Effects of extraction methods on molecular characteristics, antioxidant properties and immunomodulation of alginates from *Sargassum angustifolium*

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Abstract

The relationship between molecular structure and bioactivity was evaluated for alginates obtained under different extraction methods (water, acid, **alcalase** and **cellulase**) from *Sargassum angustifolium*. The use of enzymes considerably reduced protein (from 14.58% to < 0.4%) and polyphenol (from 16.0% to < 1.7 mg GA/ g sample) contaminations of alginates compared to those of water and acid. **The FT-IR spectrum revealed that extraction method did not affect the structure of the recovered alginates.** The highest **molecular weight (M_w)** (557.1×10^3 g/mol) was found in acid treated alginate while the M_w of **cellulase assistant alginate** (356.2×10^3 g/mol) was the minimum. The SV_g values varied from 2.79-5.17 cm³/g revealing the loosed conformational structures of **alcalase and cellulase assistant alginates.** **Alcalase assistant alginate** stimulated RAW264.7 cells to release nitric oxide and inflammatory cytokines TNF- α , IL-1, IL-6, IL-10 and IL-12. **Enzyme treated alginates showed maximum DPPH radical scavenging activity and reducing power.** Therefore, the present results showed the determinant effect of pretreatment during the extraction process of alginate and the beneficial influence of enzymatic process when biological functions of alginates are of high interest in the industry.

Keywords: Alginates, Extraction Process, Molecular weight, Immunomodulatory, *Sargassum angustifolium*

1. Introduction

Alginates are a group of hydrocolloids extensively found in both algal and bacterial resources [1]. These hydrocolloids are generally considered as being healthy food ingredients that are not digestible by the endogenous enzymes in the small intestine of humans [2]. Alginates are localized in the cell wall and matrix of brown seaweeds and play an important structural role in providing mechanical resistance and flexibility to seaweeds against currents [3].

The most exploited brown seaweeds to extract commercial alginates are *Laminaria* spp., *Ascophyllum* spp., *Sargassum* spp. and *Fucales* spp. The seaweed species, climate conditions during growth or harvesting and manufacturing process have pivotal effect on the quality of extracted alginates [4]. Accordingly, the price and application of alginates vary from one type to another. So far, most of the studies conducted on alginates were related to their rheological properties in food systems or pharmaceutical products [5]. It has been stated that any changes to the ratio and sequence of building blocks, molecular weight and carboxylic groups would significantly affect the rheological properties of alginates [5, 6]. The peculiar rheological characteristics of alginates in association with other advantages such as being widely accessible in nature, easy to extract and industrially inexpensive to manufacture, make them one of the most exploited hydrocolloids in the food industry [4]. However, in terms of physiological characteristics, there are only a very limited number of studies reporting the effectiveness of alginates in native form. For instance, alginate has shown to be capable of inhibiting pancreatic lipase activity *in vitro* and therefore could be incorporated into different types of breads as a complementary treatment for obesity [7]. The structural alterations of alginates were performed by various chemical and biological means. Chemically sulfated alginates suggested that the derivatization of these acidic polysaccharides could further broaden their applications into

functional food and nutraceutical industries where anticoagulant, anti-inflammatory and anti-tumor agents are highly desirable [8,9,10]. Selenylation and depolymerization are other forms of structural manipulations applied to alginates in order to introduce new bio-functionalities [11,12].

Despite all the efforts made to better understand the correlation between the structure and functional properties of alginates after isolation, only a few studies were devoted to investigate the impact of extraction process on their molecular properties [14].

Basically, seaweed alginates are found in an insoluble state bound with cations such as Na^+ , Mg^{2+} and Ca^{2+} . In conventional methods, prior to extraction, a pretreatment using hydrochloric acid is used to remove non-target compounds such as fucoidans, laminarins and polyphenols, and provide acidic condition up to pH 4 where alginate salts are converted to alginic acid [14]. The extraction process is performed by solubilization of alginate molecules using Na^+ and successive dissolutions and precipitations to eliminate impurities [15]. Since, the rheological properties of alginates were highly favorable from the very early days of alginate discovery, most of the studies were conducted to maintain or improve physical characteristics in an economic system [16]. But, the more recent reports on process evaluation of alginate extractions at initial pretreatment and post extraction steps indicated the necessity of conducting further studies [13,14]. Besides, in spite of tremendous amount of work conducted on the rheological properties of alginates, the studies on their physiological characteristics are underrated. Therefore, the aim of current study was to evaluate the effect of various pretreatment methods on the chemical composition and molecular properties of alginates extracted from *Sargassum angustifolium*. The immunostimulatory and antioxidant properties were also examined for alginates obtained under different extraction procedures.

