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The immunomodulatory effect of Glagah consortium microalgae polysaccharides based on splenocyte proliferation and cytokine production in vitro

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Abstract. *Afililla Z, Suwanti LT, Mufasirin, Koerniawan HP, Suyono EA, Budiman A, Siregar UJ. 2022. The immunomodulatory effect of Glagah consortium microalgae polysaccharides based on splenocyte proliferation and cytokine production in vitro. Biodiversitas 23: 5205-5209.* Indonesia is known as a maritime country that has various organisms, including microalgae. Some microalgae have many bioactivities, such as immunomodulatory activity. This study aimed to determine Glagah consortium microalgae polysaccharide (GCMP) and polysaccharide of *Spirulina platensis* (PSP) as an immunomodulator based on its effect on splenocyte proliferation and cytokine production in vitro. Crude polysaccharides were extracted by lye extraction method. Splenocytes were obtained from spleen of healthy mice and cultured in RPMI 1640 medium with and without Concanavalin A (Con A). Cells (3×10^5 per well) were treated with polysaccharide with dose gradually 0, 1.25, 2.5, 10 and 20 µg/mL. Splenocytes proliferation were observed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The supernatant was taken for the detection of cytokines using enzyme-linked immunoassay (ELISA). The results showed that GCMP, both with and without Con A at a concentration of less than 10 mg/mL decreased cell viability, on the other hand, a concentration of 10-20 mg/mL had a proliferative effect. Splenocytes were shown a proliferative effect at all concentrations of PSP, both with and without Con A. The concentration of polysaccharides, both GCMP and PSP, did not affect cytokine levels. In conclusion, based on the proliferation of splenocytes, GCMP has an immunomodulatory effect depending on its concentration.

Keywords: Glagah consortium microalgae, immunological diseases, immunomodulatory, polysaccharides

INTRODUCTION

Currently, there is an increase in immunological diseases, such as cancer and autoimmune diseases. This situation necessitates the development of a substance to overcome it that can enhance or suppress the immune response in immune system-mediated disease or immunomodulator. An immunomodulator is a compound that can modify the activity of the immune system by enhancing or suppressing the host response and are categorized as an Immunostimulant, immunosuppressant and immunoadjuvant (Sindhu et al. 2021). Several products of marine organisms, including microalgae, exhibit immunomodulatory activity (Singh and Krishna 2019). Indonesia has been known as a maritime country that is rich in biodiversity of organisms, including marine organisms and microalgae.

The bioactive components contained in microalgae are numerous and varied, including various pigments, hydrogen, hydrocarbons, carbohydrates, polysaccharides, vitamins and proteins (Randrianarison and Ashraf 2017; Khan et al. 2018; Mahendran et al. 2021). According to Lauritano et al. (2016), marine microalgae are considered as a potential and valuable source of biologically active molecules for applications in the food industry as well as in the pharmaceutical, nutraceutical, and cosmetic sectors. Several researchers have tested the biological activities of microalgae for the treatment of human pathologies, including as anti-viral (Mahendran et al. 2021), anti-cancer (Chen et al. 2019), anti-bacterial (Lauritano et al. 2016), anti-inflammatory (Rodriguez-Luna et al. 2019), and anti-fungal (Martinez et al. 2019).

Polysaccharides have been studied, examined, and explored for decades and have provided information that polysaccharides can help to modify the immune system and effectively fight infectious diseases (Sindhu et al. 2021). Natural polysaccharides are a class of natural or biological macromolecules isolated from plants, fungi, algae, animals, archaea and bacteria (Torres et al. 2019). One of the important components contained in microalgae is

