Enzymatic Fucoidan Extraction and Processing

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Preface and Acknowledgements

Preface

This thesis is submitted to fulfill the requirement for obtaining the PhD degree at Technical University of Denmark. The PhD work was carried out at Section for Protein Chemistry and Enzyme Technology, Department of Biotechnology and Biomedicine, Technical University of Denmark and at the Nhatrang Institute of Technology Research and Application, Vietnam from September 2018 to August 2021.

The PhD study was supervised by Professor Anne S. Meyer, head of Section for Protein Chemistry and Enzyme Technology and co-supervised by Maria Dalgaard Mikkelsen, Section for Protein Chemistry and Enzyme Technology. Moreover, the work was also supervised by Associate Professor Tran Thi Thanh Van from the Nhatrang Institute of Technology Research and Application, Vietnam.

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I would like to thank my big family: my parents, my parents in law, brothers and sisters for love and steady support. Finaly, a special thanks to my closest: my husband Pham Son Tung, our sons Pham Phu Bach and Pham Phu Loi.

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification and that it is the result of my own work.

Thuan Thi Nguyen

Full Name (Ph.D. Candidate)

31.01.2023

Date

Summary

Marine macroalge are renewable sources of interesting polysaccharides, which show diversity in structure as well as bioactivity. The exploitation of macroalgal polysaccharides for various commercial applications is essential to generate new potential products. Based on the hypothesis that efficient and gentle extraction of fucoidans can be achieved by enzymatic processing, the primary objective of this PhD study was development of a new enzyme-assisted method to extract sulfated polysaccharides (fucoidans) from minimum three different types of brown macroalgae. Then, to prepare and purify fucoidan oligosaccharides by enzymatic hydrolysis to deliver those to various bioactivity assessments (done by others).

Moreover, since alginate lyase is used for enzymatic fucoidan preparation, the study of alginate lyases, and notably characterization of a new alginate lyase, able to degrade alginate from different brown macroalgae was also studied.

The classical extraction methods of fucoidans are based on the use of acid and alkaline solvents, which effect the yield and chemical features of fucoidans. In this study, the enzyme-assisted extraction of fucoidans from *Fucus evanescens*, *Saccharina latissima* and *Sargassum mcclurei* using combinations of glucanase and an alginate lyase was developed and compared to a chemical extraction method. The yield, monosaccharide composition, sulfate content and molecular weight of crude fucoidans was determined. The yield of fucoidans based on extracted fucose amount in enzyme-assisted and chemical extraction was not statistically significantly different. However, the monosaccharide composition of enzymatic and chemical crude fucoidans were quite different in fucose, glucose and alginate level. Since the enzyme-assisted method showed high selectivity in degrading the surrounding cell wall components without harming the fucoidans, the sulfate content and molecular weight of fucoidans were conserved compared to the chemical method. The structure of purified fucoidan fractions from enzymatic extracts of *F. evanescens* and from both enzymatic and chemical extracts from *S. latissima* was also confirmed by nuclear magnetic resonance (NMR) spectroscopy.

The fucoidan oligosaccharides from *F. evanescens* and *S. latissima* were prepared by using specific fucoidanase enzymes. Hydrolysis products were separated into medium molecular weight and low molecular weight products. The native fucoidans extracted by enzymatic method from *F. evanescens* and *S. latissima* and fucoidan oligosaccharides from *F. evanescens* showed interesting bioactivity. Hydrolysis products from *S. latissima* were further separated and the structures were investigated. This is the first time the branched oligosaccharides from *S. latissima* was reported. Especially, based on the NMR data of fucoidan oligosaccharides, the new branch point at C4 of fucose residues was found in fucoidans from cultivated *S. latissima* compared to reported C2 branch point in fucoidans from wild *S. latissima*.

The characterization of a new alginate lyase PALy1 from sea cucumber gut marine bacteria *Pseudoalteromonas sp* was studied. The enzyme was found active on three different substrate alginates, polyM and polyG, in which the activity on polyG was highest. The optimal conditions for the alginate lyase PALy1 were 40 °C, pH 7, 256 mM NaCl. The enzyme was inhibited by alginate, polyM and polyG at high substrate concentrations.

Furthermore, it was inhibited by ethanol extracts from brown macroalgae, indicating inhibition by polyphenolic compounds. The alginate lyase PALy1 was able to hydrolyze alginate from *F. evanescens*, *S. latissima* and *S. mcclurei* by an endo-hydrolytic mechanism, which was confirmed by Carbohydrate - Polyacrylamide gel electrophoresis (C-PAGE).

In conclusion, the obtained data showed that the enzyme-assisted extraction using a combination of glucanase and an alginate lyase can extract fucoidans from brown seaweeds without affecting the chemical features of fucoidans. The study furthermore provided evidence that these fucoidans as well as fucoidan oligosaccharides with low molecular weight prepared by fucoidanase enzymes are promising for biomedical applications and also useful for determination of the chemical structures of fucoidans. Beside this, the new alginate lyase has potential for production of alginate oligosaccharides.

Dansk Sammenfatning

Marine makroalger, dvs. tang, er en kilde til interessante polysakkarider, som viser mangfoldighed i struktur såvel som bioaktivitet. Udnyttelsen af makroalgepolysakkarider til forskellige kommercielle anvendelser er afgørende for at generere nye potentielle produkter. Formålet med dette ph.d.-studie var udvikling af en ny enzym-assisteret metode til at udvinde sulfaterede polysakkarider (fucoidaner) fra brune makroalger og fremstilling af fucoidan oligosakkarider ved enzymatisk hydrolyse – of disse produkter skulle dernæst testes for bioaktivitet af forskellige projektpartnere for at vurdere fucoidan struktur-funktion. Siden alginat lyase indgår ved enzymatisk ekstraktion af fucoidan, indgik alginate lyaser i afhandlingens genstandsområde, og herunder indgik særligt karakteriseringen af en ny alginat lyase, der er i stand til at nedbryde alginat fra forskellige brune makroalger, også undersøgt.

