

Accelerating Sulfated Polysaccharides Extraction from Fast-Growing *Ulva* Green Seaweed by Frequency-Controlled Microwaves

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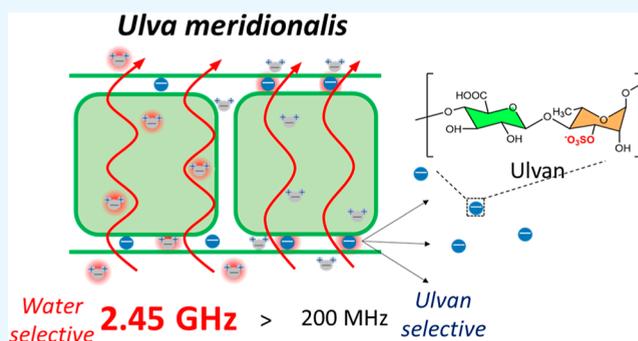


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Supporting Information

ABSTRACT: *Ulva* sp. is a type of green algae and is widely distributed in coastal areas around the world due to eutrophication. Effective utilization of *Ulva* sp. is expected for the establishment of carbon-neutral biochemical production. Microwave-assisted hydrothermal extraction is one of the most efficient ways of extracting highly functional polysaccharides called ulvan. Here, we demonstrate the importance of microwave frequency in enhancing ulvan extraction from *Ulva meridionalis*. We found that microwaves (2.45 GHz) selectively heat water solvent, while radio frequency (200 MHz) selectively heats ionic ulvan. Moreover, 2.45 GHz was more effective for extracting ulvan than 200 MHz. Then, we analyzed the conformational change in ulvan during microwave irradiation using in situ small-angle X-ray scattering. Microwaves initiated the loosening of ulvan bundles at temperatures lower than those of conventional heating. As a result, microwaves at 2.45 GHz selectively heat water and initiate ulvan structural change to enhance the extraction of ulvan from *U. meridionalis*.



INTRODUCTION

Seaweeds are noted as a blue carbon resource that absorbs CO₂ and is widely distributed in the coastal areas.^{1,2} Primary production by seaweeds reaches 0.19–0.64 Pg C in a year, and their carbon fixation is estimated as 4.2–8.4 Pg.^{1,2} *Ulva* sp. is a green alga that grows rapidly. For instance, edible *Ulva prolifera* grows twice a day.³ Even more, *U. meridionalis* is a newly found *Ulva* sp. in 2011, and it grows 4.1 times in a day.^{3,4} The green tide of *Ulva* sp. occurs around the world from North America to Europe to Asia due to the eutrophication of coastal areas.^{5–8} *Ulva* rapidly decomposes, so it damages the coastal environment and landscapes, thus affecting the aquaculture industry.⁹ However, the high growth rate of *Ulva* sp. makes it attractive as a raw material for biochemicals by efficiently fixing carbon dioxide. *Ulva* sp. has been used for biogas production¹⁰ and organic fertilizers¹¹ and as an additive to feed.¹² In addition, chemical components of *Ulva* sp. have been used to synthesize edible films,¹³ pharmaceuticals,¹⁴ and biomembranes.¹⁵ Therefore, efficient exploitation of *Ulva* sp. contributes to sustainable biochemical production from carbon dioxide.

Seaweed contains various polysaccharides with functional properties.¹⁶ Particularly, *Ulva* sp. contains 9–36% dry weight of a sulfated polysaccharide called ulvan. Ulvan is attractive for its biological activities, such as antitumor and immunomodulatory properties.¹⁷ Ulvan contains rhamnose (16.8–45.0%), xylose (2.1–12.0%), and glucuronic acid (16.0–23.2%) as well as iduronic acid. Ulvan mainly consists of repeating

disaccharide units of 1,4-linked β -D-glucuronic acid and α -L-rhamnose 3-sulfate.^{17,18} Ulvan is localized in an intercellular matrix outside the cell wall with proteins.¹⁹ The conventional ulvan extraction process requires an extraction time of 1–3 h by heat convection (conventional heating; CH) using acids or chelating agents such as HCl and ethylenediaminetetraacetic acid (EDTA).^{17,20} However, extraction by heat convection is inefficient in terms of energy and time.

