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Characteristics and anti-inflammatory effects of the enzymatically extracted polysaccharides of *Sargassum fulvellum* using crude enzyme from *Shewanella oneidensis* PKA 1008

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Abstract

Alginic acid is a polysaccharide obtained from brown algae, and its oligosaccharide has various functions such as antiviral, antitumor, immunoregulation, and antioxidant. However, because of its high viscosity, numerous studies have degraded the alginic acid by enzymes to improve its utilisation. In the present work, we characterised *Sargassum fulvellum* enzymatic extract (SFEE) using polysaccharide-degrading enzyme obtained from *Shewanella oneidensis* PKA 1008, and investigated its anti-inflammatory potential. *S. fulvellum* powder and crude enzyme were mixed at a ratio of 1:1 (v/v), and reacted at 30°C for 0 - 48 h to obtain the optimum degrading time. The changes in pH, colour, reducing sugar, and viscosity of SFEE were determined. The anti-inflammatory activity of SFEE was confirmed by measuring the expression level of nitric oxide (NO) and pro-inflammatory cytokines (IL-6, TNF-α, and L-1β) in RAW 264.7 macrophage cell line. The reducing sugar content was found to increase 2.75-fold at 24 h as compared to that at the initial reaction point, but pH and viscosity decreased significantly with increasing reaction time. SFEE showed a high inhibitory effect on the levels of NO and pro-inflammatory cytokines. SFEE thus has great potential for development as a functional food and therapeutic material owing to its anti-inflammatory effect.

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Introduction

Marine algae are considered as vital components of the marine ecosystem. Since marine algae are present abundantly, and comprise a large variety of species, their uses are mainly in the food, cosmetic, and fertiliser industries (Chapman, 1981). However, recently, it has been found that marine algae are a rich source of proteins, vitamins, and minerals, and also contain essential trace elements such as iodine and zinc. Additionally, it has been shown that marine algae contain various physiologically active substances and useful components, unlike those in land plants. Several studies on marine algae have suggested its potential as a useful food resource (Mabeau and Fleurence, 1993; Funahashi *et al*., 2001; Bae *et al*., 2012). Additionally, studies on the antioxidation (Seo and Yoo, 2003), anti-inflammatory (Kim *et al*., 2017), anticancer (Kong *et al*., 2008), and antibacterial activities (Lee *et al*., 2000; Kim *et al*., 2002; Lim *et al*., 2008) of algae have shown its potential as a functional material in the treatment of several diseases.

Sargassum fulvellum is one of the most common algae found in the coastal waters of Korea. A previous study showed that water extract of *S. fulvellum* is effective in the prevention of inflammatory diseases by inhibiting the expression of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, and tumour necrosis factor-α (TNF-α) (Jeong *et al*., 2012). In addition, studies have shown about the anticoagulation effects of fucoidan from *S. fulvellum* (Koo *et al*., 2001) and its antioxidant properties (Cho *et al*., 2007).

The brown algae *S. fulvellum* are rich in sulphated polysaccharides including alginic acid fucoidan. *S. fulvellum* contains approximately 15 -

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35% alginic acid (Gacesa, 1988). Alginic acid is a linear hetero-polysaccharide consisting of two uronic acid residues namely 1, 4-linked β-d-mannuronic acid (M) and 1, 4-linked α-l-guluronic acid (G) (Mizuno *et al*., 1983). These uranic acids are arranged in block structures that may be homopolymeric (polyguluronate, poly G; polymannuronate, poly M) or heteropolymeric (Gacesa, 1992). Alginic acid is widely used in the industry due to its advantageous physical properties; however, their industrial applications are limited owing to long dissolution time at room temperature, and insolubility in an alcohol-containing solvent (Rehm and Valla, 1997). In recent years, some studies showed that lyases produced by marine bacteria can degrade alginic acid to compensate for the limitations of alginic acid, and are applicable to the food and pharmaceutical industries (Murata *et al*., 1993; He *et al*., 2018).