De klassiske ekstraktionsmetoder for fucoidaner er baseret på brugen af sure og alkaliske opløsningsmidler, som påvirker udbyttet og de kemiske egenskaber af fucoidanerne. I denne these blev den enzym-assisterede ekstraktion af fucoidaner fra *Fucus evanescens*, *Saccharina latissima* og *Sargassum mcclurei* ved hjælp af kombinationer af glucanase og en alginatlyase udviklet og sammenlignet med en kemisk ekstraktionsmetode. Udbyttet, monosakkaridsammensætningen, sulfatindholdet og molekylvægten af fucoidanerne blev bestemt. Udbyttet af fucoidaner baseret på ekstraheret fucosemængde i enzym-assisteret og kemisk ekstraktion var ikke statistisk signifikant forskellige. Imidlertid var monosakkaridsammensætningen af enzymatiske og kemiske rå fucoidaner forskellige i fucose-, glucose- og alginatniveau. Da den enzym-assisterede metode viste høj selektivitet til at nedbryde de omgivende cellevægskomponenter uden at skade fucoidanerne, blev sulfatindholdet og molekylvægten af fucoidaner bedre bevaret sammenlignet med den kemiske metode. Strukturen af oprensede fucoidan-fraktioner fra enzymatiske ekstrakter af *F. evanescens* og fra både enzymatiske og kemiske ekstrakter fra S. latissima blev også bekræftet ved kernemagnetisk resonans (NMR) spektroskopi.

Fucoidan-oligosakkariderne fra *F. evanescens* og *S. latissima* blev fremstillet ved anvendelse af specifikke fucoidanase-enzymer. Hydrolyseprodukter blev adskilt i produkter med middel molekylvægt og lav molekylvægt. De native fucoidaner ekstraheret ved enzymatisk metode fra *F. evanescens* og *S. latissima* og fucoidan oligosakkarider fra *F. evanescens* viste interessant bioaktivitet. Hydrolyseprodukter fra *S. latissima* blev yderligere adskilt, og strukturerne blev undersøgt. Dette er første gang, de forgrenede oligosakkarider fra *S. latissima* blev rapporteret. Især, baseret på NMR-data for fucoidan-oligosakkarider, blev det nye forgreningspunkt ved C4 af fucoserester fundet i fucoidaner fra kultiveret *S. latissima* sammenlignet med rapporteret C2-forgreningspunkt i fucoidaner fra vildt-høstet *S. latissima*.

Karakteriseringen af en ny alginatlyase PALy1 fra havagurke-tarmbakterien *Pseudoalteromonas sp.* blev undersøgt. Enzymet blev fundet aktivt på tre forskellige alginat substraert, polyM og polyG, hvor aktiviteten på polyG var højest. De optimale betingelser for alginatlyasen PALy1 var 40 °C, pH 7, 256 mM NaCl. Enzymet blev hæmmet af alginat, polyM og polyG ved høje substratkoncentrationer. Desuden blev det hæmmet af ethanolekstrakter fra brune makroalger, hvilket indikerer hæmning af PALy med

polyphenoliske forbindelser. Alginatlyasen PALy1 var i stand til at hydrolysere alginat fra *F. evanescens*, *S. latissima* og *S. mcclurei* ved en endo-hydrolytisk mekanisme, hvilket blev bekræftet ved Carbohydrat - Polyacrylamid gelelektroforese (C-PAGE).

Som konklusion viste de opnåede data, at den enzym-assisterede ekstraktion ved hjælp af en kombination af glucanase og en alginatlyase kan udvinde fucoidaner fra brune tang uden at påvirke de kemiske egenskaber af fucoidaner. Undersøgelsen gav desuden indikationer for at disse fucoidaner, såvel som fucoidan-oligosakkarider med lav molekylvægt fremstillet af fucoidanase-enzymer, er lovende til biomedicinske anvendelser og også nyttige til bestemmelse af fucoidaners kemiske strukturer. Udover dette har den nye alginatlyase potentiale til produktion af alginatoligosakkarider.

Publications

Paper:

I. Nguyen, Thuan Thi, Maria Dalgaard Mikkelsen, Vy Ha Nguyen Tran, Vo Thi Dieu Trang, Nanna Rhein-Knudsen, Jesper Holck, Anton B. Rasin, Hang Thi Thuy Cao, Tran Thi Thanh Van, and Anne S. Meyer. 2020. “Enzyme-Assisted Fucoidan Extraction from Brown Macroalgae *Fucus Distichus* Subsp. *Evanescens* and *Saccharina Latissima*.” Marine Drugs 18(6). doi: 10.3390/md18060296. (Published).

II. Tran, Vy Ha Nguyen, Thuan Thi Nguyen (co-first author), Sebastian Meier, Jesper Holck, Hang Thi Thuy Cao, Tran Thi Thanh Van, Anne S. Meyer, and Maria Dalgaard Mikkelsen. 2022. “The Endo-α(1,3)-Fucoidanase Mef2 Releases Uniquely Branched Oligosaccharides from *Saccharina Latissima* Fucoidans.” Marine Drugs 20(5). doi: 10.3390/md20050305. (Published).

Additional contribution (not defended within this thesis)

III. Ohmes, Julia, Yuejun Xiao, Fanlu Wang, Maria Dalgaard Mikkelsen, Thuan Thi Nguyen, Harald Schmidt, Andreas Seekamp, Anne S. Meyer, and Sabine Fuchs. 2020. “Effect of Enzymatically Extracted Fucoidans on Angiogenesis and Osteogenesis in Primary Cell Culture Systems Mimicking Bone Tissue Environment.” Marine Drugs 18(9):1–18. doi: 10.3390/md18090481. (Published).

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V. Nielsen, Mads Suhr, Maria Dalgaard Mikkelsen, Signe Helle Ptak, Eva Kildall Hejbøl, Julia Ohmes, Thuan Nguyen Thi, Vy Tran Nguyen Ha, Xavier Fretté, Sabine Fuchs, Anne Meyer, Henrik Daa Schrøder, and Ming Ding. 2021. “Efficacy of Marine Bioactive Compound Fucoidan for Bone Regeneration and Implant Fixation in Sheep.” Journal of Biomedical Materials Research - Part A (July):1–12. doi: 10.1002/jbm.a.37334. (Published).

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VIII. Tran, Vy Ha Nguyen, Valentina Perna, Maria Dalgaard Mikkelsen, Thuan Thi Nguyen, Vo Thi Dieu Trang, Andreas Baum, Hang Thi Thuy Cao, Tran Thi Thanh

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Abbreviations list

C-PAGE	Carbohydrate - Polyacrylamide gel electrophoresis
Cetavlon	Hexadecyltrimethylammonium bromide
DEAE	Diethylaminoethyl cellulose
IPTG	Isopropyl β-D-1-thiogalactopyranoside
HIV	The human immunodeficiency virus
MMP	Medium molecular weight product
HPEAC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HP-SEC	High Performance Size Exclusion Chromatography
LMFs	Low molecular weight fucoidans
LMP	Low molecular weight product
NMR	Nuclear Magnetic Resonance
MAE	Microwave-assisted extraction
UAE	Ultrasound assisted extraction
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction and Hypotheses

Introduction:

Brown macroalgae are a source of valuable polysaccharides, including fucoidans, alginates and laminaran, which have been intensively investigated for nutrient, cosmetic and pharmaceutical applications. Fucoidans are sulfated polysaccharides with a backbone mainly consisting of fucose residues. Nowadays, besides the traditional methods, new green technologies of fucoidan extraction from brown macroalgae have been developed to reduce the use of harmful chemical as well as to avoid a negative influence on the fucoidan chemical features, e.g. bioactivity. Enzyme-assisted methods has gained interest due to high selectivity and mild extraction conditions. Until now, only limited research has focused on using enzymes to extract fucoidans from brown macroalgae. Since the cell wall of brown macroalgae is built up from the main polysaccharides alginates, fucoidans and cellulose, the use of specific alginate lyases in combination with glucanase will selectively degrade the cell wall and release intact fucoidans.