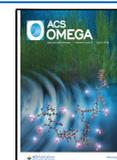
Dielectric heating using microwaves or radio frequency efficiently generates heat by dielectric relaxation of materials. The direct and selective heating of materials enables a high heating efficiency. The dielectric properties of a material depend on the frequency; thus, selective heating of material can be achieved by changing the applied frequency. Microwaves range from 300 MHz to 30 GHz and initiate dielectric losses due to the dipole rotations. Microwaves are more effective in heating water since water exhibits the maximum microwave absorption at 18 GHz.²¹ On the other hand, the radiofrequency ranges from 3 MHz to 300 MHz, and it promotes the ionic conduction of charged particles by the

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conduction losses.²² Therefore, radiofrequency is more suitable for selectively heating ionic components. Dielectric heating is widely used in various food industry processes such as cooking, thawing, drying, sterilization, and extraction.^{23,24} Microwave-assisted extraction has been widely studied for the production of essential oil, polyphenols, protein, and polysaccharides.^{25–28} Microwaves heat water within the cell and facilitate diffusion of the substance by effectively breaking down the cell wall.²⁹ As a result, shorter extraction times and higher yields can be achieved. For example, fucoidan, a polysaccharide from brown algae, can be extracted in a minute without using any acid or alkali agents.³⁰ We have previously reported that microwave irradiation effectively improves the extraction efficiency of ulvan from *U. meridionalis*.³¹ However, their acceleration mechanism is not yet well understood.

Here, we demonstrate the acceleration of ulvan extraction by selective heating of ulvan or water by varying the applied frequency from radiofrequency (200 MHz) to microwaves (915 and 2.45 GHz). In addition, we studied the mechanism of enhanced ulvan extraction by microwave irradiation by using *in situ* small-angle X-ray scattering (SAXS). We observed the microwave-induced conformational changes of the ulvan molecule, which enhance the ulvan extraction from the *U. meridionalis*.

MATERIALS AND METHODS

Materials. *U. meridionalis* (strain name E16) was cultivated in the outdoor tank at the Usa Marine Biological Institute, Kochi University (Kochi, Japan),³² rinsed with water, and air-dried. Thermogravimetry analysis using DTG-60A (Shimadzu Corporation, Kyoto, Japan) gave a moisture content of 23%. CHN elemental analysis was performed by Yanaco CHN coder MT-6 (Yanaco Technical Science, Tokyo, Japan) at the Service Center of the Elementary Analysis of Organic Compounds, Kyushu University, and the approximate composition of *U. meridionalis* was H = 4.89%, C = 29.67%, N = 2.44%, and ash = 12.34% in dry weight. Dried *U. meridionalis* was further milled by using a blender.

Microwave-Assisted Extraction by a 2.45 GHz Multimode Reactor. Milled *U. meridionalis* powder (0.1–3 g) was suspended in ultrapure water (20 mL) and allowed to swell for 5 min. Microwave heating was performed by a MicroSYNTH, which is a multimode microwave oven equipped with a magnetron (Figure 1, . GHz, maximum power: 1 kW, Milestone Inc., CT, USA). A high-pressure reactor (HPR-100) was used to conduct hydrothermal extraction at 100–180 °C with a 5 min heating time and a 5 min holding time. A

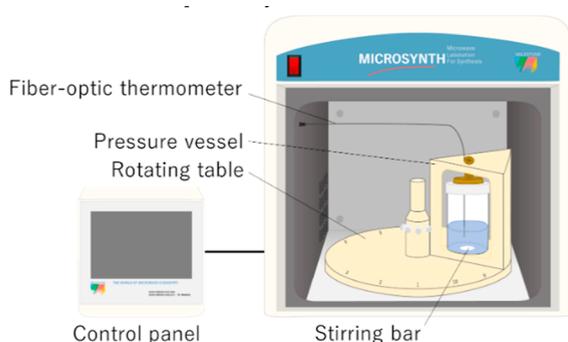


Figure 1. Schematic of ulvan extraction by a multimode 2.45 GHz reactor.

stirrer and a rotating table were used for uniform heating. The temperature was controlled by monitoring with a fiber-optic thermometer. After cooling, the extract was centrifuged and separated into the supernatant and residue. The supernatant was further concentrated, and polysaccharides were precipitated with the addition of ethanol. The precipitate was, then, lyophilized, and the yield was obtained using eq 1.

$$\text{Yield (\%)} = \left(\frac{\text{extract weight (g)}}{\text{dried sample weight (g)}} \right) \times 100 \quad (1)$$

The contents of total sugar, uronic acid, and reducing sugar in the supernatant were measured by the phenol sulfuric acid method, the *m*-hydroxy diphenyl method, and the DNS method, respectively.

Measurement of Complex Dielectric Permittivity of Ulvan. The complex dielectric permittivity of the ulvan aqueous solution (0.5–3.0%) was measured by the coaxial probe method using an N1500A and a P9371A Network Analyzer (Keysight Technologies, CA, USA). The measurement range was from 100 MHz to 6 GHz for relative permittivity and dielectric loss measurements.