2. Materials and methods

2.1. Materials

Sargassum angustifolium was collected from the coast of Bushehr, Iran. Then, fresh alga was washed with tap water and dried at 60 °C. The dried alga was pulverized using a blender, sieved (< 0.5 mm) and stored in plastic bags at -20 °C. Cellulase was purchased from Beijing Solarbio Science & Technology Co., Ltd. (China). Alcalase was acquired from Sigma-Aldrich (USA). All other chemicals and reagents used in this work were of analytical grade.

2.2. Isolation of alginates

Fig. 1 summarizes the extraction procedure employed to obtain different alginates. Briefly, to remove non-target compounds such as pigments and lipids, 50 g of milled sample was treated with 85% ethanol (EtOH, 200 mL) overnight at room temperature ($22 \pm 2^\circ\text{C}$). The supernatant was discarded after centrifugation at 10 °C and 8000 rpm for 10 min. The residual part was rinsed with acetone and dried at room temperature ($22 \pm 2^\circ\text{C}$). The dried biomass was treated with distilled water (400 mL, 65°C, 3 h), HCl (0.1M, pH 2, 65°C, 3 h), alcalase (5% w/w, pH 8, 50 °C, 24 h) or cellulase (5% w/w, pH 4.5, 50 °C, 24 h). The supernatants were discarded after centrifugation at 10 °C and 10000 rpm for 10 min. The residue was extracted with 3% Na₂CO₃ (1000 mL) at pH 11, 65 °C for 3 h. The supernatants were collected after centrifugation and then concentrated by evaporation under vacuum at 60°C.

2.3. Purification of alginates

To recover and purify the alginates, EtOH (99%) was added into the supernatant to obtain a final EtOH concentration of 70%. The suspension was kept at 4°C overnight. The precipitate was separated by centrifugation and consecutively washed with EtOH (99%) and acetone for several times. The resulting precipitates were dried at room temperature ($22 \pm 2^\circ\text{C}$) in a fume hood and designated as DW-A (distilled water), AC-A (acidic), AL-A (alcalase) and CE-A (cellulase). The yield of alginate was calculated according to the dried biomass obtained after treating the milled sample with 80% ethanol. Protein content of alginates was measured by the Lowry method (17) using a DC protein assay kit (Bio-Rad, CA, USA). The total phenolic content of alginates was measured using the Folin-Ciocalteu (18).

2.4. ATR-FTIR analysis

Fourier transform infrared (FT-IR) spectra of alginates were obtained to determine the functional groups. The samples were scanned at wavenumbers ranging from 500 to 4000 cm^{-1} at a resolution of 2 cm^{-1} using a Tensor 27 spectrometer (Bruker Instruments, Billerica, USA). Alginates were mixed with KBr as a 0.5–1 mm thick film and analyzed by ATR-FTIR using absorbance mode.

2.5. Nuclear magnetic resonance spectroscopy (NMR)

The ^1H NMR spectra of sodium alginates were recorded on a JEOL ECA-600 spectrometer (JEOL, Akishima, Japan), equipped with a 5mm multi-nuclear auto-tuning TH tunable probe, at a base frequency of 600 MHz. Alginates (were deuterium-exchanged by lyophilisation with 99.9% D_2O and subjected to the spectrometer at 80°C).

2.2. Determination of molecular properties

Alginates were dissolved in distilled water (2 mg/ mL) and heated for 30 sec in a microwave bomb prior to molecular measurement (#4872; Parr Instrument Co., Moline, IL, USA). The samples were immediately filtered through a cellulose acetate membrane (3.0 μ m120 pore size; Whatman International). A high performance size exclusion chromatography column (TSK G5000 PW, 7.5 $\times$ 600 mm; Toso Biosep, Montgomeryville, PA, USA) linked to a UV detector (Waters, 2487), multi-angle laser light scattering (HELEOS; Wyatt Technology Corp, Santa Barbara, CA, USA) and refractive index detection (Waters, 2414) system (HPSEC-UV-MALLS-RI) were used to analyze the molecular characteristics. The mobile phase, flow rate of 0.4 mL/min, was composed of an aqueous solution of 0.15 M NaNO₃ and 0.02% NaN₃. Bovine serum albumin (BSA) was employed to determine the volume delays among the UV, MALLS and RI detectors.

