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Fungal chitin–glucan derivatives exert protective or damaging activity on plasmid DNA

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Abstract

Water-soluble derivatives of the chitin–glucan (Ch–G) complex isolated from the fungal mycelium of the industrial strain of *Aspergillus niger* have been previously shown to possess potent antimutagenic protective activity in vivo. Their direct action on DNA has not been yet evaluated. Using carboxymethylation, sulfoethylation and subsequent ultrasonic treatment, lower molecular weight water-soluble derivatives were obtained from the crude fungal Ch–G. The biological effects of the prepared compounds were evaluated in direct interaction on plasmid DNA in vitro. Monitoring of electrophoretic mobility of different conformers of plasmid DNA implied that carboxymethyl chitin–glucan (CM-Ch–G) induced single- and double-strand breaks into supercoiled DNA in a concentration-dependent manner. On the other hand, sulfoethyl chitin–glucan (SE-Ch–G) alone did not induce any DNA breaks in plasmid DNA. However, process of DNA damaging induced by free-radical oxidation initiated with Fe^{2+} was inhibited, while the process of DNA breakage induced by H_2O_2 was increased in the presence of SE-Ch–G.
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1. Introduction

In recent years much attention has been paid to research on the naturally occurring substances that are able to boost the immune defence mechanisms of the organism. High-molecular-weight β-D-glucan is a common component of yeast and fungal cell walls. β-D-Glucans belong to the group of compounds known as biological response modifiers, which stimulate the immune system of the host and exert an amazing range of immunopharmacological activities that include protection against viral, bacterial, fungal, and parasitic infections, antitumour effects and metastasis inhibition, radioprotection and stimulation of the hematopoiesis, etc.[1,2] Chitin and chitosan originating either from crustacean shells, insect exoskeleton, or from the microbial cell walls also exert a wide array of biological effects on animal and human organisms.[3] In the mycelia of the filamentous fungus *Aspergillus niger*, β-D-glucan is covalently associated with chitin[4] and presence of the two biologically active polysaccharides in the complex may enhance its pharmacological effect. Moreover, chitin–glucan (Ch–G) can be easily prepared from the waste material left upon the industrial preparation of citric acid by *A. niger* and therefore represents an inexpensive natural source of a potentially active, non-toxic immunostimulatory drug.

Since β-D-glucan and Ch–G are water insoluble, a special attention should be paid to the preparation of their water-soluble derivatives that would enable their parenteral administration, because intravenous or intraperitoneal application of an insoluble microparticulate glucan can cause adverse side effects such as microembolization and granulomatosis.[5,6] Previous studies involving water-soluble derivatives of yeast β-D-glucan prepared in our laboratory revealed their pronounced antimutagenic protective activity.[7–11] Since the antigenotoxic activity of chitin and chitosan was previously described as well,[12] it can be assumed that in

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Ch–G both polysaccharide components could play roles in its protective effect. Previously, our collaborators have proved this assumption by demonstrating the antimutagenic activity of high-molecular-weight carboxymethyl chitin–glucan (CM-Ch–G) against cyclophosphamide in mice.[10] The lower molecular weight fractions of CM-Ch–G obtained by means of ultrasonication of the original complex was protective, not only under parenteral, but also under oral administration, which proved that decreased molecular weight helped the CM-Ch–G molecule to pass into the bloodstream through the wall of gastrointestinal tract.[13]

In the present paper we describe the diverse activities of the soluble preparations—CM-Ch–G and sulfoethyl chitin–glucan (SE-Ch–G)—on plasmid DNA in the presence and absence of DNA-damaging agents.

2. Results and discussion

Using the conversion of the supercoiled pBR322 DNA to the relaxed and linear forms, we witnessed the capacity of CM-Ch–G to introduce single- and double-strand nicks into plasmid DNA in a concentration-dependent manner (Fig. 1(A and B)). We observed the formation of relaxed and linear forms of DNA in approximately 1:1 ratio.

We therefore concluded that DNA damage was caused not only by single-strand breaks, but by double-strand breaks as well. CM-Ch–G presented itself in our experiments as a direct inductor of DNA damage; however, the mechanisms involved in the observed DNA-damaging processes are not understood and need further investigation. On the other hand, SE-Ch–G alone did not induce any topological changes in supercoiled plasmid DNA (Fig. 2(A), lane 1). This characteristic is the important difference between SE-Ch–G and CM-Ch–G (Fig. 1). Since both chitosan and β-D-glucan were demonstrated to possess radical-scavenging activities,[14–16] it is quite improbable that the DNA damage was inflicted through the radical degradation.

