Radioprotective property of polysaccharide in *Tinospora cordifolia*

Mahesh Subramanian, Gajanan J Chintalwar and Subrata Chattopadhyay*

Bio-Organic Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

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Radioprotective activity of a polysaccharide preparation from the Indian medicinal plant, *Tinospora cordifolia* Miers has been established using *Saccharomyces cerevisiae* X2180 strain as the *in vivo* test model. The entire activity could be attributed to the radical scavenging capacity of the preparation, as it did not enhance the expression of the protective enzymes, catalase and superoxide dismutase in the yeast cells.

The plant, *Tinospora cordifolia* Miers (Menispermaceae) is considered to be a Rasayana, Medhya and anti-aging drug in Ayurveda and is used for the treatment of various ailments including jaundice, skin diseases, diabetes, anemia, emaciation and infections. The aqueous stem extract of the plant is reported to prevent abdominal infections and sepsis, improve Kupffer cell function and polymorphonuclear cell (PMN)-mediated phagocytosis in rats with chronic liver damage and patients of surgical jaundice, respectively. Recently, from the aqueous extract of the dried stem of the plant, we isolated a pure arabinogalactan polysaccharide (TP), which was found to be polyclonally mitogenic to mouse B-lymphocytes. Since many of the radioprotecting compounds operate via their immunomodulatory activity, it was of interest to assess the potential of the TP-enriched polysaccharide preparation (CTP) from *T. cordifolia*, in preventing radiation-induced damages to biomacromolecules and organism. Herein, we report the results of our study.

**Materials and Methods**

**Materials**

Ascorbic acid, 2-thiobarbituric acid (TBA), 2-deoxyribose (DR), yeast extract and peptone were obtained from Himedia Lab. Pvt. Ltd., India. EDTA and sodium carbonate were purchased from Sarabhai Chemicals, India, while FeCl₃ and trichloroacetic acid were from Thomas Baker, India. pBR 322 DNA was obtained from Bangalore Genei Ltd, India. H₂O₂ (35%) was purchased from Lancaster, England. Epinephrine, mannitol and agarose were purchased from Sigma Chemicals, U.S.A.

Stock solutions of ascorbate, FeCl₃, EDTA and H₂O₂ were prepared in deaerated water just prior to use. The test compounds TP or the semi-purified polysaccharide (CTP) were isolated as described previously and used as an aqueous solution. Stock solutions (1% w/v) of TBA were prepared in 50 mM NaOH solution and used within a week. All solutions were made with triply distilled water.

**Isolation of TP and CTP**

The test compounds were isolated from the aqueous extract of the powdered dry stem of the plant as described previously. In brief, the dry stem powder was extracted in boiling methanol followed by boiling water and the aqueous extract was successively treated with acetone and trichloroacetic acid to remove proteins and semi-polar compounds. Further treatment of the aqueous extract with acetone, followed by dialysis, centrifugation, lyophilization gave CTP from which TP was obtained by gel permeation chromatography over a Sephacryl S-400 column. TP (average molecular weight ~10⁶ Da) was found chemically homogenous by HP-GPC (Polysep GFC-D 5000 column, Phenomenex, water 0.7 ml/min, RI detector) analysis. The yield of CTP from the dry stem powder was 0.001%, while its TP content was 0.26%.

**γ-Ray induced DNA strand break assay**

This assay was carried out as described using pure TP, CTP and two other polysaccharides viz. soluble starch and guar gum. The DNA samples in presence or absence of the test samples were prepared in a final...
volume of 20 μl and irradiated at 25°C up to a dose of 1 Gy (dose rate 8 Gy/min) using a 60Co source. In all experiments, the concentrations of supercoiled pBR322 DNA was 10 mg/l in a 10 mM potassium phosphate buffer pH 7.4. The test compounds were added as aqueous solution to achieve the final concentration. After irradiation, the resulting forms of the plasmid, Form I (supercoiled) and Form II (open circular) were separated by electrophoresis, stained with ethidium bromide and visualized under UV light. The relative intensities of the bands were determined with a Bio-Rad gel documentation system.

2-Deoxyribose assay for hydroxyl radical scavenging assay
The reaction mixture contained 2.8 mM 2-deoxyribose (DR), 2.8 mM H2O2, 20 μM FeCl3 and 100 μM EDTA without or with (1-5 mg/ml) CTP in 10 mM potassium phosphate buffer pH 7.4. The reaction was triggered by addition of 100 μM ascorbic acid and the mixture incubated at 37°C for 60 min. The extent of DR degradation by hydroxyl radical was measured by the TBA method9 using appropriate blanks. Mannitol was used as a positive control in the experiments.

Yeast strain and cell culture
The study was conducted using Saccharomyces cerevisiae X2180 strain. In order to obtain single cell suspension, cells were grown on solid YEPDA plates containing yeast extract (1%), peptone (2%), dextrose (2%) and agar (2%). A single colony from plate was taken and suspended in 0.5 ml of normal saline and the cells were counted on a hemocytometer. Approximately 10⁶ cells were plated onto a fresh YEPDA plate and incubated at 30°C for 24 hr to get a lawn. Cells from the lawn were scraped and suspended in phosphate buffered saline and stored at 4°C till further use. This procedure gave single cell suspension with less than 2% budding cells and could be stored for more than 24 hr without any change in radiation response.

