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Chemical characterization of a new sulfated polysaccharide from *Gracilaria chouae* and its activation effects on RAW264.7 macrophages

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This study aimed to characterize the chemical composition of a new sulfated polysaccharide from the red alga *Gracilaria chouae* and evaluate its activation effects on RAW264.7 macrophages. It showed that the obtained *G. chouae* polysaccharide (GCP-3A) was a sulfated acidic polysaccharide with a molecular weight of 11.87 kDa. GCP-3A was composed of xylose, galactose, glucose, and mannose with a molar ratio of 3.00:29.28:0.63:0.45, and it contained α,β-glycosidic linkages. Scanning electron microscopy (SEM) and a Congo red test showed that it was a heterogeneous polysaccharide with irregular interwoven sheets and rods, and did not have a triple-helix conformation. Furthermore, GCP-3A significantly promoted the proliferation of RAW264.7 macrophages and the secretion of nitric oxide (NO) in tests of 3-(4,5-dimethylthiahiazo-2-yl)-2,5-diphenytetrazoliumromide (MTT) and NO.

G. chouae, an economically important alga, is widely found in the low-tide or subtidal zones along the southeast coast of China (Chi et al., 2016). In recent years, it has been principally exploited for water quality improvement, abalone culture, and agar extraction; however, it has not been fully developed or utilized (Torres et al., 2019). Previous research reported that the polysaccharides from *G. chouae* exerted anti-oxidation and antitumor effects, raising the possibility of applications in functional food and medicine (Ju et al., 2016; Khan et al., 2019).

The biological activity of polysaccharides is closely linked to their structural characteristics, such as monosaccharide composition, molecular weight, substituents, branching, and conformation (Zhang et al., 2020). However, there are no clear rules connecting the structure and activity of polysaccharides, and they are all affected by various separation and purification processes. Consequently, more research is required to elucidate polysaccharide structures to define the relationship between structure and activity.

We then systematically studied its structural characteristics with gel permeation chromatography (GPC), gas chromatography-mass spectrometry (GC-MS), Fourier transform-infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR). SEM and Congo red were used to explore the surface morphology of the polysaccharides. The activation effects of GCP-3A on RAW264.7 macrophages were studied by MTT and NO assays.

We found that GCP-3A was a polysaccharide with relatively uniform molecular weight and charge. It was first purified by cellulose diethylaminoethyl-52 (DEAE-52) chromatography column (Fig. 1a) and then by Sephadex G-100 chromatography (Fig. 1b). GCP-3A is a sulfated acid polysaccharide, and its monosaccharide composition is mainly 3,6-anhydrogalactose, xylose, and galactose, with minor amounts of glucose and mannose (Table 1). The GPC elution profile indicated that GCP-3A had a relatively broad molecular weight distribution, with a weight-average molecular weight and number-average molecular weight of

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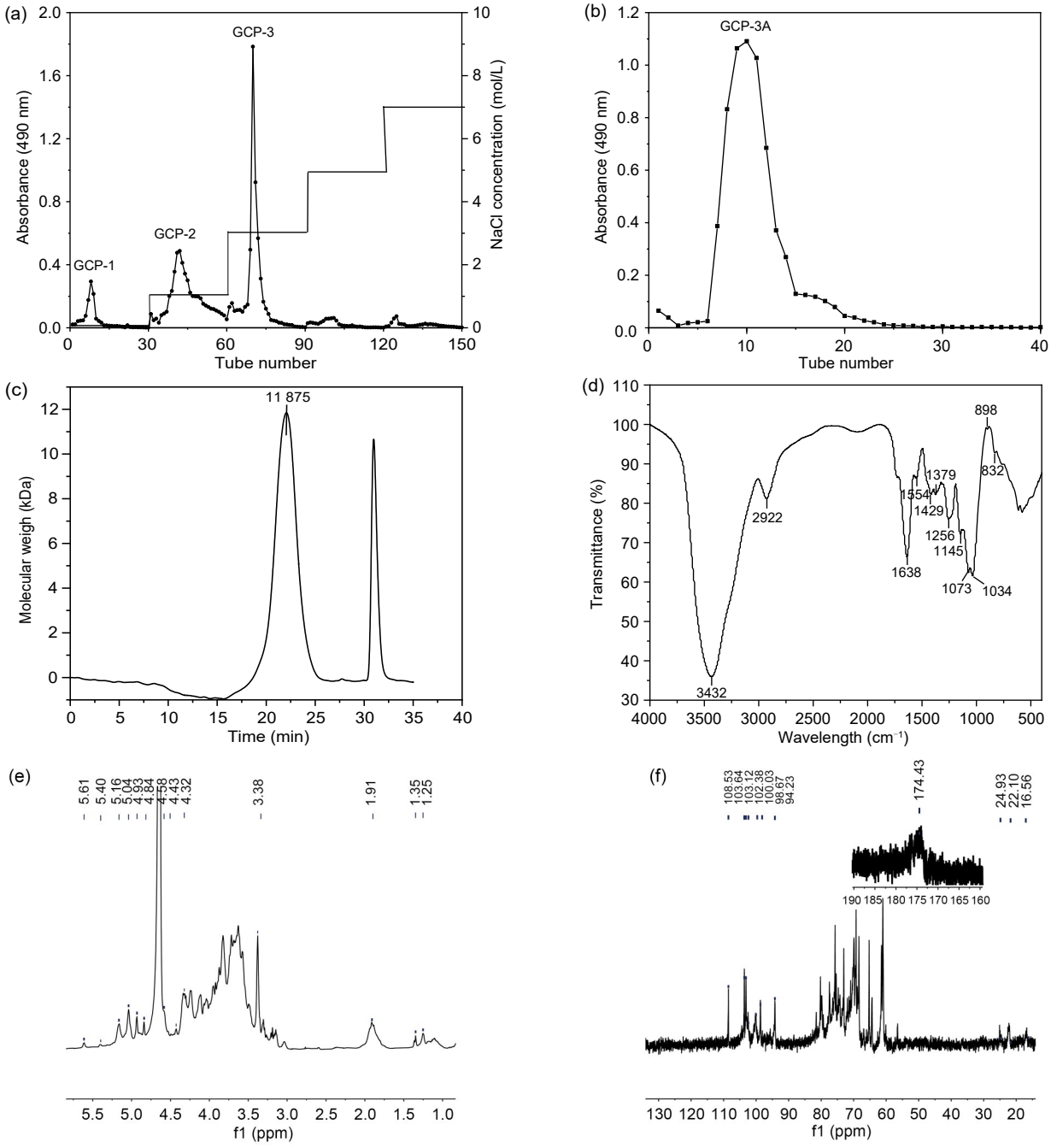

