Porphyran Reduces Cytotoxicity Induced by Two Carcinogens in *Allium sativum* Root Cells

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Abstract: Studies have shown that porphyran extracted from the red algae *Porphyra haitanesis* has a potential antioxidant activity. The effect of porphyran and its sulfate derivatives on cyclophosphamide (CP)- and mitomycin C (MMC)-induced changes in micronucleus rate, mitotic index, chromosome aberration rate, and lipid peroxidation in *Allium sativum* root cells was investigated. The results indicated that the polysaccharide significantly inhibited the micronucleus rate and chromosomal aberration rate, and significantly enhanced the mitotic index in a dose dependent manner. The polysaccharide exhibited the highest inhibitory and enhancing effects at 100 mg/L concentration. Additionally, the sulfate derivative exhibited superior resistance towards lipid peroxidation in cells compared to that exhibited by the unmodified polysaccharide. The analysis reveals that the anti-carcinogenic effect of porphyran has a definite relationship with anti-lipid peroxidation activity, and shows that the sulfate group has an indirect effect on anti-carcinogenic activity. The results showed that porphyran has an anti-carcinogenic potential.

Key words: porphyran; *Allium sativum* root; cyclophosphamide; mitomycin C

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坛紫菜多糖对两种突变剂诱导的大蒜根尖细胞损伤的保护作用

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摘要：研究发现从红藻坛紫菜中提取的多糖具有优异的抗氧化活性。本文研究了坛紫菜多糖及其硫酸衍生物对两种突变剂环磷酰胺和丝裂霉素 C 诱导的大蒜根尖细胞微核率、有丝分裂指数和染色体畸变率的影响作用，以及抗脂质过氧化作用。结果表明，两种多糖均表现出明显地抑制微核和染色体畸变作用，明显地提高有丝分裂指数。其影响作用具有明显的剂量依赖性。当多糖浓度为 100 mg/L 时，多糖表现出最大抑制效应或增强效应。同时，硫酸化衍生物表现出比原料多糖更优异的抗细胞内脂质过氧化作用。分析表明坛紫菜多糖表现出的抗突变作用与抗脂质过氧化作用具有一定关系。同时也表明硫酸基对于抗突变作用的间接影响。结果显示坛紫菜多糖具有成为抗突变活性物质的潜力。

关键词：坛紫菜多糖；大蒜根尖；环磷酰胺；丝裂霉素-C

1 Introduction

DNA mutations include point (base pair) mutations caused by different mutagenic agents, including alkylating agents, ions, enzymes and ultraviolet radiation [1]. Among these mutagenic agents, alkylating agents are commonly used in cancer chemotherapy to damage the DNA of cancer cells. Cyclophosphamide (CP) and mitomycin-C (MMC) are common DNA alkylating agents used in the treatment of various types of tumors, organ transplant rejection, as well as in the treatment of autoimmune diseases[2,3]. However, this two drug therapy is often very toxic[4,5]. Alkylated DNA does not coil or uncoil properly, and cannot be processed by information-decoding enzymes. These mutations arise from DNA damage that is not repaired or to RNA genomes (typically caused by radiation or chemical mutagens)[6]. Therefore, a study on the inhibition of mutations induced by alkylating agents is important in the discovery of antitumor drugs.

CP has been reported to be excreted as 4-hydroxy metabolites, in particular acrolein, which cause an increase in ROS (superoxide radical $O_2^{-}\cdot$) production in the bladder epithelium[7]. MMC, on the other hand, can

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react rapidly with molecular oxygen to regenerate the parent prodrug under aerobic conditions, but at the same time produce superoxide ($O_2^{-}\cdot$) and hydrogen peroxide (OH·), which can cause oxidative damage[8]. Several reports have recently described the positive correlation between high free radical scavenging activity and high antimutation activity[9]. Antioxidants can prevent the damage caused by free radicals in the body. Therefore, many chemical antioxidant drugs are used against the oxidative stress caused by CP and MMC. Recently many natural antioxidants from plant or seaweed sources were shown to be chemoprotective agents against the DNA damage mediated by reactive oxygen species[4,10,11].

Much research has shown that polysaccharides possess excellent antioxidant activity[12]. Much attention has also been given to understanding the relationship between antimutation and free radical scavenging activity of polysaccharides. Several polysaccharides with high antioxidant activity have also shown high antimutation activity[13,14]. In a previous study, porphyran extracted from the red algae, *Porphyra haitanensis*, showed excellent antioxidant activity[15]. To the best of our knowledge, however, no study has been carried out to investigate the antimutation activity of porphyran. Therefore, the aim of the current study was to investigate the effect of porphyran on DNA damage induced by the carcinogens CP and MMC *in vitro*.