Frequency-Dependent Heating Behavior of *U. meridionalis* at 200 MHz, 915 MHz, and 2.45 GHz. Heating behavior of *U. meridionalis* at 200 MHz, 915 MHz, 2.45 GHz, and CH was compared to evaluate the heating efficiency at different frequencies (Figure 2). A semiconductor generator with a single-mode applicator was used to precisely control the microwave output and frequency.^{33,34} A radiofrequency heating at 200 MHz was conducted by using a semiconductor MR-0.2G100 generator (Ryowa Electronics Co., Ltd., Miyagi, Japan) equipped with a parallel plate applicator and a variable capacitance condenser (Fuji Electronic Industrial Co., Ltd., Saitama, Japan) (Figure 2A). A microwave heating at 915 MHz was conducted by a semiconductor MR-0.9G-300 oscillator (Ryowa Electronics Co., Ltd., Miyagi, Japan), equipped with a TM₀₁₀ mode cavity resonator and a slug tuner (Figure 2B). A microwave heating at 2.45 GHz was conducted with a semiconductor MWPS-2450050-01 generator (Plasma Applications Co., Ltd., Shizuoka, Japan) with a TM₁₁₀ mode cavity resonator and a slug tuner (Figure 2C). Data logger was used to collect the power and frequency. CH was performed with a heat block placed on a hot stirrer (Figure 2D). All temperatures during heating were measured by a fluorescent fiber-optic thermometer FL-2000 (Anritsu Meter Co., Ltd., Tokyo, Japan).

U. meridionalis (0.06 g) was suspended in ultrapure water (5 mL) and allowed to swell for 5 min. CH and heating at different frequencies were used to measure the heating behavior (0–10 min, max temperature: 100 °C, 20 W, 500 rpm). Extraction was performed at 200 MHz, 915 MHz, 2.45 GHz, and CH using the same equipment used for the heating behavior tests. We used unmilled *U. meridionalis* samples to limit the extraction rate and observe the differences depending on the applied frequency.

Total sugar content in the extract was measured by the phenolic acid method. Size exclusion chromatography was performed for analyzing the molecular weight distribution equipped with a column of YMC-Pack Diol-300 (YMC Co., Ltd., Kyoto, Japan), the RI detector of LC-20AT (Shimadzu Corporation, Kyoto, Japan), and UV (280 nm) using SPD-20A (Shimadzu Corporation, Kyoto, Japan), respectively. The column temperature, injection volume, mobile phase (ultra-

