Antioxidant and Antimicrobial Activities Measurement of Brown Seaweed (*Sargassum honeri*) Hydrolyzate using Subcritical Water

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ABSTRACT

Seaweeds are potential renewable resource in the marine environment. Seaweed provides for an excellent source of bioactive compounds, such as carotenoid, dietary fiber, protein, vitamins, essential fatty acid, and minerals.Interestingly, seaweeds are a rich source of phytochemicals having anti-oxidant and antimicrobial properties. Among the functional effects of the seaweed, nutritional and health-related benefits have been widely studied. Most of seaweed has the anti-tumor, antifungal, anti-inflammatory, antioxidant and a wide range of biological activities.In this study, seaweed (*Sargassum honeri*) was extracted using an environmental friendly solvent, supercritical carbon dioxide (SC-CO_2). The SC-CO_2 will be carried out at constant temperature 45 ^oC and pressures ranging from 200 bar. It will be produced by the subcritical water (SWH) with the reaction temperatures for hydrolysis will be maintained from 180 to 260 ^oC. The SWH will be done with condition 16-220 bar for the reaction pressure and 1:25 (*w/v*) for the ratio of material to water. High pressure pump will be applied to flow water from tank to reactor for getting initial pressure 1 bar.The antioxidant activities produced will be determined by using the TPC, TFC, DPPH andABTS method. The antimicrobial activities produced will be compared against the common food spoilage and pathogenic bacteria (e.g. *Bacillus cereus*↵ *Staphylococcus aureus*↵ *Escherichia coli*↵ *Salmonella typhimurium,*).

INTRODUCTION

Marine algae have a significant attraction as an important resource of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological behavior such as antibacterial, antioxidant, anticancer, anticoagulant and antiviral properties [1↵ 2]. Seaweeds or marine macroalgae are the renewable living resourceswhich are also used as food, feed and fertilizer in many parts of the world. Seaweeds are of nutritional interest as they arelow calorie food and are rich in vitamins, minerals proteins, polyphenols, polysaccharides and dietary fibers [3↵ 4].A variety of *in vitro* studies have demonstrated that algal derived polyphenols and flavonoids exhibit antimicrobial and

antioxidant activity [5-7]. They have been consumed from time immemorial in the whole world, especially in Asia, where they constitute an alternative to vegetables in human food. In our diet, many products are manufactured with seaweeds or their derivatives, such as sauces, creams, toothpaste and milk shakes of fruits.According to the CE 258/97 Regulation, algae are considered as new foods, and they could also be considered as functional foods because they have what defines this kind of aliment [8]. This regulation, in addition to the potential nutritional properties of seaweeds, allows the food industry to include seaweeds as raw or semi-processed materials in the formulation of seafood products [3]. Apart from functional food products, newer applications of seaweeds and their bioactive compounds in different sectors, such as nutraceuticals, cosmetics, biomedicine and biotechnology, are constantly under development. Therefore, the short-termgoal of functional foods, nutraceuticalsand dietary supplements is to improve the quality of life and enhance health status while its long-term goal is to increase lifespan while maintaining health.

The purpose of this study was to obtain the optimal condition in depolymerization process of brown seaweed *Sargassum honeri* by the subcritical water hydrolysis. The parameters used in this work were the antioxidant activity (DPPH, ABTS, Total phenolic content (TPC), Total flavonoid content (TFC)) and the antimicrobial activity.

MATERIALS AND METHODS

Materials

The brown seaweed *Sargassum honeri* was collected from Guemil-eup, Wando-gun, Jeollanam, South Korea.High-puritycarbon dioxide gas (99%) was supplied by KOSEM (Yangsan, Republic of Korea). Folin-Ciocalteu reagent, sodium carbonate (≥99.9% purity) waspurchased from Sigma Aldrich Chemical Co. (St. Louis, Mo. USA). Gallic acid (98% purity),trolox (97% purity),methanol (≥99.8% purity), (+)-catechin hydrate (≥98% purity), potassium persulphate (≥98% purity), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, ≈98% purity) and 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95% purity),absolute ethanol (≥99.4% v/v), aluminium chloride-6-hydrate (>99% purity) and sodium hydroxide were purchased from Sigma Aldrich Chemical Co. (St. Louis, Mo. USA).Distilled water was used in these experiments.