Fig. 1 Chemical characterization of GCP-3A. (a) Cellulose DEAE-52 chromatogram; (b) Sephadex G-100 chromatogram; (c) GPC chromatogram; (d) FT-IR spectrum; (e) ^{1}H NMR; (f) ^{13}C NMR. ppm: parts per million; GCP-3A: *Gracilaria chouae* polysaccharide; DEAE: diethylaminoethyl; FT-IR: Fourier transform-infrared spectroscopy; NMR: nuclear magnetic resonance. (d) was adapted with permission from Li and Liu (2021), copyright 2021, *J Shandong Agric Univ (Nat Sci Ed)*.

31.32 kDa and 10.13 kDa, respectively (Fig. 1c). As shown in Fig. 1d, FT-IR revealed the typical polysaccharide characteristic peak information for GCP-3A, and confirmed the presence of uronic acid and sulfate groups (You et al., 2020). ^{1}D NMR (^{1}H, ^{13}C) spectra indicated that GCP-3A had α- and β-configuration (Figs. 1e and 1f). In addition, GCP-3A contains several complex sugar residues, and exhibits substitution of methyl, acetyl, and sulfate groups (Liu et al., 2018).

SEM analysis showed the surface morphology of GCP-3A at 350× and 1500× magnifications (Figs. 2a and 2b). GCP-3A was predominantly interwoven by

Table 1 Chemical composition and neutral sugar composition of GCP-3A

Name	Content (%, mass fraction)					Neutral sugar (Xyl:Gal:Glc:Man)
	Total sugar	Uronic acid	Protein	Sulfate group	3,6-Anhydrogalactose	
GCP-3A	63.80±0.05	10.23±0.12	0.11±0.06	7.37±0.63	15.75±0.49	3.00:29.28:0.63:0.45

Data are expressed as mean±standard deviation (n=3). GCP-3A: *Gracilaria chouae* polysaccharide; Xyl: xylose; Gal: galactose; Glc: glucose; Man: mannose. Data about chemical composition content of GCP-3A were adapted with permission from Li and Liu (2021), copyright 2021, *J Shandong Agric Univ (Nat Sci Ed)*.

irregular sheets and rods, and the surface was granular. A Congo red experiment suggested that GCP-3A did not have a triple-helical conformation in solution (Fig. 2c).

An MTT assay indicated that GCP-3A promoted the proliferation of RAW264.7 cells at concentrations of 25–300 μg/mL (Fig. 3a). GCP-3A did not exhibit any detectable cytotoxicity. In fact, it significantly promoted the proliferation of RAW264.7 cells compared with the control group (P<0.05). In the NO assay, GCP-3A significantly stimulated NO secretion in RAW264.7 cells compared with the control group, and this stimulation was in a dose-dependent manner in the range of 25–200 μg/mL (Fig. 3b). The production of NO reached the highest level (6.64 μmol/L) at 200 μg/mL.

In this study, the total sulfate content of GCP-3A was 7.37% (mass fraction), which was similar to previously reported levels (Sudharsan et al., 2015; Imjongjairak et al., 2016). In addition, the results showed that the galactose content was much higher than that of other monosaccharides in GCP-3A, which was consistent with other polysaccharides from red algae. However, the types and proportions of monosaccharides varied, which may be related to species, growth environment, and treatment methods (de Oliveira et al., 2020; Han et al., 2020; Li et al., 2020). We found that there were granular substances on the surface of GCP-3A, which was also observed previously in *G. chouae* (Khan et al., 2019).

