Carbohydrate Polymers 98 (2013) 108–115

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

In vitro evaluation of antioxidant defense mechanism and hemocompatibility of mauran

Sreejith Raveendran[a], Vivekanandan Palaninathan[a], Neha Chauhan[a], Yasushi Sakamoto[b], Yasuhiko Yoshida[a], Toru Maekawa[a], P.V. Mohanan[c,*], D. Sakthi Kumar[a,*]

[a] *Bio-Nano Electronics Research Centre, Graduate School of Interdisciplinary New Science, Toyo University, Kawagoe, Saitama 350-8585, Japan*
[b] *Saitama Medical University, Biomedical Research Centre, Division of Analytical Science, Saitama 350-0495, Japan*
[c] *Sree Chitra Tirunal Institute for Medical Sciences and Technology (Government of India), Toxicology Division, BMT Wing, Thiruvananthapuram 695012, Kerala, India*

ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form 3 April 2013
Accepted 12 April 2013
Available online xxx

Keywords:
Mauran
Extremophiles
Oxidative stress
Antioxidant
Antithrombogenic
Antihemolytic
Halomonas
Polysaccharides

ABSTRACT

Mauran (MR), a highly polyanionic sulfated exopolysaccharide was extracted from moderately halophilic bacterium; *Halomonas maura* and characterized using X-ray photoelectron spectroscopy and Fourier transform infrared spectroscopy. Purified MR was evaluated for antioxidant defense mechanisms under *in vitro* conditions using L929, mouse fibroblast cell line and mice liver homogenate. It was demonstrated that MR could impart protective effect against oxidative stress in both cells and tissue up to a concentration of 500 μg, which is found to be safe under laboratory conditions. Various enzymatic and non-enzymatic parameters of antioxidant mechanisms were evaluated and concluded that MR has the tendency to maintain a balance of antioxidative enzymes with in the test systems studied. Also, hemocompatibility assay performed revealed that MR has a lesser hemolytic index and exhibited a prolonged clotting time, which shows both antihemolytic, and antithrombogenic nature respectively. Furthermore, absorption studies performed using fluorescent-labeled MR confirmed that MR accumulated within the cell cytoplasm neither induced cellular lysis nor affected the cell integrity.

1. Introduction

Extreme habitats are well known for its endless source of industrially important molecules (Galinski & Tindall, 1992). Microorganisms that are able to thrive under extreme stress conditions are studied utmost in the field of modern pharmaceutical research. *Halomonas* are a class of moderately halophilic bacteria that can with stand 5–25% of salt concentration (Llamas et al., 2006; Mata et al., 2006; Argandona et al., 2005). *Halomonas maura* can produce a versatile, polyanionic, sulfated exopolysaccharide called mauran (MR) (Bouchotroch, Quesada, Moral, Llamas, & Bejar, 2001; Arco et al., 2005). This acidic polysaccharide has unique physicochemical properties like stable structure, composition, fluid dynamics, extreme stability, biodegradability and biocompatibility (Arias et al., 2003); they are widely exploited in modern biotechnology, material science and more recently in nanotechnology (Raveendran, Dhandayuthapani, et al., 2013). Owing to their exceptionally best viscoelastic properties and fluid dynamics, mauran has been successfully employed in the nanoparticle synthesis and application for sustained drug delivery, cancer chemotherapy and bioimaging (Raveendran, Poulose, Yoshida, Maekawa, & Kumar, 2013). MR based tissue-engineering nanofiber scaffolds were investigated for enhanced cell proliferation and migration under *in vitro* conditions using various mammalian cell lines (Raveendran, Dhandayuthapani, et al., 2013). As a widely accepted biomolecule, with a lot of potential applications in the biomedical and pharmaceutical industries, mauran can be studied as a vital candidate biodrug for evaluation of various bioactivities (Llamas et al., 2006).