Furthermore, we have attempted to study the potential of SE-Ch–G in iron chelation and in scavenging hydroxyl radicals produced from H_2O_2 in separate experiments. The ability of phosphate buffer to catalyze rapid Fe^{2+} oxidation to Fe^{3+} in the presence of DNA at low ionic strength has been well documented.[17,18] In our experiments, the quantitative analysis of the different bands showed that addition of Fe^{2+} alone into the phosphate buffer containing supercoiled DNA caused conversion of supercoiled DNA to relaxed form DNA (Fig. 2(A), lane 3). In the presence of SE-Ch–G, however, this process of DNA damaging induced by Fe^{2+} oxidation, was inhibited (Fig. 2(A), lane 4). In order to investigate the role of iron chelation or radical scavenging activity of SE-Ch–G in such a DNA-protective

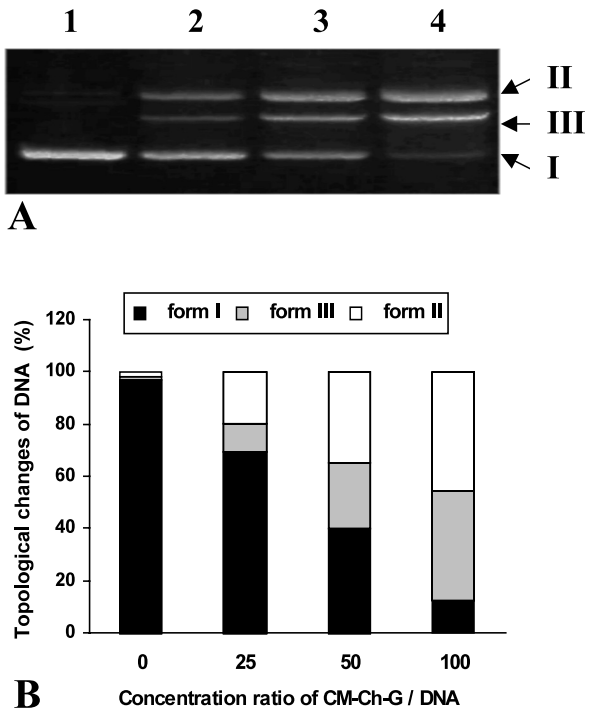

Fig. 1. The effect of CM-Ch–G on single- and double-strand break formation in pBR322 DNA. Samples contained 0.2 g DNA and 0.00; 0.25; 0.50; 1.00 mg/mL of CM-Ch–G (concentration ratio of CM-Ch–G/DNA was 0.0; 25; 50; 100) in 10 mmol sodium phosphate buffer (final volume 20 μL, final pH 7.0) were incubated at 37 °C for 30 min. Cleavage of the supercoiled DNA (form I) to the relaxed (form II) and linear (form III) forms was assessed by electrophoresis (A) and determined by densitometry (B). The alterations induced in the conformation of plasmid DNA were plotted against the concentration ratio of CM-Ch–G/DNA. Lanes in the Fig. A correspond to the respective bars in Fig. B.

effect (since both these effects could account for the observed phenomenon), we used H_2O_2 as another inductor of DNA damaging conditions. In the previous studies, DNA nicking mediated with H_2O_2 has been examined in a temperature range of 20–50 °C.[19,20] Now our experiments confirmed the induction of DNA breaks after exposure of the plasmid DNA to H_2O_2 at 50 °C for 30 min (Fig. 2(A), lane 6). Interestingly, it was observed that SE-Ch–G was able to increase this effect of H_2O_2 on DNA nicking (Fig. 2(A), lane 7). Thus, SE-Ch–G presented itself as a chelator of Fe^{2+} (inhibitor of Fe^{2+} induced DNA damage; sample 4), but also as an inductor of DNA breakage in the presence of H_2O_2 alone (sample 7).