polysaccharides because, according to Son et al. (2017), polysaccharides are the most basic stimulus in the immune system. In addition, polysaccharides play a role in various biological processes of an organism, such as immune responses to intercellular communication, ability as antioxidants and have immunostimulant properties (Lordan et al. 2011; Costa et al. 2021). Gugi et al. (2015) also added that microalgae function as immunostimulators and can protect the liver, eyes, blood vessels, skin and lungs. Immunostimulators are compounds that can stimulate the immune system by increasing the activity of components of the immune system to fight infection and disease. According to a review by Yin et al. (2021), the immunomodulatory effects of polysaccharides from edible mushrooms include the proliferation of splenic cells and increased production of cytokines tumor necrosis factor-α (TNF-α) and interferon-ɣ (IFN-ɣ). In vitro immunostimulator test conducted by Wu et al. (2020) showed that polysaccharides of *S. platensis* (PSP) were shown to increase the proliferation of splenocytes and macrophages as well as increase the production of nitric oxide (NO), TNF-α, and interleukin 6 (IL-6).

Suyono et al. (2016; 2018) have successfully explored and identified mixed microalgae from Glagah Beach, Yogyakarta, Indonesia, hereinafter referred to as the Glagah consortium microalgae. However, the immunomodulating effect of Glagah consortium microalgae polysaccharides has never been discussed. Studies on the Glagah consortium microalgae are still limited to the development of biodiesel materials by examining the lipid content profile (Suyono et al. 2016). The present study aimed to determine Glagah consortium microalgae polysaccharide (GCMP) as an immunomodulator based on its effect on spleen cell proliferation and proinflammation cytokine profile in vitro. This study also explained about the polysaccharide of *S. platensis* (PSP) as an immunomodulator.

MATERIALS AND METHODS

Materials

The microalgae biomass of *Spirulina platensis* and the Glagah consortium was obtained from the Faculty of Biology, Universitas Gadjah Mada. Splenocytes were taken from the spleen of healthy mice. Mouse Interferon- γ Elisa Kit 967 (E0056Mo, BTLAB) and Mouse Tumor Necrosis Factor-α Elisa Kit 967 (E0117 Mo, BTLAB) were used for determination cytokine level.

Microalgae polysaccharide extraction

Polysaccharides from Glagah consortium and *S. spirulina* were extracted by an alkaline extraction method with pH 10, as described by Wang et al. (2018). Microalgae biomass was grounded into a powder. Forty grams of microalgae powder was dissolved in 1.6 L of distilled water and added with 1 mol/L NaOH until the pH reached 10. Then the solution was stirred and put in at 80°C water bath for 8 hrs. The solution was centrifuged at 4000 rpm for 20 min and the supernatant was taken. The supernatant was added with 95% ethanol and incubated in the freezer overnight, than centrifuged with a speed 4300 rpm for 10 min. The precipitates were washed by acetone. The precipitates were collected then lyophilized with a freeze dryer.

Preparation of splenocyte

The preparation of splenocytes was carried as a method of Yamanaka et al. (2012). The splenocyte was provided from healthy mice. Mice were sacrificed, and spleens were removed. The spleens were sliced into smaller sizes and put in the petri dish containing 5 mL of PBS-PS [mixture of phosphate buffer saline (PBS) and Penicillin-Streptomycin (PS), with the concentration of penicillin was 100 units/mL and streptomycin was 50µg/mL]. The sliced spleens were put in between 2 object glasses then pressed to gain the splenocytes. The suspension was filtered through a 200 mesh sieve and put into a 50 mL conical tube. Than suspension was centrifuged with 1000 rpm for 5 min (4ºC). Supernatant was discarded and the pellets were added by 5 mL Tris-NH_4Cl lysis buffer to lyse the erythrocyte. The suspension was washed with 5 mL of PBS-PS. Then the cells were put in the RPMI 1640 medium added with 10% FBS. Cells were counted with a hemocytometer for 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT assay).