Fucoidans and fucoidan oligosaccharides are attractive molecules due to their various health benefits, including antitumor, antiviral, anti-inflammatory, antithrombotic, anticoagulant activities, gastroprotective (Stefaniak-Vidarsson et al. 2020; Tran et al. 2021). Fucoidan oligosaccharides would be prepared by fucoidanases, fucoidan degrading enzymes. Recently, many fucoidanases including endo (1-3) and endo (1-4) fucoidanases have been discovered and numbers are increasing. So, using these enzymes for preparing fucoidan derivatives, to study structure and bioactivities of fucoidans and fucoidan oligosaccharides, is essential for their effective utilization.

Besides fucoidans, alginates are also functional polysaccharides, mainly as hydrocolloids. Brown macroalgae are rich in alginates and they are widely used in food, cosmetic and biomedical industry. However, the high molecular weight and viscosity limit their direct application in biomedicine (Wang et al. 2021b). Enzymatic hydrolysis of alginate provides specific low molecular weight products.

This PhD thesis developed a gentle green enzyme-assisted method for extraction of fucoidans from brown macroalgae. The preparations of fucoidan oligosaccharides for structure determination and bioactivity evaluation was also performed. In addition, the new alginate lyase with potential use for alginate oligosaccharides from brown macroalgae was studied.

Background:

Fucoidans are sulfated polysaccharides, consisting of a backbone of fucose residues and they are primarily found in brown macroalgae. Fucoidans are bioactive molecules with a wide array of bioactivities. Fucoidans are classically obtained from brown seaweeds by extraction technologies that can negatively influence the fine-structure and possible bioactivities.

Hypotheses: The following hypotheses were set:

H1 - Enzymatic treatment of brown macroalgae using selected enzymes for degrading glucans and alginate can be used to extract intact sulfated fucoidan polysaccharides from brown macroalgae.

H2 - Optimal reaction parameters and enzyme selection for enzyme assisted fucoidan extraction may differ for different types of seaweed types, and for different types of fucoidans; the enzyme assisted extraction of galactofucans may in particular require judicious selection of endoglucanases to maximize yields.

H3 - Sea cucumber gut bacteria may harbor genes that encode hitherto uncharacterized enzymes, including alginate-degrading enzymes.

H4 – Fucoidans have a complex structure. Different fucoidans will therefore require treatment with different fucoidanases to produce oligomeric products. In addition, due to their differential substrate selectivity, especially with regard to target bond (α 1,3 or α 1,4), backbone (fucoidan or galactofucan), and sulfation pattern, structural analysis of fucoidan oligosaccharides from new fucoidanase reactions may contribute to build a new understanding of fucoidanase specificity and pave the way for using enzymes to obtain unique fucoidan oligomer products. Hence, fucoidan oligomers of different structure may have unique bioactivities (it is thus believed that fucoidan bioactivity is related to fucoidan structure, including size, backbone type, and sulfation).

Objectives and project phases

To validate the hypotheses, the specific objectives were given in this study:

1- Development of new enzymatic fucoidan extraction technology using glucan and alginate degrading enzymes. Optimization of the extraction parameters.
2- Investigate a new alginate lyase enzyme that can degrade alginate from different taxonomic brown macroalgae
3- Fucoidan oligosaccharide preparation using fucoidanases, elucidation of the specific oligosaccharide structures

The work was organized into the following project phases:

Phase 1: Enzymatic extraction and purification of fucoidans	- Enzymatic extraction of crude fucoidans from three different types of brown macroalgae (*F. evanescens*, *S. latissima* and *S. smcclurei*) by using glucan and alginate degrading enzymes - Chemical extraction of crude fucoidan from *F. evanescens*, *S. latissima* and *S.mcclurei* - Purification of fucoidans by ethanol precipitation and chromatography - Analysis of monosaccharide composition, sulfate content and molecular weight of crude fucoidans and fractions - Elucidation of primary fucoidan structure by NMR analyses - Optimization of enzymatic extraction parameters
Phase 2:	- Degradation of fucoidans from *F. evanescens* and *S. latissima* by endo-fucoidanase enzymes

Preparation of fucoidan oligosaccharides and structural elucidation.	- Separation of hydrolyzed products - Elucidation of fucoidan oligosaccharide structure by NMR analyses - Analysis of monosaccharide composition, sulfate content and molecular weight of hydrolysed products
Phase 3 Discovery, cloning and expression of new alginate degrading enzymes for application oligoalginate preparation. Assessment of possible alginate lyase inhibitors - phenolic compounds from brown macroalgae	- Expression of alginate lyase enzymes from sea cucumber gut bacteria - Characterization of a selected alginate lyase - Enzymatic extraction of crude extract of phenolic compounds from brown macroalgae *F. evanescens*, *S. latissima* and *S. mcclurei* - Screening of inhibitory effect of a crude extract of phenolic compounds from brown macroalgae on alginate lyase activity

Chapter 2: Literature review, State-of-the-Art

2.1 Fucoidans from brown macroalgae: structure diversity

Macroalgae (marine macroalga) are eukaryotic photosynthetic organisms. They are divided into three main macroalgae groups *Clorophyta* (green), *Rhodophyta* (red) and *Phaeophyta* (brown), which differ in their pigmentation and cell wall polysaccharides (Synytsya et al. 2015). Green and red macroalgae belong to the *Plantae* Kingdom, while brown macroalgae belong to *Chromista* Kingdom. Macroalgae are known as a source of various interesting polysaccharides, which are specific for each macroalgae group. Sulfated galactans, agars and carrageenans are red macroalgae specific polysaccharides, sulfated glycans (ulvans) are green macroalgae polysaccharides, while alginate and fucoidans are specific for brown macroalgae (Synytsya et al. 2015). Among these polysaccharides, fucoidans have attracted a lot of attention due to various bioactivities such as antioxidant (Koh et al. 2019), anticancer (Kim et al. 2010a), anti-inflammatory (Asanka Sanjeewa et al. 2019) and anticoagulant (Jin et al. 2013). However, the fucoidan content and chemical structure is species dependent. The fucoidan content in macroalgae species belonging to the *Fucaceae* family in the *Fucales* order is high, while fucoidan content in species in the order *Laminariales* is low and in family *Sargassaceae* in the *Fucales* order is lowest (Zvyagintseva et al. 2021).