ASTRA 5.3 software (Wyatt Technology Corp.) was used to calculate the weight average molecular weight (M_w), number average molecular weight (M_n), radius of gyration (R_g) and polydispersity. The specific volume of gyration (SV_g) was calculated based on the following equation [19]:

$$SV_g = 4/3\pi(R_g \times 10^8)^3/(M_w/N) = 2.522 R_g^3/M_w$$

in which N is Avogadro's number (6.02×10^{23} /mol) and the units for SV_g , M_w and R_g were cm³/g, g/mol and nm respectively.

2.7. RAW264.7 macrophage proliferation and nitric oxide production assays

RAW264.7 macrophage cells in an RPMI-1640 medium containing 10% FBS were plated in a 96-well microplate (1×10^4 cells/well, 100- μ L volume; ATCC). The cells were incubated with 100 μ L of the polysaccharides at different concentrations (10, 25 and 50 μ g/mL) in triplicate. The cell cultures were kept in humidified atmosphere containing 5% CO₂ at 37 °C for 72 h. Then, the WST-1 solution (20 μ L) was added to the wells and the solution was further incubated for 4 h at 37 °C. The optical density was measured at 450 nm using a microplate reader (EL-800; BioTek Instruments, Winooski, VT, USA). The absorbance (A) was translated into a macrophage proliferation ratio, (%) = $A_t/A_c \times 100$, where A_t and A_c are the absorbance of the test group and control group, respectively.

The nitric oxide (NO) released by macrophages into culture supernatants was measured as an indicator of immunoenhancing activity of alginates. RAW264.7 cells were plated in a 96-well plate (1×10^5 cells/well) and incubated in the presence of alginates (10, 25 and 50 μ g/mL) and LPS (1.0 μ g/mL) at 37 °C for 18 h. The amount of NO production was measured using the Griess reaction [20]. NaNO₂ (1-200 μ M in culture medium) was used as reference to quantify the amount of NO produced by macrophages.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Alginates or LPS were added to RAW264.7 cells (1×10^5 cells/well) and incubated at 37 °C for 18 h. The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from RAW264.7 cells according to the manufacturer's protocol. After quantification of RNA concentration using a spectrophotometer, construction of cDNA was performed with an oligo-

(dT)₂₀ primer and Superscript III RT (Invitrogen, Carlsbad, CA, USA). PCR amplification was then performed by GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) and specific primers. The primers used were as follows: iNOS, 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' (forward) and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (reverse); IL-1 β , 5'-ATGGCAACTATTCCTGAACTCAACT-3' (forward) and 5'-CAGGACAGGTATAGATTCTTTCCTTT-3' (reverse); IL-6, 5'-TTCCTCTCTGCAAGAGACT-3' (forward) and 5'-TGTATCTCTCTGAAGGACT-3' (reverse); IL-10, 5'-TACCTGGTAGAAGTGATGCC-3' (forward) and 5'-CATCATGTATGCTTCTATGC-3' (reverse); TNF- α , 5'-ATGAGCACAGAAAGCATGATC-3' (forward) and 5'-TACAGGCTTGTCACCTCGAATT-3' (reverse); IL-12, 5'-CCACAAAGGAGGCGAGACTC-3' (forward) and 5'-CTCTACGAGGAACGCACCTT-3' (reverse) and β -actin, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (forward) and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3' (reverse). Reverse transcriptase amplification was conducted with an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 40 s) and extension (72 °C for 1 min), and then a final extension step at 72 °C for 10 min. The electrophoresis of the PCR products was run on a 1% agarose gel and visualized by ethidium bromide staining.