Splenocytes proliferation

Cells were cultured in 96-wells microplate with density of 3×10^5/100 µL each well and divided into two groups. Group A consists of cells which added with 2.5 µg/mL Concanavalin A (Con A), while group B consists of cells without Con A. Each group was divided into 5 subgroups, where in each subgroup polysaccharides were added at a graded dose (0, 1.25, 2.5, 10, and 20 mg/mL). Cells were incubated in 37 ºC, 95% humidity with 5% CO_2 for 72 hrs. After 72 hrs, the supernatant of cultured cells was collected to detect the production of cytokines (TNF-α and IFN-ɣ) with the enzyme-linked immunoassay ELISA method. The remaining cells added with 20 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution in each well. The microplate then closed with aluminum foil to protect from the light then incubated at 37ºC for 4 hrs. Then dimethyl sulfoxide (DMSO) was added to each well, and incubation was continued for 10 min (37ºC). The absorbents were counted in a spectrophotometer (560 nm). The cell viability formula used for this research was mean OD sample/mean OD blank x 100%.

Cytokine level secreted by splenocytes

Cytokine secretion was determined based on the cytokine levels in the splenocyte culture supernatant. The cytokines measured in this study were TNF-α and IFN-ɣ. The method used to determine cytokine levels was ELISA, with the protocol following the manufacturer's manual (from the Bioassay Technology Laboratory/BT LAB). The collected supernatant from cultured splenocytes was put in a microtube then centrifuged with 2000-3000 rpm for 20 min to separate if there were still cells collected. The

supernatant was used as a sample. Samples and standard reagents were prepared as instructed. Fifty microliters (50 µL) of standard solution were put into the standard well and 40 µL samples were put to sample wells. Into the sample well was added 10 µL of anti-antibody of cytokine (TNF-α and IFN-γ). Then standard and sample wells (but not blank wells) were added with 50 µL of streptavidin-HRP and mixed well. The microplate was covered with a sealer and incubated for 60 min at 37°C. The sealer was removed and the microplate was washed with a washing buffer 5 times. Washing was carried out by adding 300 µL of washing buffer for 30 sec to 1 min for each washing. Thereafter, 50 µL of substrate A and 50 µL of substrate B were added to each well. The microplate was covered with a new sealer and incubated for 10 min at 37ºC in the dark. Each well was added with 50 µL stop solution to stop the reaction. The optical density (OD) Value was determined with a microplate reader set to 450 nm within 10 min after the stop solution was added. The OD of the standard was used to construct the regression equation line and the standard regression equation line was used to calculate the cytokine levels of the sample. The average OD value for each standard is on the vertical (Y) axis, while the concentration of the standard is on the horizontal (X) axis. The sample cytokine levels were calculated by entering the formula in the regression equation.

Statistics analysis

Quantitative data were presented as mean ± SD. One-way ANOVA (analysis of variance) test followed by Duncan's test was conducted to determine statistical comparisons. The value $p<0.05$ was stated to be statistically significant.

RESULTS AND DISCUSSION

Splenocytes proliferation

Splenocyte proliferation was determined using the MTT assay method as a representative indicator of the immunomodulatory activity of polysaccharides of microalgae, GCMP and PSP. Splenocytes were treated with polysaccharides with or without mitogen (Con A). Mitogen-stimulated lymphocyte proliferation may serve as an indicator for immunoactivities and a mitogen for detecting T lymphocyte proliferation can be evaluated by challenging splenocytes with Con A (Lan et al. 2010). There have been many research reports on the use of polysaccharides from natural ingredients as immunomodulators which observed the proliferative activity or viability of splenocytes, either without or with mitogens (Sung et al. 2015; Su et al. 2020; Zhang et al. 2021).

The concentration of polysaccharides, both GCMP and PSP, affects the viability of splenocytes ($p<0.05$) compared with the control group (medium, without polysaccharide administration or concentration 0 mg/mL) (Table 1). Both GCMP and PSP modulate splenocyte viability with or without Con A. The polysaccharides showed dose-dependent activity on splenocyte viability with or without mitogens (Con A).

Administration of 2.5 µg/mL Con A in the absence of polysaccharides increased the viability of splenocytes 3% (2.84%-3.10%). These results indicate that the addition of Con A had an effect on cell proliferation. Con A is an antigen-independent mitogen and has a function as a signal inducer, leading T cells to proliferate (Ando et al. 2014). Similar results were reported by Yu et al. (2016), which stated that the viability of splenocytes increased by about 6% after being stimulated with Con A 7.5 µg/mL. Higher results were shown by Ang et al. (2014), namely, Con A 5 µg/mL stimulation increased splenocyte viability by about 15%.