Fucoidans or fucose-rich sulfated polysaccharides (FSPs) a group of heterogeneous marine polysaccharides with complex structure. α-L-fucose residues are the main component of fucoidan molecules. In addition, other sugars e.g. galactose, glucose, xylose, mannose and uronic acids are also found in fucoidans in various proportions. The term fucoidin referring to sulfated polysaccharides was firstly reported in 1913 by Kylin (Kylin, 1913), where polysaccharides were isolated from brown macroalgae from *Fucaceae* and *Laminariaceae* families (Ale et al. 2011). However, not until 1950 was the first fine-chemical structure of fucoidans determined. Results suggested that fucoidans were built of 1→2 linked L-fucopyranose units in the fucoidan polysaccharide from *F. vesiculosus* (Conchie and Percival, 1950). Later this structure was revised by Patankar et al. (1993) suggesting that the backbone was made of (1→3)-linked α-L-fucose residues and (1→2)-linked residues were found in branches. Further studies on fucoidans from *F. vesiculosus* elucidated that the polysaccharides consisted of alternating (1→3)- and (1→4)-linked fucose residues [→3)-α-L-Fuc(2SO_3^-)-(1→4)-α-L-Fuc(2,3diSO_3^-)-(1]$_n$ (Chevolot et al. 2001). To date, several studies on isolation, structure characterization and biological activities of fucoidans from various brown macroalgae species, have been reported (Usov and Bilan 2009; Zvyagintseva et al. 2021). However, structure determinations of fucoidans is still challenging, due to the complex structure of fucoidans with branching, high sulfation degree and random arrangement of different structure motifs in the polymer (Usov and Bilan 2009).

Now it has been well established that fucoidans from brown macroalgae can be divided into two main groups according to the basic backbone structures, 1. α-(1→3)-linked L-fucosyl residues or 2. alternating α-(1→3)- and α-(1→4)-linked L-fucosyl residues. The content, position of functional groups (sulfate, acetyl), and the structure of branches vary depending on the macroalgae species (Usov and Bilan 2009).

2.1.1 Fucoidans from the order *Laminariales*

Fucoidans from brown macroalgae of the *Laminariales* order have been elucidated as α-L-fucans, where the backbone mainly consisted of α-(1→3)-linked L-fucose residues (Kusaykin et al. 2008; Usov and Bilan 2009). Fucoidans isolated from *Saccharina cichorioides* are composed of a backbone of α-(1→3)-linked L-fucosyl units with a small degree of branches of single α-L-(1→2)-fucose residues and sulfate groups at both C-2 and C-4 positions (Zvyagintseva et al. 2003). Similarly, fucoidans from *Laminaria hyperborea* have the backbone structure of α-(1→3)-linked L-fucose units with a high degree of branches of single α-L-fucose at C-4 or short fucose chains (di- and tri-saccharide) at C-2 and C-4 (Kopplin et al. 2018).

One species of macroalgae can synthesize different types of fucoidans. The brown macroalgae *S. latissima* contain four sulfated polysaccharides of different chemical structure (Bilan et al. 2010), the main structure was α-L-fucans, containing a backbone of 3-linked α-L-fucopyranose residues with sulfates at C-4 and/or at C-2 and branch points at C-2 with a single sulfated α-L-fucopyranose residue (Figure 2.1). The second structure was fucogalactan, having a backbone of 6-linked β-D-galactopyranose residues with branches at C-4 as single or short chains of β-D-galactose or disaccharides α-L-Fuc*p*-(1→3)-β-D-Gal*p*-(1→ (Figure 2.1). The third structure was fucoglucuronomannan, the backbone consisting of alternating 4-linked β-D-glucopyranosyluronic acid and 2-linked α-D-mannopyranose residues with branches at C-3 of α-D-Mannose by single α-L-fucopyranose residues. The fourth was fucoglucuronan with the (1→3)-β-glucuronan backbone and braches at C-4 as single α-L-fucopyranose residues (Bilan et al. 2010).

In addition, the α-L-fucans with α-(1→3)-linked L-fucosyl residues was also found in macroalgae from other orders, such as fucoidans from *Chorda filum* (family *Chordaceae*, Order Chordales) (Chizhov et al. 1999) and *Cladosiphon okamuranus* (family *Chordariaceae*, *Ectocarpales*) (Lim et al. 2019). The fucoidan backbone consisted of α-(1→3)-linked L-fucose units with branch points as single α-(1→2)-linked fucose units. The sulfate groups were found mainly at C-4 and less at C-2 (Chizhov et al., 1999, Lim et al., 2019).

2.2.2 Fucoidans from the order Fucales

Fucoidans from brown macroalgae belonging to the family *Fucaceae* and *Sargassaceae* in the order Fucales have been mostly studied. They can be divided into two groups: α-fucans with the backbone of alternating α-(1→3)- and α-(1→4)-linked L-fucosyl residues from macroalgae in *Fucaceae* and fucoidans with complex structure from macroalgae in *Sargassaceae*. The most simple fucoidans of the first group is *Fucus disticus* with a linear backbone built up of →3)-α-L-Fuc*p*-(2,4-di-SO_3^-)-(1→4)-α-L-Fuc*p*-(2SO_3^-)-(1→ regular repeating structure unit (Figure 2.1) (Bilan et al. 2004). Fucoidans from *Fucus evanescens* were demonstrated to have a backbone of the repeating structure sequence →3)-α-L-Fuc*p*-(2-di-SO_3^-)-(1→4)-α-L-Fuc*p*-(2SO_3^-)-(1→ with additional sulfate groups at C-4 of (1 → 3)-linked residues (Bilan et al. 2002). However, in another study fucoidans from the same macroalgae species was reported having branches at C-4 of α-(1→ 3)-linked fucose residues of the main chain by single α-L-fucose; these results were obtained from enzymatic hydrolysis products (Silchenko et al. 2014).