Irradiation conditions
An aliquot (1 ml) of yeast cell suspension (2×10⁵ cells/ml) was irradiated at ambient temperature with 60Co γ-rays in a γ-chamber at a dose rate of 11 Gy/min for the required period of time. After irradiation, cells were appropriately diluted and plated on YEPDA plates in triplicates. Plates were incubated at 30°C for at least 3 days and the numbers of colonies were counted.

Assay of the antioxidant enzyme levels in untreated and CTP untreated yeast
Yeast cells (5 mg/ml) were incubated for 12 hr in phosphate buffer pH 7.0 without or with CTP (10 mg/ml) under shaking conditions (120 rpm). The cells were harvested and lysed by grinding with glass beads. The extract was used for assay of different enzyme activities. The protein content of the extract was determined by Lowry’s method.

Estimation of superoxide dismutase (SOD)
The method of Sun and Zigman10 based on inhibition of auto-oxidation of epinephrine was followed. The absorbances at 320 nm were read continuously over a period of 5 min. The difference between ΔOD (standard) -ΔOD (enzyme) was taken as ΔA and SOD activity was calculated by considering 0.1 ΔA as three units of SOD.

Estimation of catalase
The assay of catalase was carried out following a known procedure11. Briefly, the decomposition of a fixed amount of H₂O₂ (1 ml, 10 mM) by the lysates (with same protein contents) obtained from untreated and CTP treated cells were measured from the decrease in absorbance at 240 nm. The difference in absorbance (ΔA₂₄₀) per unit time is a measure of the catalase activity. Concentration of the stock solution of H₂O₂ was measured using ε₂₄₀ = 0.00394±0.00021 l mmol⁻¹ mm⁻¹.

Results and Discussion
The deleterious biological consequences of both ionizing and non-ionizing radiations, especially with respect to causing mutation and carcinogenesis are well documented. The main cause of these effects is believed to be scission of DNA molecules by radiation12. A pre-requisite of radical-mediated DNA strand break is the degradation of its sugar moiety via a direct or indirect process13. Due to the high concentration of water in metabolizing cells, radiation exposure of biological systems primarily leads to its radiolysis furnishing e⁻, •OH and H•14. These primary radicals which subsequently damage the sugar moiety finally leading to DNA strand breakage. In aerobic cells, the electron is readily accepted by the easily reducible oxygen molecule generating various reactive oxygen species (ROS), all of which can damage DNA. However, amongst these, the hydroxyl...
radicals are believed to contribute maximum in DNA cleavage\textsuperscript{16,17}.

Based on our recent findings\textsuperscript{18} on the extraordinary high reactivity of TP with hydroxyl radicals, it appeared to be a promising candidate in protecting radiation-induced DNA damage. Hence, its protective capacity against the $\gamma$-ray induced single strand break (ssb) of plasmid pBR322 DNA was studied.

Exposure of pBR322 plasmid DNA to $\gamma$-radiation, as a function of dose, resulted in a significant increase in the single strand break (ssb) of DNA (Fig. 1). A linear increase in ssbs, assessed as average strand breaks per DNA molecule, was observed upto a dose of 1 Gy. The radiation dose of 1 Gy was sufficient to convert the entire supercoiled DNA to open circular form. Hence, this dose was chosen for further studies.

Fig. 1—Gel-electrophoretic pattern of $\gamma$-irradiated pBR322 DNA as a function of dose [Lane 1, DNA without irradiation; lanes 2-5, DNA after irradiation for 10, 20, 30 and 40 sec, respectively at a dose rate of 0.75 Gy/min. The detailed experimental conditions are given in Materials and Methods]

Addition of TP in increasing concentrations to the DNA, prior to irradiation, progressively reduced the intensity of the band due to the open circular form. Even at a very low concentration of 6 mg/ml, TP could provide 39.2±3.2% (n=4) protection of plasmid DNA against $\gamma$-ray induced ssb as revealed from the increase in its supercoiled form.

Thus, TP could effectively prevent $\gamma$-ray induced damage to plasmid DNA in a concentration-dependent manner. Considering its solubility in water and its wide utility in oriental system of medicine, we studied its radioprotecting capacity using \textit{Saccharomyces cerevisiae} X2180 yeast strain as the \textit{in vivo} test model. However, practical application of TP as an effective radioprotectant is considerably handicapped due to its low natural abundance and the tedious process of its isolation in pure form. In contrast, a semi-pure TP-enriched polysaccharide fraction (CTP) can be easily obtained from the aqueous extract of the stem of the plant by protein removal, dialysis and lyophilization. Hence, from practical point of view, we chose CTP for the \textit{in vivo} study.