Although statistically not significantly different, the GCMP concentration of less than 10 mg/mL (1.25 mg/mL and 2.5 mg/mL) had an effect on the decrease in cell viability when compared to the control group. The viability of splenocytes at a concentration of 10 mg/mL GCMP was the same as the control group and significantly increased at a concentration of 20 mg/mL. These results indicate that the effect of GCMP on splenocyte proliferation depends on the concentration. These results are similar to Liu et al. (2012), who induced splenocyte proliferation with polysaccharides from *Lycium barbarum* leaves. Their study showed that the increased proliferative effect of splenocytes depended on the concentration of the polysaccharides. Slightly different results were shown in the treatment of PSP. The results showed that PSP was nontoxic to splenocytes, which was indicated by constant cell viability at concentrations less than 10 µg/mL and tended to significantly increase at concentrations of 10 mg/mL and 20 mg/mL. This study supports the results reported by Wu et al. (2020) that PSP increases splenocyte proliferation.

Table 1. Effect of polysaccharides of Glagah consortium microalgae (GCMP) and polysaccharides of PSP on viability of splenocytes in the medium with and without Con A

Polysaccharide concentration in the medium (mg/mL)	Viability cell in GCMP (mean ± SD)		Viability cell in PSP (mean ± SD)	
	ConA- (%)	ConA+ (%)	ConA- (%)	ConA+ (%)
0	$100.00^{ab} \pm 0.00$	$103.10^{ab} \pm 1.99$	$100.00^{ab} \pm 0.65$	$102.84^{ab} \pm 0.98$
1.25	$96.20^{a} \pm 2.59$	$95.92^{a} \pm 2.19$	$100.92^{ab} \pm 1.09$	$102.00^{ab} \pm 2.61$
2.5	$93.94^{a} \pm 0.60$	$96.20^{a} \pm 6.97$	$106.91^{bc} \pm 3.04$	$102.76^{ab} \pm 2.39$
10	$102.68^{ab} \pm 0.60$	$103.94^{abc} \pm 10.36$	$113.52^{cd} \pm 0.22$	$119.51^{de} \pm 5.21$
20	$117.75^{d} \pm 0.80$	$123.66^{de} \pm 9.16$	$127.96^{e} \pm 0.65$	$129.03^{e} \pm 8.69$

Note: ^{a,b,c,d,e} Different notation shows statistically significant difference ($p<0.05$)

Among the two polysaccharides, PSP showed the highest activity on splenocyte proliferation. The difference in splenocyte proliferation in the medium containing GCMP and PSP was probably due to the different types and origins of microalgae. The Glagah consortium microalgae consisted of 6 species of microalgae and 6 bacteria originating from Glagah Beach, Yogyakarta (Suyono et al. 2016; 2018), while *S. platensis* was a single species. According to Wang et al. (2016), differences in culture, origin, and batch have been shown to have a significant influence on the physicochemical and structural properties of polysaccharides.

Cytokine level secreted by splenocytes

Tumor necrosis factor-alfa (TNF-α) and interferon-gamma (IFN-γ) are pro-inflammatory cytokines involved in various infections and the immune system (Monastero and Pentyala 2017). Several researchers have observed the immunomodulatory effect of polysaccharides from a natural substance by measuring TNF-α and IFN-γ levels both in vitro and in vivo with varying research results. Okra raw polysaccharide extract increased the level of TNF-α but not IFN-γ (Hayaza et al. 2022). Hu et al. (2022) explained in their review that polysaccharides from *Panax* species have immunomodulatory activity with the effect of increasing cytokines (including TNF-α and IFN-γ), splenocyte proliferation, increased phagocytosis and NO production.