2 Materials and methods

2.1 Sampling

Red algae samples (*Porphyra haitanensis*) were collected in the Wenzhou region, China in 2014. The samples were rinsed with sea water followed by distilled water, were dried in the shade and ground to a fine powder.

2.2 Chemicals

Cyclophosphamide (CAS: 6055-19-2) was purchased from Sigma Chemical Co. Mitomycin-C (CAS: 50-07-7), was purchased from Sinopharm Chemical Reagent Co., Ltd, China. All other chemicals and reagents, unless otherwise specified, were of analytical grade.

2.3 Polysaccharide and derivatization

The natural polysaccharide porphyran was extracted by the hot-water method as described previously [16]. The degraded porphyran was prepared with the method of H_2O_2/Vc (named LP). Then LP was sulfated with $HClSO_3$ dissolved in formamide to give the sulfated polysaccharide (SLP)[17].

Total sugar content was determined by phenol-sulfuric acid method[18]. Sulfate content was determined by barium chloride method[19]. 3, 6-Anhydrogalactose content was determined as described previously[20].

2.4 Root tips preparations

In present study garlic (*A. sativum*) cloves were selected. Garlic heads of the same size were immersed and germinated at 22 ℃ for 24 h. Subsequently, they were placed between two layers of moist cotton and allowed to germinate. When the roots tips of 1.50~2.00 cm in length emerged, they were ready to move onto the next step.

2.5 Treatments

Preliminary dose selection experiments were conducted for each carcinogen with concentration ranges derived from the literature [2]. The appropriate concentration for each compound was determined from a curve of percentage of cloves that germinated against dose.

There were three treatment categories including a negative control group and a positive control group. Each group consisted of at least 6 roots and three replicates were used per treatment. All experimental groups were kept in an incubator at 22±1 ℃. After each treatment for 4 h, the roots were rinsed 3 times with distilled water and then maintained in tap water for a 24 h recovery period. The root tips of 1.50~2.00 cm in length were cut off and fixed overnight in Carnoy's solution (glacial acetic acid/ethanol=1:3 *V/V*) for 24 h and were subsequently stored in 70% ethanol[21].

2.6 Micronucleus, mitotic index and chromosomal aberration assay

For evaluating genotoxicity, all roots tips were hydrolyzed in 1 N HCl at 60 ℃ for 10 min. Five apical parts of each root tip from each garlic plant were placed on a slide and stained with Schiffs' reagent. The slides were coded and examined blind. A total of 5000 cells from five separate slides per experimental group were scored to determine the mean micronuclei frequencies (MCN ‰). For calculating the mitotic index (MI), the treated roots were fixed immediately after treatment without a recovery period. The MI was determined by counting the number of mitotic cells among the total number of scored cells per seedling. The chromosomal aberration rate (CAR) was expressed in terms of 1/1000 scored cells. Approximately 5000 cells were scored from five separate seedlings for each treatment and control group. The following aberrations were scored: bridges, fragments, vagrant chromosomes, multipolarity and c-mitoses.

2.7 Lipid peroxidation

This assay was carried out using the 2-thiobarbituric acid (TBA) method. In brief, after being washed in water, the treated roots were cut into small pieces and then homogenized. Trichloroacetic acid (TCA) solution (5%, *m/V*) was added to the treated roots, which were then incubated at 37 ℃ for 1 h, followed by centrifugation. TBA (0.68%, *m/V*) was added to the supernatant and each sample was incubated at 95 ℃ for 30 min. After centrifugation, the absorbance of the mixture was recorded at 532 nm. The malonaldehyde (MDA) content was expressed as μmol per gram fresh weight (mol/g FW) [22].

2.8 Statistical analysis of data

Sample means and standard deviations were analyzed statistically with the *F*-test for analysis of variance (ANOVA). Dunnett's test was used to determine the level of significance against the negative control values in each experimental series.

3 Results

3.1 The chemical characteristics of LP and SLP

The polysaccharide and its sulfated form, LP and SLP, were selectively prepared and their chemical analysis results are shown in Table 1. From the table, the sulfate content increased after mollification.

Table 1 The chemical characteristics of LP and SLP

Sample	Yield/%	Total sugar/%	Sulfate/%	3,6-Anhydrogalactose/%
LP	---	79.65±4.22	15.11±1.94	9.57±0.37
SLP	75.7	68.58±5.10	28.40±2.65	6.82±0.63

