Structural and functional analysis of cell wall polysaccharides from macroalgae

(大型藻類の細胞壁多糖の構造および機能解析)

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AIR: alcohol insoluble residue

AO: ammonium oxalate

CL: cellulose

CPC: cetylpyridinium chloride

CPC-P: cetylpyridinium chloride precipitation

CPC-S: cetylpyridinium chloride supernatant

DS: desulfated polysaccharide

DSP: desulfated SP

FCSP: fucose containing sulfated polysaccharide

HC: hemicellulose

HW: hot water

RD: carboxyl reduced polysaccharide

HWE: hot water extraction

SP: sulfated polysaccharide from *C. lentillifera*

$SP_{0.1}$: partially hydrolyzed SP by 0.1 M TFA

$SP_{0.5}$: partially hydrolyzed SP by 0.5 M TFA

Mp: major peak

MW: molecular weight

NT: native polysaccharide

SAXS: small angle X-ray scattering

TFA: trifluoroacetic acid

UA: uronic acid

Abstract

Macroalgae are well-known producers of bioactive polysaccharides which are abundant in algal cell wall, so that they play key roles in the different fields of industries. However, function of polysaccharides is highly affected by their structure which can vary due to the species, age, and growth place. Structural variation will be challenging to produce uniform bioactive compounds. Therefore, I mainly focused on structural and functional analysis of macroalgal polysaccharides from *Caulerpa lentillifera* and *Cladosiphon okamuranus* which are extensively utilized and abundantly cultivated in Okinawa.

In the first experiment, I extracted sulfated polysaccharide (SP) from *C. lentillifera* and examined its hyaluronidase (HAase) inhibitory activity. Results showed that SP was a promising inhibitor of HAase with IC_{50} of 163 μg/mL. Then we analyzed, the relationship between the structural properties of SP and HAase inhibitory activity. Having significantly higher activity in native SP than desulfated and partially acid hydrolyzed SP suggested that HAase inhibitory activity extremely depends on its structural properties: sulfate content and molecular weight (MW).

Since I found structural properties are important for activities, my second study analyzed the structure of polysaccharide from *C. okamuranus* which is an excellent source of fucoidan with numerous activities and contains the highest fucoidan among any brown algae spp. Many previous studies described the individual structure and activity of soluble fucoidan from *C. okamuranus* but it was poorly understood whether insoluble residue of cell wall contains fucoidan and how different its structure is. Thus, aside from soluble fucoidan, I analyzed polysaccharide in the residues after extraction of soluble fucoidan. Hence, cell wall was sequentially treated with hot water (HW), ammonium oxalate, KOH

and fractionated into five fractions. I found that cell wall from *C. okamuranus* was mainly consisted of HW and hemicellulose (HC)-I fractions which occupied 80% in cell wall. Furthermore, it was found that both of them contained fucoidan. Particularly, fucoidan in HC-I was structurally different from fucoidan in HW in terms of sulfate content, MW, and sugar residue which was 1,4-linked xylose and 1,4-linked fucose. I also showed for the first time that fucoidan in HC-I may be involved in reinforcing cell wall structure.

In third experiment, I analyzed the structural variation of polysaccharide of *C. okamuranus* from 8 different habitats in Okinawa prefecture. Although yield, chemical composition and MW of fucoidan in HW differ slightly at different habitats, the results showed relatively uniform structural cell wall in *C. okamuranus* from any geographical location in Okinawa prefecture at peak harvesting period.

In conclusion, structure of macroalgal polysaccharide is very important on their activities and knowledge on variation of structural properties will help to improve the quality of polysaccharides for industrial application in the future.

概要

大型藻類は細胞壁多糖に富み、それら多糖は生理機能を有することから様々な工業分野で重要な役割を果たす。しかし、多糖の機能性は多糖自身の構造の影響を大きく受け、その多糖構造は藻類種によって異なるだけでなく、同じ種でも年齢や生育環境の違いによって変化する。それゆえ、多糖構造の均質化は機能性多糖としての質を保証するために重要である。そこで、沖縄で広く養殖され、且つ利用されている藻類のウミブドウとオキナワモズク由来細胞壁多糖の構造と機能性に着目し研究を行った。

初めに、ウミブドウから硫酸化多糖（SP）を抽出し、SP のヒアルロニダーゼ（HAase）阻害活性について検討した。その結果、SP は HAase 活性を阻害した。さらに、SP の構造と HAase 阻害活性との関連を調べるために、SP を脱硫酸化または低分子化し、阻害活性試験を行った。その結果、脱硫酸化した多糖と低分子化した多糖の HAase 阻害活性は著しく低下した。このことより、SP の HAase 阻害活性は、多糖の構造、即ち、硫酸化度と分子量の影響を大きく受けることが明らかとなった。

次に、我々は多糖構造が生理活性に重要であることを見出したことから、オキナワモズク由来細胞壁多糖の構造解析を行った。オキナワモズクは他の褐藻類に比べて機能性多糖のフコイダンが豊富に含まれており、フコイダンの原料として利用されている。それゆえ、オキナワモズク由来フコイダンの構造解析について多くの報告があるものの、その多くはフコイダン単体を対象に行われているものであり、細胞壁の全体構造についてはほとんど明らかでなかった。そこで、オキナワモズクの細胞壁構造を把握するために、これまで解析されてきたフコイダンに加えて、フコイダンを抽出した残渣に含まれる多糖にも着目して研究を行った。オキナワモズクの細胞壁を熱水（HW）、シュウ酸アンモニウム、KOH で順次処理し、5 つの画分に分画し解析を行った。その結果、オキナワモズクの細胞壁は主に HW とヘミセルロース I（HC-I）の 2 つの画分から成り、細胞壁多糖の 80%を占めることが分かった。また、HW および HC-I のどちらにもフコイダンが含まれていることが明らかとなった。特に、HC-I のフコイダンは硫酸含量や分子量、1,4-結合のキシロースおよび 1,4-結合のフコース残基を含む点で HW のフコイダンの構造と異なっていた。さらに、HC-I のフコイダンが細胞壁構造を強固にしているという、藻類の細胞壁内におけるフコイダンの機能について初めて示唆した。

最後に、養殖産地別のオキナワモズクの細胞壁多糖の構造について解析した。沖縄県内の 8 カ所の地点で養殖されたオキナワモズクを解析した結果、フコイダンの構造に若干の差は見られたものの、産地が異なってもオキナワモズクの細胞壁構造はほぼ同じであることが分かった。

以上のことより、藻類の細胞壁多糖の構造は機能性成分としての質を考える上で重要であり、本研究の成果は、今後、藻類の機能性多糖の品質改善のために役に立つと思われる。

CHAPTER I

General Introduction

1.1 Macroalgae and their distribution in the world.

The algae are a heterogeneous group of organisms that occur in diverse body types and cellularity. The organisms having small algal bodies that a microscope is needed to observe are referred to as microalgae including unicellular prokaryotic and eukaryotic species, while algal bodies that are large enough to be seen with the unaided eye are known as macroalgae including only eukaryotic species [1], [2]. The macroalgae, generally called “seaweed" are multicellular photosynthetic organisms, consisting of a leaf-like thallus instead of roots, stems, and leaves. Based on the thallus color derived from pigments and chlorophylls, macroalgae are broadly classified into three taxonomic groups: brown algae (around 2,000 spp. in Phaeophyceae), red algae (over 7,200 spp. in Rhodophyta) and green algae (more than 1,800 macroalgae spp. in Chlorophyta), representing the three independently evolved lineages as shown in Figure 1.1 [3]–[5].

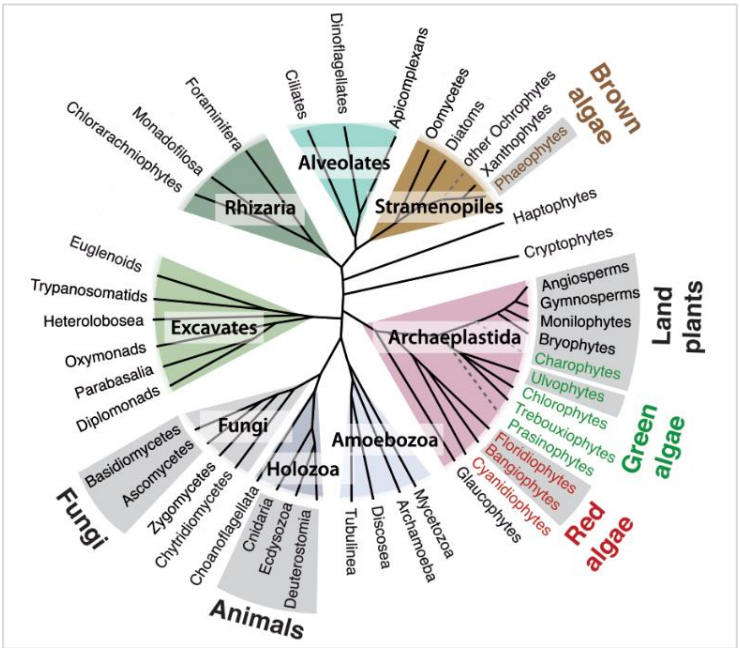

Figure 1.1 Schematic tree of the eukaryotes showing the phylogenetic positions of the brown, green, and red macroalgal lineages. Gray sectors mark lineages that have given rise to complex multicellular species. [5]

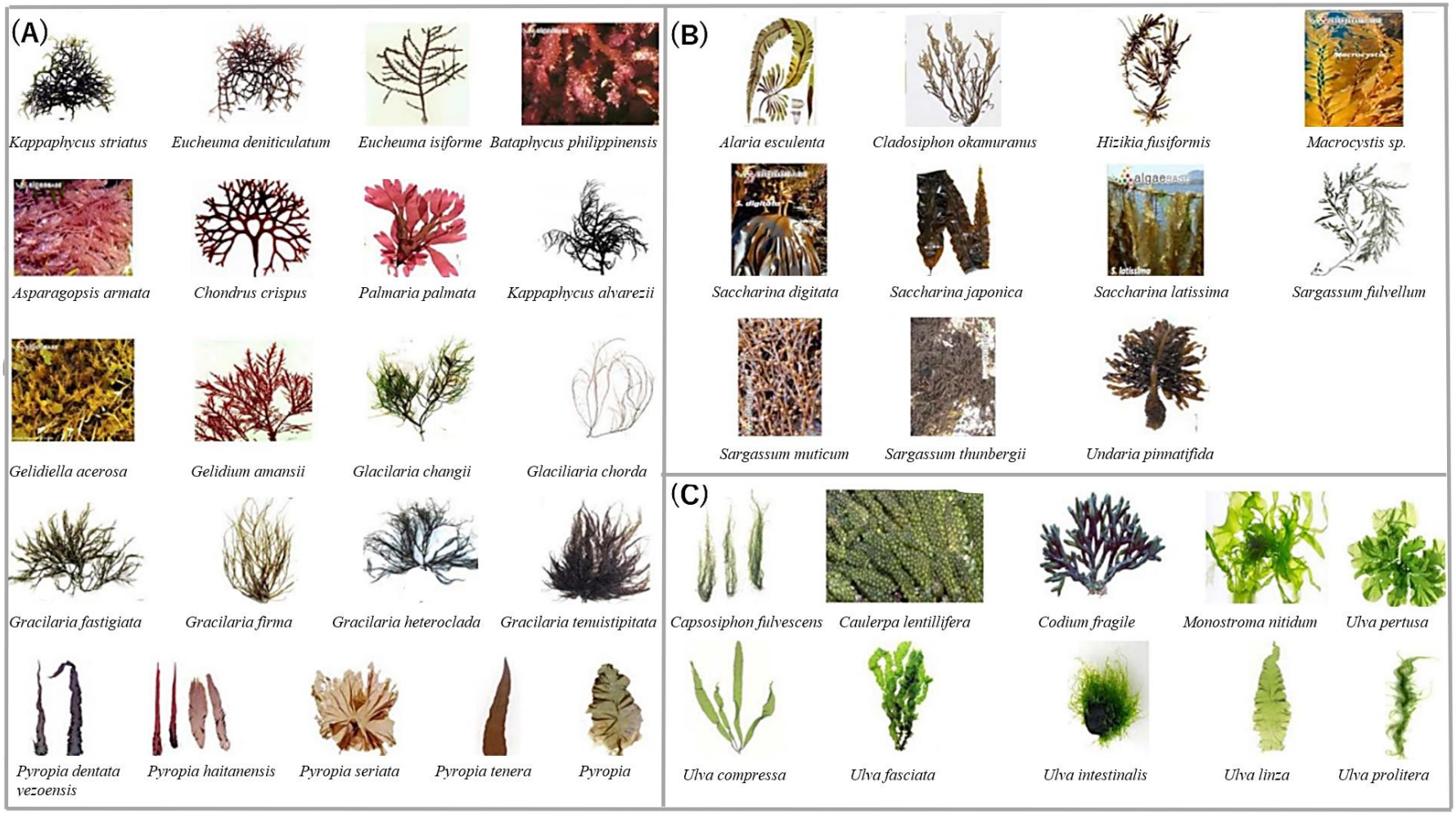

Figure 1.2 Commercially farmed (A) red macroalgae (B) brown macroalgae and (C) green macroalgae spp. in the world [6].

Macroalgae is one of the world's vital marine sources which have been harvested and grown mainly for food and food ingredients by many countries including Europeans, Asians, and South Americans for decades. They have multiple other uses in food and non-food industries, such as food additives, animal feeds, pharmaceuticals, nutraceuticals, cosmetics, textiles, biofertilizer/ biostimulants, bio-packaging, and biofuel [3]. The usage of macroalgae can be categorized into direct human consumption (77.6%), phycocolloid sector (11.4%) and phycosupplement industry (11.0%) [7]. Although there are large number of macroalgae spp., cultivation is focused on a relatively small number of spp. as shown in Figure 1.2 and Table 1.1. In 2019, red, brown and green algae accounted for 47.3, 52.6 and 0.05% of world cultivation in terms of tonnage respectively (Table 1.1).

Table 1.1 World macroalgae cultivation in 2019

Type of macroalgae	Cultivation (Wet tonnes)
Red macroalgae (Rhodophyta: 47.3%)	
Kappaphycus/*Eucheuma*	11,622,213
Porphyra	2,984,123
Gracilaria	3,639,833
Brown macroalgae (Phaeophyta: 52.6%)	
Laminaria/*Saccharina* (Kelp)	12 273 748
Undaria (Wakame)	2 563 582
Sargassum	270 000
Alaria esculenta (bladderlocks or winged kelp)	105
Cladosiphon okamuranus	90
Green macroalgae (Chlorophyta: 0.05%)	
Caulerpa spp.	1090
Monostroma nitidum	6321
Capsosiphon fulvescens	3386
Ulva spp.	2155
Codium fragile	3258

Source: FAO. 2021c. Fishery and Aquaculture Statistics. Global production by production source 1950–2019 (FishStatJ) [3].

World red macroalgae cultivation is concentrated on two warm-water genera: *Kappaphycus/Eucheuma* (33.6% of all macroalgae) and *Gracilaria* (10.5%), and one cold-water genus *Porphyra* or nori (8.6%). *Gracilaria* are primarily used for agar production and abalone feed, whereas *Kappaphycus/Eucheuma* are mostly used to extract carrageenan, and *Porphyra* are mainly used as human foods. World brown macroalgae cultivation is concentrated on two cold-water genera: Laminaria/Saccharina (mainly *Laminaria japonica:* 35.4% of all macroalgae) and *Undaria* (primarily *U. pinnatifida*: 7.4%), while minor spp. includes *Sargassum* (primarily *S. fusiforme*), *Alaria esculenta* and *Cladosiphon okamuranus*. Cultivation of green macroalgae has been considerably lower than red and brown algae, which include *Caulerpa* spp., *Monostroma nitidum*, *Enteromorpha* [Ulva] *prolifera*, *Capsosiphon fulvescens*; and *Codium fragile* [3].

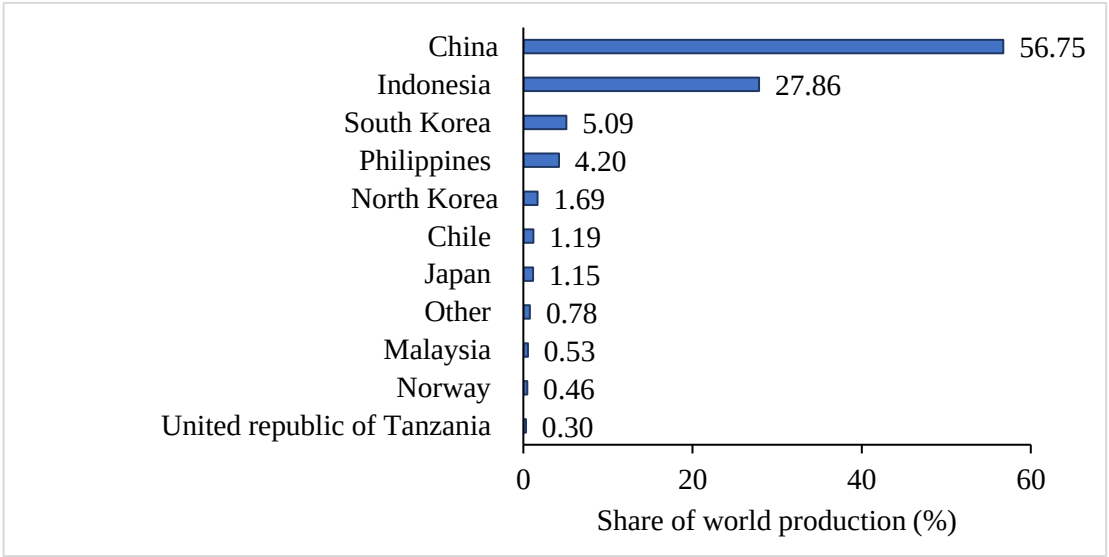

Figure 1.3 Global seaweed production and the top 10 seaweed producing countries, 2019, **Source:** FAO. 2021c. Fishery and Aquaculture Statistics. Global production by production source 1950–2019 (FishStatJ) [3].

There is a strong regional imbalance in global macroalgae production, because

97.4% of the production occur in Asia, where seven of the top ten producing countries located in Eastern or South- eastern Asia in 2019 as shown in Figure 1.3 [3]. The largest macroalgae producer is China (56.75%), while Americas and Europe contributed 1.4% and 0.8% respectively. However, macroalgae have been a food source since the fourth century in Japan and the sixth century in China.

1.2 Production and distribution of macroalgae in Japan.

Japan is the largest island country in East Asia, consisting of over 6000 islands extending along the Pacific coast. It composed of subtropical, temperate and subarctic climates and it has the sixth longest coastline in the world that stretches around 30 000 km [8]. It was reported that geography of Japan and the flow directions of the ocean currents along this coastal region allow the development and persistence of several temperate and subarctic macroalgae.

Japan has approximately 1500 species of seaweeds belonging to the three major algal groups: Chlorophyta (249), Phaeophyceae (343) and Rhodophyta (985). Some of these species are cultivated widely in many coastal areas around the archipelago, especially, the cultivation methods for most of the important edible seaweeds, namely *Pyropia, Undaria, Saccharina, Monostroma, Ulva* and *Cladosiphon* are now well-developed. Most commonly used macroalgae types for foods in Japan are shown in Table 1.2. Particularly, high annual production (300,000 tons wet wt.) was recorded by *Pyropia* which was processed into about 7.8 billion sheets of nori in 2017 [9]. Therefore, “nori” industry in Japan has developed into huge scale for commercialization. The first, second, third and fourth most cultivated species in Japan are *Neopyropia* spp. (251,362 tons, 94.2 billion JPY = 864 million USD), *Undaria* spp. (45.099 tons, 12.1 billion JPY = 111 million USD), *Laminaria* spp.(21,812 tons, 10.4 billion JPY = 95 million USD) and Mozuku (16,470 tons, 4.3 billion JPY = 40 million USD) respectively [10].

Table 1.2 Japanese seaweeds and their use for human food and as a raw material for hydrocolloid extract (agar) for various applications [9], [10].

Species name	Japanese name	Production, 2018 (Wet tons)	Food use
Rhodophyta			
Pyropia complex	Nori	251,362	Rolled with rice
Gelidium complex	Tengusa	−	Gel, agar
Grateloupia filicina	Mukadenori	−	Salad
Eucheuma gelatina	Kirinnsai	−	Salad
Meristotheca papulosa	Tosakanori	−	Salad
Gracilaria complex	Ogonori	−	Salad
Phaeophyta			
Undaria pinnatifida	Wakame	45,099	Soup
Saccharina japonica	Kombu	32,812	Soup
Cladosiphon okamuranus	Okinawa mozuku	16,470	Salad
Nemacystis decipiens	Mozuku		Salad
Ecklonia kurome	Kurome	−	Soup
Sargassum fusiforme	Hijiki	−	Soup
Chlorophyta			
Monostroma complex	Hitoegusa	−	Soup
Ulva complex	Aonori	−	jam Powder
Caulerpa lentillifera	Kuberizuta, umibudou	−	Salad

−, Production is less than 1% of the total macroalgae production in Japan

Although contribution of Japan is only 1.15% of world macroalgae production, about 50 species of algae are used daily as foods, with an estimated high annual per capita consumption ranging from 9.6 (2014) to 11.0 (2010) g macroalgae per day and up to 2 kg per year [2], [11]. As a result of health benefits of these algae consumption and the absence of long-term side effects, previous studies found that Japanese people have one of the lowest rates of obesity in the world, a very long-life expectancy, low rate of cardiovascular diseases, and a very low rate of several types of cancer [2]. Therefore,

macroalgae has major concern as essential part of Japanese diet and study on macroalgae grown in Japan is generating considerable interest in terms of the structure and activity of compound presented in macroalgae.

1.3 Polysaccharide from macroalgae

In recent years, much attention in research and industry has been focused on polysaccharides from macroalgae for their interesting physiochemical and biofunctional properties rather than nutritional value, so that they play key roles in the fields of industries [12]. Although macroalgae can synthesize wide range of bioactive compounds composed of primary and secondary metabolites: polysaccharide, lipids, amino acids, polyphenol, pigments and vitamins, many countries in the world cultivate the macroalgae for the extraction of polysaccharide, because polysaccharides are abundant, approximately 4–76% of the dry weight of algae and show many bioactive and physiochemical properties [13].