The results of the statistical analysis of cytokine levels (TNF-α and IFN-γ) showed no significant difference (Table 2 and Table 3). This indicates that neither GCMP nor PSP had any effect on TNF-α and IFN-γ levels if compared to the control group (cultures that were not given polysaccharides). In contrast to the research of Wu et al. (2020), who reported that PSP increased TNF-α levels. This difference is probably due to differences in the research model. In the research of Wu et al. (2020), TNF-α levels were measured by culturing cell line Macrophages RAW264.7 while mice splenocyte culture was only to determine the effect of proliferation. This difference in results was probably due to the different types of induced cells. This opinion was supported by the results of research by Zhao et al. (2011) on the effects of ginseng polysaccharide on 3 lymphocytes of enteric mucosal lymphocytes, namely Peyer's patches lymphocyte (PPL), intraepithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) in rats. Ginseng polysaccharide could increase the protein expression of TNF-α and IFN-γ in the supernatant of PPL but decreased the protein levels of both TNF-α and IFN-γ in the supernatant of LPL and had no significant effect on IEL supernatant in normal rats.

Based on the proliferative effect of splenocytes, Glagah microalgae polysaccharides can be developed as an alternative therapy. This finding supported our previous study that Glagah microalgae polysaccharides had anti-malarial activity in vitro (Pujiyanto et al. 2022).

In conclusion, this study confirmed that the polysaccharides from the GCMP and PSP have immunomodulatory activity based on the effect of splenocyte proliferation in vitro. Microalgal polysaccharides (GCMP and PSP) stimulate splenocyte proliferation, either in a medium without or with Con A. The modulating effect is concentration-dependent. The results of this study are expected to be scientific evidence that the Glagah consortium microalgae can be developed as natural resources to be used as functional food ingredients and potential alternative medicines. Further research on experimental animals is needed both for dose determination and to determine the effect of in vivo immunomodulation.

Table 2. Effect of polysaccharides of Glagah consortium microalgae (GCMP) and polysaccharides of PSP on Concentration of TNF-α of splenocytes in the medium with and without Con A

Polysaccharide concentration In the medium (mg/mL)	Concentration of TNF-α of splenocytes in GCMP (mean ± SD)		Concentration of TNF-α of splenocytes in PSP (mean ± SD)	
	ConA- (pg/mL)	ConA+ (pg/mL)	ConA- (pg/mL)	ConA+ (pg/mL)
0	154.83 ± 8.10	140.90 ± 11.17	162.59 ± 8.58	144.86 ± 0.98
1.25	146.13 ± 8.62	162.01 ± 12.66	122.18 ± 25.88	172.47 ± 4.14
2.5	187.83 ± 6.33	172.91 ± 22.56	134.44 ± 42.89	148.41 ± 6.63
10	147.55 ± 0.60	141.04 ± 7.38	137.24 ± 7.55	184.11 ± 1.89
20	166.73 ± 0.12	144.29 ± 8.41	152.39 ± 23.63	153.39 ± 22.65

Note: Statistically not significantly different, the value of F: 1.816 and p: 0.097

Table 3. Effect of polysaccharides of microalgae (GCMP and PSP) on Concentration of IFN-γ of splenocytes in the medium with and without Con A

Polysaccharide concentration in the medium (mg/mL)	Concentration of IFN-γ of splenocytes in GCMP (mean ± SD)		Concentration of IFN-γ of splenocytes in PSP (mean ± SD)	
	ConA- (pg/mL)	ConA+ (pg/mL)	ConA- (pg/mL)	ConA+ (pg/mL)
0	111.73 ± 24.95	155.00 ± 10.02	187.68 ± 46.06	154.79 ± 58.54
1.25	150.02 ± 21.04	147.34 ± 10.76	160.86 ± 27.36	147.34 ± 29.37
2.5	136.14 ± 9.13	148.37 ± 23.89	154.90 ± 6.38	133.72 ± 6.25
10	170.19 ± 3.02	139.17 ± 18.64	127.36 ± 15.99	170.83 ± 7.55
20	124.37 ± 8.64	120.25 ± 50.22	145.08 ± 15.07	164.24 ± 15.07

Note: Statistically not significantly different, the value of F: 1.051 and p: 0.456

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