Both activities of SE-Ch–G were verified in the dose-dependent experiments. The results presented in Fig. 3 are consistent with our previous findings (Fig. 2) and demonstrate that the DNA protective effect (experiment with Fe^{2+}) and the enhancing effect on DNA nicking (experiment with H_2O_2) are dependent on the applied concentrations of SE-Ch–G. These results also imply that SE-Ch–G probably does not function as a

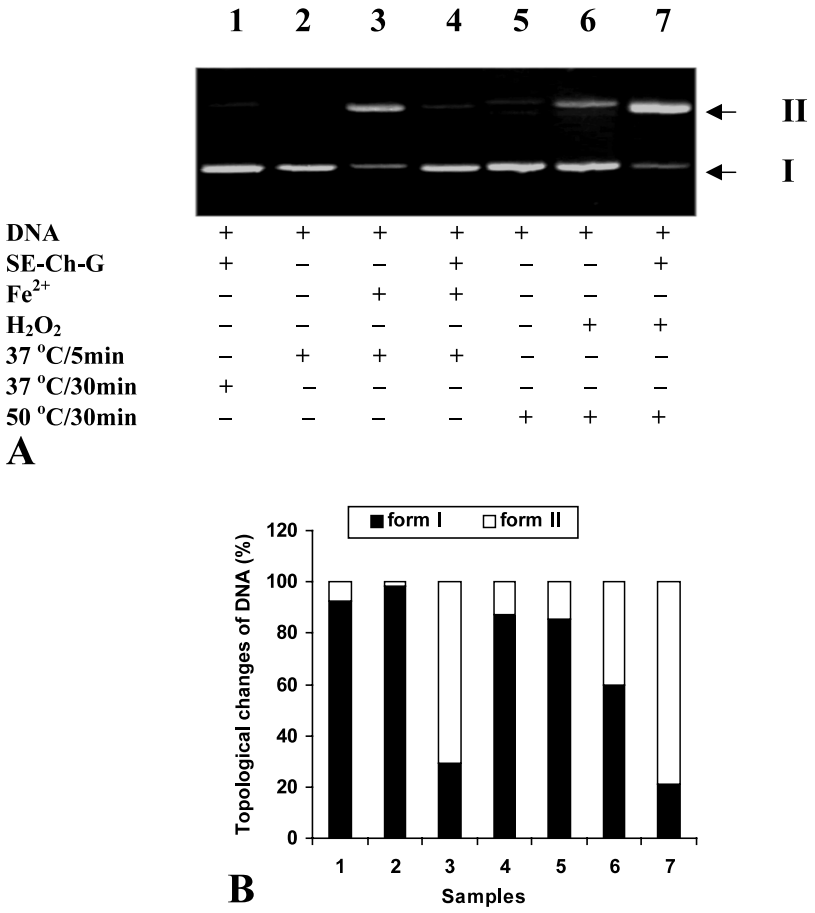

Fig. 2. Effects of SE-Ch–G on DNA breakage induced by Fe^{2+} and H_2O_2 were assessed by agarose gel-electrophoresis (A) and densitometry analysis (B). Plasmid DNA (0.2 g) was incubated in 10 mmol sodium phosphate buffer containing 1 mg SE-Ch–G/mL (samples 1, 4, and 7) in the presence of either 10 μmol Fe^{2+} (samples 3 and 4) or 100 μmol H_2O_2 (samples 6 and 7). The samples (final volume 20 μL, final pH 7.0) were incubated at 37 °C (samples 1–4) or 50 °C (samples 5–7) for 5 min (samples 2–4) and 30 min (samples 1 and 5–7). Lanes in the Fig. 1(A) correspond to the respective bars in the Fig. 1(B).

free-radical scavenger. Its DNA-protective activity can therefore be assigned to chelating of ferrous ions only. The observed difference between CM-Ch–G and SE-Ch–G can be probably ascribed to the role of the sulfoethyl substituent. The mechanism by which CM-Ch–G induced DNA damage needs further clarification. Moreover, since the observed effects took place at physiological temperature (37 °C), and Ch–G is a common component of yeasts and fungi often occurring in the surrounding environment, both as saprophytes or pathogens, our observations can therefore be of biological importance.

3. Experimental

3.1. Isolation of the crude Ch–G complex

The crude, water-insoluble Ch–G complex was isolated from the cell walls of the industrial strain *A. niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovak Republic). The mycelium was subjected to a hot alkaline (1 mol NaOH, 60 °C) digestion for 1 h. The alkali-insoluble sample was subsequently five times washed with distilled water, acetone, and finally with Et_2O. The dry sample contained 2.24% nitrogen which corresponded to a content of approximately 30% chitin.