In our previous work, we isolated an alginic acid-degrading marine bacterium from the green alga *Ulva pertusa*, and identified it as *Shewanella oneidensis* PKA 1008. The characterisation of crude enzyme from *S. oneidensis* PKA 1008 was carried out, and the crude enzyme showed its highest alginic acid-degrading activity at pH 9 and 30°C (Sunwoo *et al*., 2013). In addition, the anti-inflammatory effect of alginate oligosaccharides produced by an alginate-degrading enzyme from *S. oneidensis* PKA 1008 has been reported (Kim *et al.*, 2015). However, there have been no studies investigating the enzymatically depolymerised alginate from *S. fulvellum* using *S. oneidensis* PKA 1008. Therefore, in the present work, we attempted to degrade *S. fulvellum* polysaccharide by using an alginic acid-degrading enzyme from *S. oneidensis* PKA 1008 to improve the application value of *S. fulvellum*. Further, we characterised the degradation products and investigated their anti-inflammatory activities to identify potential industrial applications.

Materials and methods

Material

The *S. fulvellum* used in the present work was collected from Songjeong, Busan, Korea. It was washed with water, allowed to air-dry, lyophilised, ground, and then vacuum-packed and stored at -20°C.

Proximate analysis

The proximate components of *S. fulvellum* powder was analysed for crude lipid, crude protein, moisture, crude ash, and carbohydrate contents according to the Association of Official Analytical Chemists (AOAC, 2000). Crude lipids and proteins were determined by Soxhlet and Kjeldahl method, respectively. Moisture content was carried out with ambient pressure drying, and crude ash was measured by dry ashing method. Carbohydrate was calculated based on 100% minus the sum of crude lipid, crude protein, moisture, and ash contents (Jung *et al*., 2012).

Isolation and crude enzyme preparation

The microorganism used in the present work was isolated from the green algae *Ulva pertusa* in Songjeong, Busan, Korea, and identified as *S. oneidensis* PKA 1008 in previous study (Sunwoo *et al*., 2013). *S. oneidensis* PKA 1008 was cultured at pH 9.0, 2% NaCl, and 30°C for 24 h in marine broth (MB, Difco, Detroit, MI, USA). Cultured strains were centrifuged at 10,000 *g* for 30 min at 4°C, and the supernatant was used as a crude enzyme. The crude enzyme was used while being stored in a -20°C refrigerator. To measure the enzymatic activity, 1 mL of crude enzyme and 1 g of 4% alginic acid dissolved in PBS (0.01 N sodium phosphate dibasic and 2% sodium chloride, pH 7.6) were reacted at 30°C for 24 h, and reducing sugar was determined by the Somogyi-Nelson method (Chitoshi and Yoshiaki, 1980). The enzymatic activity was calculated from the calibration curve obtained using glucose as a standard, which was 490 U/mL.

Preparation of S. fulvellum enzymatic extracts (SFEE)

S. fulvelum powder (12 g) and 150 mL of 10 mM phosphate buffer (pH 9) were mixed to prepare a concentration of 80 mg/mL, and mixed with the crude enzyme solution from *S. oneidensis* PKA 1008 at a ratio of 1:1 (v/v). To prevent microbial contamination, sodium azide (Junsei Chemicals, Japan) was added at a final concentration of 0.02% (w/v). To prepare SFEE, the mixture was allowed to react at 30°C for 0, 3, 6, 12, 24, and 48 h. After the reaction, in order to inactivate the crude enzyme, it was boiled at 100°C for 10 min, cooled on ice for 10 min, then centrifuged at 10,000 *g* at 4°C for 10 min, and later the supernatant was refrigerated and used for subsequent analysis.

pH and colour measurement

The pH values of SFEE were measured using a pH meter (HM-30V, TOA, Kobe, Japan).

The Tri-Stimulus Colorimeter (JC801, Color Techno System Co., Tokyo, Japan) was used to measure the lightness (L*), redness (a*), and yellowness (b*) of SFEE at different reaction times. The instrument was calibrated using a white standard plate (L* = 98.98, a* = 0.21, and b* = -0.28).

Reducing sugar content and viscosity measurement

Reducing sugars were determined by the Somogyi-Nelson method. Briefly, 0.5 mL of sample and 0.5 mL of Somogyi's alkaline copper reagent were placed in a test tube, and heated for 20 min in boiling water. After cooling, 1 mL of Nelson's arsenomolybdate reagent was added and stirred. The samples were reacted at 30°C for 10 min, and their absorbances were measured at 520 nm using a spectrophotometer (Thermo Spectronic Rochester, NY, USA).