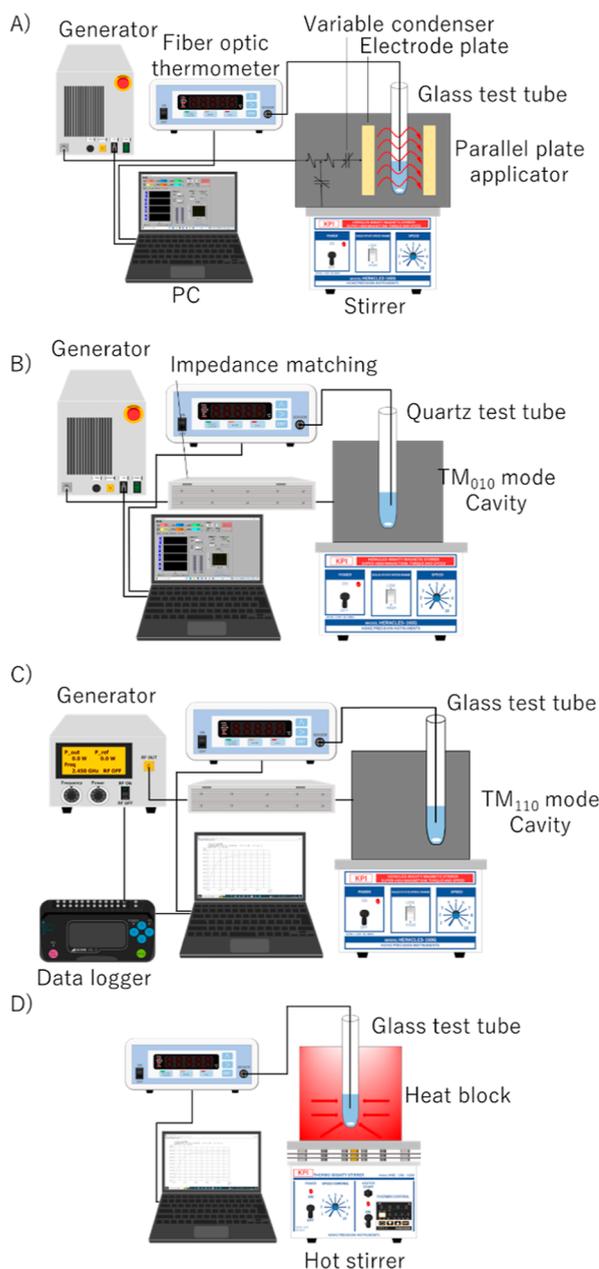


Figure 2. Schematics of ulvan extraction setup by radiofrequency at (A) 200 MHz, microwaves at (B) 915 MHz and (C) 2.45 GHz, and (D) CH using a hot stirrer.

pure water), and flow rate were 40 °C, 20 μ L, and 1 mL/min, respectively. Molecular weights were estimated using a pullulan standard of Shodex STANDARD P-82 (23.7–853 K, Resonac Holdings Corporation, Tokyo, Japan). DSC was used to observe the temperature-dependent endothermic structural change in ulvan using a DSC-60 Plus (Shimadzu Corporation, Kyoto, Japan). The ulvanic acid extraction was dissolved in ultrapure water at a concentration of 2.5%. The reference material was ultrapure water (20 μ L), and the sample was ulvan solution (2.5%, 10 μ L) in an aluminum-sealed cell ($\phi 6 \times 1.5$, Shimadzu Corporation, Kyoto, Japan). After holding at 10 °C for 15 min, the temperature was increased to 95 °C at 2.5 °C/min under a nitrogen atmosphere.

In Situ SAXS Analysis of Ulvan Molecule Conformational Changes during Dielectric Heating and CH. SAXS

was performed at SAGA Light Source (Saga, Japan) beamline BL-06. In situ SAXS measurements were performed on aqueous ulvan solution (2.5%) at 2.45 GHz heating (0–20 W) and CH (30–70 °C) (Figure 3). The SAXS patterns were

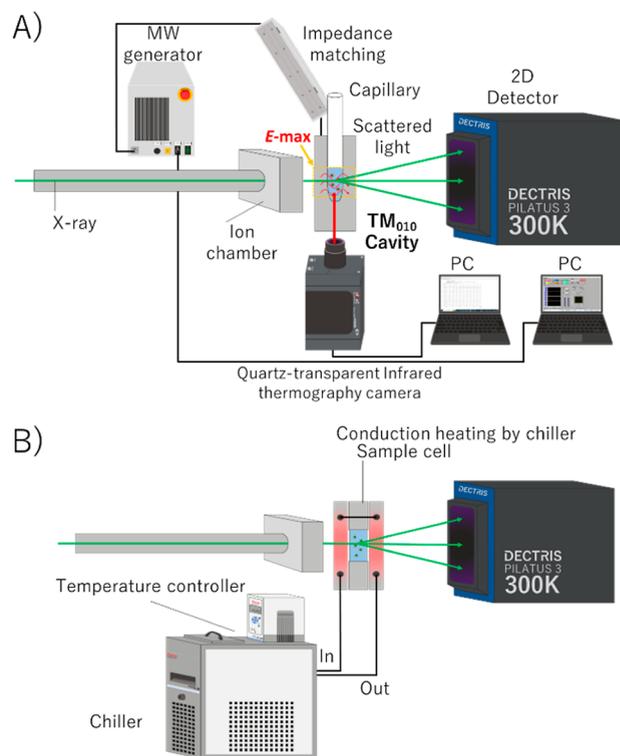


Figure 3. Schematics of in situ SAXS measurement setup by (A) MW and (B) CH.

obtained at a wavelength of 1.38 Å, a camera length of 2575 mm (CH) and 2536 mm (MW), and an exposure time of 300 s. SAXS patterns were collected using a DECTRIS PILATUS3 300 K (Rigaku Corporation, Tokyo, Japan) instrument with an effective pixel size of 172 μ m \times 172 μ m. The 2.45 GHz semiconductor generator (Ryowa Electronics Co., Ltd., Miyagi, Japan) equipped with a TM₀₁₀ mode cavity resonator was used for microwave heating. This cavity is equipped with a window at the electric field maximum (E_{\max}) position so that incident X-ray and scattered lights can pass through the cavity, which is of a similar geometry to what we used for microwave in situ Raman and X-ray fine absorption structures.^{35,36} Temperatures were measured with thermography of an InfReC H9000 Thermo HAWK (Nippon Avionics Co., Ltd., Kanagawa, Japan). Microwave heating was performed by placing the sample in a capillary of marked tube Lindemann glass 2.0 mm (TOHO Co., Ltd., Tokyo, Japan) and placed at the E_{\max} . CH was performed by heating the sample in the in situ SAXS cell (thickness 1 mm) with a Si₃N₄ window and heated by a chiller AC 28 (Thermo Fisher Scientific Inc., MA, USA) equipped with a temperature controller of AC 200 (Thermo Fisher Scientific Inc., MA, USA).