Sample preparation

After washing fresh *S. honeri* samples with fresh water, unused materials, attached salt, and minerals were removed and the samples were cut into small pieces. The small pieces of seaweeds were dried in freeze drier (Eyela FDU-2100, Tokyo Rikakikai Co., LTD, Japan) equipped by square-type drying chamber (Eyela DRC-1000, Tokyo Rikakikai Co., LTD, Japan) at temperature -80 °C for 72 hours. The dried samples of *S. honeri* were collected into sealed plastic bags. After then, the dried samples were finely ground by mechanical blender (PN SMKA-4000 mixer) and sieved by 710 μm stainless steel sieving mesh. The samples which pass though of the sieving mesh were stored at -20 °C for one day prior to use.

Removal of oil from S. honeri by SC-CO2 extraction

S. honeri samples (100 g) were put into a 500 mL stainless steel extraction vessel, so that the volume fraction of solid in the extraction vessel was 75 %. A thin layer of cotton was placed at the bottom of the extraction vessel. Before plugging with a cap, another layer of cotton was used at the top of the sample. The extraction vessel was preheated initially. After reaching the desired temperature, CO_2 was pumped at constant pressure by high pressure pump up to desired pressure which was regulated by a back pressure regulator. The experiment was performed at a temperature of 45 °C and 200bar pressure for 2 h. The flow rate of CO_2 was kept constant at 26.81 g min^{-1} for all extraction conditions [9].

Subcritical water hydrolysis

Subcritical water hydrolysis was performed in a 200-cm^3 batch reactor made of 276 Hastelloy with temperature control. A total of 6 g of material samples were loaded into the reactor. Formic acid (1%), which is used as a catalyst, was suspended separately in 150 mL of distilled water. The reactor was then closed and heated using an electric heater to the required temperature (180-260°C). Pressures were estimated based on saturated steam to be between 15 to 220bar for the temperature range studied. The temperature and pressure in the reactor were controlled using a temperature controller and pressure gauge, respectively. The sample was stirred using a four-blade stirrer at 150 rpm. The time to reach the desired temperature was 30 to 65 min. The hydrolyzate samples from the reactor were collected and filtered using Whatman nylon membrane filter (0.45 μm) and stored at 4°C.

Total phenolic content (TPC) assay

Total phenolic content (TPC) of crude extract was determined using Folin-Ciocalteu colorimetric method according to Li *et al*. (2008) [10] and Wong *etal*. (2006) [11] with slight modification. 1 mL of 50 times diluted (v/v) crude extract was mixed with 1 ml of 1:10 (v/v, in distilled water) diluted Folin-Ciocalteureagent (FCR). After 4 min, 800 μL of sodium carbonate solution (7.5%, w/v) was added into the mixture. Then, the mixture was vortexed for 5 sec and stored at room temperature in dark environment for 2 hours. Blank was also prepared by replacing 1 mL of diluted crude extract with 1 mL of distilled water. The absorbance of mixture was measured at 765 nm against blank using UV light spectrometer (UVmini-1240, Shimadzu Corporation, Japan). The measurements were carried out in triplicate and gallic acid was used for calibration of standard curve. Results were expressed as mg gallic acid equivalent per 100 g of dry weight sample (mg GAE/100 g DW). The calibration equation for gallic acid was $y = 9.779x + 0.025$ (R^2=0.999).