The viscosity of each sample was measured using a viscometer (LVLTDV-II, Brookfield Co., Middleboro, MA, USA) with the 40 cP spindle at 25°C and 30 rpm.

Degradation and thin layer chromatography (TLC) analysis

S. fulvelum powder and *S. oneidensis* PKA 1008-derived crude enzyme solution mixture was reacted at 30°C for 0, 3, 6, 12, 24, and 48 h. To inactivate the samples, they were heated in boiling water for 10 min, centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatants were lyophilised. The lyophilised samples were dissolved at a concentration of 20 mg/mL using 99.5% ethanol, and the raw sugar was precipitated for 12 h. The precipitated samples were centrifuged at 6,200 *g* at 4°C for 30 min to remove the supernatants, and dried at room temperature.

Standard mixture (cellooligosaccharides) and degradation of *S. fulvellum* polysaccharide by crude enzyme from *S. oneidensis* PKA 1008 was analysed by TLC with a solvent system of 1-butanol:methanol:water (4:1:2, v/v). The reaction products were visualised by heating the silica gel F_{254} plate at 110°C for 40 min after spraying with 10% (v/v) sulphuric acid in ethanol.

Cell culture

The murine macrophage cell line, RAW 264.7, was purchased from Korea Cell Line Bank (KCLB40071). The cells were cultured in DMEM (GIBCO, Grand Island, NY, USA) with 10% inactivated foetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 incubator (MCO-15AC, Sanyo, Osaka, Japan) at 37°C. All cells were sub-cultured when they reached a density of approximately 80% - 90%. Only cells that did not exceed 20 passages were used.

Determination of nitric oxide

To determine nitric oxide levels, a modified Griess assay was used (Kim *et al*., 2015). RAW 264.7 cells were plated in 48-well plates (5×10^4 cells/mL), and incubated for 18 h. After pre-incubation, all cells, except untreated group (UN), were cultured with lipopolysaccharide (LPS, 200 ng/mL) and SFEE (0, 3, 6, 12, 24, and 48 h) for 24 h at 37°C and 5% CO_2. Then, the supernatant was mixed with Griess reagent (Sigma-Aldrich, USA), and incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (Model 550, Bio-Rad, Richmond, CA, USA), and the quantity of NO was calculated with standard curves of sodium nitrite ($NaNO_2$).

Enzyme-linked immunosorbent assay (ELISA)

The secretion of TNF-α, IL-6, and IL-1β cytokines were determined using an ELISA kit (R&D systems, Minneapolis, MN, USA). RAW 264.7 cells were plated in 48-well plates (5×10^4 cells/mL), and incubated for 18 h. After pre-incubation, all cells, except untreated group (UN), were cultured with LPS (200 ng/mL) and SFEE (0, 3, 6, 12, 24, and 48 h) for 24 h at 37°C and 5% CO_2. Then, the concentration of IL-6, TNF-α, and IL-1β in the culture medium were measured by using the ELISA kit. Capture antibody (TNF-α, IL-6, or IL-1β) diluted in coating buffer, was coated in microplate at 100 μL per well, and incubated overnight at 4°C. After coating, it was washed three times with washing buffer (PBS containing 0.05% Tween-20), and the plates were blocked with assay diluent (PBS with 10% FBS). After washing with washing buffer, the supernatant of the cell culture solution was added to each microplate, and reacted at room temperature for 2 h. After the reaction, it was washed with washing buffer, and diluted biotinylated anti-mouse TNF-α, IL-6, or IL-1β detection antibody, and streptavidin-horseradish peroxidase conjugate were added and reacted at room temperature for 1 h. After washing with washing buffer, OPD solution was added and incubated plate for 30 min at room temperature in the dark. The reaction was stopped by adding 2 N H_2SO_4,

and absorbance was measured at 490 nm using a microplate reader.

Statistical analysis

Data were expressed as mean ± standard error of the mean. Statistical evaluation was carried out by one-way analysis of variance using SAS software (Statistical analytical system V8.2, SAS Institute Inc., Cary, NC, USA). The differences between means were determined using Duncan's multiple range test at $p < 0.05$.

