS

Material: Carboxymethyl glucan (CMG) is a derivative of β-D-glucan which was isolated from the cell walls of baker's yeast *S. cerevisiae* by extraction with diluted alkali (6% NaOH at 60 °C) followed by treatment with diluted acid (4% phosporic acid extraction at room temperature)[16]. Insoluble β-D-glucan was solubilized by carboxymethylation described in detail by Machová et al.[16]. The degree of substitution (DS) determined by potentiometric titration was 0.8 and molecular weight determined by HPCL was 250 kD.

Mutagens/carcinogens: 9-Aminoacridine (9-AA) (Serva); Methyl methanesulfonate (MMS) (Aldrich); Maleic hydrazide (Serva) and Ofloxacin (Hoechst-Biotika) were of the highest purity available.

Saccharomyces cerevisiae toxicity and antimutagenicity assay: As a testing procedure the assay according to Zimmermann[17] was used. Prior to each experiment the D7 strain (*MATa/MAT*α, *ade*2-40/*ade*2-119, *trp5-12/trp*5-27, *ilv*1-92/*ilv*1-92) was tested for the frequency of spontaneous revertants at the isoleucine locus (*ilv1*). Exponentially growing cells were treated with ofloxacin (600 μg/ml) and CMG (1×10^{-6} M; 1×10^{-5} M) for 22 h at 28 °C. After the treatment, washed cell suspensions ($1–2\times10^{6}$ cells/plate) were plated on selective medium without ilv, and 2×10^{2} to 2×10^{3} cells/plate on a synthetic medium to detect survival. The plates were incubated at 28 °C for 5–12 days. Results are means of five experiments. For statistic analysis Student's t-test was used.

Chlamydomonas reinhardtii bioprotectivity assay: Algal (recombination-repair-deficient) strain *uvs10* of the unicellular green alga *Chlamydomonas reinhardtii*, isolated at the Department of Genetics, Faculty of Science, Comenius University, Bratislava, Slovakia[18] was treated with MMS (0.1–0.5 %) and simultaneously with MMS and CMG (10^{-6} M) for 30 min in the dark, and then plated on agar dishes[19]. Survival was evaluated by microscopic method which enabled to distinguish algal cells died due to cytotoxic and due to genotoxic (lethal mutations) effect of MMS. Results are means of five experiments. For statistic analysis Wilcoxon's two sample test was used.

Simultaneous phytotoxicity and anticlastogenicity assay: This assay was carried out on plant species *Vicia sativa* (L.) according to Murín[20]. After 24 h of soaking at 25 °C in the tested CMG (10^{-5} M) and maleic hydrazide (MH), which was used as a positive control[21], the seeds of *V. sativa* were allowed to germinate on Petri dishes (diameter = 18.5 cm) with filter paper soaked with the same concentrations of tested CMG and MH as those used for soaking. Phytotoxicity was assayed after 72 h of the dark cultivation in the thermostat at 25 °C. The seedlings roots of *V. sativa* were measured, and the growth inhibition percentages were assessed. The seedlings in which the root growth was inhibited at least by 25 %, 50 % and 75 % were fixed and used for chromosome and genome mutability evaluation. The roots were fixed and permanent slides were prepared by the Feulgen method. Chromosome aberrations were determined at least in 500 ana-telophases. For statistic analysis the Student's t-test was used.

Salmonella/microsome assay (Ames assay): For the potential CMG antigenotoxicity assessment the Ames assay was performed according to the published procedure by Maron and Ames[22] . The *Salmonella typhimurium* tester strains TA97, TA98 TA100, TA102 were obtained

Table 1. The anticlastogenic effect of CMG against MH-induced clastogenicity in *Vicia sativa* L.

Test agent	Concentration	Number of cells analysed	Number of aberrations	% of aberrations
Control		1129	2	0.18±0.13
MH	1.12×10^{-3} %	1047	253	24.16±1.32
MH + CMG	1.12×10^{-3} %+10^{-5} M	1023	137	13.39±1.10**
MH	0.56×10^{-3} %	1021	179	17.53±1.19
MH + CMG	0.56×10^{-3} % +10^{-5} M	1084	72	6.64±0.79**
MH	0.28×10^{-3} %	1289	59	4.58±0.58
MH + CMG	0.28×10^{-3} % + 10^{-5} M	1151	31	2.69±0.48**

Values are mean ± SD; Control = H_2O; CMG = carboxymethyl glucan; MH = maleic hydrazide.
** significant difference between MH and MH+CMG at $p<0.01$.

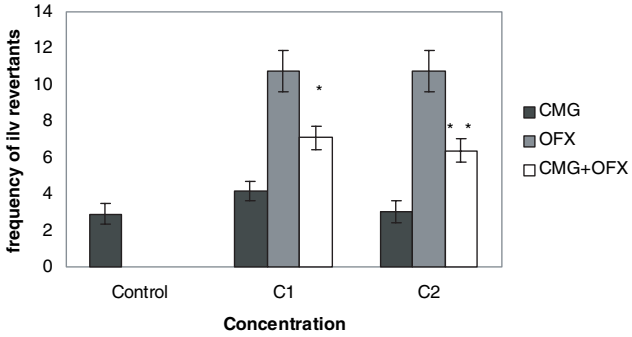

Fig. 1. Antimutagenic effect of CMG against ofloxacin-induced *ilv-1* revertants in *S. cerevisiae* D7. CMG = carboxymethyl glucan (C1 = 1×10^{-5} M, C2 = 1×10^{-6} M); OFX = ofloxacin (600 μg /ml); Control = DMSO;
* significant difference between OFX and OFX+CMG at $p<0.05$.
** significant difference between OFX and OFX+CMG at $p<0.01$.