Total flavonoid content (TFC) assay

Total flavonoid content (TFC) of crude extract was estimated using procedures described by Karadeniz*et al.* (2005) [12] and Ozsoy*et al.* (2007) [13]. 1.25 mL of distilled water was added into 0.25 mL of undiluted crude extract, followed by addition of 75μL of 5% (w/v) sodium nitrite solution. The mixture was allowed to stand for 6 min and 150 μL of 10% (w/v) aluminium chloride solution was then added. The mixture was allowed to stand for another 5 min and 0.5 mL of 1 M sodium hydroxide solution and 275 μL of distilled water were then

added, accordingly. Subsequently, the mixture was vortexed for 5 sec and its absorbance was determined at 510 nm against blank using UV light spectrometer (UVmini-1240, Shimadzu Corporation, Japan). Blank was prepared by replacing 0.25 mL of undiluted crude extract with 0.25 mL of distilled water. The measurements were done in triplicate and catechin was used for calibration of standard curve. The results were expressed as mg catechin equivalent per 100 g of dry weight sample (mg CE/100 g DW). The calibration equation for catechin was y = 0.000357x + 0.011 (R^2= 0.9965).

DPPH radical scavenging capacity

The DPPH radical scavenging capacity of crude extract was determined based on the method described by Miliauskas*et al*. (2004) [14], Saha*et al*. (2004) [15] and Cai*et al*. (2006) [16] with slight modification. 3.9 mL of ethanolic DPPH (60 μM) was firstly mixed with 0.1 mL of undiluted crude extract or ethanol (as control) and they were stored in darkenvironment at room temperature for 30 min. Subsequently, the absorbance of crude extract and control was measured against ethanol (as blank) at 517 nm using Uvi light spectrometer (UVmini-1240, Shimadzu Corporation, Japan). Absorbance measurements of crude extract and control were done in triplicate. The percentage of DPPH radical scavenging capacity was calculated using this equation: [1 – (As / Ac)] × 100% (As = absorbance of crude extract at 517 nm; Ac = absorbance of control at 517 nm). Trolox was used for calibration of standard curve and the results were expressed as μmoltrolox equivalent antioxidant capacity (TEAC) per 100 g dry weight sample (μmol TEAC/100 g DW).

ABTS radical scavenging capacity assay

ABTS radical scavenging capacity assay was carried out according to the procedures described by Cai*et al*. (2006) [16], Wetwitayaklung*et al*. (2006) [17], Guimarães*et al*. (2007) [18] and Surveswaran*et al*. (2007) [19]. ABTS radical solution was firstly prepared by mixing 10 mL of 7mM ABTS solution with 10 mL of 2.45 mM potassium persulphate solution in a 250 mL amber bottle. Subsequently, the ABTS radical solution was allowed to stand in a dark environment at room temperature for 12-16 hours to give a dark blue solution. The ABTS radical solution was diluted with denatured ethanol until its absorbance was equilibrated to 0.7 ± 0.02 at 734 nm before usage.

3.9 mL of ABTS radical solution was firstlymixed with 0.1 mL of undiluted crude extract orethanol (as control) and they were allowed to store in dark environment at room temperature for 6 min.Subsequently, the absorbance of crude extract andcontrol was measured against ethanol (as blank) at 734nm using UV light spectrophotometer (UVmini-1240, Shimadzu Corporation, Japan). The absorbance measurementsof crude extract and control were done in triplicate.The percentage of ABTS radical scavenging capacitywas calculated using this formula: [1 – (As / Ac)] ×100% (As = absorbance of crude extract at 734 nm; Ac = absorbance of control at 734 nm). Trolox wasused for calibration of standard curve and the resultswere expressed as μmol trolox equivalent antioxidant capacity (TEAC) per 100 g dry weight sample (μmol TEAC/100 g DW).