Results and discussion

Proximate components of S. fulvellum

The proximate components of *S. fulvellum* powder were determined. Results showed that *S. fulvellum* powder contained 9.06% crude lipids, 19.83% crude proteins, 2.04% moisture content, 19.58% crude ash, and 63.94% carbohydrate on a dry-weight basis.

pH and colour

The pH and colour values of SFEE were determined at different reaction times. The effect of pH values on SFEE is shown in Table 1. It appeared that the initial value of pH was 8.04 before reaction. By increasing reaction time, the pH value decreased, and showed the lowest pH value of 7.37 at 24 h. This result is similar to the results of Park (2017), who reported that the pH values of *S. coreanum* enzymatic extracts using crude enzyme produced by *S. oneidensis* PKA 1008 decreased with increasing reaction time. In addition, the pH of enzymatic extracts from *Undaria pinnatifida* roots was decreased with increasing reaction time (Xu *et al.*, 2020). This could have been due to the degradation of alginic acid, where more terminal of alginic acid were produced as well as free mannuronic acid and guluronic acid, which lowered the pH. And also, the decrease in pH might have been caused by microbial growth as a result of the increased reaction time. However, since 0.02% sodium azide was added to prevent microbial contamination during the preparation of SFEE, the influence due to microbial contamination was considered to be insignificant.

The changes in colour of SFEE with reaction time are shown in Table 1. As the reaction time increased, the redness (a*) and yellowness (b*) also increased; the redness was highest at 10.24 after 24 h of reaction, and the yellowness was highest at 75.47. Subsequently, the lightness (L*) decreased as the reaction time increased from 55.09 before reaction to 34.13 at 48 h. Algae mainly contain chlorophyll and carotenoid pigments; the discoloration was caused by physical treatments such as processing and storage conditions (Han *et al*., 1984; Lim *et al*., 2007). Shin and Jung (1998) have reported that browning occurs during drying or processing of algae, thus resulting in an overall dark colour. Therefore, the colour changes of SFEE could have been due to more and more pigments were extracted from *S. fulvellum* with increasing reaction time, and the colour of SFEE was largely affected by the pigment components of *S. fulvellum* itself.

Table 1. Changes in pH, colour values, reducing sugar, and viscometry of enzymatic extracts of *Sargassum fulvellum* with incubation time

Time (h)	pH	Reducing sugar (μg/ml)	Viscosity (cP)	Colour value		
				L*	a*	b*
0	8.04 ± 0.01$^{a\#}$	84.37 ± 4.53^{e}	1.27 ± 0.01^{a}	55.09 ± 0.01^{a}	5.41 ± 0.01^{e}	60.16 ± 0.02^{e}
3	7.59 ± 0.01^{b}	153.94 ± 0.03^{d}	1.26 ± 0.04^{ab}	38.20 ± 0.04^{b}	8.37 ± 0.15^{d}	63.77 ± 0.27^{d}
6	7.43 ± 0.02^{c}	181.48 ± 0.07^{c}	1.22 ± 0.03^{bc}	35.81 ± 0.01^{d}	8.57 ± 0.15^{c}	63.61 ± 0.62^{d}
12	7.40 ± 0.01^{c}	213.28 ± 0.68^{b}	1.20 ± 0.05^{cd}	35.30 ± 0.06^{e}	9.09 ± 0.10^{b}	64.52 ± 0.11^{c}
24	7.37 ± 0.01^{c}	231.60 ± 1.53^{a}	1.17 ± 0.02^{d}	36.32 ± 0.03^{c}	10.24 ± 0.08^{a}	69.85 ± 0.04^{b}
48	7.38 ± 0.08^{d}	216.19 ± 4.24^{b}	1.06 ± 0.03^{e}	34.13 ± 0.00^{f}	10.22 ± 0.02^{a}	75.47 ± 1.07^{a}

[#] Means in the same column followed by different lowercase superscripts are significantly different ($p < 0.05$).