The SAXS images were converted by Fit2D software (European Synchrotron Radiation Facility, Grenoble, France) to describe the circular averaged scattering pattern (Figures S8–S10). The approximated radii of the aggregated molecules were obtained by Guinier analysis using eqs 2 and 3.³⁷

$$I(q) = I(0)\exp(-1/3Rg^2q^2) \quad (2)$$

$$R_g^2 = (3/5)R^2 \quad (3)$$

The correlation length of the ulvan bundles was also obtained by the Debye–Bueche equation, as shown in 4³⁸

$$(q) = K/(1 + c^2q^2)^2 \quad (4)$$

RESULTS AND DISCUSSION

Extraction Behavior of Ulvan with Conventional Multimode 2.45 GHz. Accelerated microwave extraction of

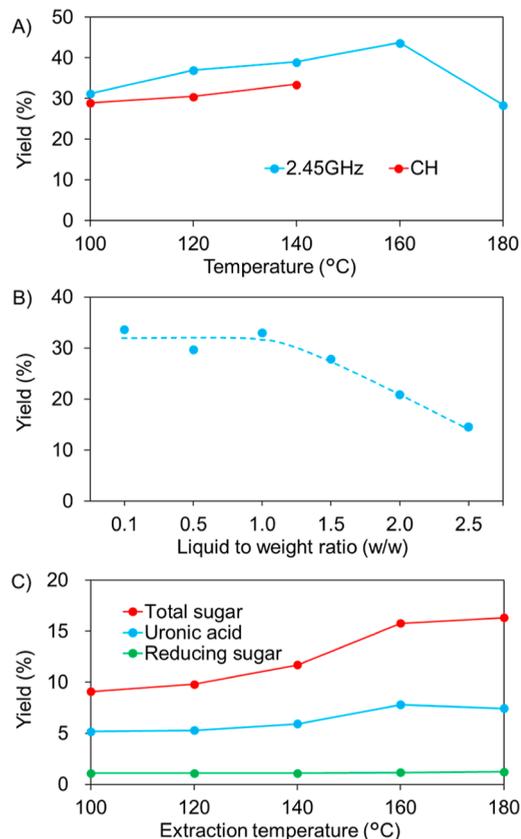


Figure 4. Ulvan yield by a multimode microwave reactor at 2.45 GHz from milled *U. meridionalis*. (A) Comparison of ulvan yield by multimode 2.45 GHz and CH. (B) Effect of the solid–liquid ratio at 100 °C extraction by multimode 2.45 GHz on ulvan yield. (C) Total sugar, uronic acid, and reducing sugar yields by multimode 2.45 GHz.

ulvan was first demonstrated by using a conventional multimode microwave oven. Figure 4A shows the polysaccharide yields obtained by multimode 2.45 GHz and CH from milled *U. meridionalis*. Temperature reached 180 °C by a multimode 2.45 GHz, whereas CH could only reach 140 °C. Extraction by 2.45 GHz yielded a maximum value of 43.8% at 160 °C.³¹ Considering the ulvan content in *Ulva* sp. (up to 36% by dry weight), other polysaccharides such as starch (storage polysaccharide), hemicellulose (cell wall polysaccharides), and extracellular matrix protein are extracted together with ulvan at higher temperatures.^{17,39} Figure 4C shows the amounts of total sugar, uronic acid, and reducing sugars at different temperatures. The total sugars and uronic acid content show the same behavior as the ulvan yield. The presence of uronic acid indicates the extraction of glucuronic acid and iduronic acid in the ulvan. Ulvan extraction was also confirmed with FT-IR spectra identified at 1262 and 1056