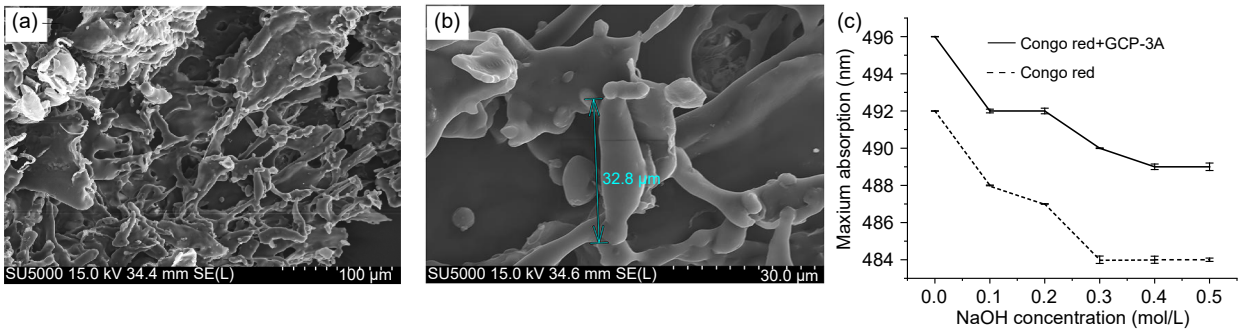

Fig. 2 Surface morphology of GCP-3A. (a) SEM images of GCP-3A at 350× magnification; (b) SEM images of GCP-3A at 1500× magnification; (c) Absorption spectra of Congo red and Congo red with GCP-3A. Data are expressed as mean± standard deviation (n=3). SEM: scanning electron microscopy; GCP-3A: *Gracilaria chouae* polysaccharide.

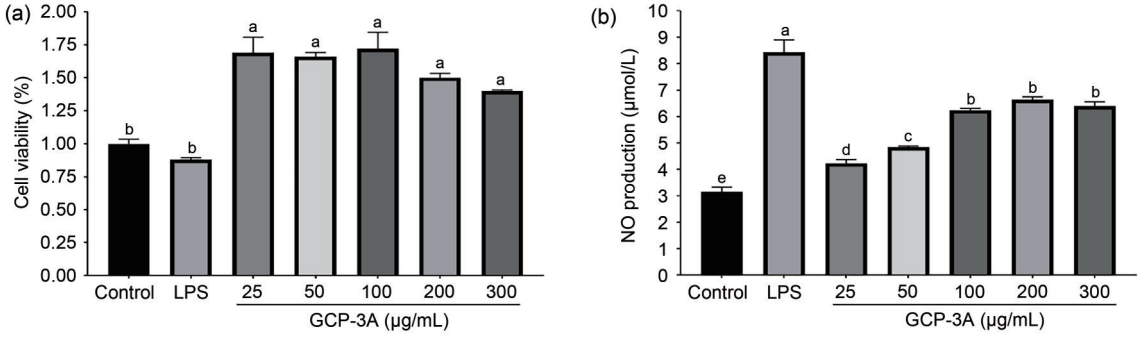

Fig. 3 Effects of GCP-3A treatment on RAW264.7 macrophages. (a) GCP-3A promoted the proliferation of RAW264.7 cells at concentrations of 25–300 μg/mL. (b) GCP-3A significantly stimulated NO secretion in RAW264.7 cells at concentrations of 25–300 μg/mL. Data are expressed as mean±standard deviation (n=6). Adjacent letters of the alphabet indicate significant differences at P<0.05. LPS: lipopolysaccharide; NO: nitric oxide; GCP-3A: *Gracilaria chouae* polysaccharide.

According to previous reports (Gu et al., 2020; Zhao et al., 2021; Zhu et al., 2021), the activated macrophages can directly kill pathogens and release effector molecules such as NO, tumor necrosis factor, and interleukin, which provide resistance to microbial infection and pathogen invasion. In addition, NO participated in various physiological and pathological reactions and is considered an important indicator of immune cell activation (Huang et al., 2017; Lan et al., 2021). Here, GCP-3A promoted the proliferation of macrophages and the release of NO, which suggested that RAW264.7 macrophages were activated. In conclusion, GCP-3A is a potential bioactive ingredient for the pharmaceutical and health product industries.

In the future, more studies should be carried out to clarify the activation effects of GCP-3A on RAW264.7 macrophages. Because of the complexity of this polysaccharide, the sequence of glycosidic bonds should be elucidated to provide a reference for the structure-activity relationship of polysaccharides in general.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Feifei LI performed the experimental research and data analysis, and wrote and edited the manuscript. Kehai LIU contributed to the study design, data analysis, and editing of the manuscript. Kewu LIU contributed to the study design and editing of the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Feifei LI, Kehai LIU, and Kewu LIU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Materials and methods