Paper disc diffusion assay

The antimicrobial activities of the oils were determined by paper disc diffusion assay according Hun et al. (1994) [20] with slight modification.Four microorganisms were used in this study. All strains were obtained from the Korean Culture Center of Microorganisms (KCCM). McFarland standard No. 0.5 was used in the preparation of suspension of microorganism. The turbidity of bacterial suspension was adjusted according McFarland standard. The accurate turbidity of the bacterial suspension was confirmed by UV-spectrophotometer (UVmini-1240, Shimadzu Corporation, Japan) on 625 nm with approximate cell density of each bacterial strain was 10^7 CFU/mL. Mueller-Hinton agar was used as growth medium for bacterial strains and sterilized at 121°Cfor 15 min. The agar was poured into sterile petri dishes and after stiff agar forming, bacterial suspension was spread on*the agar surface using sterile cotton. Then, advantec paper disc (10 mm) contains extracts or control (methanol) were then placed in the middle of the plates and incubatedfor 24 h at 37°C and the diameter of each inhibitory zone was measured (mm).

RESULTS AND DISCUSSION

Antioxidant properties

DPPH and ABTS are employed widely to evaluate the antioxidant capacity and to screen forantioxidants in Traditional Chinese Medicine. They are quick, simple and sensitive methods toevaluate the antioxidant capacity of seaweed. In this the antioxidant capacity of the subcritical water hydrolyzate products by determined by the DPPH and ABTS methods. Inhibition percentage was used asan index to compare the anti-oxidant activity.

DPPH is one of the most common methods to evaluate the free radical scavenging capacity. DPPH can absorb protons and lose its chromophoric group to become yellow. Then it shows a maximum absorption at 515 nm for essential product from *S. honeri.* The change of absorbance reflects thefree radical scavenging capacity and allows us to evaluate the anti-oxidant activity. The differentconcentrated samples and BHT were assayed and the results of the DPPH radical-scavenging assay are shown in Figure 1.

ABTS produces the blue-green cationic ion ABTS+ via activated oxygen oxidation. The reactionsystem fades when the sample that has anti-oxidant is added into ABTS, then the absorbance of free radical of ABTS is detected at the maximum absorption wavelength of 734 nm. The change of absorbance reflects the free radical scavenging capacity and allows evaluation of the anti-oxidant activity. The different concentrated samples and BHT were assayed and the results of the ABTS radical-scavenging assay are shown in Figure 1.

As shown in Figures 2, the antioxidant properties were higher in the presence. The optimal temperature for antioxidant properties was 260°C/220bar. The antioxidant properties (TPC and TFC) when *S. honeri* was used as a starting material were 0.20 ± 0.013 mg/L, 67.23± 0.05 mg/L, respectively.

The antioxidant properties of hydrolyzate water are affected by bio-compounds such as phenolics, flavonoids, and minerals. The growth environment, harvest time, and storage conditions also affected the amounts of these compounds [9]. The addition of formic acid as a catalyst is known to increase the antioxidant properties of hydrolyzate water.

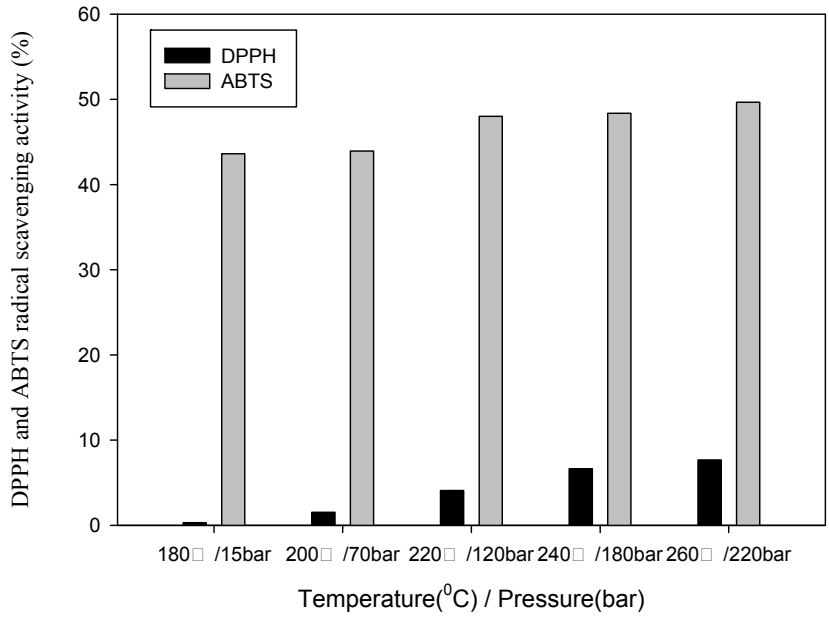

Figure 1: Antioxidant properties of *S. honeri* hydrolyzate sample on DPPH and ABTS radical scavenging activity