2.9. Antioxidant properties of alginates

2.9.1. DPPH radical scavenging activity

A slightly modified method developed by Ren et al. [21] was used to examine the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of alginates. Briefly, 100 μ L of

different alginates with various concentrations of 0.25, 0.5 and 1.0 mg/mL were added to 100 μ L of DPPH solution. The mixture was vigorously shaken and then incubated at 25 °C in the dark for 30 min. The absorbance was read at 517 nm by an UV–Vis spectrometer. The ability of alginates to scavenge the DPPH radical was calculated according to the following equation:

$$\text{DPPH scavenging activity \%} = \frac{A_c - A_s}{A_c} \times 100$$

2.9.2. Reducing power

The reducing power was performed according to the method developed by Oyaizu [22] with slight modifications. Different concentrations of alginates (0.25, 0.5 and 1.0 mg/mL) were prepared and 500 μ L of each solution was mixed with 500 μ L of sodium phosphate buffer (0.2 M, pH 6.6) and 500 μ L of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 500 μ L of 10% TCA was added to the mixture and centrifuged at 8000 rpm for 10 min. The supernatant (1.0 mL) was separated and incubated with 1.0 mL of distilled water and 200 μ L of 0.1% ferric chloride at room temperature for 10 min. The absorbance was measured at 700 nm.

2.10. Statistical Analyses

All experiments were conducted in triplicate ($n=3$) and statistical analysis performed using SPSS software (Version 16; SPSS Inc., Chicago, IL, USA). Significant differences were identified by one-way analysis of variance (ANOVA) and Duncan's multiple-range test. A probability value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussions

3.1. Yield and chemical compositions

Table 1 shows the effects of different pretreatments including water, acid, alcalase and cellulase on yield and purity of isolated alginates from *S. angustifolium*. The lowest extraction yield of alginate (3.30%) was found to be of water treated seaweeds. Only a slight increase in extraction efficiency and the amount of alginate yield (3.40%) was observed when acid pretreatment using 0.1M HCl was employed prior to extraction. The alginate yield obtained after alcalase and cellulase treatments showed a further small increase up to 3.50%. The amount of alginates isolated from different seaweeds indicates a wide range of extraction yields varying from 3.0% to 45% [6,13,14]. This significant variation in the amount of extracted alginates may be originated from differences in species, growth environment and isolation methods [23].

The extent of polysaccharide purity was determined by measuring the level of protein and phenolic compounds introduced into extracted alginates (Table 1). The employment of various pretreatments resulted in alginates having different amounts of proteins among which DW-A showed the highest level of impurity (14.58%; $p < 0.05$). Followed by AC-A, acidic condition was found to be relatively effective in producing alginates with less protein contaminations (11.92%). Surprisingly, protease and carbohydrase enzymes used before extraction could effectively reduce the amount of proteins in AL-A and CE-A alginates ($< 0.4\%$). The amount of phenolic compounds introduced into alginates during the extraction process was measured as another purity indicator of final polysaccharide. As it is shown in Table 1, most of the phenolic compounds were included in DW-A (16.03 mg GA/ g sample) and AC-A (9.16 mg GA/ g sample) alginates. In contrast, AL-A and CE-A contained less than 2.0 mg GA/ g sample of

phenolic compounds ($p < 0.05$). It has been stated that the utilization of dilute acid conditions during the extraction process disrupts hydrogen bonding and facilitates the liberation of polysaccharides [24]. Besides, the destroying process of hydrogen bonding of entanglements between cellulose and proteins of cell walls with alginates could be efficiently achieved using available commercial carbohydrase and protease enzymes [25]. Nonetheless, the introduction of proteins and low molecular weight compounds into alginates during the enzyme-assisted extraction is the main drawback [26]. On the contrary to the general perception, the present results showed that if alcalase and cellulase enzymes are employed for the purpose of extraction pretreatment, most of proteins and polyphenols can be removed in advance. Therefore, produced alginates possess higher levels of purity and their functional properties are more reliable.

The quality of alginates was assessed by the ratio between mannuronic acid (M) and guluronic acid (G). The relative areas of anomeric protons H-1 G and H-1 M correspond to the mole fractions of G and M, respectively. The ratio of M/G defines the rheological properties of alginate and it changes from one species to another (27). The NMR spectra and integral information of various alginates are presented in Fig. 2A and Table 1. As it is shown, the M/G ratio calculated from the relative area of H-1 G (5.10 ppm) and H-1 M (4.65 ppm) was 0.54 for water assistant alginate. The ratio of M/G did not changed significantly in other alginate samples indicating the inability of extraction pretreatments on the composition of mannuronic acid and guluronic acid. Similar to the present study, the M/G ratio was found to be 0.56 for alginates from *S. binderi* (28). Overall, the previous reports showed that the amounts of mannuronic acids are greater than guluronic acids in most of the alginates obtained from *Sargassum* and the M/G ratios vary between 0.19-0.82 (25).