Reducing sugar and viscosity

Table 1 depicts the changes in reducing sugar content and viscosity relative to the reaction time after mixing the crude enzyme from *S. oneidensis* PKA 1008 with *S. fulvellum* powder. Reducing sugars were determined to be 84.37 µg/mL before reaction. As the reaction time increased, the content significantly increased, and the reducing sugar content of SFEE was highest at 231.60 µg/mL at 24 h. The viscosity of SFEE was 1.27 cP before reaction. The viscosity decreased with increasing reaction time with the lowest viscosity was recorded at 1.06 cP at 48 h.

The crude enzyme produced by *S. oneidensis* PKA 1008 and the increase in reducing sugar content with the decrease in viscosity with increasing reaction time was also confirmed by Sunwoo *et al*. (2013). It has also been reported that when the alginate was heated to cause depolymerisation, the viscosity decreased sharply as a function of the increasing heating time (Kim and Cho, 2000). In the present work, the results indicated that crude enzyme from *S. oneidensis* PKA 1008 could be used to enhance the polysaccharide degradation of *S. fulvellum*, with the most efficient reaction time for producing oligosaccharide being 24 h.

Thin layer chromatography analysis

Thin layer chromatography was carried out to detect SFEE. As shown in Figure 1, no spots were found before the reaction, which indicated that *S. fulvellum* alginic acid has not been degraded. However, one spot was observed, and it was judged to be a monosaccharide at 24 h of reaction. These results are consistent with the results of Kim *et al*. (2015), that alginate was degraded into monosaccharides and oligosaccharides by crude enzyme from *S. oneidensis* PKA 1008, and degraded completely into monosaccharides after 48 h of reaction. Also, the crude enzyme from *S. oneidensis* PKA 1008 could degrade the polysaccharide of *U. pinnatifida* root into monosaccharides and oligosaccharides (Xu *et al*., 2020). Therefore, the present work suggested that the crude enzyme derived from *S. oneidensis* PKA 1008 could be used to degrade *S. fulvellum* alginic acid.

Effect on LPS-induced nitric oxide

Nitric oxide (NO), which is synthesised in large amounts by inducible nitric oxide synthase (iNOS), is known to be responsible for the vasodilation and hypotension observed during septic shock and inflammation (Ryu *et al*., 2003). As shown in Figure 2, the level of NO production induced by LPS in RAW 264.7 cells decreased significantly when treated with SFEE with different reaction times. It was confirmed that the samples after 3 h of enzyme treatment significantly decreased NO secretion as compared to the LPS treatment group. As compared to the untreated group, NO secretion was not significantly different from 6 h - 24 h of reaction. Moreover, the highest inhibitory activity was observed at 6 h after reaction, with about 79% inhibition as compared to the LPS treatment group. The reason for the decrease in NO secretion could be the monosaccharide formation by the breakdown of alginic acid polysaccharides (Figure 1).

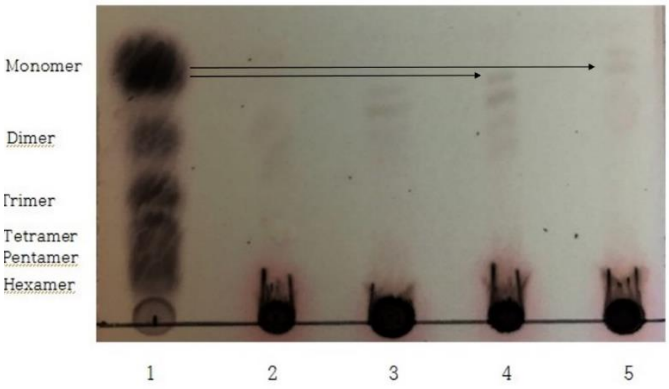

Figure 1. TLC analysis of *Sargassum fulvellum* enzymatic extracts using a crude alginate-degrading enzyme at various reaction times: lane 1, standard; lane 2, 0 h; lane 3, 3 h; lane 4, 24 h; and lane 5, 48 h.