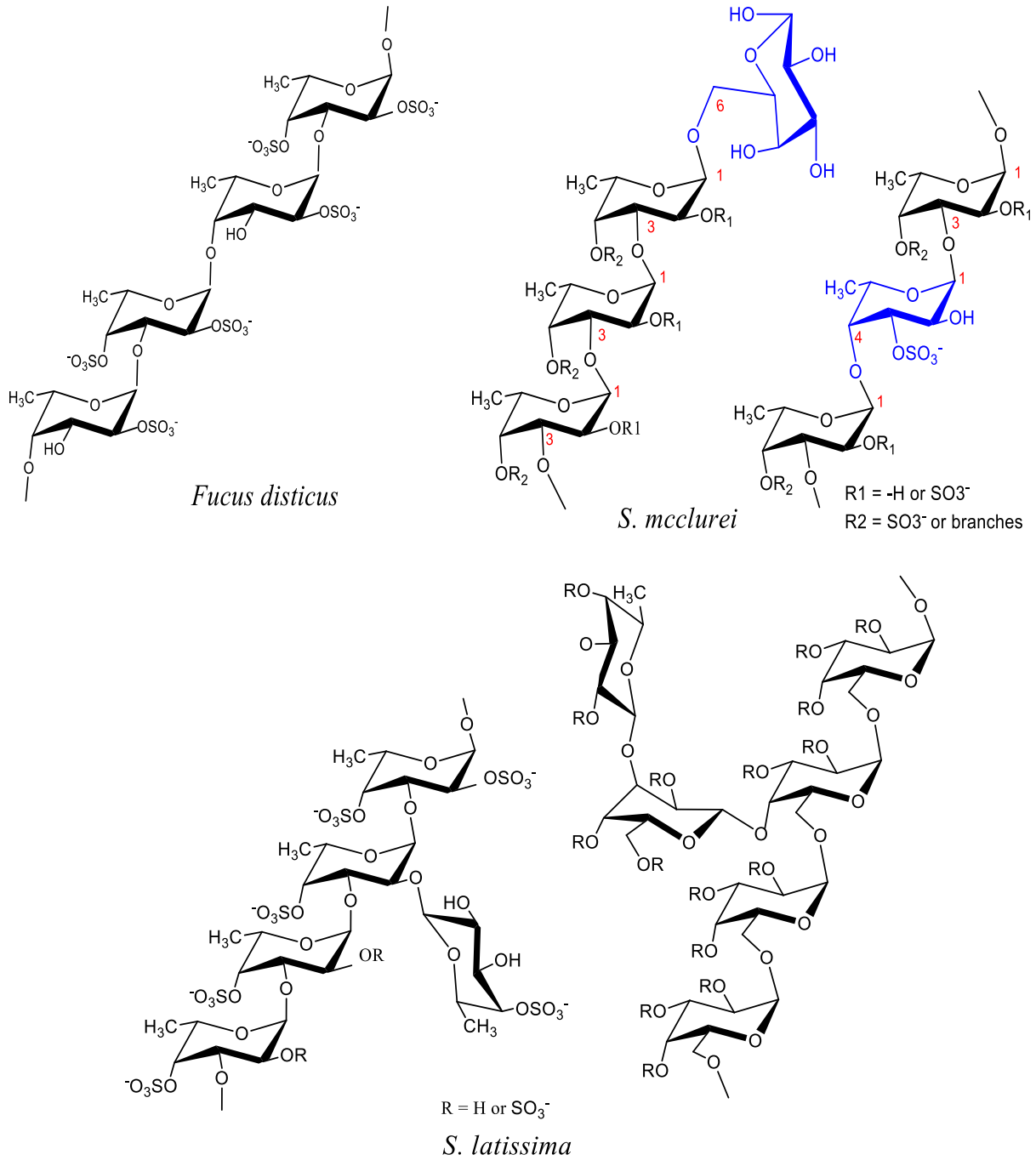


Figure 2.1 The representative main structure of fucoidans isolated from the brown macroalgae *F. disticus* (Bilan et al. 2004), *S. latissima* (Bilan et al. 2010) and *S. mcclurei* (Thinh et al. 2013); for the latter the blue color indicate a 1,4-linked 3-sulfated α-L-fucosyl insert in the main chain and the blue terminal 6-linked galactosyl unit.

Fucoidans from the *Sargassaceae* family are primarily galactofucans, which differ in their backbone and side chain structures. While sulfated fucans mainly contain fucose, these galactofucans may contain up to comparable amounts of fucose and galactose and the galactose residues may be distributed both in the backbone and in side chains (Bilan et al. 2013; Thinh et

al. 2013). Fucoidans from *Turbinaria ornata* have a backbone of 3-linked α-L-fucose residues with branches at C-4 as →4)-Gal*p*-(1→. The sulfate groups occupy mainly C-2 and less at C-4 of both fucose and galactose residues (Thanh et al. 2013). Some galactofucans have the backbone of both α-(1→3)-linked and α-(1→4)-linked L- fucose residues as fucoidans isolated from *Sargassum crassifolium* (Yuguchi et al. 2016). They have a backbone of alternating α-(1→3)- and α-(1→4)-linked L-fucose residues and branches at C-4 by 1→4-linked galactose residues. Galactose may also be found in the backbone of the galactofucans. Fucoidans presented in this group are from *Sargassum polycystum* and *S. mcclurei*. They contain a backbone of α-(1→3)-linked 4-sulfated fucose residues with inclusion of (1→2)-linked 4-sulfated α-D-galactopyranose residues for *S. polycystum* (Bilan et al. 2013) and with (1→6)-linked galactose at reducing end for *S. mcclurei* (Figure 2.1) (Thinh et al. 2013). The branches of these fucoidans vary: (1→4)-linked fucose residues and disaccharide fragments β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→ for *S. polycystum* and alternating sulfated (1→3)- linked α-L-fucose residues and (1→4)-linked β-D-galactose residues for *S. mcclurei*.

2.2 Biological activities of fucoidans from brown macroalgae

Fucoidans from brown macroalgae were, as mentioned above, reported to exhibit various pharmacological properties, including anticancer (Huang et al. 2015), anticoagulant (Jin et al. 2013), antiviral (Krylova et al. 2020), anti-inflammatory (Sanjeewa et al. 2017).

Anticancer

Fucoidans demonstrated anticancer activities against various cancers such as skin, lung (Ale et al. 2011) (Ale et al. 2011), colon (Kim et al. 2010), and breast, through different mechanisms. Fucoidans have been shown not to be toxic (Dörschmann et al. 2020; Ramu et al. 2020), their anticancer activities are in contrast based on the induction of apoptosis of tumor cells (Kim et al. 2010), enhancing immune function (Ale et al. 2011). In another system, fucoidans exerted anti-angiogenic effects by inhibition of the formation of VEGF (vascular endothelial growth factor), reducing the supply of nutrient and oxygen to tumor tissues (Huang et al. 2015).