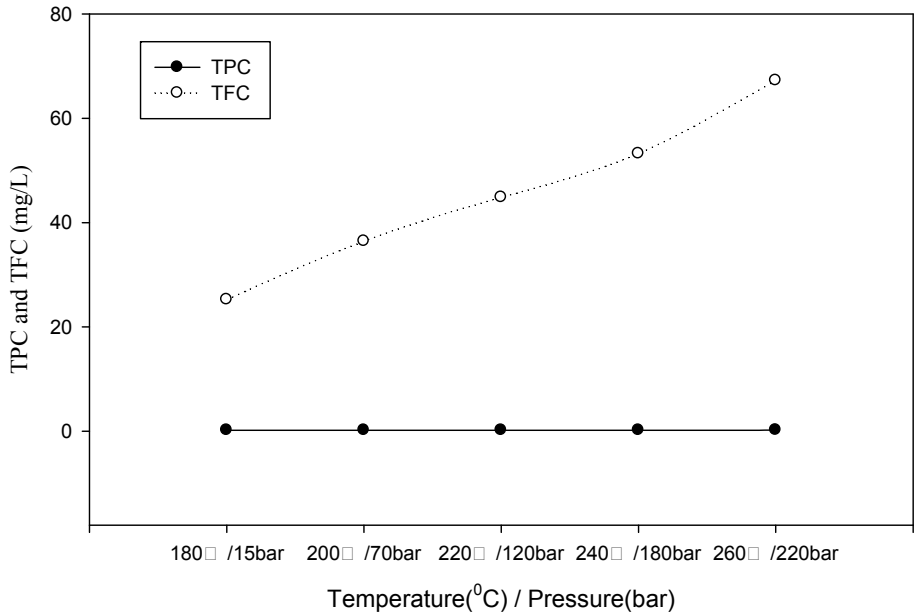

Figure 2: Antioxidant properties of *S. honeri* hydrolyzate sample on Total Phenolic Content ↖ TPC↗ and Total Flavonoid Content (TFC)

The addition of acid as catalyst during the hydrolysis process induces novel properties of water such as variations in density, polarity, and solubility. Furthermore, the presence of acid yields more protection from oxidation and damage by antioxidant compounds, and also

changes the solubility and density of water [17].

Antimicrobial activity

The antimicrobial activity in subcritical water hydrolyzate products were assessed by paper disc diffusion assay and the results were shown in Table 1. The products obtained byproducts were showed slight antimicrobial activity against *B.cereus*and *S.aureuas* on the 200 °C, showed slight antimicrobial activity against *E.coil* on the 180 °C and showed moderate antimicrobial activity against *S.typhimurium* on the 180 °C.

Table 1: Clear zone of subcritical water hydrolyzate products in different condition

	Clear zone of the SWH				
	180°C /16bar	200°C /70bar	220 °C /120bar	240°C /180bar	260°C /220bar
B.cereus	±	+	±	-	-
S.aureus	±	+	±	-	-
E.coli	+	±	-	-	-
S.typhimurium	++	+	±	-	-

- No inhibition (10mm)
± Very slight inhibition (10~11mm)
+ Slight inhibition (11~12mm)
++ Moderate inhibition (12~16 mm)

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CONCLUSIONS

Subcritical water hydrolysis was an efficient eco-friendly technology where successfully isolated antioxidative compounds from *S. honeri*. Hydrolyzate of *S. honeri* was valuable bioactive compounds, e.g. flavonoid and phenolic. Antioxidant and antimicrobial activities were found in *S. honeri* hydrolyzate. Therefore, this hydrolyzate can be incorporated as multi-functional ingredients in the food industries.

