Materials and methods

Gracilaria chouae was collected from the Ailian Bay breeding area, Shandong Province. The alga was washed with distilled water, dried at 56°C, and cut into 1 cm fragments. Cellulose DEAE-52 and Sephadex G-100 were purchased from labtop Bio-Technology Co., Ltd. (Shanghai, China). Monosaccharide standards (rhamnose, arabinose, xylose, mannose, glucose, galactose and galacturonic acid) and trifluoroacetic acid (TFA) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Murine macrophage cell line RAW264.7 were obtained from the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), Lipopolysaccharide (LPS), 3-(4,5-dimethylthiahiazo-2-yl)-2,5-diphenytetrazoliumromide (MTT), Dimethyl sulfoxide (DMSO) were purchased from Sangon Biotech Bioengineering Co., Ltd. (Shanghai, China). Nitric oxide (NO) assay kit and BCA protein assay kit were purchased from Beyotime, China. The other reagents used in this paper were analytical grade.

Isolation and purification of GCP-3A

The *G. chouae* (50 g) was extracted with deionized water at 80°C for 4 h. After filtered by gauze, the residue was re-extracted again. The filtrate was collected, cooled, and stored overnight at 4°C. After thawed supernatant, the gel and non-gel components were separated by centrifugation. Then three times the volume of anhydrous ethanol was added to precipitate the non-gel portion. Subsequently, 3% (v/v) trichloroacetic acid (TCA) was added and deproteinized overnight at 4°C. After centrifugation, the supernatant was concentrated, 3 volumes of anhydrous ethanol were added and stored overnight at 4°C. The precipitates were redissolved in deionized water, dialyzed at 4°C for 48 h (cut off at 3500 Da MW), and then concentrated and freeze-dried to obtain crude polysaccharides, which were called GCP.

GCP was fractionated on DEAE-52 cellulose chromatography column (50 cm × 1.6 cm), and eluted with a stepwise gradient of NaCl (0, 0.1, 0.3 and 0.5 mol/L) at a flow rate of 1.5 mL/min (6 mL/tube). The three elution peaks GCP-1, GCP-2, GCP-3 were obtained measured by phenol-sulfuric method (Mecozzi, 2005). The main fraction GCP-3 was further purified on Sephadex G-100 column (70 cm × 2.6 cm) at a flow rate of 1 mL/min (6 mL/tube). The eluent is 0.3 mol/L NaCl. Then, the main elution peak was collected, concentrated, dialyzed, and lyophilized to obtain GCP-3A.

Chemical and monosaccharide compositions analysis of GCP-3A

The total sugar content, protein content, sulfate content and uronic acid content were determined by the methods described previously (Cui et al., 2019). The 3,6-anhydrogalactose was quantified by colorimetric method (Yaphe and Arsenault, 1965).

The monosaccharide of GCP-3A was determined by acetylation derived GC-MS. Initially, 5 mg of purified GCP-3A was hydrolyzed in 2 mL of 2 mol/L TFA for 6 h at 100°C in a round bottom flask and blew dry with N_2. Then, acetylation of GCP-3A hydrolysates was conducted based on previous study (Wei et al., 2019). Hydroxylamine hydrochloride (5 mg) and pyridine (0.25 mL) were added to the hydrolysates of GCP-3A, and then reacted at 90°C for 30 min. Then acetic anhydride (0.25 mL) was added to acetylate for 30 min at 90°C. Finally, the final derivatives were dissolved in trichloromethane (2 mL) and the supernatant was analyzed by GC-MS instrument (Thermofisher ISQ7000, USA) equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent, USA). Helium was used as carrier gas. The column temperature was maintained at 90°C for 1 min, rose to 170°C at 8°C/min, rose to 240°C at 5°C/min, and rose to 300°C at 20°C /min for 6 min. The injection temperature was 280°C. The split ratio was 10: 1, and the injection volume was 1 μL. The monosaccharide standards (rhamnose, arabinose, xylose, mannose, glucose, and galactose) were treated as described above.

Molecular weight measurement

The weight-average molecular weight (Mw) and number-average molecular weight (Mn) of GCP-3A were measured by gel permeation chromatograph (GPC), which equipped with Tsk-Gel 3000PWXL (7.8 × 300 nm) column and SEDEX75 evaporative light scattering detector. The mobile phase was 0.1 mol/L Na_2SO_4 at a flow rate of 0.5 mL/min and the column temperature was kept at 35°C. T-series dextrans standards were used to draw the standard curve of molecular weight.

FT-IR spectrometric analysis

FT-IR (Nicolet 6700, USA) was chosen to analyze the functional groups and glycosidic bond configuration of GCP-3A. Briefly, 3 mg sample was mixed with KBr powder and pressed into thin sheets for Fourier transform infrared spectroscopy (FT-IR) scanning from 400 to 4000 cm^{-1}.

NMR spectroscopy analysis

30 mg sample was dissolved in D_2O (0.5 mL) and kept overnight at room temperature. ^{1}H, ^{13}C

NMR spectra were recorded on a Varian Inova-600 NMR spectrometer.

SEM analysis

3 mg lyophilized polysaccharide powder was affixed to conductive tape and then coated with a layer of gold. The surface morphology of GCP-3A was observed by SEM (SU5000, Hitachi, Japan) at an accelerating voltage of 5.0 kV.

Congo-red assay

Triple helix conformation of GCP-3A was performed using the method previously reported (Zhang et al., 2020).

Activation effects on RAW264.7

RAW246.7 macrophages were cultured in DMEM medium containing 10% fetal bovine serum. The cells were incubated in a humidified incubator at 37°C with 5% CO_2 and then were passaged every two days.

The RAW264.7 cells proliferation was determined using the previous described method with minor modification (Gu et al., 2020). Briefly, the density of RAW264.7 cells was diluted to 1 x 10^5/mL, and then 100 μL cell suspension was aspirated and cultured overnight in 96-well culture plates. Then the cells were treated with 100 μL GCP-3A (5, 25, 50, 100, 200, 300 μg/mL) for 24 h. The same volume of DMEM and LPS (1 μg/mL) were added as blank group and positive control. Every 6 parallel wells were set for each concentration. After removing the medium, 100 μL MTT (0.5 mg/mL) was added to each well for 4 h of additional incubation. Next, the supernatant was sucked up and 150 μL dimethyl sulfoxide (DMSO) was added to dissolve formazan. By the end of the experiment, the absorbance was measured by microplate reader at 490 nm. The RAW264.7 cells viability was calculated as the following formula:

$$Cell\ viability\ (\%) = {Abs_{sample}}/{Abs_{blank}}$$

The NO production of supernatant was detected by Griess assay (Shi et al., 2020). After RAW264.7 cells incubated overnight in 96 well plates, cells were treated with GCP-3A (0, 25, 50, 100, 200, 300 μg/mL) or LPS (1 μg/mL) for 24 h. The sample supernatant (50 μL) was mixed with Griess reagent (100 μL). After reaction for 10 min, the absorbance at 540 nm was determined by a microplate reader.

Statistical analysis

All experimental data was analyzed by one-way ANOVA analysis followed by Duncan's multiple range test. $P < 0.05$ was statistically significant differences. Statistical analysis was performed by SPSS software version 17.0 and OriginPro 9.1.

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