Macroalgal polysaccharide occur as cell wall and storage materials as categorized in Table 1.3. Algal cells are surrounded by the cell wall enriched with diverse types of cell wall polysaccharides, functioning on cell wall processes and properties. The storage polysaccharide build directly from photosynthetic mechanism and stored in algae plastids to reuse on demand for maintaining the basic metabolism in the cell [14]. These polysaccharide mainly include alginate, fucoidan, laminaran, and sargassan found in brown algae, sulfated galactans and xylans found in green algae as well as agars, carrageenans, xylans, and floridean in red algae as classified in Table 1.3 [12].

Table 1.3 Major cell wall and storage polysaccharide present in different plant and algal Taxa

Polysaccharide (PS)		Taxa				
				Chloroplastida		
		Phaeophyceae (Brown algae)	Rhodophyta (Red algae)	Chlorophyta (Green algae)	Charophyceae (Fresh water algae)	Embryophyceae (Land plant)
Matrix PS	Matrix sulfated PS	Homofucans	Agars Carrageenans Porphyran	Ulvans	—	—
	Matrix carboxylic PS	Alginate	—	Ulvans	Pectin	Pectin
Skeletal PS	Hemicelluloses	Sulfated-xylofucoglucan Sulfated-xylofucoglucuronan	Glucomannan Sulfated MLG (1→3),(1→4)-β-D-xylan	Xyloglucan Mannans Glucuronan (1→3)-β-glucan	Xyloglucan Mannans Xylans (1→3)-β-glucan	Xyloglucan Mannans Xylans MLG (1→3)-β-glucan
	Crystalline PS	Cellulose	Cellulose (1→4)-β-D-mannan (1→4)-β-D-xylan (1→3)-β-D-xylan	Cellulose	Cellulose	Cellulose
Reserve/ storage PS		Laminaran	Floridean glycogen	Inulin (Fructan) Laminaran Starch	—	Starch

Wall polymers are defined as follows: cellulose, (1→4)-β-D-glucan; MLG, (1→3), (1→4)-β-D-glucan; ulvans, sulfated xylorhamnoglucuronans. Agars, carrageenans, and porphyrans are sulfated α-(1→3), β-(1→4)-galactans differentiated by the fact that agars and porphyrans contain D-and L-galactose, whereas carrageenans contain only D-galactose, and alginates are polymers ofα-L-guluronic acid and β-D-mannuronic acid. We recommend the following reviews, which cover this topic in greater depth [15], [16], [17], [18]

1.4 Role of polysaccharide in algal cell wall

Like land plants, the cell wall of macroalgae can be described as two-phase system: a crystalline or skeleton phase embedded in more amorphous phase called matrix [19]. However, compositional and configurational differences of cell wall polymers occur in between palnts and macroalgae. Since macroalgae grow in the intertidal environment, it's cell wall is abundant with polyanionic matrix polysaccharide which are not commonly found in terrestrial biomass to protect cells from physical shocks and other stress conditions. On the other hand, it contains less amount of skeletal component compared to the land plant.

Although two networks were found in macroalgal cell wall, interactions between them appears to be vary in different algae species. Recently, Kloareg et al [5] develop the cell wall models for brown, red and green algae adapted from previous studies as shown in Figure 1.4. According to the green algal cell wall model adapted from Lahaye and Robic [20], the major polysaccharide is ulvan which can form a weak gel in the presence of both boric acid and divalent cations and two minor hemicelluloses polysaccharides: xyloglucans and glucuronans. Glucuronans was found to interact with ulvans and proteins, while xyloglucans interact with cellulose, but their role as cross-linkers of cellulose microfibrils were not understood (Figure 1.4 A). Most abundant polymers of the red algal cell wall are sulfated galactans (carrageenans), containing 3,6-anhydro-D-galactose units associate through hydrogen-bonding and hydrophobic interactions (Figure 1.4 B). The resulting helices further cluster into microfibrils by ionic interactions between their sulfate groups and cations. Glucomannans have been cross-linked with cellulose microfibrils and interactions of minor sulfated glucans with the other polysaccharides remain uncertain [5].

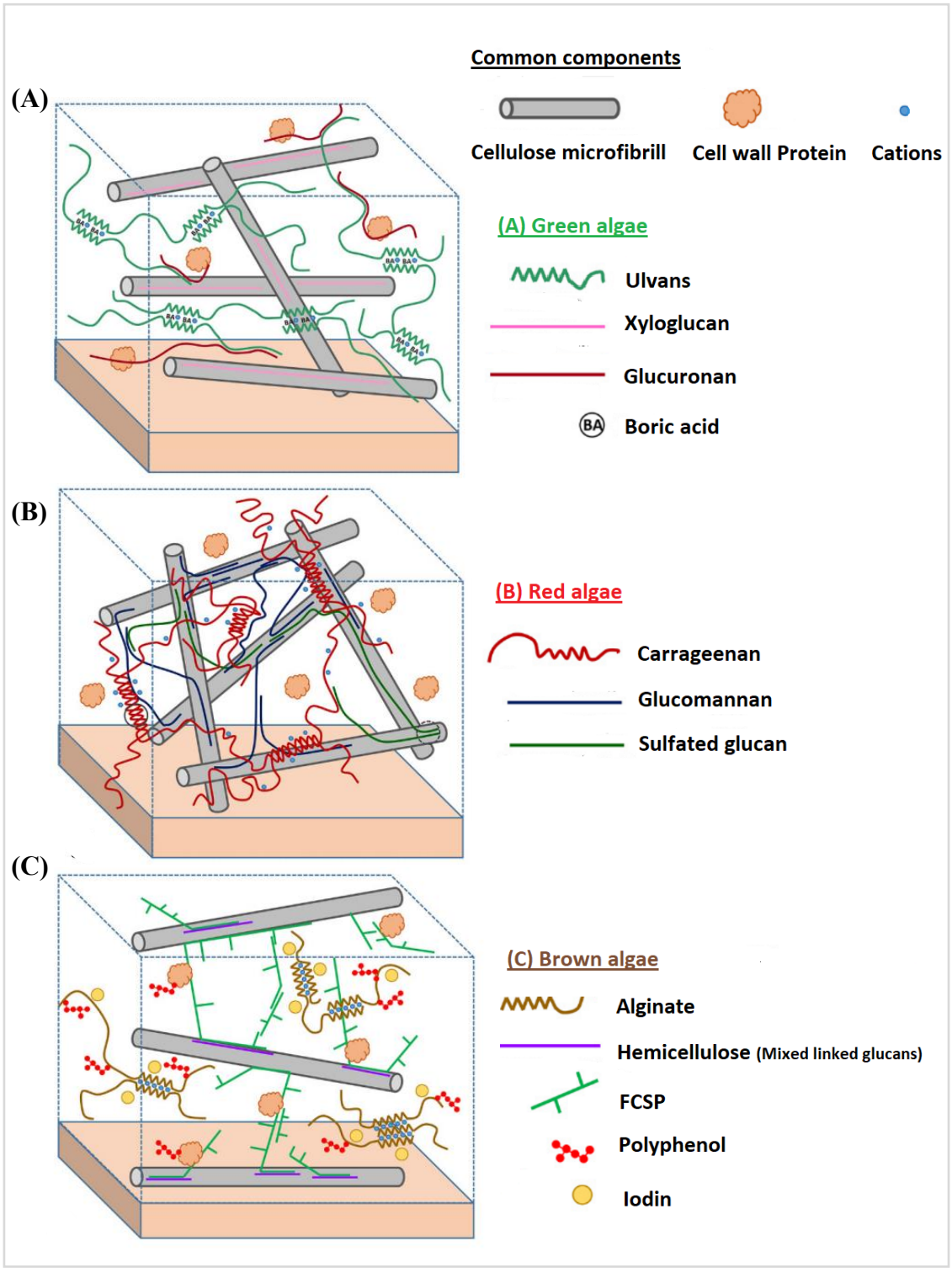

Figure 1.4 Models of macroalgal cell walls as summarized by Kloareg et al [5]. (A) Green algae (example of Ulva species) (B) Red algae (example of a carrageenophyte). (C) Brown algae (example of a Fucales species).

Deniaud-Bouët et al. [21] proposed the most significant brown algal cell wall model, describing a cell wall comprising two networks: the first of fucose-containing sulfated polysaccharide (FCSP) interlocking the cellulose microfibrils and the second of alginate, cross-linked by polyphenols (Figure 1.4 C). Proteins are likely tightly interlinked with FCSPs and also covalently attached to phenol compounds. Nevertheless, FCSP–cellulose bonds could not be explained, and short-chained hemicellulose molecules may present as intermediates between cellulose microfibrils and FCSPs. Recently, hemicelluloses such as β-1,4-1,3-glucans, β-1,3-glucans, and arabinogalactan protein were identified in the brown algal cell wall, but associations with other components are poorly understood [22]–[24]. Hence, despite the cell-wall model described above, it remains unclear how several cell wall polysaccharides are interlinked.

The algal cell wall is a vital organelle with different functions: mechanical strength, adaptation, defense responses, morphogenesis and essentially involved in growth and development. Preceding studies of brown algae found that the cell wall contributed to cell polarity [25], cell development with cell differentiation in growing filaments [26], tissue integrity, and cell adhesion [21]. Sulfated fucoidans and/or polyphenols cross-linked to alginates was found to contribute to cell adhesion [27]. Role of cell wall swelling in some Laminariales, in the pressure-driven transport of photoassimilates was explained by Knoblauch et al. [28]. In *Laminaria digitata* specific fragments of alginates are known to elicit the expression of defense responses [29]. Other functions are thickening of cell walls through deposition of β-(1–3)-glucan [30] and sulfated polysaccharides probably play a key role in the adaptation to osmotic stress [15]. Collectively, to explain more about these cell wall function related to dynamic process of growth, development, and decay of the cell wall, studying the interlinking and distribution of polymers throughout the cell wall is essential.

1.5 Role of macroalgal polysaccharide in different application

Nowadays, many industrialists and researcher consider macroalgae as a source of natural polysaccharide with numerous bioactivities and phychochemical properties. Most of bioactive compounds, characterized by bio activities are unable to synthesize or synthetize them in too low amounts in human body and external administration of these compound can promote health and suppress diseases [2]. Therefore, macroalgae are well-known producer of bioactive polysaccharide, mainly sulfated polysaccharide such as fucoidan, galactan, carrageenan and ulvan. They have been studied for wide range of physiological and biological activities rather than role of basic nutrition over the last few years, so that they play key roles in the fields of pharmaceutical, nutraceutical, cosmeceutical and functional foods [12]. Another important sector of algal polysaccharide application is phycocolloid sector, as food stabilizers and texture modifier based on their rheological properties [7], Hence, algal polysaccharides including alginate, carrageenan and agar are commonly used as texturing agents in the food, pharmaceutical and cosmetic industries.

Among these polysaccharides, fucoidans, and alginates are receiving high attention due to their interesting functional properties. Unlike alginate which is renowned for metal chelation and for its ability to form hydrogels, fucoidans are renowned for their wide range of bioactivities: anticoagulant, anticancer, antivirus, immunomodulatory, anti-inlammatory, anti-obesity, antioxidant, anticomplementary activity, and activity against uropathy, hepatopathy, and renalpathy [31]. However, these physical and biological properties are strongly influenced by their structural characteristics such as monosaccharides composition, anomeries, glycosidic bonds and branching degree, sulfate content and position, molecular weight. For instance, positive relationship was found between sulfate content of fucoidan and anticoagulant activity, rhamnan sulfate and

hyaluronidase inhibitory activity [32], whereas, negative relationship was found between fucoidan and antithrombin effects [33]. Furthermore, high molecular weight of fucoidan increase the antitumor activity [34] and anti-fibrogenesis activity [35], but anti cancer activity was high in low MW fucoidan [36]. Therefore, these variations can occur due to the influence of more than one structural characteristic on polysaccharide, revealing that study on relationship between structure and activity of bioactive polysaccharides are very important for their applications in different fields.

1.6 Causes for the variation of polysaccharide content in macroalgae

Although I extract a polysaccharide with known structure, these structural characteristics may vary significantly depending on the species, or even from the same species, depending on the age of algae, harvesting season and geographical location [37], [38]. The critical issue of the dynamic variation of the composition and structure is that it will be challenging to produce functional polysaccharides with reproducible quality [37]. Therefore, study of the influential factors on variation of the content and structure of polysaccharide is really needed.

Seasonal variation of the polysaccharide content and structure was reported by many studies [38], [39], [40] and Tsuji *et al*. [41] suggested that although the highest fucoidan content was recorded in May, algae samples harvested from March to April are most appropriate to extract bioactive compound with the highest sulfate content and molecular weight. Furthermore, geographic variation of yield of fucoidan from *Sargassum wightii* and *S. oligocystum* in India [42] and from *Laminaria. digitata* in Denmark [43] were observed. These geographical variations of algal polysaccharide were explained by prevailing diverse environmental factors such as light, temperature and salinity in sea water [43]. Effect of the maturity or age of the macroalgae on the variation of fucoidan content was found that non-reproductive tissue, fronds contain more fucoidan

than stipes and midribs [44], while older algae tend to contain more fucoidan than young algae [45]. Considering the changes in biomedical properties due to variation in composition and structure of algal polysaccharide, understanding variation in several aspects will be important for controlling the quality of compounds. Moreover, it allows determination of the optimum time and suitable place to collect algae in order to standardize the preparations of these polysaccharides for their use in medicine and food industry.

1.7 Macroalgae used in this study

In this study I mainly focused on *Caulerpa lentillifera* (Figure 1.5 A) and *Cladosiphon okamuranus* (Figure 1.6 A), which are grown and cultivated mainly in Okinawa prefecture where tropical climatic is prevail.

Caulerpa lentillifera (Caulerpaceae, Bryopsidophyceae) is green alga, called umibudou in Japanese, that grows in the southern part in Japan, particularly is cultivated in Okinawa with an annual production in 2006 of 200 tons of wet weight (approx. 5,000,000 USD [46]. It is basically used for human consumption, but in recent years, bioactivities of polysaccharides, extracted from *C. lentillifera* have been reported including antioxidant, anticoagulant and anticancer [47], [48], immune-stimulatory [49], [50], anti-inflammatory [51], anti-tumor, therapeutic microbial effects [49] and SARS-CoV-2 inhibitory activity [52]. Hyaluronidase inhibitory activity is another important activity, but it was not reported in sulfate polysaccharide from *C. lentillifera* yet. The water-soluble polysaccharides derived from *Caulerpa* spp. mainly contain glucans and sulfated polysaccharide such as sulfated xyloarabinogalactans [53], and xylogalactomannans [54] and their structure was well described in previous studies.

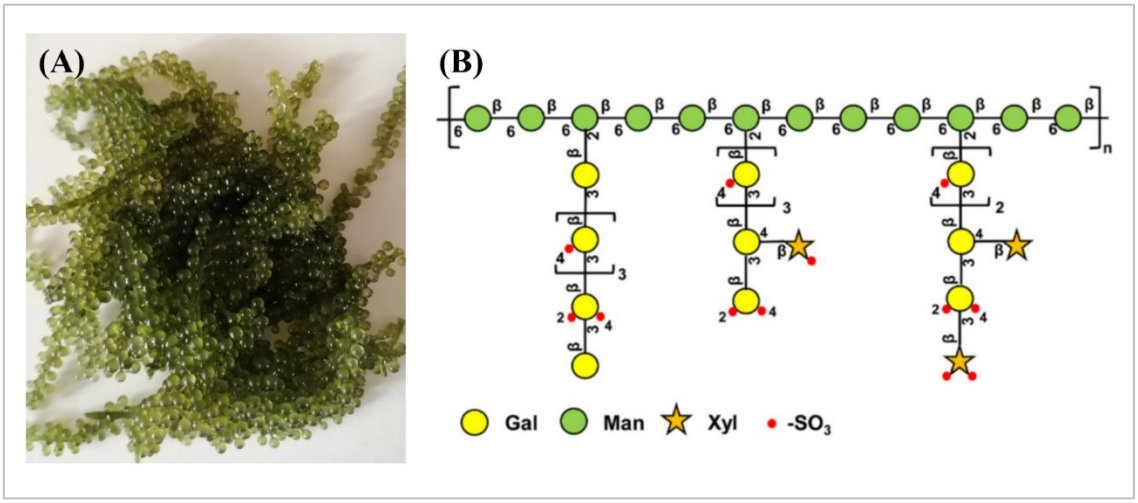

Figure 1.5 (A) Photograph of specimen of *C. lentillifera* used in this study, (B) Proposed structure of sulfated polysaccharide from *C. lentillifera* by You *et al.* in 2022 [52].

Most recently described structure of sulfated polysaccharide from *C. lentillifera* shows in Figure 1.5 B. Although effect of structural properties of sulfated polysaccharide from *C. lentillifera* on different bio activities were found, so far, the effect of sulfate content and/or molecular weight on hyaluronidase inhibitory activity were not investigated. Therefore, we investigated the hyaluronidase inhibitory activity of sulfated polysaccharides from *C. lentillifera* and evaluate the impact of influential factors: sulfate contents and molecular weight of the polysaccharides on its activity.

Cladosiphon okamuranus (Chordariceae, Phaeophyceae) Okinawa mozuku in Japanese, is an extensively utilized edible brown algae in Japan and identifies as excellent source of fucoidan in Japan. Previous reports investigated that fucoidan extracted from *C. okamuranus* possess the highest content among any brown algae species [55] with average yield of about 2.3% (w/w) from wet algae. The alginate was only 1/10 of the fucoidan content [56]. Therefore, fucoidan is the most abundant polysaccharide in *C. okamuranus* with the structure of α-13-linked fucan as the main backbone by

substituting D-glucuronic acid (GlcA) residues at *C*-2 and sulfate at *C*-4 of L-fucose (Fuc) [57]. Although Okinawa mozuku is utilized mainly as food that supplies dietary fiber for the native population since ancient time, recently, it has gained much attention by industrialists and researchers as a significant source of fucoidan. Up to date, several bioactivities of fucoidan from *C. okamuranus* have been reported such as anti-tumor [58], anti-cancer [55], antiviral [59], anti-oxidant [60], Immunomodulatory [61], and cardioprotective activities [62], so that they play a key role in the fields of pharmaceutical, nutraceutical, cosmeceutical, and functional food [12].

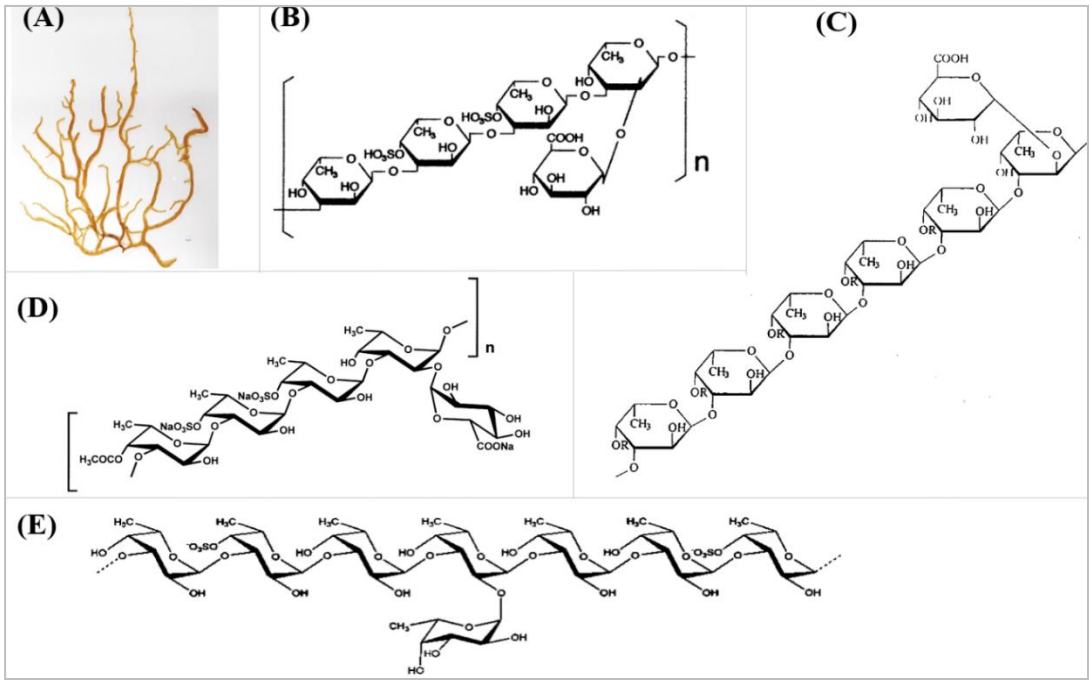

Figure 1.6 (A) Photograph of specimen of *C. okamuranus* used in this study, Structure of fucoidan extracted from *C. okamuranus* proposed by **(B)** Sakai *et al.* **(C)** Nagaoka *et al.* **(D)**Teruya *et al.* **(E)** Lim *et al.*

Typical fucoidan extracted with dilute acid or water from *C. okamuranus* were extensively studied for individual structure as shown in Figure 1.6 (B)-(E) and bioactivities, but remarkably little is known about structure of polysaccharide including

fucoidan, remained in the residue after extraction of typical fucoidan and how their structure is important for bioactivities. Therefore, we sequentially fractionated and analyzed the structure of cell wall polysaccharide from *C. okamuranus* to understand its whole cell wall structure. Recently, due to climate change-induced alterations in the coastal environment, large fluctuations in yield of *C. okamuranus*, which has ranged from 5,500 to 20,300 tons during 2007–2019 as reported by mozuku aquaculture promotion council in Okinawa [63], [64]. Therefore, we believe that study of whole cell wall from *C. okamuranus* at the structural level will provide an understanding of cell wall function, particularly process of growth and development. Furthermore, *C. okamuranus* spread over wide area of Ryukyu archipelago as shown in Figure 1.7 and geographical variation in morphological characteristics and productivity have already reported [10]. Since, it is possible to vary the structure of polysaccharide with place of growth, we evaluated the geographical variation in structure of polysaccharide from *C. okamuranus* as well. Results of this study will help to find the solutions for recently occurred yield reduction with advancement of applications in different field of industries.