References

[1] VAIRAPPAN, C.S.,DAITOH, M., SUZUKI, M., ABE,T., MASUDA, M.,Phytochemistry, Vol. 58, **2001**, p. 291.

[2]ATHUKORALA, Y., LEE, K.W., KIM, S.K., JEON, Y.J., Bioresource Technology, Vol. 98,**2007**, p. 1711.

[3]BURTIN, P., Electronic Journal of Environmental, Agricultural and Food Chemistry, Vol. 2, **2003,** p. 498.

[4]MACARTAIN, P., GILL, C.I.R., BROOKES, M., CAMPBELL, R., ROWLAND, I.R., Nutrition Reviews, Vol.65, **2007**, p. 535.
[5]HEO, S.J., PARK, P.J., PARK, E.J., KIM, S.K., JEON, Y.J., European Food Research and Technology, Vol. 221, **2005**, p. 41.
[6]CHANDINI, S.K., GANESAN, P., BHASKAR, N., Food Chemistry, Vol.107,**2008**, p. 707.
[7]ZARAGOZA, M.C., LOPEZ,D., SAIZ, M.P., POQUET, M., PEREZ, J., PUIG-PARELLADA, P., MARMOL, F., SIMONETTI,P.,GARDANA, C., LERAT, Y. *et al*., Journal of Agricultural and Food Chemistry, Vol.56, **2008**, p. 7773.
[8]CRESPO, M.O.P., YUSTY, M.A.L.,Ecotoxicology and Environmental Safety, Vol. 57, **2004**, p. 226.
[9]ROH, M.K., SALIM-UDDIN, MD., CHUN, B.S., Biotechnology and Bioprocess Engineering, Vol. 13,**2008**, p. 724.
[10]LI, H. B, WONG, C. C., CHENG, K. W., CHEN, F.,LWT- Food Science and Technology, Vol. 41 (3), **2008**, p. 385.
[11]WONG, S. P., LEONG, L. P., KOH, J. H. W., Food Chemistry, Vol. 99, **2006**, p. 775-783.
[12]KARADENIZ, F., BURDURLU, H. S., KOCA, N., SOYER, Y.,Turkish Journal of Agriculture and Forestry, Vol. 29, **2005**, p. 297.
[13]OZSOY, N., CAN, A., YANARDAG, R., AKEV, N., Food Chemistry, Vol. 110, **2007**,p. 571.
[14]MILIAUSKAS, G., VENSKUTONIS, P. R., BEEK, T. A., Food Chemistry, Vol. 85, **2004**, p. 231.
[15]SAHA, K., LAJIS, N. H., ISRAF, D. A., HAMZAH, A. S., KHOZIRAH, S., KHAMIS, S., SYAHIDA, A., Journal of Ethnopharmacology, Vol. 92, **2004**, p. 263.
[16] KOIVIKKO, R., ERÄNEN, J. K., LOPONEN, J., JORMALAINEN, V.,Journal of Chemical Ecology, Vol. 34,**2008**, p. 57.
[17] CAI, Y. Z., SUN, M., XING, J., LUO, Q., CORKE, H., Life Sciences, Vol. 78, **2004**, p. 2872.
[18] WETWITAYAKLUNG, P., PHAECHAMUD, T., LIMMATVAPIRAT, C.,KEOKITICHAI, S., NaresuanUniversity Journal, Vol. 14, **2006**, p. 1.
[19] GUIMARÃES, C. M., GIÃO, M. S., MARTINEZ, S. S., PINTADO,A. I., PINTADO, M. E., BENTO, L. S., MALCATA, F.X.,Journal of Food Science, Vol. 72, **2007**, p. 39.
[20] HUN, W.W.; HOCK, G.S.; MOI, P.S.,Algaebiotechnology in the Asia-Pacific-region, University of Malaya, Kulalumpur, **1994**, p.75.