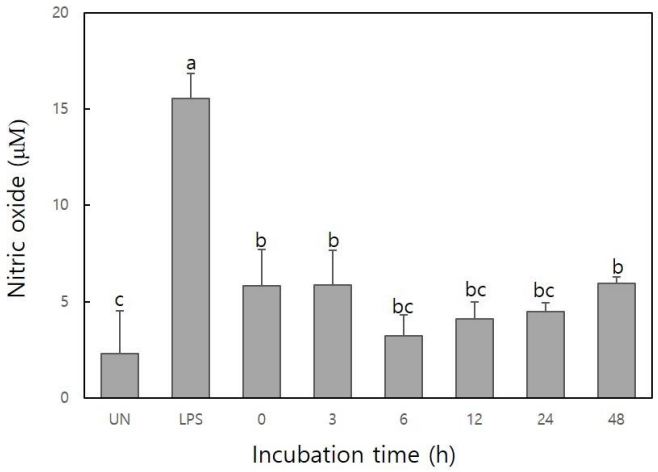

Figure 2. Inhibitory effect of enzymatic extracts of *Sargassum fulvellum* with reaction time on the production of NO in RAW 264.7 cells. Means with different lowercase superscripts are significantly different ($p < 0.05$).

These results showed that the NO inhibition activity of SFEE using crude enzyme from *S. oneidensis* PKA 1008 was higher than treatment with the *S. fulvellum* ethanolic extract at 100 μg/mL (Kim *et al*., 2013). In addition, in a study investigating the inhibitory effect of *U. pinnatifida* root (UPT) enzymatic extract on NO production in LPS-induced RAW 264.7 cells, the secretion of NO by LPS stimulation was significantly inhibited by UPT enzymatic extract treatment (Xu *et al.*, 2020). In particular, after 12 h, NO secretion was significantly suppressed, and the amount of NO secretion decreased by about 69% as compared to the LPS-treated group at 48 h. Therefore, it can be concluded that the inflammation inhibition effect was greatly enhanced after *S. fulvellum* degradation by crude enzyme from *S. oneidensis* PKA 1008.

Inhibitory effect on production of LPS-induced IL-6, TNF-α, and IL-1β

The pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β are mediators in inflammatory immune responses, and prolonged responses can lead to skin inflammation and cancer (Weisz *et al*., 1996; Choi *et al*., 2013). To test for any anti-inflammatory effect of SFEE using crude enzyme from *S. oneidensis* PKA 1008, the inhibition effect of IL-6, TNF-α, and IL-1β production in LPS-induced RAW 264.7 macrophages was investigated. Results showed that IL-6 cytokine significantly decreased after treatment of SFEE as compared toh the LPS treatment group. Particularly, IL-6 cytokine significantly decreased by 96% (Figure 3A) as compared to the LPS group at 24 h of reaction. The cause of the decrease in release of IL-6 cytokine at 24 h of reaction can be explained by the TLC analysis results (Figure 1), showing that alginic acid polysaccharides were degraded into monosaccharides at 24 h of reaction. As compared to the untreated group, the secretion of IL-6 was not significantly different from 3 h - 48 h of reaction.

In addition, the TNF-α level secreted from SFEE significantly decreased in 3 - 48 h as compared to the LPS treatment group (Figure 3B), and the maximal inhibition of TNF-α production was 39% at 12 h of reaction. The secretion of IL-1β was also significantly inhibited after treatment of SFEE as compared to the LPS group (Figure 3C). With reaction time being prolonged, the IL-1β level did not change significantly, similar to the untreated group.