Polysaccharides with high sulfate contents are generally known for various biopharmaceutical activities like anticancer, antiviral, antiparasitic, anticoagulant, and antioxidant properties (Kodali, Perali, & Sen, 2011; Raveendran, Yoshida, Maekawa, & Kumar, 2013; Toshihiko, Amornrut, & Robert, 2003). However, evaluation of a bacterial polysaccharide for specific bioactive mechanisms are always challenging due to their immunogenic nature. In spite of being a polysaccharide of bacterial origin, MR has an excellent biocompatibility and biodegradability with enhanced cell proliferation effect. Furthermore, MR has been widely employed in the commercial level for industrial, food and pharmaceutical applications. Medically they are found important since they posses'

* Corresponding authors. Tel.: +81 49 239 1375; fax: +81 49 234 2502.
E-mail addresses: mohanpv10@gmail.com (P.V. Mohanan), sakthi@toyo.jp, drsakthi@gmail.com (D.S. Kumar).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.04.050

immunomodulating and antiproliferative effect on cancer cells (Llamas et al., 2006). Enhanced accumulation of free radicals with in the cells causes abnormal necrosis and cell death. The phenomenon of an excess free radical generation and related abnormal tissue mechanisms causes oxidative stress (Surendran, Geetha, & Mohanan, 2012). As a result, various macromolecules like DNA, proteins and lipids are damaged nonspecifically (Anjana, Tinu, Geetha, & Mohanan, 2012; Mohanan & Yamamoto, 2002). Natural antioxidant defense mechanism can be restored and ameliorated with the help of various biologically active polysaccharides irrespective of their source of extraction (Guerra Dore et al., 2013; Kodali et al., 2011 Silva et al., 2005; Wang, Wang, Liu, Yuan, & Yue, 2013; Yan et al., 2012). The major enzymes that play a vital role in antioxidant defense mechanisms by catalyzing various free radicals include glutathione reductase, glutathione peroxidase and superoxide dismutase. These enzymes differ in their structure, tissue distribution and cofactor requirement (Arun, Silija, Sheeja, Geetha, & Mohanan, 2012).

In the present study, we are evaluating the protective antioxidant effect of MR by analysis of various parameters responsible for oxidative stress in mammalian cells and tissues separately. Apart from that hemocompatibility and absorption studies were also concentrated.

2. Materials and methods

2.1. Chemicals and reagents

Thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), glutathione reduced (GSH), glutathione oxidized (GSSG), dithio-bis-2-nitrobenzoic acid (DTNB), hydrogen peroxide (H_2O_2), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and ethylene diamine tetra acetic acid (EDTA) were procured from Sigma–Aldrich, USA and BCA reagent kit (Qiagen, Germany). Sypro-Ruby was purchased from Invitrogen, USA. All other chemicals and reagents used were of analytical grade.

2.2. Equipments

Rotor stator homogenizer at 1000 rpm, Refrigerated centrifuge (Himac, CF12RX), UV–Visible spectrophotometer (CU730, Beckman Coulter) and Confocal laser scanning microscope (Olympus 81 under DU897 mode).

2.3. Bacterial culture and MR production

The strain was grown in MY medium as mentioned elsewhere (Arias et al., 2003). Briefly, the growth medium composition: NaCl, 51.3 g; $MgCl_2{\cdot}6H_2O$, 9 g; $MgSO_4{\cdot}7H_2O$, 13 g; $CaCl_2{\cdot}2H_2O$, 0.2 g; KCl, 1.3 g; $NaHCO_3$, 0.05 g; NaBr, 0.15 g; $FeCl_3{\cdot}6H_2O$, traces; Glucose, 10 g; Yeast extract, 3 g; Malt extract, 3 g; Proteose peptone, 5 g; Trace salt solution, 0.00325 g. Bacto agar (2 g/L) was added for the preparation of solid medium. Liquid medium was prepared, sterilized and inoculated with 1 ml of 48 h culture grown in the same medium (OD_{520} = 2.5) and incubated at 32 °C in a rotary shaker at 110 rpm for 15 days. Bacterial growth and EPS production were monitored in batch cultures of 500 ml Erlenmeyer flasks with 100 ml of medium in each. At the end of incubation culture was centrifuged using an ultracentrifuge, Himac, CF12RX at 12,000 rpm for 1 h at 4 °C. Supernatant was precipitated out with cold ethanol and again centrifuged. Pellet was dissolved in ultra pure distilled water and purified using dialysis against distilled water (3–4 exchanges) for 48 h using Snakeskin pleated dialysis tubing from Thermo scientific of 10,000 MWCO. Purified MR was freeze dried and subjected to evaluate the antioxidant activity.