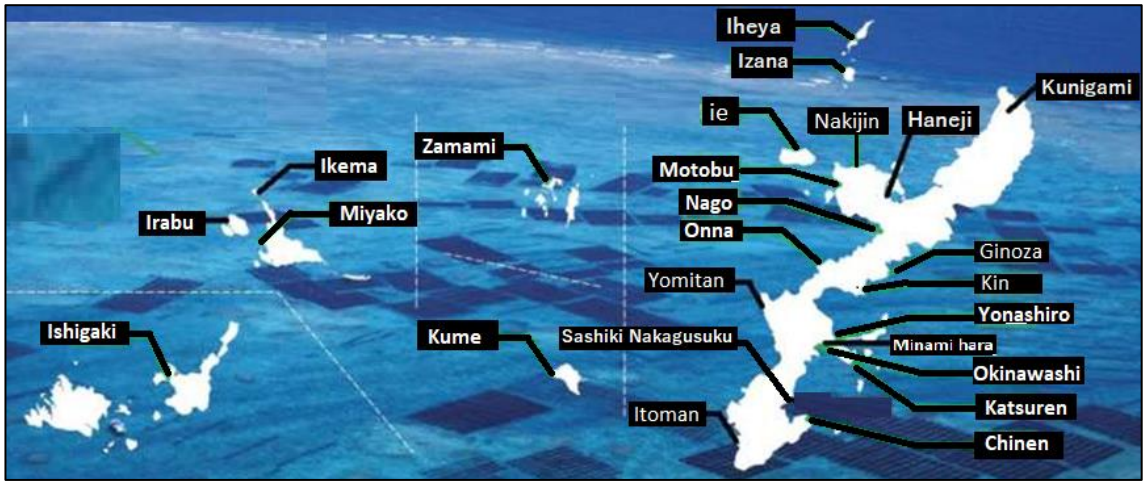

Figure 1.7 Major mozuku production area in Okinawa prefecture (Okinawa prefectural mozuku aquaculture promotion council, HP)

1.8 Objectives and outline of the study

The main objective of this study is to study on structure of bio active polysaccharide and to understand how structural properties are influenced on their activities. I analyzed polysaccharides from 2 spp. of macroalgae from *Cladosiphon okamuranus* and *Caulerpa lentillifera*, which are extensively utilized and abundantly cultivated algae in Okinawa prefecture, where processes one of the world's most interesting food culture with people who have longest life expectancies and low disability rates.

The present thesis is divided to 5 chapters. Chapter I described importance of study on macroalgae by explaining the present situation of macroalgae cultivation, production in Japan and the world, structure-activity relationship of polysaccharide and factors affecting on their structure. In chapter II, I studied on structure of functional polysaccharide from *C. lentillifera* and evaluated the relationship between structure and activity to understand which structural properties are most important for the activity. I found particularly molecular weight and sulfate content of polysaccharide are major influential factors.

In chapter III, I also analyzed the structure of polysaccharide from *C. okamuranus* to understand how the main structural properties identified in chapter II, exert in the whole cell wall. Therefore, I sequentially fractionated the cell wall polysaccharide and identify water soluble and insoluble fucoidan with different structural properties (molecular weight, sulfate content, sugar composition) which may be related to their localization in the tissue to perform the distinct roles of cell walls.

I further analyzed the structure of polysaccharide from *C. okamuranus* in chapter IV to identify whether structural variation of polysaccharide occur due to place of growth. I could obtain relatively uniform structural fucoidan in water soluble fraction from any

geographical location in Okinawa prefecture at peak of harvest. Finally general conclusions, open questions and future perspectives are summarized in chapter V.

CHAPTER II

Structural analysis of sulfated polysaccharides from *C. lentillifera* and evaluation of its structure-activity relationship

2.1 Introduction

Among 100 *Caulerpa* spp., *Caulerpa lentillifera* and *C. racemosa* are the two most popular edible green algae in the Asia-Pacific Region. *Caulerpa lentillifera* is one of the main sea products cultivated on a large scale in Okinawa, Japan, where it is known as Umibudou [13], [49], [65]. Polysaccharide is the most abundant compound in *Caulerpa* spp., containing up to 83.2 % of algal dry weight [65]. Rather than role of basic nutrition, algal polysaccharide have been studied for wide range of physiological and biological activities, so that they play key roles in the fields of pharmaceutical, nutraceutical, cosmeceutical and functional foods [12]. In recent years, bioactivities of polysaccharides, extracted from *C. lentillifera* have been reported including antioxidant, anticoagulant and anticancer [47], [48], immune-stimulatory [49], [50], anti-inflammatory [51], anti-tumor and therapeutic microbial effects [49]. Hyaluronidase inhibitory activity is another important activity, reported for algal polysaccharides. Furthermore, inhibitory activity of some enzymes such as dipeptidyl peptidase-IV and α -glucosidase were observed in *C. lentillifera* extract previously[66]. Therefore, in this study, we focused on the inhibitory activity of hyaluronidase with polysaccharide from *C. lentillifera.*

Hyaluronic acid is a principal component of the extracellular matrix in connective tissue, distributed through organs and body fluids in human body [67]. Since hyaluronic acid is extremely hydrophilic, it plays a vital role in maintaining skin smoothness and moistness, and reducing wrinkles, while involves in many fundamental physiological and pathological processes [68]. Hyaluronidase, an enzyme for hyaluronic acid hydrolysis involves in development of inflammatory diseases, tumor invasiveness, metastasis, invasive breast adenocarcinoma, metastatic human melanoma, colon carcinoma and glioblastoma cell lines [69]. Therefore, hyaluronidase inhibitors are vital to overcome

phenomena accompanied by abnormal decomposition of hyaluronic acid such as above mentioned diseases, deregulation of skin homeostasis and wounds. Particularly, hyaluronidase inhibitors likely to become increasingly important as therapeutic agent in pharmaceutical industry and antiaging agents in the cosmetic industry. Despite reporting several types of hyaluronidase inhibitors, up to date, algal acidic polysaccharides have found to be a promising potential inhibitor, for example, sulfated polysaccharide from *Cladosiphon okamuranus* [70], *Lessonia nigrescens*, *Laminaria angustata* [71], *Undaria pinnatifida* [69]*, Padina pavonica* [72], *Fucus vesiculosus* [73], *Monostroma nitidum* [32] and *Porphyridium purpureum* [74]. However, little is known about hyaluronidase inhibiting polysaccharide from *C. lentillifera,* which has already been reported to contain sulfated polysaccharide [49], [50], [51].

Although many studies measure the hyaluronidase inhibitory activity from algal sulfated polysaccharide, the mechanism and influencing factors were not widely understood. Li *et al*. suggested that sulfate content and molecular weight of the bioactive polysaccharide significantly affect their biological activities [33]. Sulfated polysaccharide from green algae *Monostroma nitidum* and brown algae *Cladosiphon okamuranus* explained a strong positive relationship between sulfate content and hyaluronidase inhibitory activity [32], [70]. Despite lack of previous data about the effect of molecular weight of algal sulfated polysaccharide on hyaluronidase inhibitory activity, some compound such as outer layer of green coffee bean and phlorotannin from Fucales showed a positive relationship between hyaluronidase inhibitory activity and molecular weight [75], [76]. In contrast, Asada *et al*. demonstrated that in despite of hyaluronidase inhibitory activity of alginate from *Lessonia nigrescens* increased with increasing molecular weight up to some extent, the highest molecular weight compound (388 kDa) had decreased inhibitory activity [71]. Therefore, it is clear that sulfate content and

molecular weight can affect diversely on hyaluronidase inhibitory activity. So far, the effect of both sulfate content and molecular weight of sulfated polysaccharides on hyaluronidase inhibitory activity were not investigated together. Here, we investigated the hyaluronidase inhibitory activity of sulfated polysaccharides from *C. lentillifera* and evaluate the impact of highly influential factors: sulfate contents and molecular weight of the polysaccharides on its activity.

2.2 Materials and methods

2.2.1 Algae materials

Caulerpa lentillifera purchased from the local market of Ishigaki island, Okinawa, in 2007, was used in this study. Alcohol insoluble residue (AIR) from *C. lentillifera* was obtained in the same manner as previously reported and kept frozen at -30 °C until start of the experiment [77]. Briefly, fresh seaweed was washed with tap water, lyophilized and powdered. The algal powder was sequentially treated with 80 % ethanol, chloroform/methanol (1:1, v/v) and acetone. The residue was collected as AIR after filtration through filter paper.

2.2.2 Extraction and purification of polysaccharides from *C. lentillifera*

Polysaccharides were extracted as described by Konishi *et al.*[77]. In brief, AIR was stirred in water, and heated at 80 °C for 1 h. Supernatants from two consecutive extractions were pooled after centrifugation and named hot water extraction (HWE). Purification of acidic polysaccharides from HWE was based on cetylpyridinium chloride (CPC) precipitation as described by Tako *et al.*[78]. In brief, HWE was kept at 37 °C for 16 h after adding 2% of CPC solution and collected CPC supernatant and CPC precipitation by centrifugation. The CPC supernatant was precipitated by the addition of 2 volume of ethanol and the CPC precipitation was re-precipitated with a three-fold

volume of ethanol after dissolving in 4 M $CaCl_2$. Then, both ethanol precipitations were stirred overnight with distilled water and freeze dried after dialysis. The final resultants from CPC supernatant and CPC precipitation were named CPC-S and CPC-P respectively.

2.2.3 Analysis of chemical composition

Total sugar and uronic acid (UA) contents were determined by the phenol–sulfuric acid method [79] using glucose (Glc) as the standard, and *m*-hydroxybiphenyl method [80] using galacturonic acid as the standard, respectively. To estimate sulfate content and sugar composition, polysaccharides were hydrolyzed in 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h. The hydrolysate was dried to remove TFA and dissolved in distilled water. The hydrolysate was subjected to high-performance liquid chromatography with an AS4A-SC column (4 mm × 250 mm, Dionex Co., Tokyo, Japan) to measure sulfate content. The column was eluted at 1 mL/min at room temperature with buffer containing 1.7 mM $NaHCO_3$ and 1.8 mM Na_2CO_3 [77]. The weight of sulfate in the sample was calculated from a calibration curve using Na_2SO_4 as a standard based on the molecular weight of HSO_3.

Monosaccharides in the hydrolysate were analyzed by high-performance anion-exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD) using Carbo Pac PA1 column (4 mm × 250 mm, Dionex, ICS-5000). The eluent flow was 1 mL/min at room temperature with buffer containing 0.5 M NaOH and 0.5 M CH_3COONa/0.1 M NaOH [77].

2.2.4 Hyaluronidase inhibitory activity

Hyaluronidase inhibitory activity of the extracted polysaccharides (HWE, CPC-S and CPC-P) was estimated following procedures outlined in previous reports with a slight modification [69], [71]. Hyaluronic acid sodium salt from rooster comb was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and molecular

weight was estimated to be 2.38 × 10^6 by size-exclusion chromatography by using a column of TSKgel G5000 PWXL (7.8 mm × 300 mm, Tosoh Co., Kyoto, Japan) as the method described in this study (Data not shown). Hyaluronidase type I-S from bovine testis (451 U/mg) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a enzyme unit (U) is defined as the amount of enzyme that liberates one micromole of N-acetylglucosamine from hyaluronic acid per minute at 37˚C and pH 4.0 [81]. First, the reaction mixture containing 100 µL of hyaluronidase (5 mg/1.5 mL) in 50 mM sodium acetate buffer (pH 4.5) and 200 µL of polysaccharide sample was incubated at 37 °C for 15 min. Then, 700 µL of hyaluronic acid (1 mg/mL) as a substrate, containing 100 µL 1.5 M NaCl and 18 µL of 1 M sodium acetate buffer (pH 4.0) was added and further incubated at 37 °C for 15 min. The reaction was stopped by boiling at 100 °C for 10 min. Quantitative analysis of *N*-acetyl-amino sugar (product) was determined by modified Morgan–Elson method [53]. Percentage inhibition was calculated as follows:

$$Inhibition\ (\%) = 100 \times \{1 - (S - B)/(C - B)\},$$

where B is the absorbance at 585 nm in the absence of an enzyme, C is the absorbance at 585 nm in the absence of an inhibitor, and S is the absorbance at 585 nm in the presence of an inhibitor [69].

In order to evaluate the dose-dependent manner of inhibitor, inhibition rate was measured at different concentrations of CPC-P (0–339 µg/mL), and also of fucoidan (0–358 µg/mL) extracted from *C. okamuranus* as described by Tako *et al.*[78] to compare the mechanism of inhibition with known hyaluronidase inhibitory polysaccharide. Furthermore, the mechanism of action of hyaluronidase inhibition was characterized using two different strategies – desulfation and partial hydrolysis – to examine the effect of sulfate content and molecular weight.

Chemical desulfation of CPC-P was performed using the method of Shiroma *et al*

[82]. Sample (100 mg) was dissolved in distilled water (20 mL) and passed through DOWEX 50W×8 (H^+, 100 mesh) resin purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). After neutralization with pyridine, the solution was lyophilized. The lyophilized pyridinium salt was dissolved in DMSO:MeOH (9:1; v/v, 20 mL). The mixture was heated at 80 °C for 4 h and then desulfated product was dialyzed against distilled water and lyophilized to obtain desulfated CPC-P (DSP).

2.2.5 Determination of molecular weight

Extracted CPC-P was partially hydrolyzed using 0.1 M or 0.5 M TFA at 70 °C for 3 h to obtain low molecular weight CPC-P. After removing TFA by drying, the hydrolysates were dissolved in distilled water. Partially hydrolyzed CPC-P in 0.1 M and 0.5 M TFA were named $SP_{0.1}$ and $SP_{0.5}$, respectively. Molecular weights of CPC-P, DSP, $SP_{0.1}$ and $SP_{0.5}$ were determined by size-exclusion chromatography (LC-6A; Shimadzu Co., Kyoto, Japan) equipped with a column of TSKgel G5000 PWXL (7.8 mm × 300 mm, Tosoh Co., Kyoto, Japan) and a refractive index detector RID-10A at 35 °C [82]. The column was eluted by water at a flow rate of 1 mL/min. Pullulan P-10 (molecular weight = 0.96×10^4), P-50 (4.71×10^4), P-200 (20.0×10^4) and P-800 (70.8×10^4) (Showa Denko Co., Tokyo, Japan) were used as the molecular weight standards.

2.3 Results

2.3.1 Preparation and characterization of *C. lentillifera* polysaccharides

The yield and chemical composition of polysaccharides extracted from *C. lentillifera* are shown in Table 2.1. Polysaccharides were initially extracted from 5 g of AIR of *C. lentillifera* by hot water treatment and HWE consisted of mainly galactose (Gal), Glc, xylose (Xyl), mannose (Man) together with UA, and a small amount of fucose (Fuc), rhamnose (Rha), arabinose (Ara) residues. After purifying the polysaccharide with

CPC, yields of CPC-S and CPC-P were 275 mg and 463 mg, respectively. Sugar composition analysis showed that CPC-S contained 91.4 % Glc, but UA, Gal, Xyl, and Man represented less than 5 %. The CPC-P contained 21.7 % sulfate and 53 % total sugar, including mainly Gal, Glc, Xyl, and Man. The molar ratio of sugar in CPC-P was estimated to be Gal, Glc, Xyl, and Man in the ratio of 0.2: 0.2: 0.12: 0.16 (Table 2.2). These data indicate that CPC-P is SP.

Table 2.1 Yield and chemical composition of polysaccharides from *C. lentillifera*

Sample	Yield (mg)	Total sugar in mg (%)	Monosaccharide (wt %)								SO_3^- (%)
			Fuc	Rha	Ara	Gal	Glc	Xyl	Man	UA	
HWE	1942	753 (39%)	0.3	0.2	0.3	21.6	39.8	20.4	14.1	3.3	11.7
CPC-S	275	247 (90%)	0.1	n.d	n.d	2.5	91.4	0.7	0.7	4.6	6.6
CPC-P	463	244 (53%)	0.6	0.3	0.8	27.7	28.9	14.6	22.5	3.4	21.7

n.d: less than 0.1 (in 5 g AIR)

2.3.2 Hyaluronidase inhibitory activity of polysaccharides extracted from *C. lentillifera*

The hyaluronidase inhibitory activities of HWE, CPC-S and CPC-P (SP) were 32.3 %, 24.7 % and 90.4 %, respectively, at a polysaccharide concentration of 339 µg/mL (Figure 2.1). It was clear that there was a low percentage of inhibition (0–2 %) at low SP concentration (0–40 µg/mL) but higher SP concentration (200–339 µg/mL) required to inhibit reaction at a higher rate (70–92 %) (Figure 2.2 a). Thus, the inhibition curve was distinctly sigmoid in shape and IC_{50} of SP was 163 µg/mL. I compared the mode of hyaluronidase inhibition of SP with known hyaluronidase inhibitors such as fucoidan and it showed a hyperbolic curve as shown in (Figure 2.2 b).

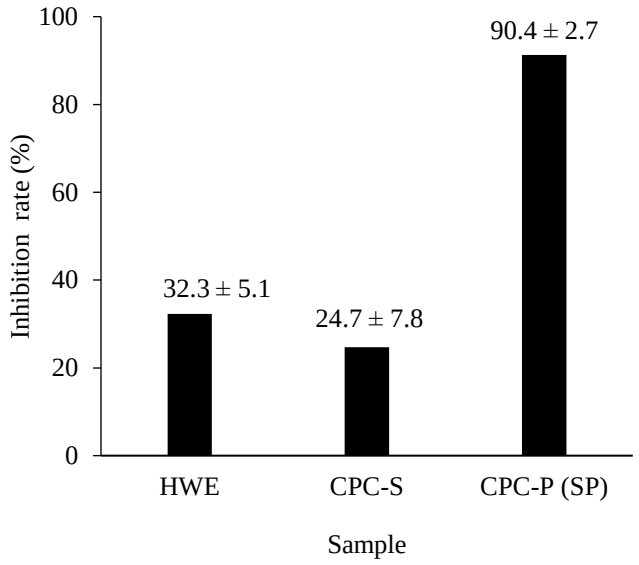

Figure 2.1 Hyaluronidase inhibitory activity of polysaccharides extracted and purified from *C. lentillifera* at a polysaccharide concentration of 339 µg/mL. Data are shown as mean ± SD

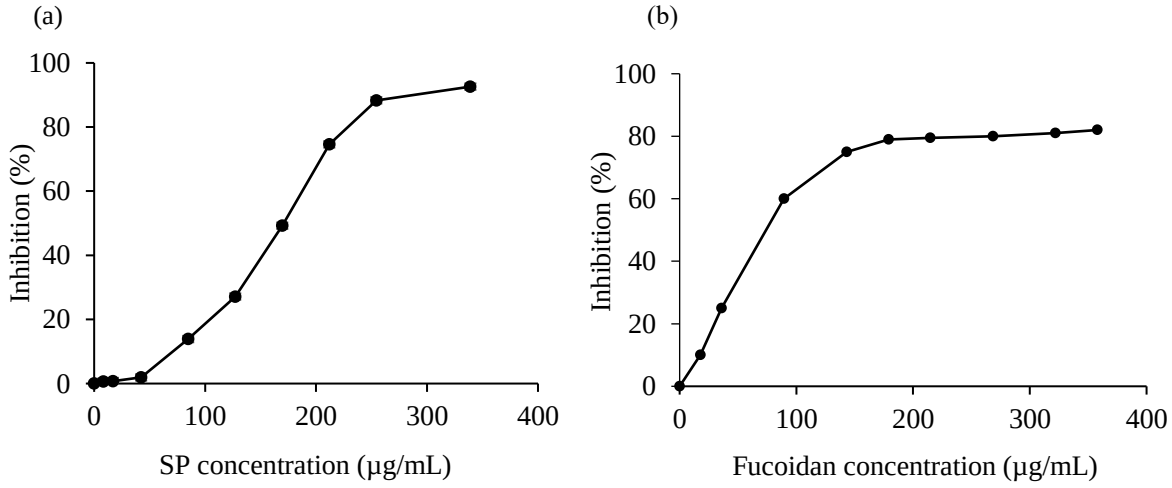

Figure 2.2 Hyaluronidase inhibitory activity of (a) SP and (b) fucoidan.

2.3.3 Effect of sulfate content of SP on hyaluronidase inhibitory activity

We prepared DSP to investigate the effects of sulfate content in the polysaccharide on hyaluronidase inhibition. After desulfation, sulfate content of DSP decreased to 1.6 %. Results highlighted that desulfation of SP decreased to −0.9 % of inhibitory activity, which showed no inhibition of hyaluronidase (Figure 2.3). Profiles of the molecular weight distribution of SP and DSP (Figure 2.4 a, d) indicated approximately similar molecular weight distribution with a retention time at 4.5–5.5 min, suggesting that the desulfation reaction didn't affect any depolymerization or unfavorable chemical changes in the polysaccharide molecules. The peak molecular weight of SP and DSP were 169 × 10^4 and 155 × 10^4 respectively.

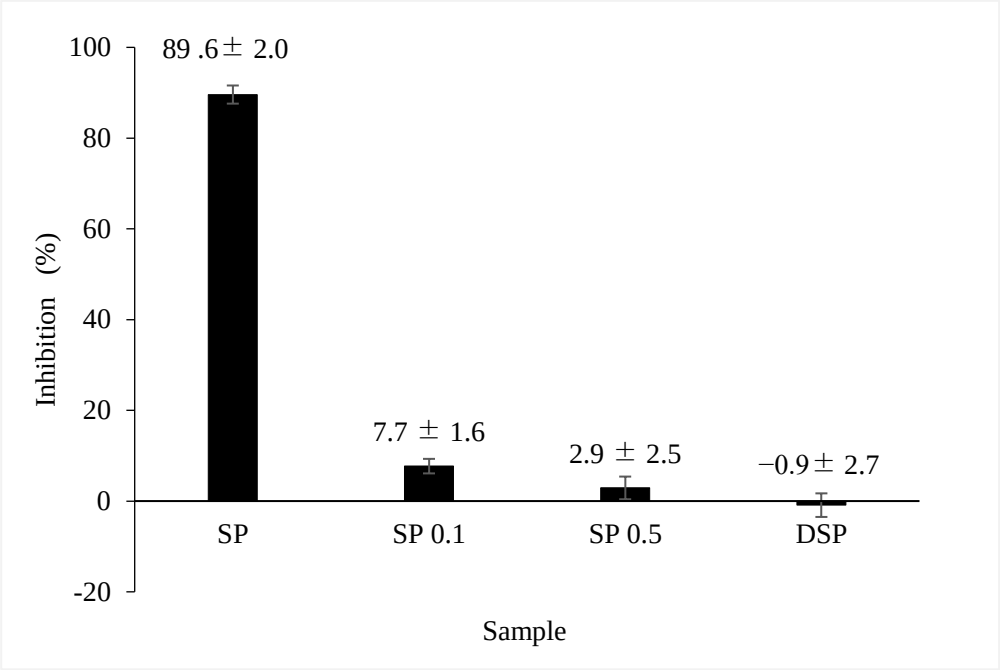

Figure 2.3 Hyaluronidase inhibitory activity of SP, $SP_{0.1}$, $SP_{0.5}$, and DSP at a polysaccharide concentration of 339 μg/mL. Data are shown as mean ± SD.