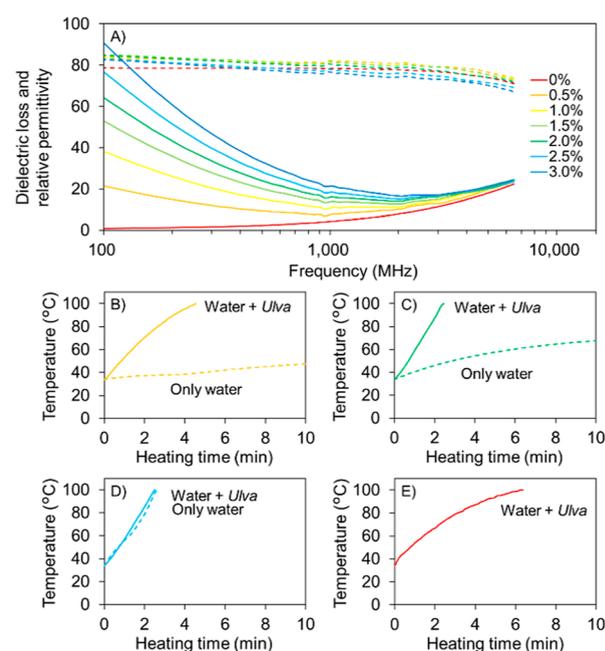


Figure 5. (A) Dielectric property of aqueous ulvan solution in different concentrations. Microwave and radiofrequency heating behavior of distilled ultrapure water and with and without *U. meridionalis* (unmilled) by using (B) 200 MHz, (C) 915 MHz, (D) 2.45 GHz, and (E) CH.

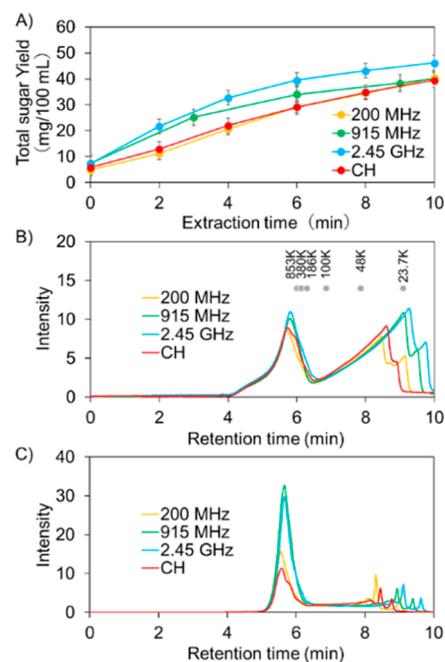


Figure 6. Component analysis extracted from *U. meridionalis* at different frequencies and CH. (A) Total sugar content. Size exclusion chromatograms measured by (B) RI and (C) UV (280 nm) detectors. Error bars indicate standard deviation ($n = 3-5$).

cm^{-1} , which are attributed to the S=O stretching vibration of the sulfate group and the C–O stretching of ulvan and glucuronic acid, respectively (Figure S1). The peaks at 1650 and 1550 cm^{-1} are attributed to C=O stretching vibrations of uronic acid and peptide bonds and N–H deformation vibration,⁴⁰ respectively, suggesting the extraction of proteins at the intercellular matrix in association with the ulvan. The