Given the importance of hydroxyl radicals in radiation-induced cellular damage, the scavenging activity of CTP against these radicals was estimated using the 2-deoxyribose assay\textsuperscript{9}. Recently, we have found that the pure polysaccharide, TP scavenges the hydroxyl radicals with a high rate constant, not commonly encountered with water soluble antioxidants\textsuperscript{18}. In the present study, in an unstimulated experiment, TBARS formation was less (Absorbance\textsubscript{532} 0.13±0.06, n = 4). However, addition of ascorbic acid triggered their generation significantly (Absorbance\textsubscript{532} 0.61±0.01, n = 4). As shown in Fig. 3, CTP inhibited the degradation of 2-deoxyribose almost linearly with respect to its concentration with an IC\textsubscript{50} value of 3.88±1.2 mg/ml (n = 4) which was almost similar to that of pure TP\textsuperscript{18}. In comparison, the IC\textsubscript{50} value of mannitol under similar experimental conditions was 8.43±1.4 mg/ml (n = 4).

Before carrying out the \textit{in vivo} study with CTP, its capacity to protect plasmid pBR322 DNA against the $\gamma$-ray induced single strand break (ssb) was also ascertained and the results compared with those of TP. The experiments were carried out using four different concentrations (0.5, 1.5, 3.0 and 4.5 mg/ml) of CTP under the same conditions as used for TP and the results are shown in Fig. 4. It was found that like TP, the polysaccharide fraction, CTP also prevented the $\gamma$-ray induced DNA strand breakage in a
concentration-dependent manner. In fact, the protective activity of CTP was better than that of TP, possibly due to the presence of some other polysaccharides. For example, the percentage protections offered by 3.0 and 4.5 mg/ml of CTP were 34.4±1.8 and 37.8±2.2%, while those obtained with the same concentrations of TP were 22.8±3.2 and 28.2±2.6%, respectively.

At this point, we wanted to ascertain whether the above DNA radioprotection was a general phenomenon with any polysaccharide or specific to those present in *T. cordifolia*. Accordingly, the protective activities of CTP, soluble starch and guar gum against γ-ray induced pBR322 DNA cleavage were also assayed at two different concentrations (0.5 and 2.0 mg/ml) of each of the test compounds. As shown in the results (Fig. 5), while CTP showed significant protective activity even at low concentrations (0.5 and 2.0 mg/ml), the protection offered by both soluble starch and guar gum were almost negligible at these concentrations. These observations show that the DNA radioprotective activity of CTP is due to its constituent polysaccharide.

In view of the above promising results, the *in vivo* radioprotecting activity of CTP was subsequently carried out. Fig. 6 shows the effect of γ-radiation dose on the extent of survival of *S. cerevisiae* X2180 cells in the absence and presence of CTP. In the absence of CTP, there was a sharp drop in the percentage of survival of the yeast cells as the radiation dose was increased from 200 to 1000 Gy. CTP at a concentration of 10 mg/ml enhanced the survival of the yeast cells significantly over the entire dose range, although the effect was more significant at the higher dose range. For example, at a dose of 200 Gy, the survival was 62.56±5.33% for the untreated yeast cells, CTP increased the survival to 76.28±5.26%. At a higher dose of 750 Gy, however, the percentage of cell survivals were 11.20±3.71 and 38.14±3.65 for the untreated and CTP treated cells, respectively. The
results of Fig. 6 indicate a significant dose-modifying factor of 1.36 for CTP. It is well known that endogenous antioxidant enzymes like SOD, and catalase act as endogenous defense mechanisms against the ROS-mediated biological damages. Any radioprotectant, thus, can exert its action by ROS scavenging and/or inducing the generation of the above enzymes \textit{in vivo}. Consequently, in order to probe into the mechanism of radioprotective action of CTP, its possible role in upregulating the levels of SOD and catalase in the yeast cells was also investigated. Earlier, it has been reported that radiation alone, does not trigger SOD level in yeasts, while it can inactivate both SOD and catalase. The effects of the 12 hr incubation of the yeast cells with CTP (10 mg/ml) on the levels of SOD and catalase are shown in Table 1. As evident from the results, the CTP treatment did not have any significant effect on the levels of the protective enzymes in the yeast cells. Thus, the radioprotective activity of CTP can be entirely attributed to its superior radical scavenging activity.

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<thead>
<tr>
<th>Enzyme Activity</th>
<th>Untreated cells</th>
<th>Treated cells</th>
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<tbody>
<tr>
<td>Superoxide dismutase</td>
<td>10.72±2.6 U/mg</td>
<td>10.20±1.8 U/mg</td>
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<tr>
<td>Catalase</td>
<td>2.76±0.93 k/l</td>
<td>2.59±0.81 k/l</td>
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Table 1—Modulation of SOD and catalase levels in \textit{S. cerevisiae} X2180 cells by CTP

[Yeast cells were incubated for 12 hr in phosphate buffer pH 7.0 without or with CTP (10 mg/ml) under shaking conditions. The harvested cells were lysed and assayed for their SOD and catalase contents. The detailed experimental conditions are given in Materials and Methods. Values are mean±S.E. (n = 4)]

References

10. Sun M & Zigman S (1978) \textit{Anal Biochem} 90, 81-89