2.3.4 Effect of molecular weight of SP on hyaluronidase inhibitory activity

In order to examine the effect of molecular weight on the inhibitory activity, we partially hydrolyzed the SP using 0.1 or 0.5 M TFA to obtain a SP of lower molecular weight than native SP. The native SP showed molecular weight of about 169×10^4 with one main peak using size-exclusion chromatography (Figure 2.4 a). Approximately, 30 % of $SP_{0.1}$ was composed of polymers of low molecular weight range of 42.2 to 0.4×10^4 (Figure 2.4 b) and 70 % of $SP_{0.5}$ contained low molecular weight polymers of range 23.7 to 0.4×10^4 (Figure 2.4 c). Increasing the concentration of TFA in hydrolysis clearly increased production of low molecular weight compounds, and inhibitory activities of low molecular weight $SP_{0.1}$ and $SP_{0.5}$ were 7.7 % and 2.9 %, respectively (Figure 2.3).

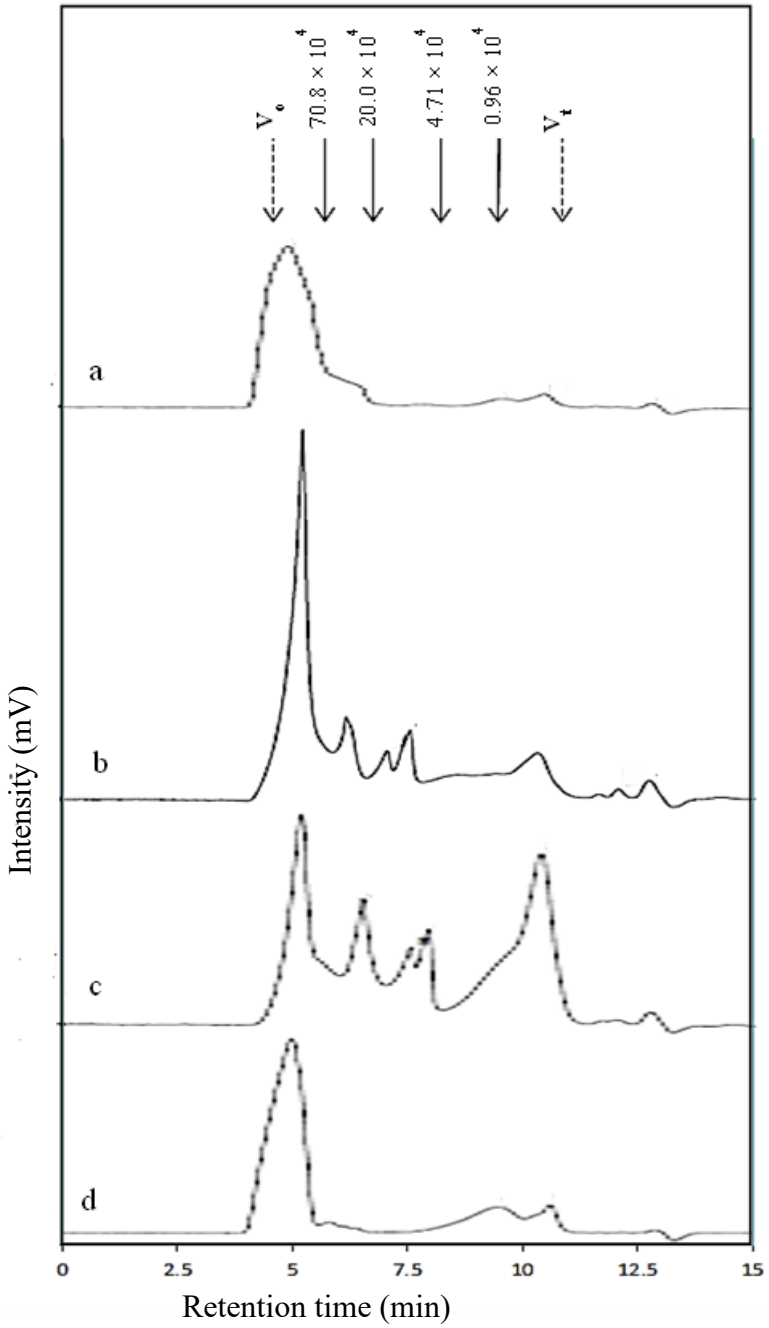

Figure 2.4 Size-exclusion chromatography of (a) native SP, (b) $SP_{0.1}$, (c) $SP_{0.5}$ and (d) DSP. Solid arrows indicate the elution positions of size standards of pullulan with molecular weight of highest peak. Dashed arrows indicate the void volume (V_o) and total column volume (V_t).

2.4 Discussion

The water-soluble polysaccharides derived from *Caulerpa* spp. mainly contain glucans and sulfated polysaccharide such as sulfated xyloarabinogalactans [18] and xylogalactomannans [54]. Among these, previous studies revealed that sulfated polysaccharides were found to be a highly bioactive compound. This time we found hyaluronidase inhibitory activity with sulfated polysaccharide from *C. lentillifera* in terms of the effect of sulfate content and molecular weight. This result confirmed that purified sulfated polysaccharide show significantly high activity of 90.4 % in 339 μg/mL of SP compared to other polysaccharide fractions. Chaiklahan *et al*. suggested that sulfated polysaccharide from *Caulerpa* spp. generally contained approximately 8–23 % sulfate [13]. Presence of 21.7 % of sulfate in CPC-P confirmed that it was a sulfated polysaccharide, mainly composed of Gal, Glc, Xyl, Man, and a minor amount of UA (Table 2.3). In past years, several sulfated polysaccharides were extracted and purified from *Caulerpa* spp. as listed in Table 2.3. Comparison of monosaccharide composition with preceding studies found that Gal, Glc, Xyl and Man are generally present in sulfated polysaccharide in *Caulerpa* spp. In addition to Gal, Glc, Xyl, and Man a considerable amount of Ara contains in sulfated heteroglycan extracted from *C. filiformis* [83], and *C. racemose* [84]. Although sulfated polysaccharide derived from *C. lentillifera* consisted mainly of Gal, Glc, Xyl, and Man, their composition and molecular weight may differ due to different source material, habitat, environmental parameters, and processes of extraction and purification as shown in Table 2.3 [37].

Table 2.2 Comparison of the hyaluronidase inhibition, composition and molecular weight of sulfated polysaccharides extracted from different algae types

Algae	IC_{50}	Molar ratio of monosaccharide									SO_3^- (%)	Molecular weight	References
	(μg/mL)	Fuc	Ara	Gal	Glc	Xyl	Man	Rha	Rib	UA			
Caulerpa lentillifera	163.3	n.d	0.02	0.20	0.20	0.12	0.16	n.d	-	0.03	21.7	169×10^4	This study
Undaria pinnatifida	13.0	0.14	-	0.14	-	-	-	-	-	n.d	0.71*	6×10^4	Katsube *et al,* 2003 [69]
Cladosiphon okamuranus	25.6	0.75	-	-	-	0.04	-	-	-	0.21	13.2	-	Tako and Minami, 2008 [70]
Fucus vesiculosus	2.9	0.49	0.02	0.03	0.08	0.04	-	-	-	0.34	27.0	735 kDa	Pozharitskaya *et al*, 2020 [73]
Porphyridium purpureum	210.0	-	-	0.31	0.14	0.43	-	-	0.03	0.09	4.5	500 kDa	Mase *et al*, 2013 [74]
Monostroma nitidum	145.0	-	-	n.d	0.08	0.02	-	0.75	-	0.15	20.0	70×10^4	Yamamoto *et al*, 2017 [32]

*: Molar ratio, n.d: less than 0.01, Rib: Ribose,

Table 2.3 Comparison of the chemical composition and molecular weight of sulfated polysaccharides extracted from *Caulerpa* spp.

Algae	Habitat	Purification method	Monosaccharide (wt%)						SO_3^- (%)	Molecular weight	References
			Gal	Glc	Xyl	Man	Ara	UA			
C. lentillifera	Ishigaki Okinawa	CPC precipitation	27.7	28.9	14.6	22.5	0.8	3.4	21.7	1.69×10^6	This study
C. lentillifera	Onna village, Okinawa	Superdex column	44.2*	2.2*	49.3*	-	-	4.3*	21.5	>100 kDa	Maeda *et al*, 2012 [49]
C. lentillifera	Changai island, China	DEAE column	43.2	-	-	38.7	-	2.3	21.3	3878 kDa	Sun *et al*, 2018 [54]
C. filliformis	Cape town, South Africa	Remove glucan by α-amylase	5†	n.d	2†	2†	1†	-	17.6	-	Macki & Percival, 1960 [83]
C. rasemosa	Gujarat, India	DEAE column	43.0	2.0	27.0	n.d	28.0	-	12.0	80 kDa	Chattopadhyay *et al*, 2007 [84]

n.d; less than 0.1, *;Molar %, †; Molar ratio

It was reported that IC_{50} value of hyaluronidase inhibitors derived from sulfated polysaccharide of seaweeds were ranging from 2.9 to 210 μg/mL as shown in Table 2.2. We also examined the substantial hyaluronidase inhibition of SP with IC_{50} of 163 μg/mL. Yamamoto et al. revealed that 100 μg/mL of natural sulfated polysaccharides such as porphyrin, rhamnan sulfate, κ-carrageenan, fucoidan and λ-carrageenan showed 25%, 40%, 35%, 70% and 80% of hyaluronidase inhibitory activity, respectively [32], while our study showed 20% of activity at the same SP concentration (Figure 2.2a). Furthermore, mode of inhibition of SP was predicted to be allosteric due to the sigmoid shape in dose-dependent curve, but it was hyperbolic shape in fucoidan. Different shapes of the hyaluronidase inhibitory curves such as sigmoid and hyperbolic were also recorded in phlorotannin from brown seaweeds *Cystoseira nodicaulis* and *Fucus spiralis* respectively [76]. Furthermore, sigmoid dose-dependent curve was reported for pectic acid and further clarified its hyaluronidase inhibitory mechanism as non-competitive [85]. Moreover, mode of hyaluronidase inhibition of SP derived from *Monostroma nitidum* [32] was found to be competitive but inhibition was of mixed uncompetitive–noncompetitive type in *Undaria pinnatifida* [69]. Taken all together, it became possible to state that different shapes of the dose-dependent inhibition curve might be due to various inhibition patterns of the inhibitors. However, further enzyme kinetic analysis is required to elucidate the exact mechanism of SP in hyaluronidase inhibition.

Regarding influencing factors of hyaluronidase activity, Li *et al*. suggested that sulfate content and molecular weight of the polysaccharide significantly affect their biological activities [33]. Therefore, I modified SP by desulfation to determine the effect of sulfate content and used TFA hydrolysis to assess the impact of molecular weight on the inhibition. It was found that existence and spatial positioning of sulfate groups on polysaccharides play key roles in their biological activity profile. Sun *et al.* found that

sulfate groups can substituted at C-3 positions of xylose and mannose, and C-6 positions of galactose of xylogalactomannan from *C. lentillifera* [54]. In our study, inhibitory activity of native SP was significantly decreased -0.9 % after desulfation as shown in Figure 2.3, suggested that the sulfate group in the inhibitor remarkably influenced hyaluronidase inhibitory activity. The present finding agreed with the finding of Yamamoto *et al*. of a strong positive relationship between sulfate content and hyaluronidase inhibitory activity in rhamnan sulfate from *Monostroma nitidum* with different sulfate contents (0.5, 7, 20 and 41 %) in different polysaccharide concentrations (0.1, 0.12, 0.16, 0.18 and 0.2 mg/mL) [32]. Moreover, Toida *et al.* found that chemically over-sulfated glycosaminoglycans (chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and hyaluronan) showed stronger hyaluronidase inhibitory activity (IC_{50}: 1.35, 1.33, 0.78, 1.28 and 1.14 µg/mL, respectively) than the original glycosaminoglycans [86]. Collectively, the present results provide support for the well-known fact that hyaluronidase inhibitory activity greatly depends on presence of the sulfate group.

Several reports have demonstrated a correlation between the molecular weight of inhibitor and hyaluronidase inhibitory activity [87], [88], [89]. Although significantly stronger inhibitory activity was reported by high molecular weight native SP in our study, it was significantly low in $SP_{0.1}$ and $SP_{0.5}$ (Figure 2.3), suggesting that decreasing of molecular weight may affect reduction of inhibitory activity. This reduction may also be due to the partial removal of the sulfate groups from the polysaccharide during hydrolysis. However, previous studies evident that occurrence of desulfation is negligible during the partial hydrolysis of sulfated polysaccharide with diluted TFA (0.1 - 0.75 M) at slightly elevated temperatures (60-100 °C) for several hours (1-2 h) [90], [91], [92]. Therefore, decreasing of molecular weight seems to cause reduction of inhibitory activity in our study. Similar results were reported by Asada *et al*. who demonstrated that hyaluronidase

inhibitory activity of alginate from *Lessonia nigrescens* increased with increasing of the inhibitor molecular weight within the range of 150–370 kDa [71]. In contrast, Asada *et al.* found that polysaccharides with the highest molecular weight (388 kDa), generally had decreased inhibitory activity [71]. Furusawa *et al*. also discovered that higher molecular weight acidic polysaccharide from outer layer of green coffee bean contributed most to the hyaluronidase inhibition [75]. All these results suggest that long chains of polysaccharide with high molecular weight are required for hyaluronidase inhibitory activity.

Nevertheless, the sulfate content of SP appeared to contribute more than molecular weight did to the inhibition, because DSP showed a significantly lower inhibitory activity despite having a high molecular weight similar to SP (Figure 2.4a, d). Consistently, Furusawa *et al*. revealed that UA content of an acidic polysaccharide contributed more to the hyaluronidase inhibitory activity than its molecular weight [75]. The present results indicate that hyaluronidase inhibitory activity of acidic polysaccharides mainly depended on action of the acidic group in the inhibitor compared to other factors.

Beside sulfate content and molecular weight, hyaluronidase inhibitory activity and the mechanisms of action of sulfated polysaccharide are influenced by a number of factors, including type of polysaccharide backbones and glycosidic linkages present in the polysaccharide [89]. This hypothesis could be further confirmed by comparison of inhibitory activity with sugar composition, sulfate content and molecular weight of SP and other sulfated polysaccharides reported in many studies (Table 2.2). Based on the result and references, it can be suggested that hyaluronidase inhibitory activity might be affected by more than single feature of the inhibitor molecule.

2.5 Conclusions

In the present study, SP, mainly consisted of Gal, Glc, Xyl, and Man found to effectively inhibit hyaluronidase activity. This activity correlated with sulfate content and molecular weight of SP. However, molecular weight alone was not likely sufficient, and the sulfate group was essential to inhibit hyaluronidase activity. It can be suggested that hyaluronidase inhibitory activity by SP might be affected by more than single feature of the inhibitor molecule.

CHAPTER III

Fractionation and characterization of cell wall polysaccharides from *C. okamuranus*

3.1 Introduction

Brown algae (Phaeophyta) represent the largest photosynthetic organisms in the marine ecosystem and include more than 250 genera and 1500–2000 species [93]. Brown algae contain a polysaccharide-rich cell wall in which alginate and fucose-rich sulfated polymer, generally called fucoidan are the most abundant and structurally well-characterized polysaccharides, representing up to 45% of algal dry weight, but cellulose (CL) is rare, representing 1–8% [94]. Unlike terrestrial plants, since many seaweeds grow in the intertidal environment, they contain distinct polysaccharides not commonly found in terrestrial biomass to protect cells from physical shocks and other stress conditions. Preceding studies of brown algae found that the cell wall contributed to cell polarity in *Fucus serratus* zygotes [25], cell development with cell differentiation in growing filaments of *Ectocarpus siliculosus* [26], tissue integrity in *E. siliculosus* protoplasts, and cell adhesion from cortical cells within *Saccharina latissimi* stipes [21]. Particularly, polysaccharides in brown algal cell walls were involved in different functions, including thickening of cell walls through deposition of β-(1–3)-glucan [30] and osmotic regulation by fucoidan [15]. The cell wall is a vital organelle with different functions, essentially involved in growth and development. Therefore, studying the interlinking and distribution of polymers throughout the cell wall is essential to understanding the process of growth, development, and decay of the cell wall.

Although chemical analyses of individual polysaccharides such as fucoidan and alginate from different brown algae are quite well described, many questions remain concerning how these polysaccharides form networks in the cell wall [21]. Few studies have investigated the linkage and interactions between brown algal cell wall polysaccharides using chemical or enzymatic fractionation [15], [21], [95], [96].

Deniaud-Bouët et al. [21] proposed the most significant brown algal cell wall model, describing a cell wall comprising two networks: the first of fucose-containing sulfated polysaccharide interlocking the CL microfibrils and the second of alginate, cross-linked by polyphenols. Enzymatic extraction proved that proteins are likely tightly interlinked with fucose-containing sulfated polysaccharide and also covalently attached to phenol compounds. Nevertheless, fucose-containing sulfated polysaccharide–CL bonds could not be explained and short-chained hemicellulose molecules may present as intermediates between CL microfibrils and fucose-containing sulfated polysaccharide. Recently, hemicelluloses such as β-1,4-1,3-glucans, β-1,3-glucans, and arabinogalactan protein were identified in the brown algal cell wall, but associations with other components are poorly understood [22]–[24]. Despite the cell-wall model described above, it remains unclear how several cell wall polysaccharides are interlinked. Therefore, the present study deals with chemical disruption and fractionation of cell wall material from *Cladosiphon okamuranus* (Chordariaceae, Phaeophyceae) [63] is in order to extend our understanding of how polymers are assembled in the brown algal cell wall.

Cladosiphon okamuranus, Okinawa mozuku in Japanese, is one of the most utilized edible brown algae and accounts for more than 90% of mozuku production in Japan. It possesses the highest fucoidan content among any brown algae species [55] with an average yield of about 2.3% (w/w) from wet algae, and alginate is only about 0.1% [56]. Typical fucoidan from *C. okamuranus* with the structure of α-1,3-linked L-fucose (Fuc) as the main backbone substituted by D-glucuronic acid (GlcA) residues at *C*-2 and sulfate at *C*-4 of L-Fuc [57] have been extensively explored for numerous therapeutic effects and bioactivities; consequently, *C. okamuranus* plays a key role in the fields of pharmaceutical, nutraceutical cosmeceutical, and functional food [12]. However, fucoidan in these studies were mostly extracted with HCl or water and analyzed as an

individual polymer. Here, I fractionated the whole cell wall of *C. okamuranus* and recognized the structural differences in predominant polysaccharide types. Particularly, I found that fucoidan is the major cell wall polysaccharide that is not only present in the hot water fraction (HW), as is typical for fucoidan, but also in hemicellulose-I fraction (HC-I). I hypothesized that fucoidan in HC-I is structurally different from HW due to their localization in the tissue to perform diverse roles in the cell wall. Therefore, study of cell wall polysaccharide from *C. okamuranus* at the structural level will provide an understanding of cell wall function, particularly process of growth and development. Consequently, it will help to find the solutions for recently occurred yield reduction of *C. okamuranus* [63] with advancement of applications in different field of industries.

3.2. Materials and methods

3.2.1. Algal material

Cladosiphon okamuranus, cultivated in Katsuren coast, Okinawa was purchased at Katsuren Fisheries Cooperative in 2016 and frozen until use.

3.2.2. Preparation of alcohol insoluble residue (AIR)

To prepare AIR, seaweed (200 g) was ground with a blender in four volumes of ethanol (800 mL) followed by centrifugation at 14,000 × *g* at 25 °C for 15 min, then precipitate was sequentially treated with 80% ethanol, twice with 100% ethanol, and with methanol:chloroform (1:1, v:v) followed by acetone. After suction filtration, residue was dried overnight at room temperature and used as AIR [77].

3.2.3 Fractionation of cell wall polysaccharides

Fractionation of cell wall polysaccharides from AIR was based on the different solubilities of polysaccharides similar to terrestrial plants, following previous procedures

[97] with slight modification as described in Figure 3.1. Briefly, AIR was sequentially treated with hot water at 85 °C for 2 h, 0.25% ammonium oxalate (AO) at 85 °C for 1 h, 4% KOH, and 24% KOH with shaking for 16 h, to produce HW, AO, HC-I and HC-II fractions respectively and final residue was collected as CL. After neutralizing HC-I and HC-II using acetic acid, AO, HC-I, and HC-II were dialyzed, lyophilized, and used for analysis. The CL was washed twice with water containing acetic acid, lyophilized, and solubilized with ice-cold 72% sulfuric acid for chemical composition analysis [77].

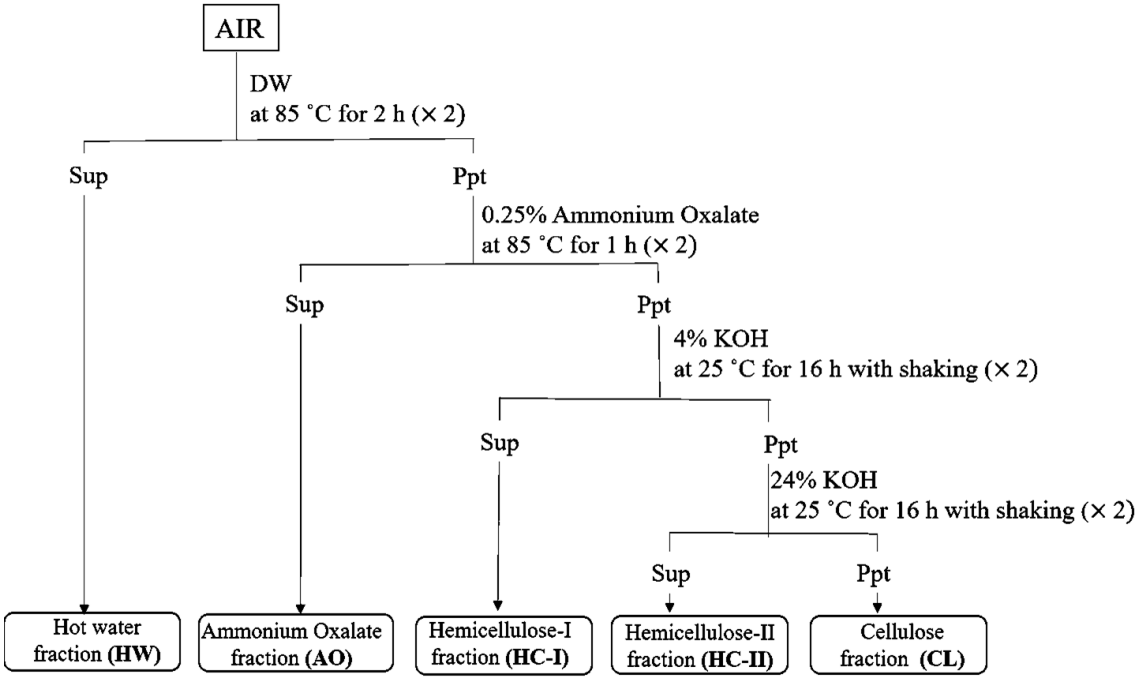

Figure 3.1 Fractionation procedure for cell wall polysaccharides from AIR of *C. okamuranus.*

3.2.4. Chemical composition analysis

Total sugar and uronic acid (UA) contents were determined by phenol–sulfuric acid method [79] using a Fuc standard and *m*-hydroxybiphenyl method [80] using a GlcA standard [80] respectively. Total polyphenols were quantified using the Folin–Ciocalteu

method using gallic acid as standard [98]. Protein content was measured using bicinchoninic acid assay (BCA) following the manufacturer's instructions of a BCA Protein Assay Kit (Takara Bio Inc., Shiga, Japan). The calibration curve was prepared using bovine serum albumin.