3.2 The MCN test

Table 2 Inhibition of micronuclei rate in cells by LP and SLP

Sample	Concentration/(mg/L)	CP Test		MMC Test	
		MCN/1000 cells	Inhibition/%	MCN/1000 cells	Inhibition/%
LP	1	5.54±1.24	1.42±0.32	20.08±6.67	7.55±2.43
	10	4.98±1.08	11.39±1.79	18.64±5.86**	14.18±4.97**
	20	4.72±0.97	16.01±3.44	14.33±6.46**	34.02±6.82**
	50	3.66±1.73**	34.88±6.63**	13.99±5.90**	35.59±7.67**
	100	3.02±1.27**	46.26±8.32**	10.71±5.45**	50.69±9.36**
SLP	1	5.31±1.16	5.52±0.90	18.23±6.14**	16.07±3.88**
	10	4.63±1.44*	17.62±4.27*	15.52±6.09**	28.55±5.30**
	20	3.87±1.21**	31.14±5.85**	10.66±6.24**	50.92±7.12**
	50	3.12±0.89**	44.48±7.94**	8.48±4.75**	60.96±8.25**
	100	2.25±1.62**	59.96±9.03**	6.55±3.37**	69.84±10.46**
Ascorbic acid	10	2.04±0.85**	63.70±9.62**	5.73±3.11**	73.53±11.29**

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Negative control	0	0.65±0.25	
CP control		5.62±1.80	
MMC control			21.72±5.36

Note: *p < 0.05, **p < 0.01 versus positive control.

Preliminary dose selection experiments showed that the two carcinogens CP and MMC could induce significant (p<0.05 and/or 0.01) levels of genotoxicity at one or more doses to garlic root tip cells. According to the results, the experimental concentrations of CP and MMC were determined to be 5 and 0.5 μg/mL.

As shown in table 2, a significant decrease in MCN frequency was observed in roots exposed to 1~100 mg/L polysaccharides and one of the carcinogens. The dose-response relationship for the MCN inhibition rate showed a large increase as expected. The differences between each group were statistically significant compared with the positive control. When the concentrations of LP and SLP were at 100 mg/L, the highest MCN inhibition rate was observed. For the inhibition in CP test, IC50 of LP was not determined while that of SLP was 72.34 mg/L. For MMC test, IC50 of LP and SLP was 97.56 and 19.22 mg/L. However, SLP showed a higher inhibition rate than LP. The above analysis showed that LP and SLP could effectively inhibit MCN frequency induced by CP and MMC.

3.3 Mitotic index

Table 3 Effects of samples on mitotic index of cells of the root tips

Sample	Concentration /(mg/L)	CP Test	MMC Test
LP	1	12.23±3.19	8.78±1.36
	10	14.52±3.77**	9.98±1.83*
	20	16.75±4.25**	11.05±2.21**
	50	17.86±4.56**	13.43±2.88**
	100	19.67±4.82**	16.70±3.35**
SLP	1	13.45±3.43*	10.02±2.42*
	10	16.65±4.90**	11.44±3.27**
	20	18.71±3.78**	14.12±3.09**
	50	20.20±5.23**	16.67±3.44**
	100	22.43±5.67**	18.53±4.50**
Ascorbic acid	10	25.08±6.34**	22.37±5.47**
Negative control	0	16.75±4.33	
CP control		12.17±3.84	
MMC control			8.54±1.29

Note: *p < 0.05, **p < 0.01 versus positive control.

Table 3 shows that LP and SLP can increase the MI in root tips. From the table, CP and MMC induced a delay in mitosis at the applied concentrations. However, MI decreased with increasing polysaccharide concentration and showed significant differences between the control and treatment groups after exposure to the carcinogen and polysaccharide. A significantly improved rate of MI versus positive control was detected after exposure to 100 mg/L LP and SLP. Therefore, two samples could significantly inhibit the mutagenesis of CP and MMC, slow genetic damage to cells, and promote the normal division of root tip cells.

3.4 Chromosomal aberration

Table 4 Inhibition of chromosomal aberration in cells of the roots tips by LP and SLP

Sample	Concentration /(mg/L)	CP Test	MMC Test
LP	1	7.43±1.65	12.21±3.59
	10	6.03±1.33**	11.50±3.26*
	20	4.32±0.86**	9.41±3.44*
	50	2.96±0.54**	6.56±2.17**
	100	2.78±0.22**	4.47±1.44**
SLP	1	6.69±1.58	11.38±3.63*
	10	4.81±1.10**	10.42±3.20**
	20	3.63±0.11**	7.73±2.41**
	50	1.79±0.32**	4.77±1.24**
	100	1.08±0.07**	2.12±1.55**
Ascorbic acid	10	0.12±0.01**	1.08±0.32**
Negative control	0	1.48±0.24	
CP control		7.64±1.15	
MMC control			12.95±3.45

Note: *p < 0.05, **p < 0.01 versus positive control.