3.2. Preparation of the water-soluble Ch–G complexes

Carboxymethylation of Ch–G was performed using the modified procedure[21] described by Sasaki and co-workers.[22] The degree of substitution of the carboxymethylated Ch–G thus obtained was 0.43 as determined by potentiometric titration with KOH.[23] Sulfoethylation was performed as described by Chorvatovičová and co-workers.[9] The content of sulfur in the prepared SE-Ch–G was 2.30%, which corresponded to the degree of substitution 0.26. In order to improve solubility of the obtained compounds, their ultrasonication was carried out using a procedure described by Chorva-

tovičová and co-workers.[24] The samples obtained upon ultrasonication were purified by gel-permeation chromatography, and their molecular weights (MW) were determined by HPLC as described in Chorvatovičová and co-workers.[24] From the original high-molecular-weight samples with MW approximately 600 kDa, the following samples were prepared: CM-Ch–G with MW 57 kDa and SE-Ch–G with MW 40 kDa. We have observed that prolonged ultrasonication led to an increase in chitin content in the resulting sonicated sample in comparison with the initial high-molecular-weight product.[21]

3.3. Chemicals and DNA

The DNA used was the plasmid pBR322 purchased from Advanced Biotechnologies Ltd. (UK) and was predominantly ($>95\%$) in the supercoiled form (as judged by agarose gel-electrophoresis). Iron sulfate ($FeSO_4 \cdot 7H_2O$) was used as a source of Fe^{2+}, hydrogen peroxide (30% H_2O_2) and mono- and disodium phosphates were of commercially available analytical reagent grade.

3.4. Reaction conditions for induction of DNA strand breakage

The reaction mixture (final volume 20 μL) contained 0.2 μg plasmid DNA (pBR322 30 μmol base-pairs) in 10 mmol sodium phosphate buffer (pH 7.0) and either CM-Ch–G alone or SE-Ch–G in combinations with Fe^{2+} or H_2O_2. Specific details of the incubation period and agent concentrations are given in the legends to the Figures. The reaction was started by the addition of the inductor of DNA breakage to the reaction mixture, and quenching of the reaction was achieved by immediate loading onto an agarose gel-electrophoretic system.

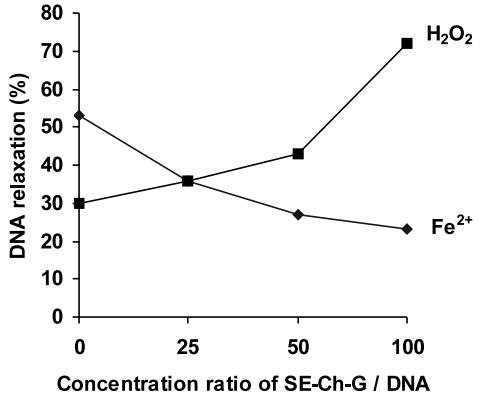

Fig. 3. Opposite effect of SE-Ch–G on the nicking of supercoiled pBR322 DNA induced by Fe^{2+} (◆) or H_2O_2 (■). Plasmid DNA (0.2 μg) was incubated in 10 mmol sodium phosphate buffer containing 0.00; 0.25; 0.50; 1.00 mg/mL of SE-Ch–G (concentration ratio of CM-Ch–G/DNA was 0.0; 25; 50; 100) plus 10 μmol Fe^{2+} for 5 min at 37 °C (◆) or 100 μmol H_2O_2 for 30 min at 37 °C (■), (final volume 20 μL, final pH 7.0). DNA strand-break formation was inhibited in an experiment with Fe^{2+} plus SE-Ch–G (DNA-protective effect) and increased in an experiment with H_2O_2 plus SE-Ch–G (DNA damaging effect). The percentage of relaxed DNA (form II) was plotted against the concentration ratio of SE-Ch–G/DNA. Results are the average from the triplicate samples (SD < 10%).

3.5. Analysis of DNA single/double-strand breaks

Plasmid DNA isomers migrated as discrete bands in agarose gel during electrophoresis (1.5% agarose, Sigma, USA; 60 min/60 V) in the minigel apparatus as described previously.[25] The DNA was made visible by staining with ethidium bromide (1 μg/mL, Sigma, USA) and UV illumination (Ultra-Lum Electronic UV Transilluminator, USA). DNA single-strand breaks were assayed by measuring the conversion of supercoiled DNA (form I) to relaxed circular DNA (form II), while double-strand breaks were estimated based on its conversion to linear DNA (form III). Percentages of supercoiled, relaxed, and linear DNA forms were calculated using a computer program (UTHSCA, Image Tool for Windows, Version 1.27). Since supercoiled DNA is restricted in its ability to bind ethidium bromide relative to relaxed circular and linear forms, it was necessary to correct values obtained for supercoiled DNA.[26,27] All experiments were repeated two or more times.