To estimate sulfate in polysaccharides, HW, AO, HC-I, and HC-II were hydrolyzed in 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h, hydrolysates were subjected to high-performance liquid chromatography with an AS4A-SC column (4 mm × 250 mm, Dionex Co., Tokyo, Japan). The column was eluted at 1 mL/min at room temperature with a buffer containing 1.7 mM $NaHCO_3$ and 1.8 mM Na_2CO_3 [77].

3.2.5. Sugar composition analysis

To analyze sugar composition, HW, AO, HC-I, and HC-II were hydrolyzed as used for sulfate estimation, while the CL fraction was treated with ice-cold 72% (w/w) sulfuric acid at 4 °C for 1 h with sonication, followed by hydrolysis with 2 N H_2SO_4 at 121 °C for 1 h [99], [100]. Monosaccharides in the hydrolysate were analyzed by high-performance anion-exchange chromatography coupled with a pulsed amperometric detector using a CarboPac PA1 column (4 mm × 250 mm, Dionex Co.). The column was eluted at a flow rate of 1 mL/min at 35 °C with 14 mM NaOH for neutral sugar, followed by a linear gradient program of 0–250 mM CH_3COONa in 100 mM NaOH for UA.

3.2.6. Determination of molecular weight (MW)

The MWs of HW, AO, HC-I, and HC-II were determined by size-exclusion chromatography (LC-6A; Shimadzu Co., Kyoto, Japan) equipped with a TSKgel G5000 PWXL column (7.8 mm × 300 mm, Tosoh Co., Tokyo, Japan) and a refractive index detector RID-10A [82]. The column was eluted by 0.2 M NaCl at a flow rate of 0.3 mL/min at 40 °C. Pullulan P-10 (MW = 0.96×10^4), P-50 (4.71×10^4), P-200 (20.0×10^4), and P-800 (70.8×10^4) (Showa Denko Co., Tokyo, Japan) were used as standards.

3.2.7. Methylation analysis

For sugar linkage analysis, methylation analysis of native (NT), desulfated (DS), and carboxyl reduced (RD) polysaccharides was carried out as previously described [101]. Briefly, the sample was methylated with CH_3I and NaOH in DMSO and hydrolyzed with 2 M TFA. The partially methylated product was converted to alditol acetates and analyzed by GC-MS spectrometry (GC-MS- QP 5000, Shimadzu Co.) with SP2330 column (0.25 mm × 30 m, Supelco Inc., Ann Arbor, MI, USA). The column temperature was maintained at 80 °C for 2 min and then increased to 170 °C at 4 °C/min, and subsequently to 240 °C at 4 °C/min, and held at 240 °C for 10 min.

3.2.8 Desulfation and carboxyl reduction of polysaccharides

Chemical desulfation of polysaccharides was performed according to Shiroma et al. [82]. The sample was dissolved in distilled water and passed through DOWEX 50W×8 resin (Sigma-Aldrich Inc., MO, USA). After neutralization with pyridine, the solution was lyophilized. The lyophilized pyridinium salt was dissolved in DMSO:MeOH (9:1, v/v), and heated at 80 °C for 4 h. The desulfated product was dialyzed against distilled water and lyophilized.

Carboxyl reduction of UA in polysaccharides was carried out according to a published method [102]. In brief, 5 mg of sample was dissolved in MES buffer (pH 4.7), followed by activation with carbodiimide reagent at 25–30 °C for 3 h. Free UA residues were reduced with $NaBD_4$ at room temperature for 3 h while maintaining pH 7.0 by adding 4 M imidazole-HCl. Glacial acetic acid was added dropwise to destroy excess reductant, and the suspensions were dialyzed and lyophilized.

3.2.9 Small angle X-ray scattering (SAXS)

SAXS measurements were carried out at BL-6A at Photon Factory in Tsukuba,

Japan. The aqueous solutions of fractions of cell wall polysaccharides were prepared and measured at room temperature by putting into a flat cell of 0.2 cm path-length. An incident X-ray was monochromatized to λ=0.15 nm and the scattered X-ray was detected by a PILATUS 1 M. The camera distance was set up with 1030 mm.

3.3 Results

3.3.1. Yield and chemical composition

Approximately 5% of the fresh weight was recovered as AIR through treatment with ethanol, chloroform:methanol (1:1, v/v), and acetone. Using sequential chemical extraction, AIR was separated into five fractions: HW, AO, HC-I, HC-II, and CL. This is a well-established method for plant polysaccharide fractionation: hot water extracts starch and not tightly bound pectin, ammonium oxalate releases polysaccharide bound in cell wall via calcium such as pectin, alkali solubilizes hemicellulose bound to CL, and CL remain in the residue. The same concept was applied to extract the polysaccharide in brown algae in our study to know how these polysaccharides exist in the whole cell wall. Particularly, this time, we mainly focus on hemicellulose fraction in brown algae, which was not clearly understood yet. According to this method, fucoidan is easily extracted with hot water as it is water soluble polysaccharide [103] and alginate can be extracted with chelating agents, such as oxalate ions which can solubilize alginate by chelating calcium ions in the same way of solubilizing low methoxylated pectin in plants [104]. After removing pectin, the common way to extract hemicelluloses in plant is treating with aqueous solutions of alkali [105]. Hence, more drastic conditions with 4% KOH and 24% KOH were used to extract HC-I and HC-II respectively and final residue was collected as CL. Results show that HW, AO, HC-I, HC-II and CL yielded 39.6%, 7.5%, 25.5%, 3.4% and 4.8% from AIR respectively, which possess 49.0%, 9.3%, 31.6%, 4.2% and

5.9% of total cell wall recovered from AIR respectively. Since over 80% of total cell wall recovered from AIR yielded HW (49.0%) and HC-I (31.6%), the *C. okamuranus* cell wall was found to be mainly composed of HW and HC-I. The sulfate contents of HW and HC-I were 17.9% and 6.1% respectively. More than 60% of the dry weight of each fraction was sugar, with the highest UA of 41.9% in AO fraction. Protein was co-extracted together with a minor amount of polyphenol in every fraction except CL, and the highest protein (30.0%) and polyphenol (3.0%) contents were for the HC-I fraction (Table 3.1).

Table 3.1 Yield and chemical composition of *C. okamuranus* cell wall fractions.

Fraction	Yield[a]	Total sugar	UA[b]	SO_3^-	Protein	Polyphenol
HW	39.6	61.3	27.5	17.9	22.3	2.3
AO	7.5	62.1	41.9	6.3	8.5	1.3
HC-I	25.5	68.4	25.9	6.1	30.0	3.0
HC-II	3.4	60.8	17.0	3.8	22.7	3.0
CL	4.8	75.5	4.8	tr	tr	tr

[a] Percentage weight of each fraction in AIR (wt%)
[b] Percentage weight of uronic acid in total sugar; other constituents in weight % of the respective fraction
tr, trace amount less than 0.1

3.3.2. Sugar composition analysis

Previous reports showed that fucoidan was the main cell wall polysaccharide extracted from *C. okamuranus* [57], [106], [107]. Therefore, Fuc was set to be the predominant monosaccharide in each fraction, once the molar ratio of sugar was calculated. As a result, HW and HC-I were mainly composed of Fuc, GlcA, and sulfate in molar ratios of 1.0:0.3:0.9 and 1.0:0.2:0.3, respectively; thus, fucoidan was predominant in both fractions. However, the sulfate content was lower in HC-I than HW. In addition to the main constituents of fucoidan (i.e. Fuc, GlcA, and sulfate), high amounts of guluronic acid (GulA) and mannuronic acid (ManA) were detected in AO, suggesting

predominance of alginate in AO; whereas HC-II comprised glucose (Glc), xylose (Xyl), and mannose (Man) as well as fucoidan (Table 3.2). High Glc content of the CL fraction confirmed abundance of CL. Overall, there was a wide spectrum of fucoidan in all fractions, mostly in HW and HC-I, but their structure may differ due to diverse compositions of sugar and sulfate.

Table 3.2 Sugar compositional analysis of *C. okamuranus* cell wall fractions.

Fraction	Neutral sugar					UA			SO_3^-
	Fuc	Xyl	Glc	Gal	Man	GlcA	GalA/GulA	ManA	
HW	1.0	0.1	tr	tr	tr	0.3	0.1	−	0.9
AO	1.0	0.1	tr	tr	tr	0.2	0.2	0.3	0.4
HC-I	1.0	0.1	0.1	tr	0.1	0.2	0.1	0.1	0.3
HC-II	1.0	0.6	1.1	0.1	0.3	0.4	0.1	−	0.2
CL	1.0	0.9	17.5	−	0.8	−	0.9	−	−

tr, trace amount less than 0.1; −, not detected (molar ratio)

3.3.3. MW analysis

The MW distribution of HW and HC-I was determined by high-performance size-exclusion chromatography. In the chromatogram, HW and HC-I mainly comprised one major peak (Mp), but HC-I showed a broad MW distribution compared to HW (Figure 3.2). Estimated average MWs of Mp of HW and HC-I were 266.3×10^4 and 36.4×10^4, respectively. These results gave more insights into the different structural properties of HW and HC-I. The peak at V_t was NaCl in the sample and eluting buffer while the small peak, occurred just before V_t was comparatively lower (<10%) than Mp in both chromatography.

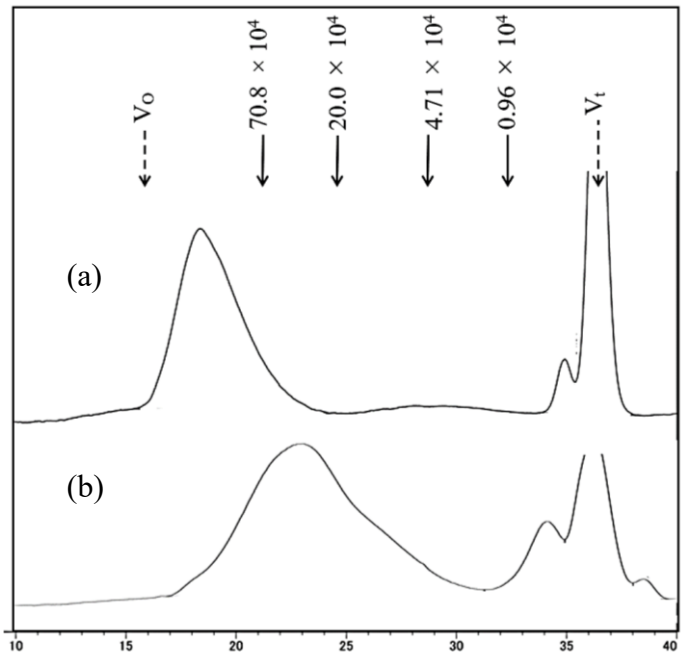

Figure 3. 2 Molecular weight distribution of HW (a) and HC-I (b). Mp is the main peak of each chromatogram. Solid arrows indicate the elution positions of size standards of pullulan with a molecular weight of the highest peak. Dashed arrows represent the void volume (V_o) and total column volume (V_t).

3.3.4. Methylation analysis

Sugar linkage was analyzed to determine the polysaccharide structure. The methylation analysis of HW showed that the most abundant sugar was Fuc, mainly composed of 28.5% of 1,3-linked, 43.8% of 1,3,4-linked, and 16.3% of 1,3,4-linked Fuc (Table 3.3). After desulfation, 1,3-linked Fuc increased to almost 70%, but 1,2,3-linked Fuc decreased to 7.8% compared to NT, suggesting that the backbone of fucoidan in HW consisted of 1,3-linked Fuc and the sulfate groups substituted at *C*-4 in 1,3-linked fucosyl residues. After carboxyl reduction, terminal Glc increased, and 1,2,3-linked Fuc remained mostly unchanged compared to NT, indicating that GlcA was present as terminal and likely to be substituted at *C*-2 in 1,3-linked fucosyl residues. Thus, HW fucoidan appeared

to be the same structure extracted using acidic condition as described in previous studies.

Table 3.3 Methylation analysis of HW and HC-I.

Partially methylated alditol acetates	Deduced linkage	HW			HC-I		
		NT*	DS*	RD*	NT*	DS*	RD*
1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol	→3)-Fuc-(1→	28.5	69.2	25.3	23.8	73.0	22.1
1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylfucitol	→4)-Fuc-(1→	1.8	2.9	1.5	13.7	4.3	3.4
1,2,3,5-tetra-*O*-acetyl-4-*O*-methylfucitol	→2,3)-Fuc-(1→	16.3	15.1	21.9	18.2	13.7	24.6
1,3,4,5-tetra-*O*-acetyl-2-*O*-methylfucitol	→3,4)-Fuc-(1→	43.8	7.8	32.8	23.4	6.2	25.4
1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol	Xyl-(1→	6.8	2.6	1.9	10.4	2.0	3.0
1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol	→4)-Xyl-(1→	–	–	–	10.5	0.8	2.4
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol	Glc-(1→	–	–	13.1	–	–	15.6
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	→4)-Glc-(1→	2.0	1.4	1.9			
1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylglucitol	→3,4)-Glc-(1→	–	–	–	–	–	3.5
1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol	→4)-Man-(1→	0.8	1.1	1.6	–	–	–

*NT, native polysaccharide; DS, desulfated polysaccharide; (mol%)
RD, carboxyl reduced polysaccharide; -, not detected

Based on the similar rationale as described above in methylation analysis, fucoidan in HC-I also contain 1,3-linked fucose as the main backbone substituted by GlcA residues at *C*-2 and sulfate at *C*-4 of Fuc. Despite methylation analysis of HC-I fraction showed a similar result to HW, it contained terminal Xyl (10.4%), 1,4-linked Xyl (10.5%), and 1,4-linked Fuc (13.7%) (Table 3.3). However, terminal Xyl, 1,4-linked Xyl, and 1,4-linked Fuc were drastically decreased after desulfation, due to the substantial precipitation in the dialysis step for desulfation. Both Xyl and Fuc were detected by sugar composition analysis of the precipitate (data not shown), suggesting that 1,4-linked Xyl and 1,4-linked Fuc were somehow insoluble by desulfation. Although 1,3,4-linked Fuc significantly decreased after desulfation of HC-I, it still remained in the DS-HC-I, suggesting that terminal Xyl could substitute at *C*-4. Another possible attachment of terminal Xyl is *C*-2

in 1,3-linked fucosyl residues like GlcA. On the other hand, the 1,4-linked Xyl and 1,4-linked Fuc may indicate 1,4-xylan or 1,4-fucan in the HC-I fraction. However, further analysis of DS-HC-I is required to confirm its detail structure. Based on the results and the references, proposed structure of fucoidan in HW and HC-I show in Figure 3.3.

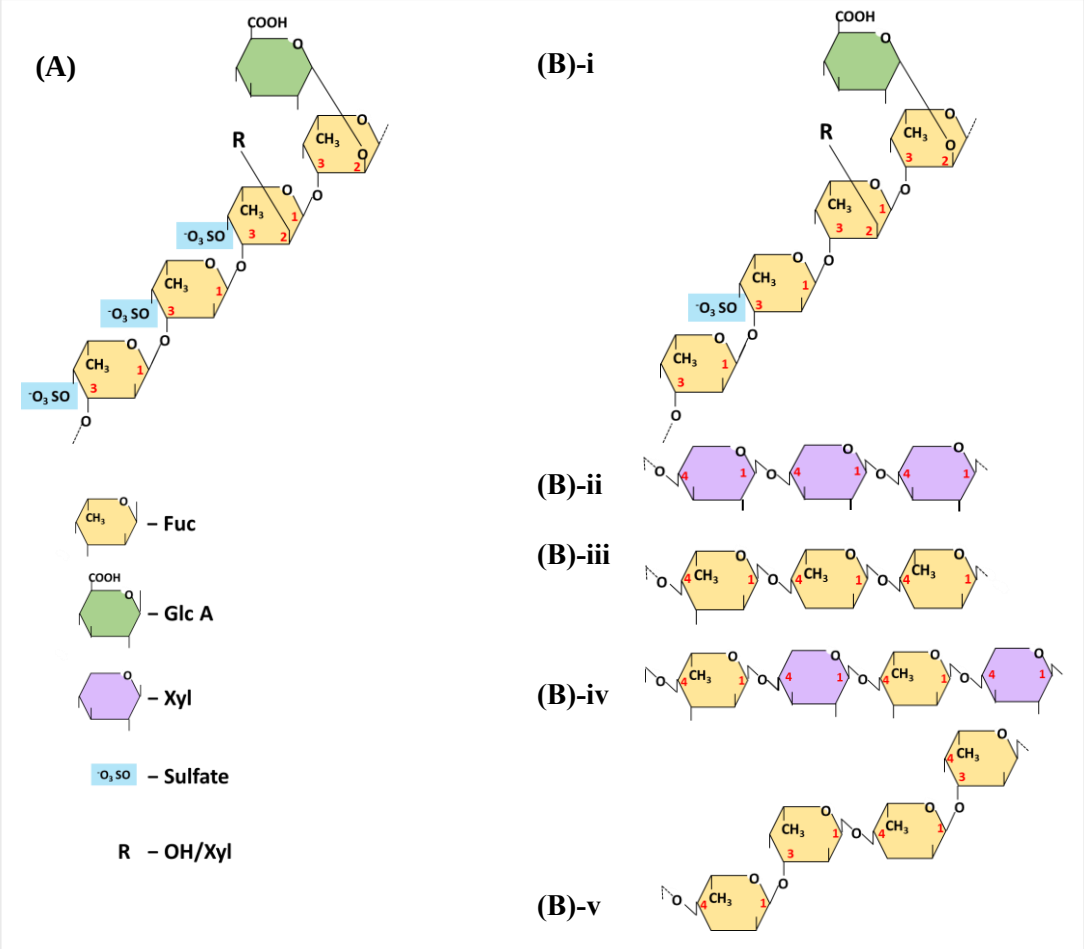

Figure 3.3 Proposed structure of fucoidan in HW (A) and HC-I (B)-i, ii, iii, iv and v.

3.3.5 Small angle X-ray scattering

The conformational structure of HW and HC-I was examined by SAXS. Figure 3.4 shows Kratky plots ($q^2I(q)$ vs q) for SAXS from 0.5% HW and HC-I aqueous solution, as I(q) is the scattering intensity and q is the magnitude of the scattering vector defined

by $(4\pi/\lambda)\sin\theta$ where λ is wavelength of incident X-ray and 2θ is a scattering angle. The two SAXS patterns of HW and HC-I revealed distinctly different profiles, especially in $q<1.6$ region. The intensity of HC-I was higher than HW and its profile included the component peak around 0.3 of q. On the other hand, HW showed a proportional function behavior in a small q region and then it seems to slightly bend at around 0.5 of q. These results suggested that fucoidan in HW is presumed to be a chain structure with a certain degree of rigidity for an electrolyte polymer.