Anticoagulant

The ability of fucoidans preventing blood coagulation was reported in several studies. The anticoagulant properties of fucoidans are mainly based on thrombin inhibition mediated by antithrombin III (AT-III) and/or heparin cofactor II (HC-II) – main control factors in blood coagulant processes (Ustyuzhanina et al. 2014). The anticoagulant properties of fucoidans are related to their monosaccharide composition, molecular weight and sulfate groups (Jin et al., 2013, Ustyuzhanina et al., 2014).

Anti-inflammatory activity

Fucoidans isolated from different brown macroalgae also exhibited anti-inflammatory activity by inhibiting the production of pro-inflammatory cytokines (Asanka Sanjeewa et al. 2019). A study showed that after two weeks of oral fucoidan administration, the level of main pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) were significantly reduced in advanced cancer patients (Takahashi et al. 2018).

Antiviral

Fucoidans having antiviral potential has been reported in several brown macroalgae. Fucoidans are able to inhibit enveloped human pathogens such as herpes viruses (HSV-1, HSV-2) and human immunodeficiency virus (HIV-1) and nonenveloped RNA viruses as enterovirus (ECHO-1) (Thuy et al. 2015; Krylova et al. 2020). Fucoidans can modify the properties of cell surfaces, preventing the entry of vira into cells and it is possible that fucoidans can also directly interact with viral enzymes or proteins on the surface of the virus (Usov and Bilan 2009). The antiviral activity of fucoidans is related to their sulfate content, molecular weight and also the fine structure of the polysaccharides (Krylova et al. 2020). Currently, the infectious disease caused by SARS-CoV-2 virus is rapidly spreading, striking worldwide with a catastrophic human and economic crisis. Fucoidans with antiviral activity were also studied against SARS-CoV-2 virus (Yim et al. 2021). Fucoidans from *Saccharina japonica* show potential activity against SARS-CoV-2 virus and were suggested to be promising for clinical use (Kwon et al. 2020).

Besides the classical bioactivities of fucoidans, recent new effects of fucoidans have been reported such as effects against diabetes and regulation of blood lipids. Interestingly, one of the new potential bioactivities of fucoidans is their use in bone disease treatment. The regulation and balance between the osteoclast-mediated bone resorption and osteoblast-induced bone formation are important contributing factors for bone health (Wang et al. 2019). The bone diseases such as osteoporosis and rheumatoid arthritis occur when this balance is broken, increasing osteoclastic bone resorption and decreasing bone formation (Boyle et al. 2003). Thus, the treatment requires the use of bone generation and osteoclast inhibiting drugs. The effect of fucoidans on bone tissue have been reported. Fucoidans from *Undaria pinnatifida* showed anti-osteoclastogenic effect when added to bone marrow macrophage cultures (Kim et al. 2014). Fucoidans extracted by enzyme-assisted method from *F. evanescens* were studied for the anti-angiogenic and anti-osteogenic properties in bone-related primary mono- and co-culture systems (Ohmes et al. 2020). Based on the determination of gene expression and protein levels of the regulatory molecules vascular endothelial growth factor (VEGF), angiopoietin-1 (ANG-1), ANG-2 and stromal-derived factor 1 (SDF-1), these fucoidans inhibited angiogenic and osteogenic processes – one of mechanisms of action of fucoidans could therefore be in treatment of bone tumors (Ohmes et al. 2020). The potential use of fucoidans in bone repair was furthermore, reported in a *in vivo* study (Kim et al. 2018). Fucoidans induced new blood vessel formation and partially promoted bone formation in a rabbit model with calvarial bone defect (Kim et al. 2018).

The bioactivity of fucoidans are related to fucoidan structures, which are influenced by extraction methods. So the next sections will include the knowledge on cell wall composition and methods of fucoidan isolation from brown macroalgae.

2.3 The cell wall of brown macroalgae

The cell wall of brown macroalgae consists of a complex network of biopolymers, mainly polysacchrides (Deniaud-Bouët et al. 2014). Like plants, brown macroalgae contain cellulose microfibrils, which are arranged in layers parallel to the cell surface. However, the content of cellulose in the cell wall is small, ranging from 1 to 8% of the macroalgae dry weight (Michel et al.

2010) compared to 40-50% in plants (Zeng et al. 2017). So in contrast to plants, brown macroalgae cell wall matrix consists of fucose-containing polysaccharides (fucoidans) and alginate.

The arrangement and interaction between the cell wall components have previously been presented in a proposed cell wall model of brown macroalgae from the *Fucus* order (Figure 2.2) (Deniaud-Bouët et al. 2014). Fucoidans interlink the cellulose microfibrils, forming the cellulose – fucoidans network (Deniaud-Bouët et al. 2014). While hemicelluloses are hypothesized to be associated with the cellulose microfibrils and bridge the fucoidans to the cellulose microfibrils (Deniaud-Bouët et al. 2014). Alginates consist of mannuronic acid and guluronic acid, which are arranged in a linear backbone and in specific blocks. Alginates can interact with calcium, forming an insoluble gel (Domozych 2019). In the cell wall, alginate is thought to associate with phenolic compounds, forming the network in which embedded cellulose – fucoidans network (Deniaud-Bouët et al. 2014). Proteins, glycoproteins and iodide were also found in the cell wall of brown macroalgae but only in small proportions (Deniaud-Bouët et al. 2017). In addition, the presence of mixed-linkage glucan (MLG) made of β-(1–3)- and β-(1–4)-linked D-glucose residues in the cell wall of brown macroalgae was reported. This component was hypothesize to be associated with alginate in the cell wall (Salmeán et al. 2017). Alginate – phenolic network mainly impacts on the rigidity of brown algal cell wall. While fucoidans not only participate in building of algal cell wall but also in algal protection since they occur both in the cell wall and amorphous intercellular matrix (Kloareg and Quatrano 1988; Mabeau et al. 1990). Fucoidans protect intertidal brown algae from drying out by forming hydrophilic coating on the surface of thallus (Skriptsova 2015). In addition, fucoidans with their sulfate groups can participate in ion exchange with environment, supporting algae in ionic regulation (Kloareg and Quatrano 1988; Kloareg et al. 2021).