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References

1. Bohn, J. A.; BeMiller, J. N. *Carbohydr. Polym.* **1995**, *28*, 3–14.
2. Kogan, G. In: *Studies in Natural Products Chemistry. Bioactive Natural Products*; vol. 23, Part D, Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 2000; pp. 107–152.
3. Muzzarelli, R. A. A. *Carbohydr. Polym.* **1993**, *20*, 7–16.
4. Stagg, C. M.; Feather, M. S. *Biochim. Biophys. Acta* **1973**, *320*, 64–72.
5. Maeda, Y. Y.; Watanabe, S. T.; Chihara, G.; Rokutanda, M. *Cancer Res.* **1988**, *48*, 671–675.
6. Seljelid, R.; Scand, J. *Immunology* **1989**, *29*, 181–192.
7. Chorvatovičová, D. *Strahlenther. Onkol.* **1991**, *167*, 612–614.
8. Chorvatovičová, D.; Navarová, J. *Mutat. Res.* **1992**, *282*, 147–150.
9. Chorvatovičová, D.; Kováčiková, Z.; Šandula, J.; Navarová, J. *Mutat. Res.* **1993**, *302*, 207–211.

10. Chorvatovičová, D.; Šandula, J. *Mutat. Res.* **1995**, *346*, 43–48.
11. Chorvatovičová, D.; Machová, E.; Šandula, J. *Mutat. Res.* **1996**, *371*, 115–120.
12. Ohe, T. *Sci. Total Environ.* **1996**, *181*, 1–5.
13. Machová, E.; Kogan, G.; Chorvatovičová, D.; Šandula, J. *Ultrason. Sonochem.* **1999**, *6*, 111–114.
14. Patchen, M. L.; D'Alessandro, M. M.; Brook, I.; Blakely, W. F.; MacVitie, T. J. *J. Leukocyte Biol.* **1987**, *42*, 95–105.
15. Matsugo, S.; Mizuie, M.; Matsugo, M.; Ohwa, R.; Kitano, H.; Konishi, T. *Biochem. Mol. Biol. Int.* **1998**, *5*, 939–948.
16. Babincová, M.; Machová, E.; Kogan, G. *Z. Naturforsch.* **1999**, *54l*, 1084–1088.
17. Miller, D. M.; Buettner, G. R.; Aust, S. D. *Free Radical Biol. Med.* **1990**, *8*, 95–108.
18. Muiras, M. L.; Giacomoni, P. U.; Tachon, P. *Mutat. Res.* **1993**, *259*, 47–54.
19. Rauko, P.; Romanová, D.; Miadoková, E.; Macáková, K.; Novotný, L.; Elford, H. L.; Szekeres, T. *Anticancer Res.* **1997**, *17*, 3437–3440.
20. Romanová, D.; Vachálková, A.; Čipák, L.; Ovesná, Z.; Rauko, P. *Neoplasma* **2001**, *48*, 104–107.
21. Machová, E.; Kogan, G.; Šoltés, L.; Kvapilová, K.; Šandula, J. *React. Funct. Polym.* **1999**, *42*, 265–271.
22. Sasaki, T.; Abiko, N.; Nitta, K.; Takasuka, N.; Sugino, Y. *Eur. J. Cancer* **1979**, *15*, 211–215.
23. Rinaudo, M; Hudry-Clergeon, G. *J. Chem. Phys.* **1967**, *64*, 1746–1752.
24. Chorvatovičová, D.; Machová, E.; Šandula, J. *Mutat. Res.* **1998**, *412*, 83–89.
25. Rauko, P.; Novotný, L.; Balážová, E. *Neoplasma* **1993**, *40*, 283–288.
26. Reed, C. J.; Douglas, K. T. *Biochem. J.* **1991**, *275*, 601–608.
27. Ohshima, H.; Yoshie, Y.; Auriol, S.; Gilibert, I. *Free Radical Biol. Med.* **1998**, *25*, 1057–1065.