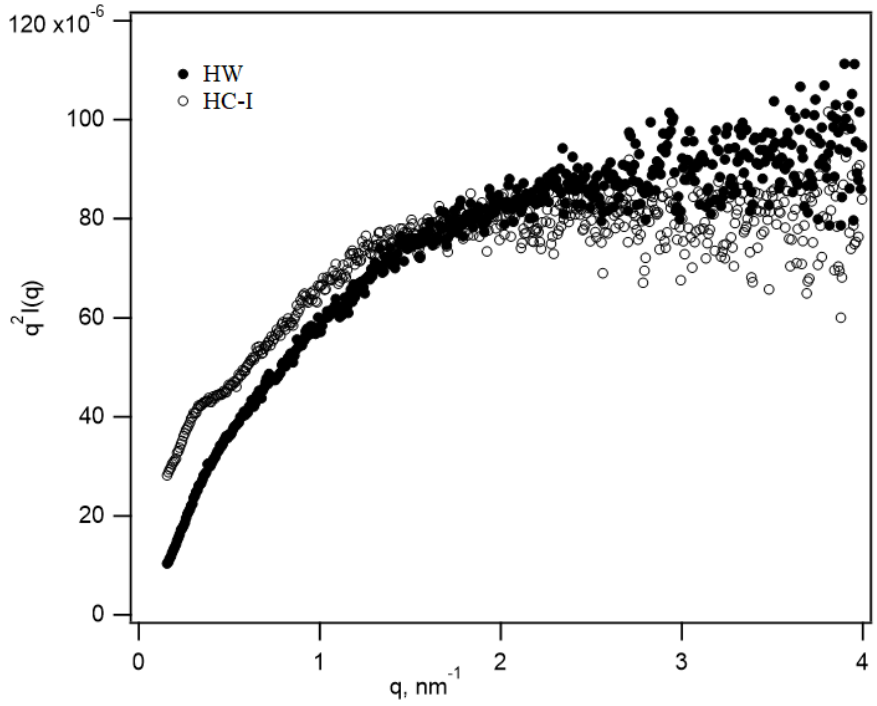

Figure 3.4 Kratky plots for SAXS from fucoidan HW and HC-I in aqueous solution

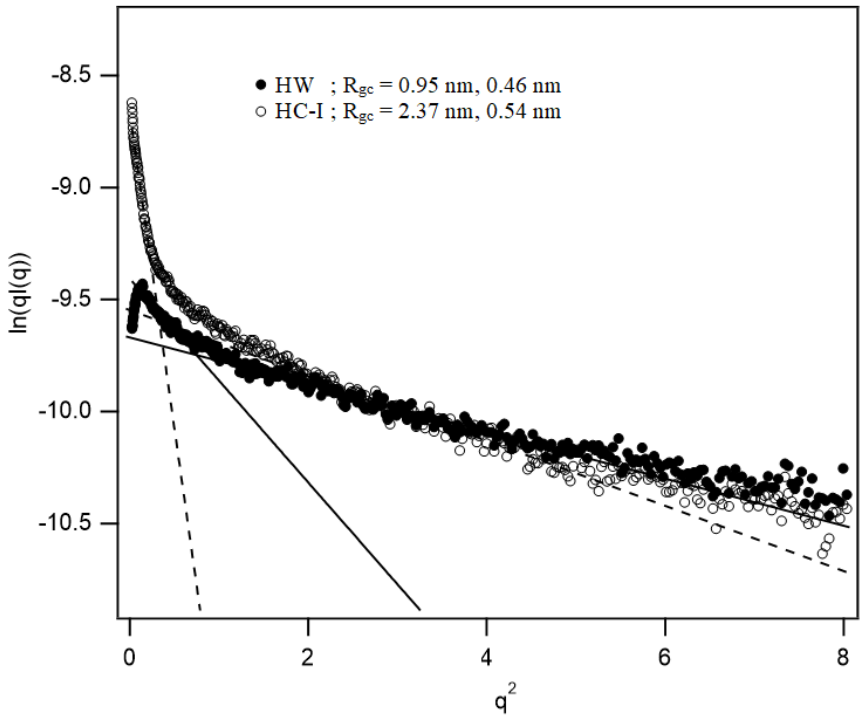

Figure 3.5 Cross-sectional Guinier plots for SAXS from fucoidan HW and HC-I in aqueous solution

The scattering from the rod-like structure can be approximated by the Guinier formula as:

$$qI(q) \sim \exp\left(-\frac{Rgc^2 q^2}{2}\right) \qquad \text{(equation 1)}$$

where R_{gc} is the cross-sectional radius of gyration. The R_{gc} can be estimated from the slope of cross- sectional Guinier plots (ln(qI(q)) vs q^2) in the linear region [108]. The cross-sectional Guinier plots for SAXS from 0.5% HW and HC-I aqueous solution are shown in Figure 3.5, assuming a scattering from the rod-like structure. Since it can be seen that there are roughly two components of slope, we tried to evaluate two components of R_{gc} at low and high angle linear regions from each profile. The smaller cross-sectional component at high angle linear region was estimated to be 0.46 nm in HW and 0.54 nm in HC-I, suggesting that it may correspond to the cross-section of fucoidan chain with the

side chains of a few saccharides, while R_{gc} of bigger components at low angle linear region was 0.95 nm in HW and 2.37 nm in HC-I, suggesting some aggregate structures. Although some size distribution in the aggregate structure is possible, the obtained R_{gc} values are informative as major values because of the relatively good linearity of the Guinier plot profiles. These association structures are speculated due to the influence of xylose and 1,4-fucose main chain, and its extent is considerably greater for HC-I.

3.4 Discussion

Sequential fractionation of AIR showed that cell wall polysaccharide in *C. okamuranus* mainly consisted of two fractions of HW and HC-I, which occupied 80% of total cell wall recovered from AIR. In contrast, CL is the most abundant major polysaccharide representing up to 50% of terrestrial plants' cell walls, but was only 4.8% in *C. okamuranus*, and HC-II was 3.4% (Table 3.1). The AO yield was 7.5% and it contained alginate and fucoidan by sugar composition analysis (Tables 3.1 and 3.2). Alginate is the major cell wall polysaccharide in most brown algae, representing up to 45% of dry weight. Unlike other brown algae, *C. okamuranus* consists mostly of fucoidan (2.3% w/w) and little alginic acid (0.1% w/w) [55]. Furthermore, sugar composition, MW, methylation and SAXS analysis results revealed the heterogeneity of fucoidan in HW and HC-I, suggesting that different fucoidans are likely due to localization in tissue to perform different roles in the cell wall. We hypothesize that since fucoidan in HW is loosely bound, it might be involved in functions related to withstanding desiccation and osmotic stress at low tide, while fucoidan in HC-I may play a similar role to hemicellulose in terrestrial plants, by keeping CL microfibrils separated and controlling cell wall expansion [105]. Having high flexibility in the algal body and 100–1000 times lower stiffness of brown

algal cell walls than for terrestrial plants can be explained by the compositional and configurational variation of HC-I and CL in brown algae. Overall, the presence of higher HW and HC-I with fucoidan and lower HC-II and CL in brown algae compared to terrestrial plants appears to be adaptation to survive in coastal habitats.

Most of the compounds were extracted in HW, and fucoidan was predominant, which appeared similar in composition to typical fucoidan extracted in most previous studies. The molar ratio of Fuc, GlcA, and sulfate of fucoidan in HW was 1.0:0.3:0.9, respectively (Table 3.2). This is quite similar to that of typical fucoidan previously extracted by aqueous dilute acid from *C. okamuranus* which contained Fuc, GlcA, and sulfate with a molar ratio of 1.0:0.2:0.5 [106], 1.0:0.3:0.6 [57], and 1.0:0.3:0.5 [107]. In contrast, the MW of fucoidan in HW was 266.3×10^4 (Figure. 3.2), which was remarkably higher than in previous reports of 50×10^4 [78], 38×10^4 [62], and 46×10^4 to 107×10^4 [41]. Since fucoidan was extracted by dilute acid instead of water in previous reports, acid degradation of native fucoidan may explain the lower MW of fucoidan in HW compared to our study. Similarly, Kawamoto et al. [55] extracted the highest MW of 320×10^4 for fucoidan from *C. okamuranus* with boiling water for 1 h. Thus, highly sulfated homo fucoidan in HW is suggested to be weakly held in cell wall matrix and may be involved in osmotic regulation in brown algae [94].

A second major component of cell walls was alkali-soluble HC-I, which also mainly contained fucoidan, in molar ratio of Fuc, GlcA, and sulfate of 1.0:0.2:0.3, respectively (Table 3.2). However, fucoidan in HC-I was not identical to fucoidan in HW, because the MW and sulfate content of fucoidan in HC-I was lower than that of HW. Furthermore, methylation analysis showed that HC-I also contained 13.7% of 1,4-linked fuc and 20.9% of Xyl which was terminal and 1,4-linked (Table 3.4). It is possible that

the terminal Xyl was substituted at *C*-2 or *C*-4 in 1,3-linked fucosyl residue in fucoidan from HC-I, and 1,4-linked Xyl was expected to derive from hemicellulose-related polysaccharide like 1,4-linked xylan. However, few studies have produced alkali-soluble fractions with the presence of Xyl from brown algae after removing alginate. Similarly, monoclonal antibodies which bind to xylosyl residues in xyloglucan, but not in xylan were found to bind in 4 M KOH extract from Fucales, Laminariales, and Ectocarpales [109]. Although Bilan et al. [110] found only traces of 1,4-linked Xyl residue in fucoidan prepared by water-soluble Ca-salt treated with hot alkaline sodium borohydride from *Saccharina latissimi*, it could be suggested that these residues are separate 1,4-linked β-xylan chains in fucoidan by NMR analysis. Consequently, fucoidan in HC-I, extracted by alkali treatment is likely involved in reinforcing cell wall structure, as hemicellulose does in terrestrial plants. However, remarkable decreases in 1,4-linked Xyl and 1,4-linked Fuc were observed with substantial precipitation in DS-HC-I compared to its native form in our study. Similarly, Fernández et al. [111] revealed that solubility of 1,4-β-D-mannan improved when it contains more sulfate groups. Therefore, we suggest that removing sulfate groups during desulfation may decrease solubility with subsequent precipitation of DS-HC-I. However, further study is required to identify the structure of the DS-HC-I precipitate related to 1,4-linked Xyl and 1,4-linked Fuc residues.

Our hypothesis was further confirmed by the conformational differences in the structure of HW and HC-I, observed using SAXS method. SAXS analysis mainly provide shape and assembly information such as aggregated and unfolded structure of molecules. The two SAXS patterns in Kratky plots and cross-sectional Guinier plots revealed distinctly different profiles of HW and HC-I. R_{gc} value of HW (0.95nm) in bigger component was within the range of previously extracted fucoidan with backbone of 1,3

or/and 1,4 linked Fuc residues and different saccharide side chains, including *Sargassum crassifolium* (0.83 nm), *Padina australis* (0.70 nm) [112], *Turbinaria ornata* (1.3 nm) [113] and 6 different brown algae Spp. (0.78 -1.51 nm) [114]. However, R_{gc} value of HC-I (2.37 nm) in bigger component was comparatively higher and we hypothesized that association structure of HC-I with high R_{gc} value appears to be influenced by abundant xylose and 1,4-fucose main chain. Moreover, aggregate structures with 1,4-xylan or 1,4-fucan might allow fucoidan in HC-I to cross-link to CL for strengthening the brown algal cell wall.

Based on our current results and understanding of the organization of polymers in *C. okamuranus* cell walls, we suggest the distribution pattern of cell wall polysaccharides in brown algae. The structure of the *C. okamuranus* cell wall can be explained as two networks in which the first network is created by interconnection between fucoidan in HC-I and CL fibrils thought to be embedded in the second matrix, built by predominantly fucoidan in HW and some alginate as shown in Figure 3.6. We show for the first time that fucoidan in HC-I may be involved in reinforcing cell wall structure, while fucoidan in HW was typical fucoidan as extracted in most previous studies.

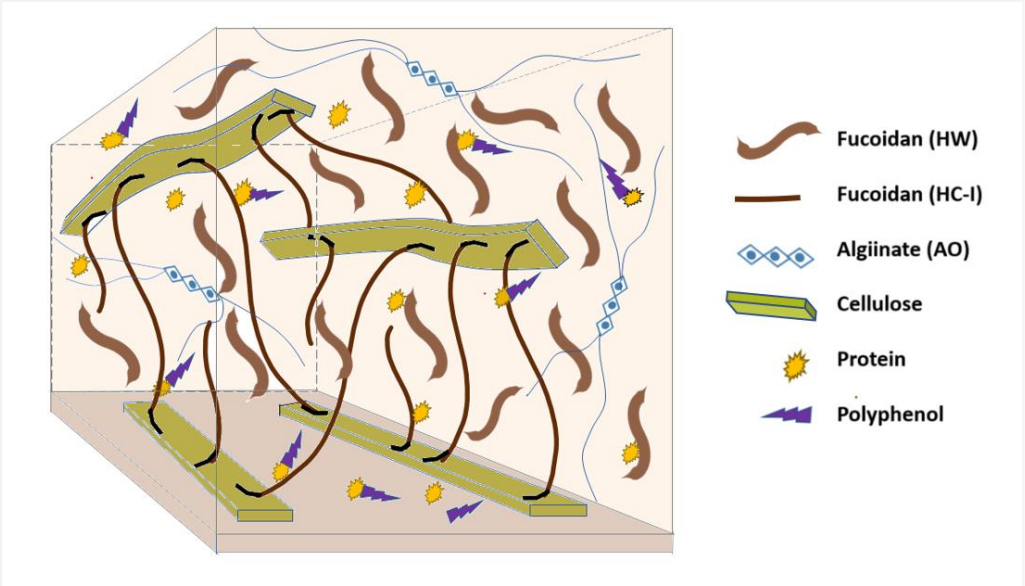

Figure 3.6 Proposed model of cell walls from *C. okamuranus* as described in this study

3.5 Conclusions

Algal cell walls from *C. okamuranus* were sequentially fractionated into five fractions and almost 80% of the total cell wall recovered from AIR was composed of HW and HC-I. Both HW and HC-I mainly contained fucoidan composed of Fuc, GlcA, and sulfate in molar ratios of 1.0:0.3:0.9 and 1.0:0.2:0.3, respectively. The fucoidan structure from HW and HC-I differed in terms of contents of sulfate and Xyl, MW and profile of SAXS. Methylation analysis revealed that HW was typical fucoidan, while HC-I contained 1,4-linked Xyl and 1,4-linked Fuc as well as components of typical fucoidan, suggesting that these are likely to be 1,4-xylan and/or 1,4-fucan. These structural heterogeneities of fucoidan in HW and HC-I may be related to their localization in the tissue to perform the distinct roles of cell walls. Particularly, if the structure of fucoidan in HC-I is 1,4-xylan and/or 1,4-fucan, it might allow to cross-link with cellulose. The details structural analysis and application of fucoidan in HC-I would be the subject of future study.

CHAPTER IV

Structural analysis of cell wall polysaccharide from *C. okamuranus* grown at different habitats

4.1 Introduction

Cladosiphon okamuranus (Chordariaceae, Phaeophyceae) Okinawa mozuku in Japanese, is an extensively utilized edible brown algae in Japan. Its annual production has been reported to be approximately 22 kilotons in 2020 and contributes 5 billion yen to the Japanese economy [115]. It is an endemic species in Ryukyu archipelago where spread over a wide area from the southern limit (24◦ N) of the Ishigaki islands in Okinawa prefecture to the northern limit (29◦ N) of Amami island in Kagoshima prefecture [106] and it was identified as a warm water sp. [116]. Although Okinawa mozuku is utilized mainly as food that supplies dietary fiber for the native population since ancient time, recently it is recognized as an excellent source of fucoidan with a wide range of bioactivities, so that, they play an essential role in the fields of pharmaceutical, nutraceutical cosmeceutical, and functional food [12]. Particularly, it was found that fucoidan extracted from *C. okamuranus* possess the highest content among any brown algae species [55] with an average yield of about 2.3% (w/w) from wet algae and alginate was only 1/10 of the fucoidan content [56].

Bioactivities are influenced by structural properties of polysaccharide including sugar composition, type of sugar backbones, glycosidic linkages, sulfate content and position and molecular weight [89]. The structure of fucoidan extracted from *C. okamuranus* was well described that α-1,3-linked fucan is the main backbone by substituting D-glucuronic acid (GlcA) residues at *C*-2 and sulfate at *C*-4 of L-fucose (Fuc) [57]. Furthermore, sulfate content and molecular weight of fucoidan from *C. okamuranus* was found to be a main factors affecting on different bioactivities [56], [70], [117]. For examples, fucoidan from *C. okamuranus* with higher sulfate content was reported to show higher hyaluronidase inhibitory activity [70] and macrophage-stimulating activity [56], while Shimizu et al. reported that high MW fucoidan extracted from *C. okamuranus* (200–

300 kDa) promotes greater increases in the proportion of murine cytotoxic T cells related to antitumor activity than middle (2–3 kDa) or low MW fucoidan (0.5–1 kDa) [34]. In contrast, Choi et al. demonstrated that anticancer activity of fucoidan from *Fucus vesiculosus* could be significantly enhanced by lowering their MW (38 -7 KDa) [36]. However, structure of polysaccharide can vary significantly due to the species, or even from the same species, due to the harvesting season, location and maturity of the algae body [37], [38]. The critical issue of the dynamic variation of the composition and structure is that it will be challenging to produce functional polysaccharides with reproducible quality [37]. Therefore, understanding of how these structural properties of bio active polysaccharide are affected by biotic and abiotic factors is particularly important.

Among different influential factors, effect of season on the variation of fucoidan content and structure in *C. okamuranus* were reported by Tsuji *et al*. [41], suggested that although the highest fucoidan content was recorded at end of peak, the peak period is most appropriate to extract bioactive compound with the highest sulfate content and molecular weight. Thus, this time we analyzed cell wall polysaccharide from *C. okamuranus* cultivated in different points to understand how geographic location affect on the structure of cell wall polysaccharide. Moreover, content and structure of fucoidan extracted from brown algae were reported to vary significantly with the place of growth in preceding studies [42], [43], [45]. For instance, Tatiana *et al*. found that fucoidan in *Fucus evanescens* from Iturup Islands had a considerably higher molecular weight (150-500 KDa) than that of fucoidan from Kraternaya Bay (20-30 KDa) and [45] Paramushir island (14-40 KDa) in Russia, but molar ratio of Fuc : sulfate was estimated to be remarkably higher in Kraternaya Bay (1 : 2) compared to other 2 islands (1 : 1) [45]. Thus, our present study was aimed to analyze the structural variation of cell wall

polysaccharides in *C. okamuranus*, with special reference to fucoidan, obtained from eight major habitats. We believe that the information provided by this study will be useful when selecting the algae samples with controlling the quality of bioactive compound in different field of industries in future. Moreover, it will help to establish *C. okamuranus* cultivation for better use of bioactive compounds in divers application.

4.2 Materials and method

4.2.1 Sampling of algae

Cladosiphon okamuranus were purchased from local markets located in 8 different places from main 3 islands (Okinawa, Miako and Ishigaki) of Okinawa prefecture, Japan, during the beginning of peak: from late March to early April 2018. The selected geographical locations are A; Bise (Motobu city), B; Katsuren (Uruma city), C; Yonashiro (Uruma city) and D; Shikiya (Nanjo city) in Okinawa island, E; Karimata, F; Nishihara and G; Hisamatsu in Miako island and H; Ishigaki in Ishigaki island (Figure 4.1).

4.2.2. Preparation of alcohol insoluble residue (AIR)

Alcohol insoluble residue from *C. okamuranus* in different habitat were prepared as described in 3.2.2 in Chapter III

4.2.3 Fractionation of cell wall polysaccharides

The cell wall of AIR prepared using *C. okamuranus* from different locations were fractionated in to 5 fractions: HW, AO, HC-I, HC-II and CL as described in 3.2.3 in Chapter III.

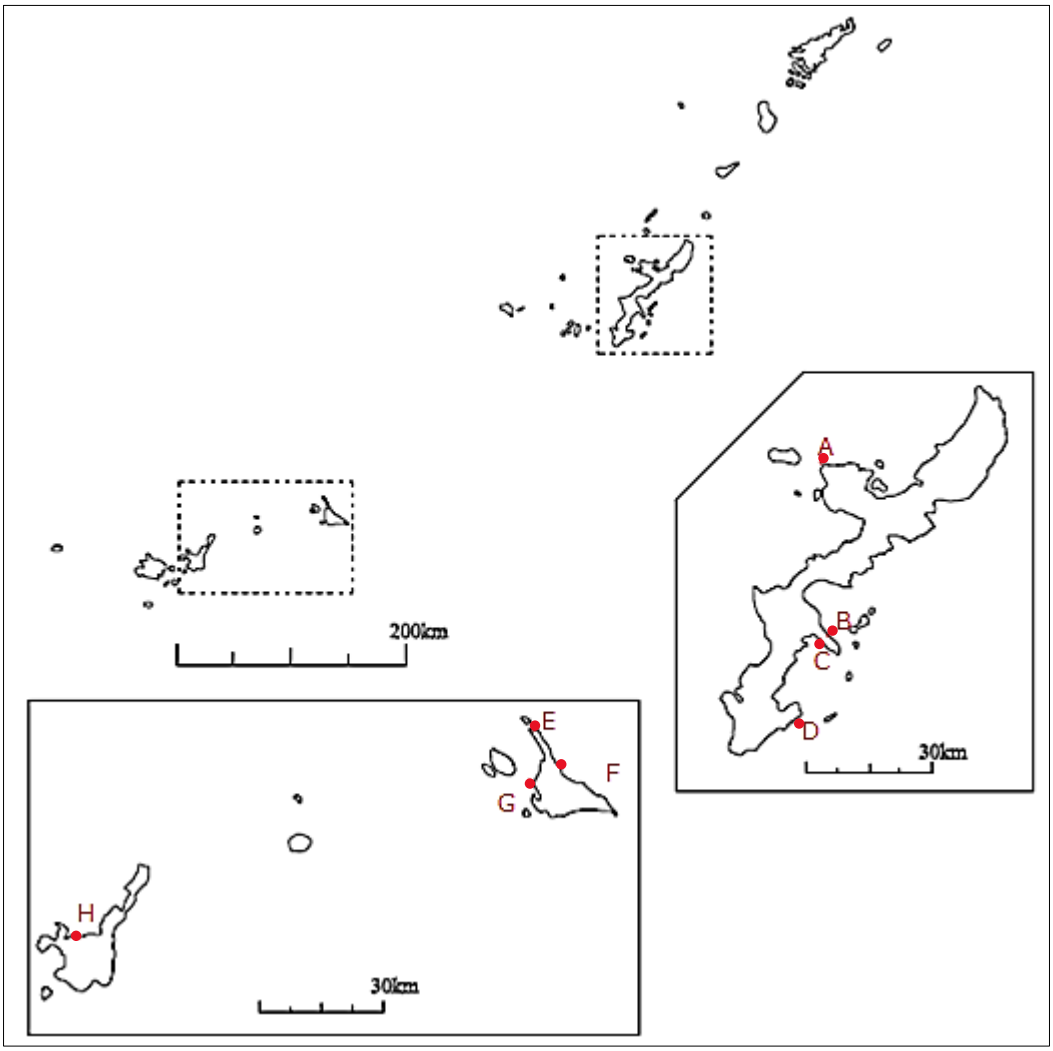

Figure 4.1 Map of Okinawa prefecture showing the sample locations of *C. okamuranus*: A, Bise (Motobu city); B, Katsuren (Uruma city); C, Yonashiro (Uruma city); D, Shikiya (Nanjo City); E, Karimata (Miako island); F, Nishihara (Miako island); G, Hisamatsu (Miako iisland); H, Ishigaki (Ishigaki island).

4.2.4 Chemical composition analysis

Total sugar and uronic acid, protein, polyphenol and sulfate content of each fraction were determined using the methods mentioned in 3.2.4 in Chapter III.

4.2.5 Sugar composition analysis

Since HW and HC-I were found as main fractions, composed of fucoidan with different structures, sugar composition analysis was conducted only for HW and HC-I, extracted from *C. okamuranus* in selected main 5 habitats and for AIR from 8 habitats as described in 3.2.5 in Chapter III.

4.2.6 Determination of molecular weight

The molecular weight of HW and HC-I extracted from *C. okamuranus* in selected main 5 habitats were determined by method described in 3.2.6 in Chapter III.

4.2.7 Statistical analysis

Data were expressed as mean ± standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Tukey's test. Probability values of less than 0.05 ($p < 0.05$) were considered as significant.