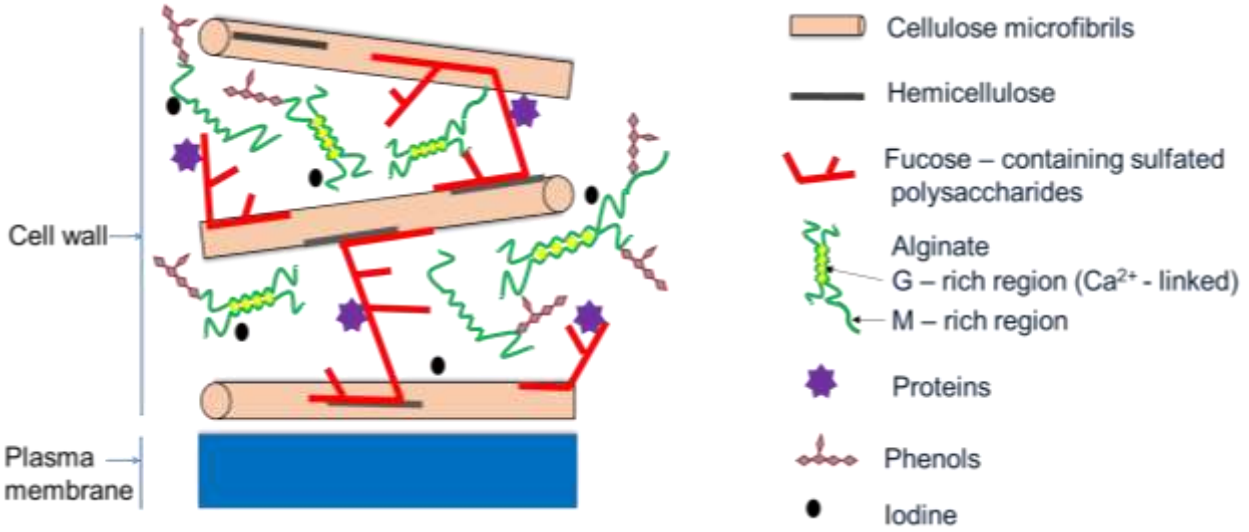


Figure 2.2 Cell wall model of brown macroalgae from the order *Fucales* (modified from Deniaud-Bouët et al. 2014)

The abundance of cell wall components, in particular fucoidans and alginates, is different among algae species, within certain species in response of part of algae (Saraswathi et al. 2003) and environmental factors. The fucoidan and alginate content was various among brown algal species, up to 10% of algal dry weight for fucoidans (Zvyagintseva et al. 2021) and 40% for alginates (Horn

et al. 1999). The amount of fucoidans in reproductive tissue is higher than in sterile tissue (Skriptsova et al. 2011; Mak et al. 2013). Brown algae *S. latissima* growing in North Atlantic with high salinity contain higher amount of fucoidans than algae which grow in the Baltic Sea (Ehrig and Alban 2015). The seasonal variation of fucoidans and alginates have been investigated. The fucoidan yield was high in summer when brown algae was at reproductive stage (Honya et al. 1999; Skriptsova et al. 2010; Mak et al. 2013; Bruhn et al. 2017). The alginate content in brown algae *Macrocystis integrifolia* and *Nereocystis luetkeana* seasonally varied from 19 to 31% of dry weight and was higher in summer. In addition, the amount of alginate in stipe tissue was found slightly higher than in blade tissue (Rosell and Srivastava 1984). Similarly, alginate yield in *Sargassum muticum* was lowest in winter (11.14%) and highest in spring/early summer (25.62%) at maximum vegetative growth (Belattmania et al. 2021). The ratio M/G in alginate from *Turbinaria conoides* was reported various in different part of algae, where M/G in entire thallus was highest, followed by leaf and stem (Jothisaraswathi et al. 2006).

Based on the understanding of the cell wall composition, different extraction methods of fucoidans from brown macroalgae was developed.

2.4 Extraction of crude fucoidans from brown macroalgae

Traditionally fucoidans from brown macroalgae were isolated in multistep procedures using hot water (Li et al. 2006), acid (Imbs et al. 2014) or alkaline solutions (Bilan et al. 2006) at high temperatures and long extraction times (Figure 2.3). These methods may alter the fucoidan structure, effecting the biological properties. For conserving the fucoidan structure, bioactivity and furthermore reducing the use of chemicals, new green extraction techniques were developed such as subcritical water extraction (Saravana et al. 2018), microwave–assisted (Ptak et al. 2019), ultrasound-assisted (Hmelkov et al. 2018), and enzyme-assisted extractions (Wijesinghe et al. 2011; Alboofetileh et al. 2018; Nguyen et al. 2020) (Table 2.1). In general, fucoidan extraction procedures include following steps: 1- preparation of macroalgae (wash, dry, and grind the macroalgae), 2 - pretreatment of macroalgae to remove impurities, 3- extraction of crude fucoidans and 4- purification of fucoidans (Hahn et al. 2012).

2.4.1 Preparation and pre-treatment of macroalgae

In the initial steps, the fresh macroalgae are washed with distilled or tap water to remove salt and others impurities from the macroalgae surface and then dried (Imbs et al. 2014). Since fucoidans are cell wall polymers, to release fucoidans into the solution the cell wall must be loosened/destroyed. Therefore, to disrupt the cell wall and increase the contact surface between macroalgae and extraction solvent, improving the extraction yield, the dried macroalgae are most often grinded into small-sized homogeneous macroalgae particles (Hahn et al. 2012; Imbs et al. 2014).

In the next step, macroalgae are treated with different organic solvents to remove other contaminating compounds, increasing the purity of the crude fucoidans. Since the polarity of the impurities are various, mixtures of organic solvents with different polarity are applied for the macroalgae treatments. In several studies a mixture of methanol/chloroform/water (4:2:1) were used at room temperature to remove lipids, pigments and low molecular weight components from the macroalgae (Bilan et al. 2006; Mak et al. 2013). In another study macroalgae were treated by

ethanol 70–96% for different time durations and temperatures (Anastyuk et al. 2017). In these aqueous-ethanolic solutions, manitol (the major storage carbohydrate in brown macroalgae) and chlorophyll were extracted. Lipids and pigments from macroalgae were removed by a mixture of acetone and ethanol (Dinesh et al. 2016). The pretreated macroalgae were dried before continuing to the extraction of the fucoidans.

2.4.2 Classical methods of fucoidan extraction

Since fucoidans are polyanionic and water soluble polymers, they can be isolated in water (Figure 2.3). In most of the methods, fucoidans were extracted with water at high temperatures 70–90°C for 1 to 4 hours (Duarte et al. 2001; Li et al. 2006; Cong et al. 2014). To avoid using high temperatures in the extraction process, in some other studies the isolation of fucoidans at room temperature for longer time (12 hours) was performed (Duarte et al. 2001). However, extraction with water is not a selective method, not only fucoidans but also other polysaccharide as alginate, laminaran and water soluble compounds are extracted as well. The obtained crude extracts contain fucoidans and high amount of impurities and need more purification steps to increase the purity of the fucoidans. Ethanol was added to the crude extract in a 3:1 ratio, precipitating all polysaccharides (Duarte et al. 2001; Li et al. 2006). The precipitate was dissolved again in water and $CaCl_2$ was added to the solution to gelate and remove alginate. The supernatant containing fucoidans was dialyzed and dried (Duarte et al. 2001; Li et al. 2006).