3.2. FT-IR analysis

The FT-IR spectrum of extracted alginates from *S. angustifolium* showed a broad signal at 3430 cm^{-1} attributing the stretching vibrations of hydroxyl groups OH and a small signal at 2939 cm^{-1} attributing the stretching vibration of CH (Fig. 2B) [29]. The peak at 1618 cm^{-1} attributed to COO^- asymmetric stretching vibration of carboxyl groups of alginate [30]. The strong peak at 1419 cm^{-1} corresponded to COO^- symmetric stretching vibrations of uronic acids [31]. It is reported that there are two characteristic peaks in the IR spectra of alginates; one at 1093 cm^{-1} assigned to β -mannuronic acid and the other one at 1030 cm^{-1} assigned to α -L-guluronic acid [32]. The appearances of peaks corresponding to mannuronic acids and guluronic acids have been also reported for alginate from *S. vulgare* (29). In general, the FT-IR analysis revealed that the employment of different pretreatments did not cause any shifts in the characteristic peaks of the alginates and therefore the chemical structure was not changed subsequently.

3.3. Molecular weight properties

The RI (refractive index) chromatograms of various extracted alginates are shown in Fig. 3. The RI profile of DW-A showed the presence of alginate molecules in three distinct populations eluted from 24-28 min (Peak I), 28-40 min (Peak II) and 40-46 min (Peak III) (Fig. 3A). The use of acidic condition to treat *S. angustifolium* led to a similar RI profile where, compared to DW-A, more molecules with middle and small molecular weights were produced (Fig. 3). When alcalase employed (AL-A), a significant loss of high molecular weight alginates took place and peak I sharply decreased while peaks II and III remained the same (Fig. 3C). The molecular

distribution of CE-A alginate showed a small shoulder followed by two large peaks indicating a further decrease of high molecular weight alginates in cellulase-treated samples (Fig. 3D).

The average molecular weight (M_w) of DW-A alginate obtained by the multi-angle laser light scattering technique was 480.3×10^3 g/mol (Table 2). The M_w determination of alginates from various species such as *Ecklonia cava*, *Sargassum vulare* and *Macrocystis pyrifera* suggests a broad spectrum of M_w ranging from $190\text{--}798 \times 10^3$ g/mol [13,14,26]. The M_w of AC-A alginate showed a notable increase in M_w (557.1×10^3 g/mol). The increase in alginate M_w of acidic pretreatment compared to water treated one has been previously reported in *S. wightii* [33]. As it was shown in RI chromatograms, treating samples with alcalase and cellulase enzymes dramatically reduced the molecular weight of isolated samples to less than 357.0×10^3 g/mol. Number average molecular weight (M_n) values of alginates were also analyzed in order to calculate the level of molecular heterogeneity (Polydispersity index) of various alginates. Overall, the results revealed that the lowest polydispersity was achieved for DW-A alginate while significantly higher polydispersity values were respectively obtained for AC-A, AL-A and CE-A alginates.

The specific volume of gyration (SV_g) was calculated from the values of M_w and R_g . The SV_g provide the theoretical gyration volume per unit of molar mass and is inversely proportional to the degree of molecular compactness [19]. The SV_g values for different alginates showed considerable variations and ranged from 2.79 to 5.17 cm³/g (Table 2). The calculations suggested that DW-A alginate had the most compact molecular conformation while acid treatment resulted in alginates with slightly more expanded structure. AL-A and CE-A alginate molecules were found to have more loosed conformational structures. The above results indicated that the pre-

extraction treatment of *S. angustifolium* powders leads to final alginate polymers with significant variations in their M_w , polydispersity and conformation.