4.3 Results

4.3.1 Morphological observation

Since more than 95% production of *C.okamuranus* was reported in Okinawa prefecture, we selected 8 places considering most northern, central and southern area from main 3 islands including Okinawa, Miako and Ishigaki islands (Fig 4.1). Although morphologies of algae appear to be similar during sample collection, detail observation revealed some variations among them in size and level of branching as shown in (Fig 4.2). Algal samples collected from Bise and Katsuren has intermediate lateral branches, but they are considerably thicker in samples in Bise than all other locations. Slight consistence was observed in morphology of algae collected from Yonashiro and Shikiya that contains more fibrous branches compared to Bise and Katsuren in Okinawa Island. However, more hairy appearance with thin and dense branches was clearly appear in both

Karimata and Nishihara from Miako Island. Longer lateral branches were present in Hisamatsu and Ishigaki, but it was long dense in Ishigaki. Taken all together, morphological variation can be categorized mainly in to 2 groups: First group comprised the algae in Okinawa island with intermediate size less hairy lateral branches (A, B, C, D) and second group included algae in Miako and Ishigaki island with long and highly hairy lateral branches (E, F, G, H). Particularly, having very strong algal body with thicker lateral branches in algae from Bise compared to all other locations reinforced us to conduct further chemical analysis for understanding how geographical location affect on the structure of cell wall component.

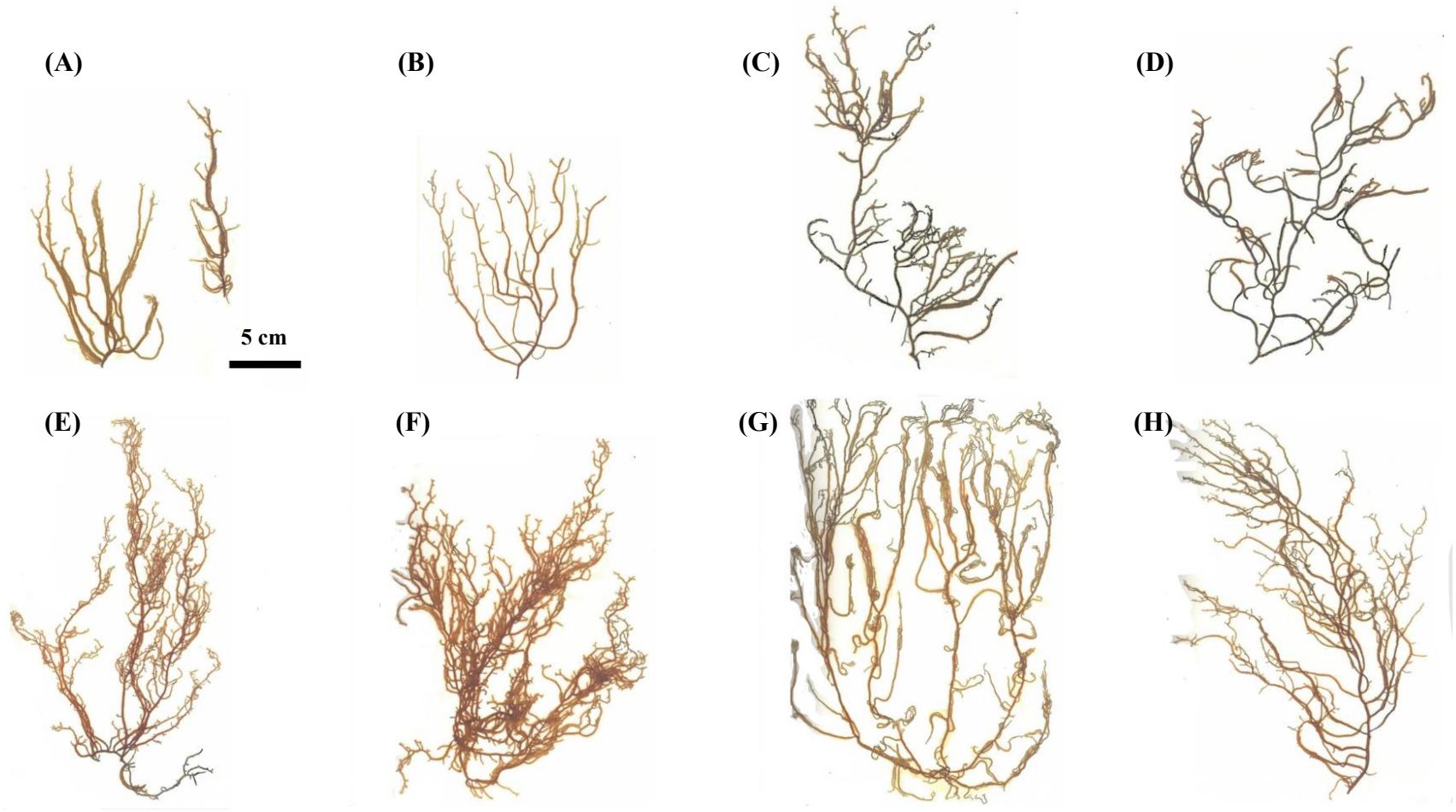

Figure 4.2 Photograph of specimens of *C. okamuranus* (scale = 5cm) in different habitats:(A) Bise, (B) Katsuren), (C)Yonashiro, (D) Shikiya, (E) Karimata, (F) Nishihara, (G) Hisamatsu, (H) Ishigaki

4.3.2 Yield and composition of AIR

Results of the yield and composition of the AIR extracted from *C. okamuranus* is shown in Table 4.1.

Table 4.1 Yield and chemical composition of AIR from *C. okamuranus* in different habitats

Location	Yield [†] (%)	Total [‡] sugar (%)	Uronic [§] acid (%)	Molar ratio						
				Fuc	Glc	Gal	Man	Xyl	UA	SO_3^-
(A) Bise	2.6	53.3±0.6	29.9±1.1	1.0	0.2	tr	tr	0.1	0.5	1.0
(B) Katsuren	3.3	56.5±1.9	28.4±1.6	1.0	0.3	tr	tr	0.1	0.5	0.9
(C) Yonashiro	3.8	56.7±4.3	27.3±1.9	1.0	0.3	tr	tr	0.1	0.5	0.8
(D) Shikiya	5.3	56.5±3.4	26.4±2.0	1.0	0.3	tr	tr	0.1	0.4	0.8
(E) Karimata	4.0	56.6±0.4	26.5±2.6	1.0	0.4	tr	tr	0.1	0.5	0.8
(F) Nishihara	2.8	54.2±1.7	28.3±4.0	1.0	0.3	tr	tr	tr	0.5	0.9
(G) Hisamatsu	2.6	51.7±0.9	30.0±1.3	1.0	0.3	tr	tr	tr	0.5	0.9
(H) Ishigaki	3.1	56.6±1.0	27.9±1.0	1.0	0.3	tr	tr	0.1	0.5	1.0

[†]weight % in wet algae; [‡] weight % in AIR; [§]weight % in total sugar
values were means ± SD of 3 independent experiments; tr, trace amount less than 0.1

The overall yield of AIR indicates that 2.6 -5.3% of the total algal wet weight was recovered the cell wall materials through the isolation process. Sugar compositional analysis of AIR was performed to make a rough estimation of variation of cell wall components in different habitats. Approximately half of the weight of AIR consist of sugar in the range of 51.7 - 56.7%. Fucose is the most abundant monosaccharide in AIR and GlcA and sulfate also display a significant portion as shown in Table 4.1, suggesting that cell wall material of *C. okamuranus* appears to be rich in fucoidan, reported previously [33], [57]. Content of GlcA of AIR is ranging from 26.4 to 30.0% and other neutral sugars including Glucose (Glc), Galactose (Gal), Mannose (Man) and Xylose (Xyl) were detected in minor quantities. However, there were no significant difference

between the habitats with respect to the content of total sugar and other monosaccharides in AIR ($P>0.05$). Next step was to fractionate AIR into 5 main fractions and analyze their structure related to different locations.

4.3.3 Yield and composition of different cell wall fractions.

Using sequential chemical extraction, AIR of each habitat was fractionated into five fractions: HW, AO, HC-I, HC-II, and CL. The extracted yield of 5 fractions in different locations displayed in (Figure 4.3). Remarkably, HW fraction highlighted the highest in every location, ranged between 60–70%. The second largest fraction, HC-I ranged from 15 to 20%. Yield of the AO fractions were in the range of 10 -14%, while HC-II and CL fractions were 2-4% and 3-6% respectively. Since there were no more variations in the yield of fractions among different habitats, we selected 5 habitats: (A) Bise, (B) Katsuren, (D) Shikiya, (G) Hisamatsu and (H) Ishigaki, among 8, representing 3 islands to identify the geographical variation of different type of polymers with special reference to the fucoidan as described below. Bise and Shikiya were selected considering most northern part and southern part in the Okinawa island respectively. Being a larger mozuku producer and relatively central part of the Okinawa island is basis for selecting Katsuren. Hisamatsu in Miako island was selected due to reporting the lowest yield of HW in our study and Ishigaki was from Ishigaki island.

Further analysis of yield and chemical composition of each fraction in 5 major habitats were listed in (Table 4.2). Results show that almost 80% of the cell wall in *C. okamuranus* consisted of HW fraction (58.2–67.7%) and HC-I fraction (14.5 – 18.7%) in every location. The highest yielded HW fractions were rich in total sugar, ranging between 52.5 and 64.3%, including uronic acid in the range from 30.9 to 35.5% in 5 habitats. It is important to note that sulfate content of the HW fraction didn't vary significantly among the habitats, ranging from 13.8 to 17.2% and it was significantly

greater than that of AO, HC-I and HC-II. Proteins in HW were estimated to be in the range from 8.8 to 16.5% and it was low in the content of polyphenol, ranged between 2.5 and 4.8%.

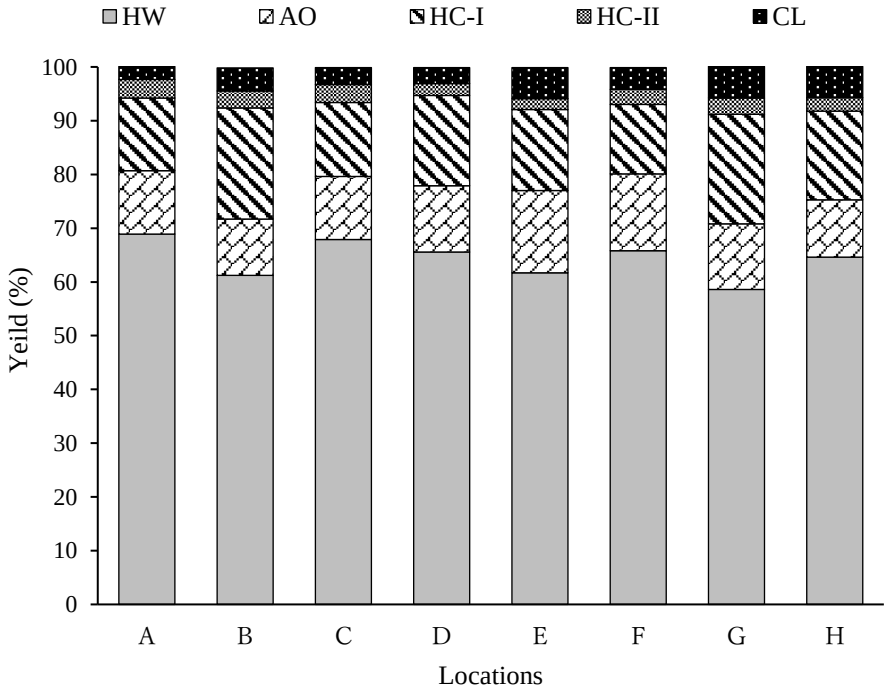

Figure 4.3 Yield of different polysaccharide fractions: HW, Hot water; AO, Ammonium oxalate; HC-I, Hemicellulose-I; HC-II, Hemicellulose -II; CL extracted from *C. okamuranus* in different habitats: (A) Bise, (B) Katsuren), (C)Yonashiro, (D) Shikiya, (E) Karimata, (F) Nishihara, (G) Hisamatsu, (H) Ishigaki.

The second highest yielded fraction, HC-I also composed of sugar, more than 50% of its dry weight and significant difference were not found among different habitats ($P > 0.05$). Uronic acid content of HC-I ranged from 21.9 to 24.5%, while sulfate content was in the range of 5.1 and 9.1%. Result of the composition of HC-I fraction highlighted higher protein content, ranged from 39.3 to 54.8% and higher polyphenol content, ranged

between 11.0 and 15.1%, compared to all other fractions as shown in Table 4.2 and no any significant variation was found among different habitats ($P > 0.05$).

Table 4.2 Yield and chemical composition of cell wall polysaccharide fractions from *C. okamuranus* in different habitats

Location	Fraction	Yield†	Total Sugar	Uronic acid‡	SO_3^-	Polyphenol	Protein
(A) Bise	HW	67.7±1.0^{a}	54.9±2.3^{bc}	35.5±2.0^{a}	13.8±2.4	3.5±0.3^{b}	16.5±1.6
	AO	12.4±1.3^{ab}	54.1±10.1	52.0±5.4	2.4±0.3^{c}	2.5±1.5	11.4±5.9
	HC-I	14.5±1.2^{b}	54.0±4.3	22.9±1.9^{c}	5.1±0.4^{c}	15.1±3.3	54.8±9.1
	HC-II	3.0±0.7	83.4±15.2^{ab}	8.8±0.9^{b}	1.0±0.3^{c}	1.9±0.6^{b}	8.6±3.3^{b}
(B) Katsuren	HW	63.0±1.7^{b}	59.3±1.3^{ab}	33.6±0.7^{ab}	14.2±1.8	3.7±0.1^{b}	13.9±3.1
	AO	11.4±1.3^{ab}	63.8±10.2	49.4±5.2	4.4±0.6^{bc}	1.9±0.5	9.1±1.0
	HC-I	18.7±2.0^{a}	59.0±0.8	22.9±0.9	7.4±0.7^{b}	11.0±1.4	42.4±6.0
	HC-II	2.7±0.5	85.1±18.9^{ab}	12.0±2.4^{b}	2.1±0.3^{bc}	2.8±0.4^{b}	12.9±2.5^{b}
(D) Shikiya	HW	66.0±1.1^{ab}	64.3±3.2^{a}	30.9±2.1^{b}	14.0±0.5	2.5±0.2^{c}	8.8±0.7
	AO	12.0±0.5^{ab}	70.5±15.1	43.8±8.5	7.3±1.7^{a}	1.9±0.5	9.8±1.5
	HC-I	16.7±0.6^{ab}	61.0±5.7	22.4±0.6	9.1±0.2^{a}	11.0±2.0	39.6±6.1
	HC-II	2.2±0.4	77.6±14.7^{ab}	13.1±1.5^{b}	3.3±0.6^{a}	2.0±0.3^{b}	9.3±2.1^{b}
(G) Hisamatsu	HW	58.2±0.4^{c}	52.5±2.2^{c}	35.2±0.5^{a}	17.2±1.1	2.7±0.0^{c}	14.4±4.1
	AO	14.4±2.0^{a}	69.6±12.6	48.9±6.2	5.2±0.7^{ab}	1.6±0.6	10.1±1.6
	HC-I	18.7±2.0^{a}	57.7±3.2	24.5±0.2	8.7±0.5^{a}	10.4±1.4	39.3±4.1
	HC-II	2.8±0.4	54.2±2.2^{b}	20.3±2.8^{a}	3.3±0.7^{ab}	4.8±0.5^{a}	24.0±4.4^{a}
(H) Ishigaki	HW	65.7±1.5^{ab}	53.3±1.6^{c}	34.4±0.8^{ab}	15.7±0.2	4.8±0.2^{a}	15.9±5.4
	AO	10.6±0.7^{b}	65.2±7.9	49.5±6.8	3.4±0.7^{bc}	2.6±1.4	11.9±3.6
	HC-I	16.3±0.4^{ab}	63.1±4.3	21.9±1.2	5.9±0.5^{c}	12.1±0.8	45.3±1.7
	HC-II	2.1±0.4	107.6±9.2^{a}	12.9±0.6^{b}	3.3±0.1^{ab}	2.7±0.7^{b}	11.6±2.0^{b}

†Weight % in total weight fraction recovered in AIR (wt %)

‡Weight % in total sugar; other constituents in weight % of the respective fraction

values were means ± SD of 3 independent experiments: tr, trace amount less than 0.1

values in a column of same fraction in different locations sharing a common superscript (same color) are not significantly different (Tukey's test; $p < 0.05$; $n = 3$)

Approximately, half of the total sugar in AO fraction was represented by uronic

acid, ranged between 43.8 and 52.0% which shows the highest among all polysaccharide fractions, suggested that this fraction contains most of alginate from *C. okamuranus* cell wall. However, significant difference was not found in alginic acid among various locations ($P>0.05$). This polysaccharide fraction exhibited low content of polyphenol (1.6 -2.6%), protein (9.8 - 11.9%) and sulfate (2.4 - 7.3%) as well. Yield of HC-II fraction was less than 3% and no significant difference of yield was observed in different populations as indicated in Table 4.2 ($P > 0.05$).

Most of the compounds in cell wall from *C. okamuranus* were extracted in HW and HC-I whereas, it has already been found that fucoidan is predominant in these 2 fractions [118]. Therefore, Further analysis were conducted only for HW and HC-I fractions to study their structural variation in different habitats.

4.3.4 Molecular weight distribution of HW and HC-I fractions

Molecular weight distribution of HW and HC-I was determined by high-performance size-exclusion chromatography as illustrated in Figure 4.4. The chromatogram showed that HW and HC-I mainly comprised one major peak (Mp), but HC-I was eluting later and broader than HW. Although a significant difference was recorded in peak MW of HW in various locations, it was ranging between 235.7×10^4 and 290.0×10^4. However, there were no significant difference in the MW of HC-I ($P > 0.05$), which ranged from 37.6×10^4 to 30.2×10^4. The peak at V_t is NaCl in the sample and eluting buffer while the small peak, occurred just before V_t was comparatively lower (<10%) than Mp in all chromatography.

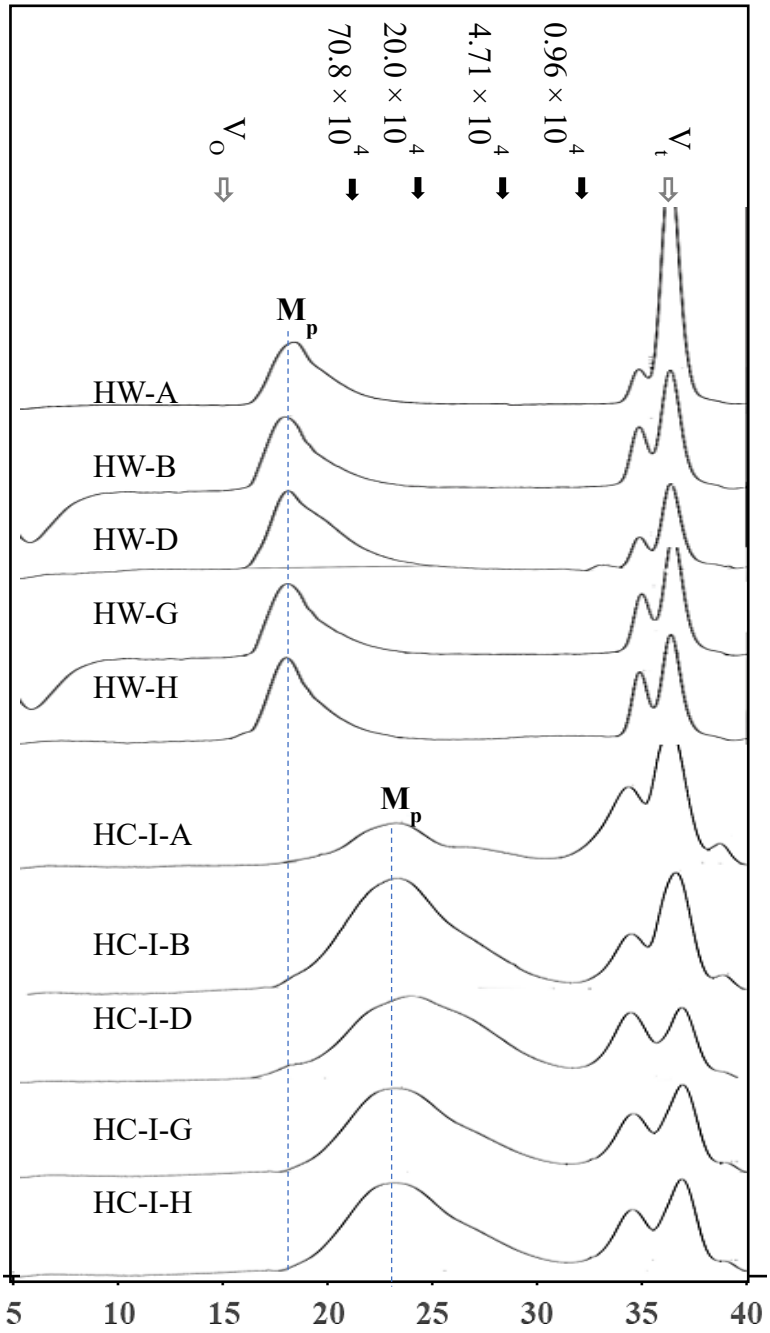

Figure 4.4 Gel filtration HPLC of Hot water fraction (HW) and Hemicellulose-I (HC-I) fractions from *C. okamuranus* at (A) Bise, (B) Katsuren, (D) Shikiya, (G) Hisamatsu and (H) Ishigaki. M_p is the main peak of each chromatogram. Black arrows indicate the elution positions of size standards of pullulan with molecular weight of highest peak. White arrows indicate the void volume (V_O) and total column volume (V_t).

4.3.5 Sugar composition analysis of HW and HC-I fraction.

The HW fraction is likely to be a most important fraction from the cell wall of *C. okamuranus*, because it obtained the highest yield and we found it composes of most of the fucoidan in cell wall. Since, total sugar content, uronic acid content and molecular weight of HW fraction showed slight significant difference among the different location, structure of the fucoidan may be place dependent. Therefore, sugar component analysis was performed for further clarification of its structure. Result of the sugar component analysis revealed that Fuc and UA are the main sugar with the small quantities of Gal, Glc, Man, Xyl in HW and there was no significant difference in monosaccharide profile and sulfate between different location ($P > 0.05$) as shown in Table 4.3. The molar ratio of Fuc: UA: sulfate was 1.0: 0.5: 0.7~1.0 in every samples as listed in Table 4.3, while Gal, Glc, Man and Xyl presented less than 0.1.