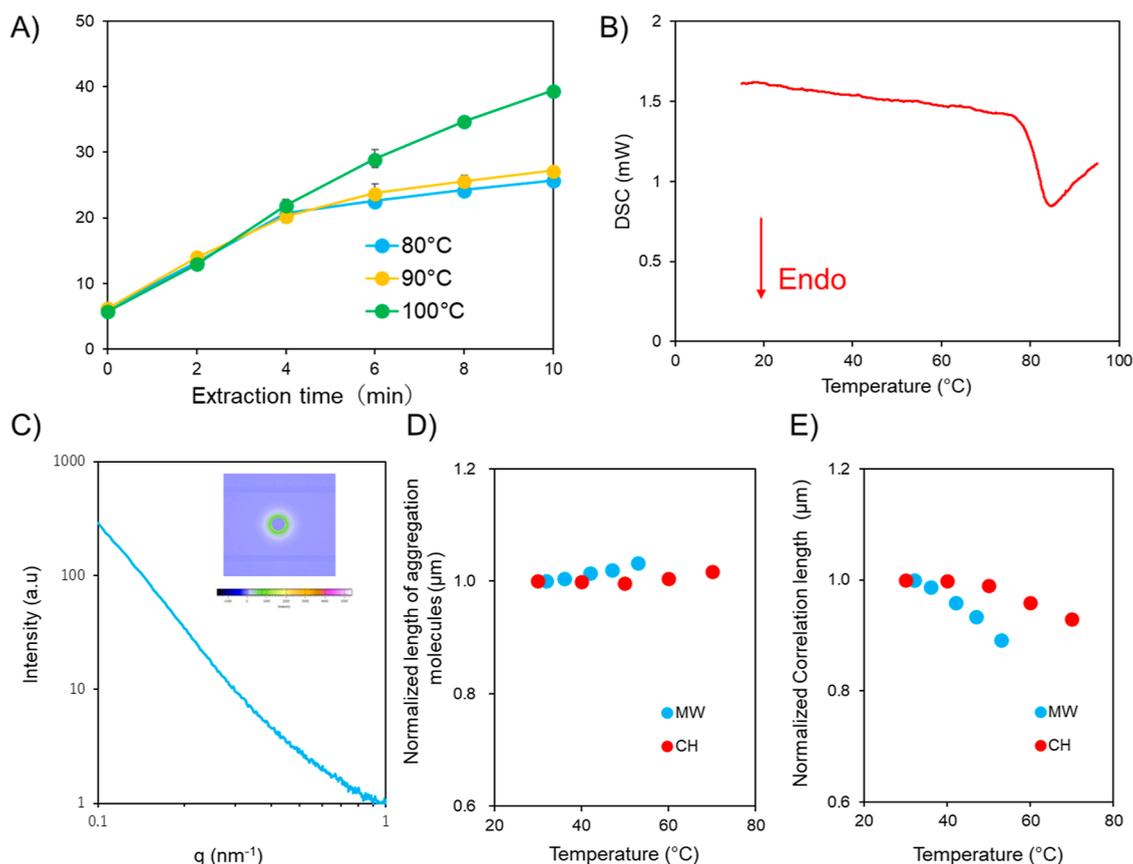


Figure 7. Mechanistic analysis of microwave-accelerated ulvan extraction. (A) Total sugar yield at different extraction temperatures by CH. (B) DSC thermogram of 2.5% ulvan solution. Structural changes in ulvan solution (2.5%) by in situ SAXS measurement. (C) SAXS patterns of ulvan (1D and 2D). (D) Length of aggregated ulvan molecules and (E) correlation length of the ulvan molecule bundles. Error bars indicate standard deviation ($n = 2-3$).

peak around 1650 cm^{-1} also originates from the asymmetric stretching of COO^- of uronic acid.⁴¹ The extracted ulvan showed a brown discoloration after $160\text{ }^\circ\text{C}$, where ulvan starts thermal decomposition,⁴² and the ulvan yield started to decrease at $180\text{ }^\circ\text{C}$. Figure 4B shows the ulvan yields with different solid/liquid ratios at $100\text{ }^\circ\text{C}$. High ulvan yields of 33% were obtained for up to $1.0\text{ g}/20\text{ mL}$ of solid/liquid ratio, but the yield decreased above 1.5 g . This is due to an increase in the viscosity by water swelling of the *U. meridionalis* powder. Thus, $1.0\text{ g}/20\text{ mL}$ of solvent is optimal for ulvan extraction from powdered *Ulva* sp.

Frequency-Dependent Acceleration of Ulvan Extraction. The complex dielectric permittivity of aqueous solution-extracted ulvan (0–3 wt %) was measured to understand the effects of selective heating of ulvan and water. Figure 5A shows the relative permittivity (ϵ') and dielectric loss (ϵ'') measured by the coaxial probe method at frequencies between 100 MHz and 6 GHz. Ultrapure water exhibited larger dielectric loss above 1 GHz (corresponding to microwaves), which is related to the dielectric relaxation of water molecules. Meanwhile, the dielectric loss of ulvan solutions was higher at lower frequencies (corresponding to radiofrequency) and enhanced with the increase in ulvan concentration. This is attributed to the conduction losses occurring at the counterions of the sulfate and carboxyl groups of rhamnose and uronic acids in the ulvan.⁴³ Therefore, microwaves selectively heat the water, while radiofrequency selectively heats the ulvan.

Next, we characterized the heating property of water and unmilled *U. meridionalis* at different frequencies of 200 MHz, 915 MHz, and 2.45 GHz (Figure 5B–E). Water did not heat at 200 MHz, while the addition of the *U. meridionalis* increased the heating rate by 11 times, indicating that radiofrequency selectively heats ulvan by conduction loss. In contrast, there was no change in the heating rate of water with or without *U. meridionalis* at 2.45 GHz. This is attributed to the selective heating of water at 2.45 GHz, and conduction loss by ulvan is unlikely to occur at 2.45 GHz. Water was slightly heated at 915 MHz, and the addition of *U. meridionalis* increased the heating rate by 8.8 times. This is an intermediate behavior between 200 MHz and 2.45 GHz. These results indicate that the ulvan and water can be selectively heated by radiofrequency (200 MHz) and microwaves (2.45 GHz), respectively.