3.5. Immunomodulation of alginates

The effect of alginates from *S. angustifolium* was examined on the proliferation of RAW264.7 cells at concentrations ranging from 10 to 50 $\mu\text{g/mL}$ (Fig. 5A). The addition of alginates into macrophage cells medium effectively increased the cell proliferations in the range of 15-40%. Among the alginates, AL-A exerted the greatest increase in RAW264.7 cell proliferation at 50 $\mu\text{g/mL}$ ($p < 0.05$). These results suggested that not only none of the extracted alginates were toxic to macrophage cells but also RAW264.7 cells were positively signaled to proliferate. Fig. 5B shows the amount of NO released from RAW264.7 cells by different alginate samples at 10, 25 and 50 $\mu\text{g/mL}$. The level of NO produced by macrophage cells remarkably varied among alginates and ranged from 4.9 to 36.1 μM ($p < 0.05$). Only a maximum amount of 15.0 μM of NO was produced when DW-A and AC-A alginates used to stimulate the macrophage cells ($p > 0.05$). In contrast, enzymatically treated alginates resulted in high NO production where AL-A exhibited the NO release of more than 36 μM at 50 $\mu\text{g/mL}$. This amount of NO production from *S. angustifolium* alginates was comparable to that of LPS (1.0 $\mu\text{g/mL}$) used as positive control and therefore could be considered as strong stimulants of RAW264.7 cells. So far, only alginate oligomers were found to show immunostimulatory effects. These oligomers that were mostly produced through chemical or enzymatic reactions could stimulate human monocytes and mouse macrophage cells to release pro-inflammatory cytokines [34,35]. In addition, it has been reported

that the molecular weight of alginates has a determinant role in the magnitude of cell induction in which smaller molecules exhibit higher activity [11].

To answer the question whether the NO production from RAW264.7 cells is enzymatically generated and not introduced by external contamination, the mRNA expression of inducible nitric oxide synthase (iNOS) was examined. The AL-A alginate was selected as the most potent macrophage stimulant in order to search for mRNA expression of iNOS by agarose gel electrophoresis of RT-PCR products using primers of iNOS mRNA. As shown in Fig. 6A, the sharp and thick bands were observed on the agarose gel for different concentrations of AL-A alginate and LPS which was consistent with the results of NO production. Thus, the results confirmed the activation of RAW264.7 cells by AL-A alginate through the enhanced mRNA expression of iNOS and formation of nitric oxide. The mRNA expression of other cytokines including IL-1B, **TNF- α** , IL-6, IL-10 and IL-12 was also studied in RAW264.7 cells exposed with AL-A alginate (Fig. 6A and 6B). The AL-A alginate was able to significantly increase the mRNA expressions of these cytokines in a dose dependent manner. Basically, the pro-inflammatory cytokines such as TNF- α , IL-1B and IL-6 are thought to be pivotal for the survival of humans from infections and abnormal cells through the stimulation of immune system [36,37]. If the secretion of pro-inflammatory cytokines reaches beyond the normal level, severe inflammation symptoms can be seen as a result of harmful effects on the repair process. Therefore, there are anti-inflammatory cytokines, such as IL-10 and IL-12, in the immune system that are released to initiate the adverse mechanism and prevent detrimental effects of excessive macrophage activation [38]. The mRNA expression of anti-inflammatory cytokines was also remarkably occurred upon the addition of AL-A alginate into cell medium. This indicates that

AL-A alginate might also be able to inhibit the potential harmful effect of pro-inflammatory cytokines through IL-10 and IL-12.

3.6. Antioxidant properties of alginates

The DPPH radical scavenging activity of different alginates from *S. angustifolium* is shown in Fig. 7A. The employment of different pretreatment techniques produced alginates with diverse DPPH scavenging activities. All alginate samples were able to produce the stable form of DPPH in a dose-dependent manner. The scavenging effects of DW-A alginate was found to be the minimum (39.9%) while those of AL-A (66.5%) and CE-A (66.7%) were at the highest levels and comparable to that of BHT (commercial antioxidant). Fig. 7B demonstrates the ability of isolated alginates to reduce ferric to ferrous in a redox linked colorimetric reaction. Similarly, the DW-A alginate exhibited the lowest reducing power while AL-A alginate appeared as the strongest sample ($p < 0.05$). The previous findings have also revealed the high DPPH radical scavenging activity and reducing power of alginates obtained from *Turbinaria conoides* and *Dictyopteris polypodioides* [39,40]. In accordance with the current results, the antioxidant evaluation of alginate isolated from *S. vulgare* and its depolymerized derivatives prepared using acidic condition (1.5M HCl) showed the higher efficacy of lower molecular weight alginates [29].

4. Conclusions

The present study examined the effects of pretreatments on the quality of alginates extracted from *S. angustifolium* for the purpose of biofunctional properties. Although, the differences between the extraction yields of various methods were small, alginate polymers with higher purity were obtained after enzyme treatments. A significant extent of changes was observed in the molecular properties of alginates obtained under different extraction methods. There was a considerable loss of high molecular weight polymers for alginates isolated after alcalase and cellulase treatments while a greater amount of low molecular weight alginates were recovered. alcalase treated alginate was the most potent immunoenhancer polymer which could stimulated macrophage cells to release NO, pro-inflammatory and anti-inflammatory cytokines. All isolated alginates showed antioxidant activity in which enzymatically treated seaweeds produced alginates with higher activity. The current study, therefore, demonstrates the crucial effect of different extraction methods on purity, molecular characteristics and biological properties of extracted alginates.