2.4. Electron microscopy of H. maura

Bacterial cells were taken from mid exponential phase culture of *H. maura* for electron micrographic study of the EPS layering the bacterial cell. Ultra thin sectioning was performed as mentioned elsewhere (Bouchotroch et al., 2001) with slight modifications in the proto-col and viewed through transmission electron microscope (TEM) (JEOL, JEM-2200FS) and images were recorded.

2.5. Preparation of mammalian cell and tissue homogenates

L929, mouse fibroblast cell line homogenate and mice liver homogenates were used for testing the *in vitro* antioxidant activity of MR. L929 cells were cultured and maintained using DMEM, supplemented with 10% of FBS at 37 °C in a 5% CO_2 atmosphere. Cells were trypsinized after attaining confluent growth and collected separately. L929 cells were diluted using normal saline, in an order of 2×10^5 cells/ml and mixed with various concentrations of MR. Liver homogenate was prepared using fresh liver isolated from rat. 10% liver homogenate were made in ice-cold normal saline using a glass-homogenizing vessel in a rotor stator homogenizer at 1000 rpm. The suspended mixture was maintained in an ice bath until used for various antioxidant assays and total protein estimation. Standard protocols with slight modifications were used for assaying antioxidant enzymes. Effect of oxidation stress and the amount of its inhibition by MR was evaluated using optical density (OD) measurement by UV–visible spectrophotometer.

2.6. Total protein assay

Determination of total protein concentration in cell and liver homogenates were performed using bicinchonic acid assay method (Smith et al., 1985) with bovine serum albumin as standard and expressed in mg/ml.

2.7. Lipid peroxidation (LPO) assay

Cell membrane damage is characterized with the oxidation of the lipids, released from the tissue or cells. As a result of free radical generation, electrons will be removed from the cellular membrane, causing a rupture that result in the lipid peroxidation. LPO assay characterizes the degradation of the TBA and the concentration of melondialdehyde reacted *in vitro* to give a fluorescent product that can be measured at 532 nm. LPO assay or thiobarbituric acid reactive substances (TBARS) assay were performed using L929 cell and mice liver homogenate, as described by Ohkawa, Ohishi, and Yagi (1979). Briefly, 1.5 ml of 0.8% of TBA was mixed with 0.2 ml of 8.1% of SDS and 1.5 ml of 20% acetic acid. Above mixture was added with 0.2 ml of test sample and volume was adjusted using deionized water. The reaction mixture was kept in a boiling water bath for 1 h and cooled using tap water. 1 ml of deionized water was added and centrifuged at 3500 rpm for 10 min. Supernatant was collected to measure the OD value.

2.8. Reduced glutathione (GSH) assay

Moron et al., method was used to assay the GSH level present in the cell and tissue homogenate with a slight modification (Moron, Depierre, & Mannervik, 1979); DTNB was used to react with GSH to form a spectrophotometrically detectable product. Briefly, 0.5 ml of the supernatant of cell and tissue homogenate obtained after centrifugation at 3500 rpm for 10 min at 4 °C were mixed with 4 ml of 0.2 M Phosphate buffer solution (PBS), respectively and the total volume was adjusted using deionized water. 0.5 ml of DTNB was added to the mixture, prior to the measurement of absorbance at 412 nm. The change in absorbance is a linear function of the GSH