However, results of the sugar composition analysis of HC-I in different habitat were not similar as shown in Table 4.4. Although HC-I contains the main components of fucoidan including Fuc, UA and sulfate, presence of Ara, Gal, Glc, Man and Xyl in considerable amount show the structural differences of HW and HC-I in every location. It is highlighted that sugar composition and sulfate content of HC-I fractionated from Bise was estimated to be Fuc, Ara, Gal, Glc, Man, Xyl, UA and sulfate in the molar ratio of 1.0: 0.1: 0.4: 1.0: 0.5: 0.8: 0.9: 0.5, which was comparatively higher than those of HC-I in other locations.

Table 4.3 Sugar composition analysis of fucoidan in HW fraction extracted from *C. okamuranus* in main 5 habitats

Location	Monosaccharide content						SO_3^-
	Fuc	Gal	Glc	Man	Xyl	UA	
(A) Bise	1.0	tr	tr	tr	tr	0.5	0.9
(B) Katsuren	1.0	tr	tr	-	tr	0.5	0.8
(D) Shikiya	1.0	tr	tr	tr	tr	0.5	0.7
(G) Hisamatsu	1.0	tr	tr	-	tr	0.5	1.0
(H) Ishigaki	1.0	tr	tr	-	tr	0.5	1.0

tr, trace amount less than 0.1; -, not detected (Molar ratio)

Table 4.4 Sugar composition analysis of fucoidan in HC-I fraction extracted from *C. okamuranus* in main 5 habitats

Location	Monosaccharide content							SO_3^-
	Fuc	Ara	Gal	Glc	Man	Xyl	UA	
(A) Bise	1.0	0.1	0.4	1.0	0.5	0.8	0.9	0.4
(B) Katsuren	1.0	0.1	0.1	0.3	0.2	0.2	0.5	0.3
(D) Shikiya	1.0	tr	0.1	0.1	0.2	0.1	0.4	0.3
(G) Hisamatsu	1.0	tr	0.1	0.1	0.1	0.1	0.4	0.3
(H) Ishigaki	1.0	0.1	0.2	0.3	0.5	0.2	0.6	0.3

tr, trace amount less than 0.1; -, not detected (Molar ratio)

4.4 Discussion

Habitat, harvesting time and maturity stage of brown algae was often found to be a considerable influencing factor on the yield, composition, structure and biological activity of sulphated polysaccharide [37], [42], [43]. Variability in cell wall composition and structure of algae was reported to link with complex environment exogenous factors such as temperature, salinity, wave exposure, desiccation and light in different geographical locations [119], [120]. This time we mainly focused on effect of geographical location on the structure of polysaccharide from *C. okamuranus,* collected at peak harvesting time [41]. It is well known that brown algae contain a polysaccharide-

rich cell wall which is abundance with fucoidan and alginate, containing up to 45% of the algal dry weight, while with less amount of cellulose, ranging between 1-8% of its dry weight [94]. Although alginate is the major cell wall polysaccharide in most of the brown algae, *C. okamuranus* was found to consist mostly of fucoidan (2.3% w/w) and little amount of alginic acid (0.1% w/w), suggesting *C. okamuranus* is an extremely excellent raw material in terms of industrial extraction of fucoidan [56]. In previous study, we characterized the polysaccharide in HW, AO, HC-I, HC-II and CL from *C. okamuranus* in similar manner, found that HW and HC-I mainly contained fucoidan with different structure, while AO mostly composed of alginate and CL was almost cellulose [118]. In this study, we analyzed the variation of these cell wall fractions in different habitat, with special reference to fucoidan in HW and HC-I fraction.

First, AIR was prepared from algal samples in 8 locations and their sugar composition analysis was performed to understand first characterization of cell wall polysaccharide variation. However, a significant variation of monosaccharide composition was not observed among AIR prepared from various locations (Table 4.1). Presence of abundant Fuc and sulfate with UA in AIR, suggesting that cell wall material of *C. okamuranus* appears to be rich in fucoidan, as reported previously [33], [57]. The Glc in AIR appears to be from cellulose, which is rare in brown algae cell wall [94]. Since sugar composition analysis of AIR provide rough estimation of cell wall polysaccharide in *C. okamuranus*, AIR was fractionated in to 5 fractions to identify the geographical variation of different type of polymers with special reference to the fucoidan. Despite their morphological variation, we found that there were no more variations in the yield of fractions among different habitats, so that we selected 5 habitats among 8 from 3 islands to identify the geographical variation. Selected 5 places are representing most northern

part (Bise), most southern part (Shikiya) and central part (Katsuren) in the Okinawa island, lowest HW yielded place (Hisamatsu in Miako Island) and most southern limit in Okinawa prefecture (Ishigaki from Ishigaki island).

Almost 80% of the cell wall in *C. okamuranus* in different habitat consisted of HW and HC-I that mainly contained fucoidan, while about 10% of AO, mainly composed of alginate and about 4% and 6% of HC-II and CL respectively. This result is consistence with our previous study conducted in *C. okamuranus* from Katsuren, showed that HW and HC-I yielded 80.6% and AO, HC-I, HC-II and CL possessed 9.3%, 4.2% and 5.9% respectively [118]. Having high sulfate content in HW is evident the presence of highly sulfated polysaccharide (Table 4.2). However, slight statistically significant differences were found between different locations in yield, total sugar, polyphenol, sulfate and protein content of most fractions. Therefore, detail structural analysis was conducted for 2 main fractions: HW and HC-I to observe the geographical variation related to structure of fucoidan in *C. okamuranus.*

The HW is the most important fraction in this study because it was reported to be the highest yield and highest content of fucoidan which appeared similar in composition to typical fucoidan extracted in most previous studies. I suggested that highly sulfated homo fucoidan in HW may be involved in osmotic regulation, since they weakly held in cell wall matrix [118]. Despite little variations in the total sugar and uronic acid percentage in HW from different places, sulfate content did not reveal any significance difference among them. Furthermore, quite similar content of sulfate in fucoidan from *C. okamuranus* at same cities were reported previously (Table 4.5). For instance, sulfate content of fucoidan from Chinen village, Nanjo city such as 13.2 [70], 13.5 [56] and 13.6% [121] were quite similar to that of HW fucoidan from Shikiya, Nanjo city in our

study (14%). Sulfate content of the fucoidan from Ishigaki island also almost similar in our study and Lim et al., which was estimated 15.7 and 15.2% [57] respectively. Molecular weight analysis of fucoidan in HW shows a slight significant difference between different locations (Figure 4.4), but it can be categorized as a higher molecular weight fucoidan, ranged from 235.7 × 10^4 and 290.0 × 10^4 compared to most of the fucoidan extracted from *C. okamuranus* using acidic condition precedingly in Shikiya (MW: 46 × 10^4 -107 × 10^4) [41], Ishigaki island (MW: 21.1 KDa) [58], Ginoza (MW: 50 × 10^4) [78] and so on. This MW variation likely not to be due to geographical variation, but may be due to extraction process, because extraction with acidic condition instead of water in previous reports might cause to degradation of native fucoidan into low molecular weight fucoidan. This explanation was further supported by extracting the highest molecular weight (320 × 10^4) fucoidan with boiling water from *C. okamuranus* in Ishigaki island by Kawamoto et al. [55].

However, showing a slight significant difference in the content of total sugar, uronic acid and molecular weight of HW fraction reinforced us to determine its more structural detail among the different locations. Therefore, sugar component analysis was performed. As a result, we found no significant difference in monosaccharide profile and sulfate in HW fucoidan among different locations. The molar ratio of Fuc, UA, and sulfate of fucoidan in HW was 1.0 : 0.5 : 0.7~1.0, respectively (Table 4.3), which is correspond well with fucoidan previously extracted by aqueous dilute acid from *C. okamuranus* in the molar ratio of 1.0:0.2:0.5 from Okinawa island [106], 1.0:0.3:0.6 from Ishigaki island [57], and 1.0:0.3:0.5 [107]. Beside Fuc and UA, minor quantities of Glc, Gal, Man and Xyl contained in fucoidan of HW. Furthermore, molar ratio of Fuc, Xyl and GlcA of fucoidan from *C. okamurnus* collected every two weeks from late January to early May

in Shikiya were estimated to be 1.00 : 0.17~0.21 : 0.14 ~ 0.19 [41]. These results suggested that polysaccharide structure in the composition of sugar of fucoidan in HW is likely to be quite similar in different locations, although there is a slight difference in yield and molecular weight.

Second major fraction extracted from cell wall of *C. okamuranus* in diverse habitat is HC-I which contains the main components of fucoidan including Fuc, UA and sulfate, and considerable amount of Ara, Gal, Glc, Man and Xyl as well (Table 4.4). Fucoidan in HC-I shows lower sulfate content (5-9%), molecular weight (37.6×10^4 - 30.2×10^4) and higher Xyl (Fuc:Xyl, 1.0 : 0.1 ~0.8) compared to fucoidan in HW in every locations. Similarly, our earlier study found that fucoidan structure of HC-I from Katsuren was found to be different from HW in terms of the contents of sulfate and Xyl, MW and profile of small angle X-ray scattering [118]. Furthermore, methylation analysis revealed that HC-I contained 1,4-linked Xyl and 1,4-linked Fuc other than components of typical fucoidan, suggesting that these were 1,4-xylan and/or 1,4-fucan, might allow fucoidan in HC-I to cross-link to cellulose. On the other hand, despite having quite similar sulfate content and molecular weight in HC-I from different location, it was reported significantly higher Gal Glc, Man, Xyl and UA in fucoidan from HC-I extracted from Bise. Since tissues containing Galactan/ xylan-rich polysaccharide cell walls are expected to be mechanically stiffer and stronger than those that are lack on these sugar [122], cell wall structure of *C. okamuranus* from Bise would be different from others. This hypothesis is further supported by existing of thicker lateral branches in the morphology of HC-I of *C. okamuranus* collected from Bise compared to other places. Therefore, structure of HC-I may play a more significant role for strengthening the cell wall. Similarly, we suggested that fucoidan in HC-I with 1,4-xylan and/or 1,4-fucan may play

a similar role to hemicellulose in terrestrial plants through reinforcing cell wall structure by cross-linking to cellulose [118]. Furthermore, plant polysaccharide in fibrillar cell wall and their orientation were found to be the major determinants of cell morphogenesis, while mechanical strength largely relies on the load bearing network consisting of multiscale cellulose microfibrils and their interactions with xylan [123]. So, structure of HC-I likely to be related with morphological characteristics. Consequently, sugar composition of the HC-I and morphology appears to vary among different locations, although most of the structural properties of HW were same. However, except for factors such as seasonal, temperature or salinity and age, Charrier et al. explained that brown algae can induce rapid morphological changes including their size, shape and stiffness as an adaptation strategy to withstand for mechanical forces: waves, currents, tides and crowding [124].

To sum up, we found that structural properties including sulfate content, molecular weight and sugar composition of the main fucoidan fraction in HW extracted form different locations were fairly constant. However, previous studies investigated that content and structure of typical fucoidan extracted from brown algae can alter significantly with the place of growth [42], [43], [45]. For instance, Zvyagintseva *et al*. [45] found that fucoidan in *Fucus evanescens* from Iturup Islands had a considerably higher molecular weight (150-500 KDa) than that of fucoidan from Kraternaya Bay (20-30 KDa) and Paramushir Island (14-40 KDa) Sea of Okhotsk, but molar ratio of Fuc : sulfate was estimated to be remarkably higher in Kraternaya Bay (1 : 2) compared to other 2 islands (1 : 1). These locations are spanning over more than 800 Km, but this study locations cover around 350 - 400 Km. Furthermore, Shinzato *et al*. stated that although southern Ryukyu Archipelago is located in southwestern Japan, spanning over 600 km

and has unique geographic, hydrodynamic, and historical features [125]. Therefore, spreading over close area with unique geographic and hydrodynamic characteristics would be the reason for structural similarities of fucoidan in *C. okamuranus* compared to other studies. I believe that existing approximately similar sulfate content, sugar composition and higher molecular weight in HW fucoidan from different habitats would be an important finding in terms of controlling the quality of fucoidan as a functional polysaccharide. Many studies investigated that these structural properties of fucoidan plays a pivotal role in bio functional properties [31], [52], [126]. Particularly, fucoidan from *C. okamuranus* with higher sulfate content was reported to show higher activities: hyaluronidase inhibitory activity [70] and macrophage-stimulating activity [56], while higher the molecular weight of fucoidan from *C. okamuranus*, the stronger the activities: antitumor [34] and anti-fibrogenesis in DEN-induced liver cirrhosis [35]. Finally, these results confirmed that identical fucoidan can be obtained with known structural properties in HW fraction from any location from Okinawa prefecture at peak harvesting time of the year. This is particularly advantageous to produce uniform bioactive compound without considering the cultivated location in industrial applications.

4.5 Conclusion

The geographical variations in the content of cell wall material from *C. okamuranus,* harvested at peak time in Okinawa prefecture were evaluated. Results show that almost 80% of the cell wall in *C. okamuranus* in all habitats consisted of HW and HC-I, mainly contained fucoidan. Although yield, chemical composition and molecular weight of fucoidan in HW differ slightly at 5 different places, sulfate content and sugar composition were fairly constant, while considerable variation in the composition of

sugar from fucoidan in HC-I was vary in Bise compared to other places. Therefore, this study suggested that relatively similar structural fucoidan in HW from *C. okamuranus* could be obtained at any geographical location from Okinawa prefecture in peak harvesting time of the year. This finding would be beneficial for production of functional fucoidan in diverse industrial application. Nevertheless, there is more research required to fully understand the variation of its functionality in various habitats for accurate recommendation for therapeutic use.

Table 4.5 Review of analysis of extracted fucoidan from *C. okamuranus* in different habitats in literature

Habitat/Institute	Extraction method	Monosaccharide (%)							SO_3^- (%)	Molecular weight	Remarks	References
		Gal	Glc	Man	Xyl	Ara/ Rha	Fuc	UA				
Chinen village,	0.05 M HCl, Ethanol ppt		-	-	0.03†	-	4†	13.5 (1†)	13.6	-	Nov 1995- May 1996	Tako *et al,* 2000 [121]
Chinen, fishery cooperation association	HCl, $CaCl_2$, Acetyl fucoidan	-	-	-	0.2†	-	4.0†	1.1†	13.2	-	Hyaluronidase inhibitory activity	Tako and Minami, 2008 [70]
Chinen village,	Tako *et al,* 2000	-	-	-	-	-	-	-	13.5	-	March 2003, Antiproliferative activity	Teruya *et al*, 2007 [56]
Shikiya, Chinen, Nanjo,	AIR, 0.1 M HCl	0.03-0.05	0.19-0.73		0.17-0.21		1	0.14-0.19	1.75-2.17	46×10^4 - 107×10^4	Jan – May 2006	Tsuji *et al,* 2013 [41]
Ishigaki island	Citric acid	-	-	-	-	-	72	24	8	21.1 kDa	Anti-tumor activity	Haneji *et al,* 2005 [58]
Ishigaki island,from Yaizu Suisankagaku (YSK) IndustryCo., Ltd	Purified by DOWEX 50Wx8	0.8	0.31	0.54	1.8	0.34/ 0.17	70.3	9.27	15.1	-	-	Lim *et al, 2019* [57]
Southern part of Okinawa Island	Tako *et al,* 2000, Acetyl fucoidan,	-	-	-	-	-	4†	12.1 (1.0†)	13.5 (1.8†)	-	March 2005, Anti-inflammatory activity	Teruya *et al*, 2009 [127]
Okinawa	Nagaoka *et al,* 1999	-	-	-	-	-	556.7 mg/g	179.7 mg/g	14.1	41.4 kDa	Anti- fibrogenesis activity	Nakazato *et al,* 2009 [35]
Harvested in Okinawa, Kadoya & Co., Kobe, Japan (lot A03012)	Tako *et al,* 2000	-	-	-	-	-	38.6	23 with Xyl	15.9	92.2 kDa	Anti-Newcastle-virus-activity	Trejo *et al*, 2014 [59]
Iheya island	Hot water, Na_2SO_4, KCl, Ethanol ppt	-	-	-	-	-	-	-	9.8	320×10^4	Anticancer activity	Kawamoto *et al,*2006 [55]
Yaizu Suisankagaku Industry, Shizuoka, Japan	-	39.6	◄	15.6		►	39.6	9.9	16.9	$20\text{-}30 \times 10^4$	Antitumor activity,	Shimizu *et al,* 2005 [34]
Ginoza Fisheries corporation, Kunigami,	CPC ppt	-	-	-	0.1†	-	3.9†	1.0†	23	50×10^4	June 1992	Tako *et al,* 1996 [78]
South product Co. LTD, Suzaki, Japan	Nagaoka *et al,* 1999		2.2		0.7		30.9	23.4	15.1	-	Anti-inflammatory activity	Cumashi *et al*, 2007 [128]

Yaizu Suisankagaku Industry, Shizouka, Japan	Fucoidan YSK-NB	◄		24.7	►		40.2	3.7	18.6	38×10^4	Cardioprotective activity	Thomes *et al,* 2010 [62]
Tropical Technology Center Co. (Okinawa,)	HCl, $CaCl_2$, AEC	-	-	-	-	-	6.1	1.0	2.9	56 kDa	-	Nagaoka *et al,* 1999 [106]
South Product Co., Ltd. (Uruma, Japan)	-	-	-	-	-	-	52	18	17.6	49.8 kDa	Immunomodulatory Effect	Tomori *et al*, 2019 [61]
Marine Products Kimuraya Inc. (Tottori, Japan	-	-	-	-	-	-	-	-	-	300-330 kDa,	Antitumor activity,	Azuma *et al,*2012 [117]
-	-	-	-	-	-	-	1.00 †	0.25 †	0.50 †	$2\text{-}200\times 10^4$	-	Sakai *et al*, 2013 [107]
-	Ion exchange purification	3	-	-	1	-	50	30	16	95.7 kDa	-	Sichert *et al*, 2021 [129]

-, not mentioned or not detected; † Molar ratio

CHAPTER V

General conclusion and future direction

Polysaccharides from macroalgae are well-known producer of bioactive compound, that play a bigger role in the different fields of industries than direct use for human consumption. Over the last three decades many studies have shown remarkable growth in the macroalgae industry and increasing global demand for macroalgae products. However, improving quality and production yield is a challenge for future application of these bioactive polysaccharides. It was found that structure of polysaccharide is a highly influential factor on their function. Therefore, this study is mainly focused on structural and functional analysis of algal polysaccharide and how they are varied due to arrangement in the cell wall and prevailing biotic and abiotic factors. I studied on algal polysaccharide from *Cladosiphon okamuranus* and *Caulerpa lentillifera*, which are extensively utilized and abundantly cultivated algae in Okinawa prefecture, where processes one of the world's most interesting food culture with people who have longest life expectancies and low disability rates.

In first study, I extracted bioactive sulfated polysaccharide from *C. lentillifera,* mainly consisted of Gal, Glc, Xyl, and Man and found to effectively inhibit hyaluronidase activity. Since hyaluronidase inhibitors likely to become increasingly important as therapeutic agent in pharmaceutical industry and antiaging agents in the cosmetic industry, the results obtained from this study will be very important in future industrial applications. Although plenty of bioactivities are found in the compound extracted from *C. lentillifera,* hyaluronidase inhibitory activity poorly understood. Except for activity I found that this activity correlated with structural properties of the polysaccharide mainly sulfate content and molecular weight. However, molecular weight alone was not likely sufficient, and the sulfate group was found to be essential to inhibit hyaluronidase activity. These results suggested that sulfated polysaccharide from *C. lentillifera* would be a promising potential agent for

development of new pharmaceutical formulas to prevent skin aging, inflammatory state, allergic state and tumor cell invasion. Forthcoming studies should focus on the molecular mode of action through *in vitro* study.

In my second study, I analyzed the structure of polysaccharide from *C. okamuranus* which is an excellent source of fucoidan with numerous activities and contains the highest fucoidan among any brown algae spp. Many previous studies described the individual structure and activity of water soluble fucoidan from *C. okamuranus* but it was poorly understood whether insoluble residue of cell wall contains fucoidan and how different its structure is. Therefore, common plant cell wall extraction method was applied to extract the polysaccharide from *C. okamuranus*. Results show that almost 80% of the total cell wall recovered from AIR from *C. okamuranus* was composed of HW and HC-I and both fractions mainly contained fucoidan but, their structure differed in terms of contents of sulfate and Xyl, MW and profile of SAXS. Methylation analysis revealed that HW was typical fucoidan, while HC-I contained 1,4-linked Xyl and 1,4-linked Fuc as well as components of typical fucoidan, suggesting that these are likely to be 1,4-xylan and/or 1,4-fucan. Particularly, if the structure of fucoidan in HC-I is 1,4-xylan and/or 1,4-fucan, it might allow to cross-link with cellulose. This interaction could not be explained yet in previous studies and it was hypothesized that short-chained hemicellulose molecules may present as intermediates between CL microfibrils and fucose-containing sulfated polysaccharide. Hence, I show for the first time that fucoidan in HC-I may play a similar role to hemicellulose in terrestrial plants, by keeping CL microfibrils separated and controlling the expansion growth of cell wall. Finally, this study showed the structure of the *C. okamuranus* cell wall as two networks in which the first network is created by interconnection between fucoidan in HC-I and CL

fibrils thought to be embedded in the second matrix, built by predominantly fucoidan in HW and some alginate. The details structural analysis and application of fucoidan in HC-I would be the subject of further study.

In Chapter IV, we mainly studied on the geographical variations in the content of cell wall material from *C. okamuranus,* collected at the peak time from 8 locations in Okinawa prefecture. Results show that although yield, chemical composition and molecular weight of fucoidan in HW differ slightly at 5 different places, sulfate content and sugar composition were fairly constant, while considerable variation in the composition of sugar from fucoidan in HC-I was found in Bise compared to other places. Therefore, this study suggested that relatively similar structural fucoidan in HW from *C. okamuranus* could be obtained from any location in Okinawa prefecture at peak harvesting time of the year. Therefore, I believe that the result provided by this study will help to select any samples of *C. okamuranus* in Okinawa for different fields of industries without considering variation due to habitat. Nevertheless, there is more research required to fully understand the variation of its functionality in various habitats for accurate recommendation for therapeutic use.

To sum up, this study will improve our understanding of how structure of macroalgal polysaccharide is important for activities. Consequently, it can be utilized to open new insights for improving quality and production yield of polysaccharide in diverse industrial application in future.

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