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509 **Figure captions**

510 **Fig. 1.** Extraction and fractionation of the alginates from brown seaweed *S. angustifolium*.

511 **Fig. 2.** ¹H NMR spectra (A) and ATR-FTIR spectra (B) of DW-A (Water), AC-A (Acid), AL-A
512 (alcalase) and CE-A (cellulase) alginates.

513 **Fig. 3.** Refractive index (RI) chromatograms of DW-A (A), AC-A (B), AL-A (C) and CE-A (D)
514 alginates dissolved in distilled water using a TSK G5000 PW column with 0.15 M NaNO₃ and
515 0.02% NaN₃ at a flow rate of 0.4 mL/min.

516 **Fig. 4.** FE-SEM image and EDX analysis of DW-A, AC-A, AL-A and CE-A alginates.

517 **Fig. 5.** Proliferation activity (A) and nitric oxide production (B) of the RAW264.7 macrophage
518 cells after treatment with DW-A, AC-A, AL-A and CE-A alginates. The RAW264.7 cells were
519 plated in an RPMI-1640 medium containing 10 % FBS in a 96-well microplate (1×10⁴
520 cells/well) and incubated with 100 µL of 10, 25 and 50 µg/mL different alginates or 1 µg/mL
521 LPS (positive control). The letters a,b,c,d indicate a significant difference (p < 0.05) between the
522 concentrations of the alginates, with A, B, C, D indicating a significant difference (p < 0.05)
523 between the alginates at each concentration.

524 **Fig. 6.** Effects of AL-A alginate on cytokine mRNA expression in RAW264.7 cells. (A) The
525 cytokine mRNA expressions for interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α),
526 interleukin-10 (IL-10), interleukin-6 (IL-6) and interleukin-12 (IL-12) were determined by RT-
527 PCR. (B) Graphic analysis of the polymerase chain reaction products for RNA levels of different
528 cytokines. The letters a,b,c,d indicate a significant difference (p < 0.05) between the
529 concentrations of the cytokines. β-actin was used as a control.

Fig. 7. DPPH radical scavenging activity (A) and reducing power (B) of DW-A, AC-A, AL-A and CE-A alginates. The letters a,b,c,d indicate a significant difference ($p < 0.05$) between the concentrations of the alginates. BHT (100 $\mu\text{g/mL}$) was used as a commercial antioxidant.

Table 1. Yield and chemical contaminations of DW-A (Water), AC-A (Acid), AL-A (alcalase) and CE-A (cellulase) alginates from *S. angustifolium*.

Alginates	Yield (%)	Protein (%)	Total phenol (mg GA/ g sample)	F_G	F_M	M/G
DW-A	3.30	14.58 ± 0.50^a	16.03 ± 0.84^a	0.87	0.47	0.54
AC-A	3.40	11.92 ± 0.69^b	9.18 ± 0.64^b	0.75	0.40	0.53
AL-A	3.50	0.36 ± 0.33^c	1.77 ± 0.55^c	0.77	0.42	0.54
CE-A	3.47	0.30 ± 0.33^c	1.40 ± 0.32^c	0.79	0.44	0.55

Table 2. Weight average molecular weight (M_w), number average molecular weight (M_n), radius of giration (R_g), specific volume of gyration (SV_g) and polydispersity of different alginates.

Alginates	$M_w \times 10^3$ (g/mol)	$M_n \times 10^3$ (g/mol)	R_g (nm)	SV_g (cm ³ /g)	Polydispersity (M_w/M_n)
DW-A	480.3 ± 5.93	255.1 ± 3.53	81.05 ± 7.56	2.79	1.79 ± 0.12
AC-A	557.1 ± 1.48	294.7 ± 3.32	87.85 ± 0.07	3.06	1.85 ± 0.02
AL-A	357.0 ± 3.67	153.5 ± 1.62	80.4 ± 0.70	3.67	2.15 ± 0.09
CE-A	356.2 ± 3.81	120.95 ± 0.35	90.05 ± 2.33	5.17	2.94 ± 0.14