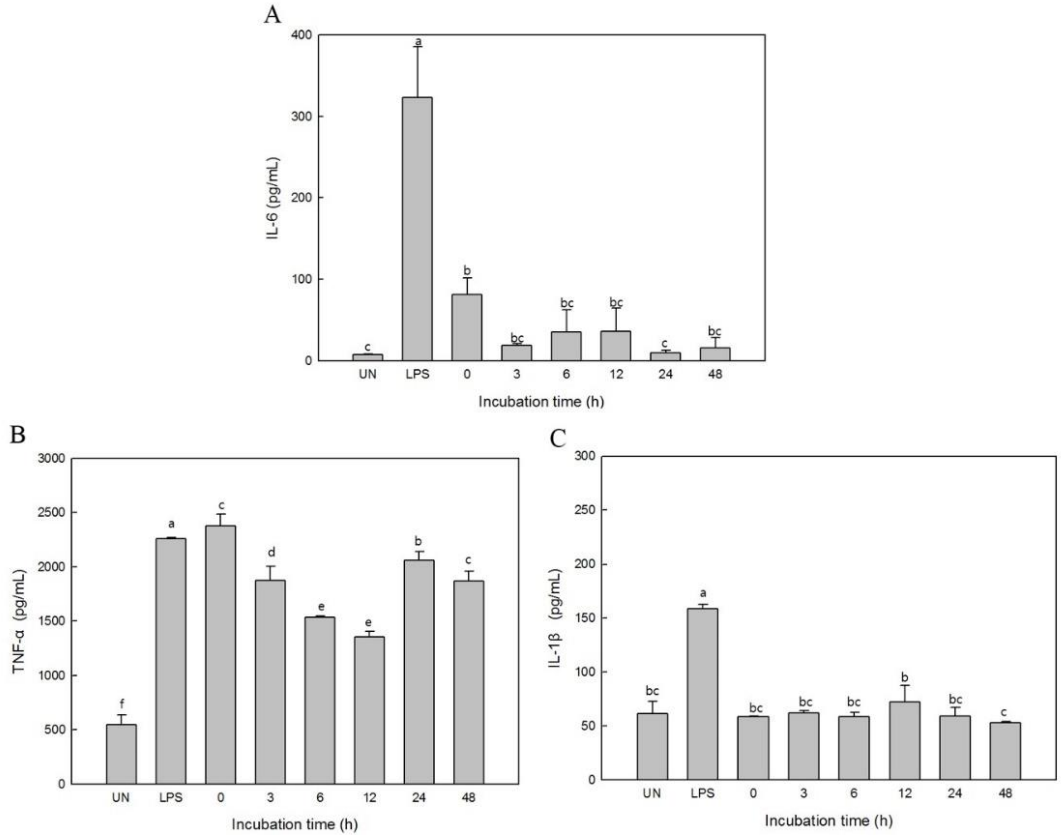

Figure 3. Inhibitory effect of enzymatic extracts of *Sargassum fulvellum* with reaction time on the production of IL-6 (a), TNF-α (b), and IL-1β (c) in RAW 264.7 cells. Means with different lowercase superscripts are significantly different ($p < 0.05$).

It has been proposed that the water extract and/or ethanolic extract from *S. fulvellum* could promote a decrease in TNF-α, IL-6, and IL-1β secretion (Weisz *et al*., 1996; Jeong *et al*., 2012). In a study by Xu *et al*. (2020), the inhibitory effect of UPT enzymatic extract on inflammatory cytokines (IL-6, TNF-α, and IL-1β) was confirmed. As a result, it was confirmed that the secretion of IL-6, TNF-α, and IL-1β significantly decreased by the treatment of the UPT enzymatic extract as compared to the LPS alone treatment group. In particular, the secretion of IL-6 and TNF-α decreased by approximately 97% and 54% in the 24 h incubation sample, respectively. The secretion of IL-1β was shown to decrease by approximately 62% in 12 h incubation samples. In addition, in a study by Lee *et al*. (2021), the secretion of IL-6, TNF-α, and IL-1β cytokines was significantly inhibited in a concentration-dependent manner when *S. patens* C. Agardh enzymatic extracts were used from 0.1, 1, 10, 50, or 100 μg/mL.

Therefore, we observed that crude enzyme from *S. oneidensis* PKA 1008 could degrade the polysaccharides of *S. fulvellum* to get alginic acid oligosaccharide, which could inhibit the LPS-induced IL-6, TNF-α, and IL-1β production in RAW 264.7 cells.

Conclusion

In the present work, the characterisation and anti-inflammatory effect of SFEE using crude enzyme from *S. oneidensis* PKA 1008 was investigated. Crude enzyme from *S. oneidensis* PKA 1008 could degrade *S. fulvellum* polysaccharides into oligosaccharides. The pH and viscosity of SFEE decreased with the increase in reaction time; colour measurement showed that the redness and yellowness increased and the brightness decreased, as the reaction time increased. Additionally, SFEE could inhibit the production of NO and pro-inflammatory cytokines (IL-6, TNF-α, and L-1β), and showed excellent anti-inflammatory effects. In summary, SFEE can be used and processed as an anti-inflammatory functional food. Future research is necessary for developing potential therapeutic materials using SFEE with excellent anti-inflammatory effects.

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