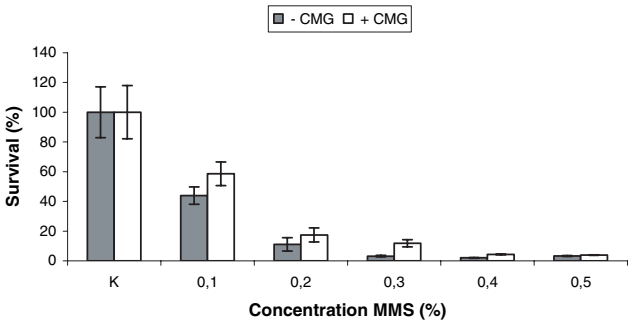

Fig. 2. Bioprotective effect of CMG against MMS-induced cytoxicity and mutagenicity in recombination-repair-deficient strain *uvs10* of *Chlamydomonas reinhardtii.* C = control; MMS = methyl methanesulfonate (0.1–0.5 %)
* significant difference between variats with and without CMG at $p<0.05$.

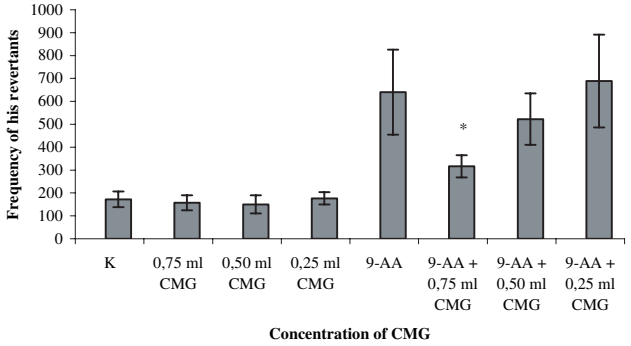

Fig. 3. Antigenotoxic effect of CMG against 9-AA-induced mutagenity *in Salmonella typhimurium* TA97
* significant difference between 9-AA (100 μg/plate) and 9-AA+CMG (0.25-0.75 ml/plate) at $p<0.05$.

from the Masaryk Univerzity Collection, Brno, Czech Republic. Test tubes containing CMG in three concentrations (750 μg/plate, 500 μg/plate, 250 μg/plate) either alone or with relevant diagnostic mutagen were placed on minimal bottom agar plates. His^+ revertants were counted after 72 h of incubation at 37 °C on Biotran III Colony Counter (New Brunswick Scientific Co). Results are means of seven experiments. For statistic analysis the Student's t-test was used.

RESULTS

In this study potential antigenotoxic properties of CMG were investigated using yeast, algae, plant and bacteria as model genetic systems.

CMG significantly reduced the frequency of ofloxacin-induced revertants at the *ilv1* locus in the toxicity and antimutagenicity assay in *S. cerevisiae* D7 (Fig. 1). It can be suggested that the antimutagenic effect of CMG against ofloxacin may be based on its ability to scavenge reactive oxygen species.

Results obtained after simultaneous treatment of algal recombination-repair-deficient strain *uvs10* with MMS and CMG documented that CMG exerted bioprotective effect (Fig. 2) because it reduced cytoxicity and mutagenicity (lethal mutations) of MMS.

Data obtained from the simultaneous phytotoxicity and anticlastogenicity assay revealed that 10^{-6} M CMG significantly reduced clastogenic effect of MH applied on *V. sativa* seeds (Table 1).

However, in the Ames assay CMG did not exert statistically significant antimutagenic/anticarcinogenic effect (so that data are not shown), despite of the fact that it reduced 9-aminoacridine-induced mutagenicity in *S. typhimurium* TA97 (Fig. 3), but his^+ revertants decreasing was statistically significant only at the highest concentration of CMG.

DISCUSSION

The conventional treatment of surgery, radiation, and chemotherapy has been the cornerstone of cancer treatment over the past 50 years. Today, the clinical success of these treatments has reached a plateau. There is an urgent need to break through this cure plateau by trying fresh approaches. Acceptance and utilization of BRMs, including CMG, is one of them. As β-D-glucan isolated from *S. cerevisiae* is water insoluble, has higher molecular weight, and is resistant against alkali-acid treatments, it was processed by carboxymethylation[15] on purpose to change these properties and facilitate its potential utilization in medicine.

For *in vitro* screening of antimutagens/anticarcinogens various prokaryotic and eukaryotic model organism, which enable to monitor different genetic endpoints, have been used[23]. Reduction of the genotoxic effect of MMS applied on recombination-repair-deficient strain of *Chlamydomonas reinhardtii* by CMG could be either a result of their interaction resulting in the MMS inactivation (CMG acting as desmutagen), or result of stimulation of non-damaged repair mechanism(s) (e.g., excision repair) by CMG (CMG acting as bioantimutagen). But, antigenotoxicity explanation due to acting in a desmutagenic manner, is more probable. Moreover, its antioxidative capacity based on efficient free-radical scavenging was also revealed by other authors[6, 7, 24]. In our experiments on yeast performed with ofloxacin, which is known to be inhibitor of DNA gyrase[25] and producer of reactive oxygen species (ROS)[26–28], a decreased number of revertant colonies (Fig. 1) may be based on ability of CMG to prevent the oxidative damage to DNA induced by ROS which play important role in the multistage carcinogenesis and mutagenesis. Thus, CMG possesses high antioxidative activity as well as expressive antimutagenic/bioprotective effects, exerted through combined application with other biologically active compound (mutagen/carcinogen). CMG as a representative of fungal polysaccharides is of pharmacological importance due to its potential employment in cancer prevention and therapy.

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