Results of genotoxicity tests along with the chromosomal aberration test are given in Table 4. The results showed that the polysaccharides had no effect on the anaphase aberrations at the bottom of the root tip cells. It was noteworthy that CP and MMC showed significant chromosomal aberration, among which fragmentation was the most prominent type. From the table, LP and SLP also possessed significant inhibition (p<0.05 and/or 0.01) on CAR in a dose dependent manner compared to the negative control. These two samples could inhibit teratogenic effects of CP and MMC on root tip cells.

3.5 Lipid peroxidation

The antioxidant activities of LP and SLP evaluated by the lipid peroxidation assay in *A. sativum* root cells and the results are shown in Table 5. As shown in the table, MDA content decreased significantly in roots exposed to a low concentration of polysaccharide (20 mg/L). SLP showed higher anti-lipid peroxidation activity than LP and the differences of between each group were statistically significant.

Table 5 Effect of samples on MDA content in cells of the root tips

Sample	Concentration /(mg/L)	CP Test	MMC Test
LP	1	5.66±1.34	6.08±1.88
	10	5.12±1.25	5.89±1.04
	20	4.60±0.78*	5.23±1.66*
	50	4.22±1.31*	4.70±0.82*
	100	3.67±0.74*	4.16±1.58**
SLP	1	4.85±1.92	5.76±1.69
	10	3.77±1.41**	4.38±1.22**
	20	2.37±0.83**	3.94±0.63**
	50	1.99±0.79**	3.30±0.08**
	100	1.50±0.16**	2.79±0.67**
Ascorbic acid	10	0.34±0.01**	0.95±0.04**
Negative control	0	1.25±0.05	
CP control		5.83±1.68	
MMC control			6.73±1.31

Note: *p<0.05, **p<0.01 versus positive control.

4 Discussion

Micronuclei are the result of acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of mitotic cells[23]. MI is an important prognostic factor predicting both overall survival and response to chemotherapy in most types of cancer[24]. CAR tests play a central role in testing for the mutagenic/carcinogenic potential of chemicals in most countries and CAR is an important index to judge normal or aberrant cell mitosis[25]. In present study, MCN and CAR decreased gradually with a gradual increase in MI in the treatment group. At a concentration of 100 mg/L, LP and SLP showed the strongest repair activity, suggesting that MI was the highest and MNR and CAR were the lowest at this concentration. This suggests that the two polysaccharides can enhance the cellular replication and DNA repair to inhibit MNR and CAR.

Reactive radicals in oxidative stress caused by chemotherapeutics can damage cells and tissues[26]. For example, CP increased the levels of acid phosphatase (ACP), alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) in the liver, which is indicative of toxic effects on a critical metabolic organ[27]. MMC may also be responsible for the generation of free radicals, such as superoxide and hydroxyl radicals, which can lead to DNA strand breaks[28]. CP significantly increased the extent of lipid peroxidation in the livers of experimental animals[29], with MDA as the most common breakdown product noted after lipid peroxidation. Induced ROS can attack purine and pyrimidine bases in DNA causing DNA strand breaks, which can increase the probability of chromosome/chromatid fragmentation, thereby leading to MN formation[30]. In the present study, MN frequency was associated with increased lipid peroxidation, indicating that MN formation is dependent upon carcinogen-induced oxidative stress. Previous studies indicated the antioxidant had activity probably due to either direct antioxidant effects or effective free radical scavenging[31] or its protection against lipid peroxidation and ROS were due to replenishing the GSH pool and decreasing ROS formation[32]. In this study, treatment with polysaccharide and carcinogen restored the level of MDA to almost control levels, most likely by the latter mechanism.

Biological membranes contain polyunsaturated fatty acids (PUFAs) and OH^- ions can remove hydrogen (H) atoms from PUFA, forming $PUFA^-$ radicals. O_2 attacks such radicals, creating peroxyl radicals ($PUFAOO^-$),

which in turn extract H atoms from adjacent PUFAs, triggering a chain reaction[11]. The polysaccharides used in this study may donate an H atom to $PUFAOO^{\cdot}$, forming a stable lipid species, which preserved the PUFA in the cell membrane and protected low-density lipoproteins from lipid peroxidation. In addition, many studies indicated that the antioxidant activity of different polysaccharide fractions correlates directly with increasing sulfate group content[33, 34]. In this study, SLP had higher anti-lipid peroxidation activity probably due to strong proton-donating ability and could serve as a free-radical inhibitor or scavenger, acting possibly as a primary antioxidant.

5 Conclusion

Evidence obtained from the present study indicates that treatment with polysaccharides was effective in partially preventing CP and MMC-induced damage (lipid peroxidation and restore MCN, MI in testes), and its effect is probably due to high anti-lipid peroxidation activity. The sulfated polysaccharide SLP showed higher inhibition rate of DNA damage than LP due to strong proton-donating ability. However, more studies are needed to understand the mechanism of porphyran in relation to its beneficial effects and its possible interaction with anticancer drugs.

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