Then, we measured the total sugar yield in the extract at different frequencies (Figure 6A). The total sugar content was 1.4 times higher at 2.45 GHz and 1.1 times higher at 915 MHz than that during CH. However, no difference was observed between 200 MHz and CH. The results indicate that the selective heating of water by 2.45 GHz significantly increased the total sugar yield. Meanwhile, the selective heating of ulvan by 200 MHz did not affect the total sugar content. Therefore, water-selective heating by 2.45 GHz is important to improve the ulvan yield of *U. meridionalis*.

The molecular weight distribution of ulvan was analyzed by using SEC (Figure 6B). The extracted ulvan contained a high-molecular-weight component (853 K) and a low-molecular-

weight component (23.7 K). Higher intensities were observed for both high- and low-molecular-weight components at 2.45 GHz and 915 MHz than at CH. This behavior coincides with their higher total sugar yield (Figure 6A). Meanwhile, 200 MHz does not improve ulvan extraction because there was no difference in the total sugar yield between 200 MHz and CH. In addition, UV absorption ($\lambda = 280$ nm) peaks in HPLC chromatograms suggest that ulvan extraction is associated with protein (Figure 5C) since ulvan forms an extracellular matrix with proteins.¹⁸ Quantification of the protein content by the Lowry method (Figure S2) showed that the extracted ulvan contains slight protein (2.4–3.0%) in the extract.

In Situ SAXS Analysis of Ulvan Molecule Conformational Changes during Dielectric Heating and CH. Ulvan forms a thermoreversible gel, suggesting that the heat-induced structural change of ulvan can contribute to the microwave-enhanced extraction of ulvan.¹⁸ Considering the ulvan yield, it increases above 80 °C (Figure 7A). We have also observed an endothermic peak at 80–90 °C by DSC analysis of aqueous ulvan solution. Therefore, acceleration in ulvan extraction is associated with the structural change of ulvan at 80–90 °C (Figure 7B). Therefore, we directly analyzed the structural changes in ulvan and their bundles by in situ SAXS under microwave heating (Figure 7C).⁴⁴ We evaluated the differences in the normalized values of the approximated radius of the aggregated ulvan molecules and correlation length of the ulvan molecule bundles since we used different in situ cells for CH (rectangle cell with Si₃N₄ windows) and MW (quartz capillary). CH slightly increased the length of aggregated ulvan molecules with increasing temperatures above 60 °C (Figure 7D). Meanwhile, the correlation length of the ulvan bundles more obviously decreased with increasing temperature, which is attributed to the loosening of the ulvan bundles (Figure 7E). Therefore, the endothermic peak in DSC should be attributed to the loosening of the ulvan bundle structure.⁴⁵ In the case of microwaves, the length of aggregated ulvan and the correlation length of the ulvan bundles started to change at around 40 °C, indicating that microwaves promote conformational change in ulvan at lower temperatures (Figure 7D,E). Ulvan has been reported to stabilize the helical structure through hydrogen bonding.¹⁸ In particular, water-selective microwave heating at 2.45 GHz may promote the breakdown of hydrogen bonds to initiate the loosening of the ulvan bundles. As a result, microwaves promote the conformational change of ulvan and initiate its enhanced extraction into the water solvent.

CONCLUSIONS

The present study demonstrated the frequency-dependent acceleration of ulvan extraction from *U. meridionalis* and revealed the mechanism of extraction acceleration by microwaves. Selective heating of ulvan occurs at 200 MHz due to conduction loss, while selective heating of water is enhanced at 2.45 GHz due to the dielectric loss of water. We found that 2.45 GHz was more effective for extracting ulvan than 915 MHz, 200 MHz, and CH. Therefore, selective heating of water by 2.45 GHz due to dielectric loss of water is important for enhancing the extraction of ulvan. In addition, we developed microwave in situ SAXS and directly observed the structural change in ulvan under microwaves. We found that loosening of ulvan bundles is enhanced at lower temperatures compared to CH. Therefore, we conclude that microwave-accelerated extraction of ulvan from *U. meridionalis* is attributed to the

selective heating of water by 2.45 GHz microwaves, enhancing the structural change of ulvan at lower temperatures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.4c04080>.

Sample culture conditions, materials and methods for the quantitative analysis of sugars and proteins, FT-IR spectra of extracts at different temperatures, different extraction methods and analysis of extracts, and in situ SAXS setup and results (PDF)

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Author Contributions

K.M.: conceptualization, data curation, investigation, methodology, writing—original draft, and writing—review and editing; D.T.: SAXS data analysis; M.H.: Ulva cultivation; T.S.: in situ SAXS measurement; N.I.: conceptualization and investigation; and S.T.: investigation, conceptualization, writing—original draft, writing—review and editing, funding acquisition, project administration, and supervision.

Notes

The authors declare no competing financial interest.

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