Fucoidan extraction from brown macroalgae have also frequently been performed using dilute acid solutions (Figure 2.3). The use of acids disrupts the cell wall of macroalgae, enhancing the fucoidan extraction (Lim and Wan Aida 2017). In acid solutions, alginate is converted into alginic acid, which is water insoluble (DJ. McHugh 1987) . This alginic acid can be removed together with macroalgae residues after extraction. Therefore, the obtained crude fucoidans have high purity. Hydrochloric acid with concentrations of 0.01 to 0.2 M, pH ranging from 1 to 3 have commonly been used (Lim and Wan Aida 2017). The extraction processes was performed at high temperatures, at different time durations and was in many studies repeated several times (Thinh et al. 2013; Imbs et al. 2014; Fletcher et al. 2017). The acid concentration was reported to have an effect on the yield of fucoidans (Ale et al. 2012), the classical multi-step extraction with acid (≥0.2 M HCl) at high temperature and extended time had a harmful effect on the yield of fucoidans, since the structural integrity of the polysaccharides was compromised (Ale et al. 2012). After acid extraction of fucoidans, the extracts were neutralized with NaOH and dialyzed to remove salts (Hemmingson et al. 2006). To get higher purity of fucoidans, the extracts were further treated with $CaCl_2$ to remove alginate and the fucoidans were then precipitated with ethanol and dried (Nguyen et al. 2020).

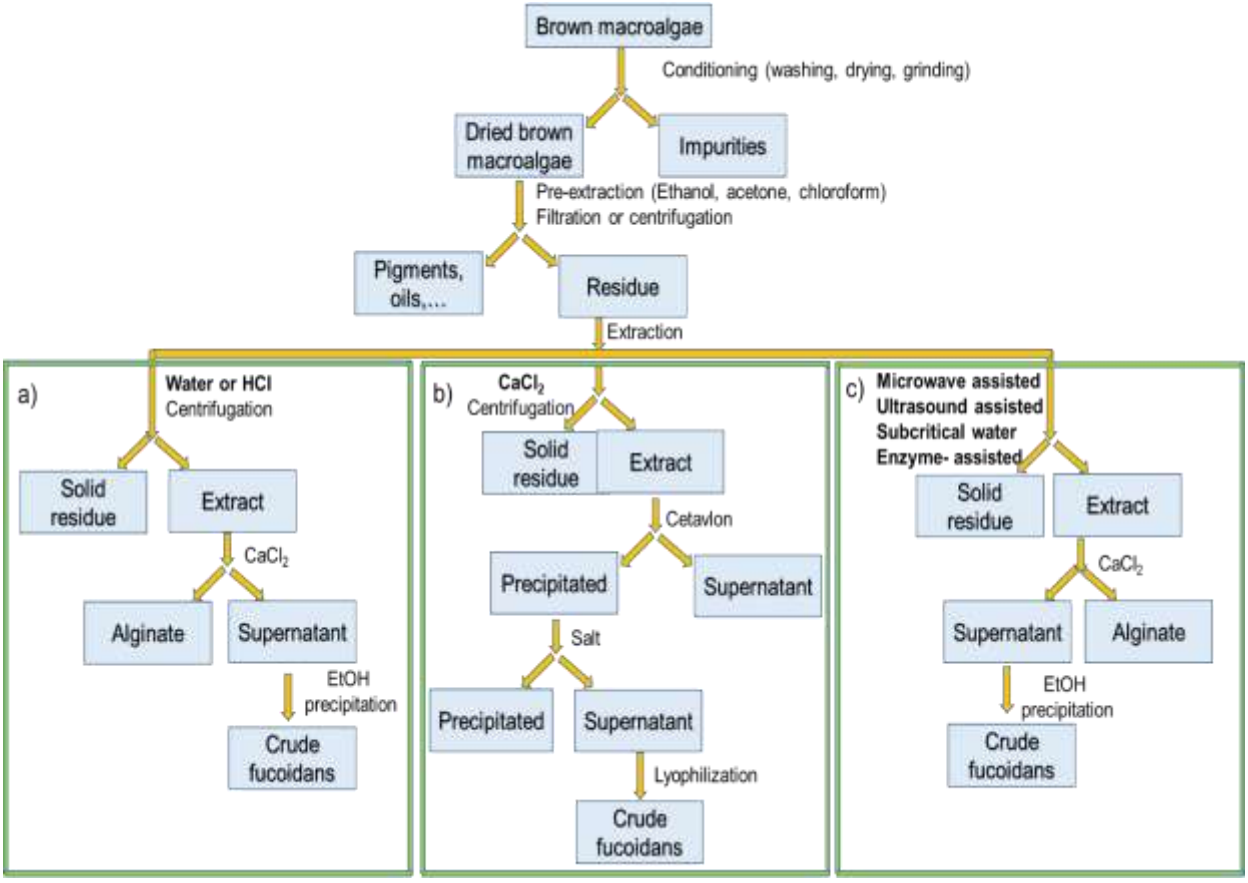


Figure 2.3 Flow chart of different fucoidan extraction procedures: a) water (van Weelden et al. 2019) and acid (Fletcher et al. 2017) extraction, b) Alkaline extraction (Bilan et al. 2006) and c) novel extraction techniques (Yuan and Macquarrie 2015; Hmelkov et al. 2018; Saravana et al. 2018; Nguyen et al. 2020)

In the alkaline extraction method, calcium chloride ($CaCl_2$) solutions has been applied to extract fucoidans from macroalgae (Figure 2.3). Commonly, the macroalgae were treated with 2 % aqueous solution of $CaCl_2$ at high temperatures and the process was often repeated (Bilan et al. 2006; Hmelkov et al. 2018). Mechanical agitation and high temperature was used for the extractions to increase the rate of extraction and dissolution of fucoidans. During the extraction process alginates were often found co-extracting with the fucoidans. The use of aqueous $CaCl_2$ solution as extraction solvent selectively precipitated alginate during the extraction process and the precipitate was removed with macroalgae residues by centrifugation. For increasing the fucoidan purity, the supernatant was treated by the cationic hexadecyltrimethylammonium bromide (Cetavlon) (Bilan et al. 2010; Thanh et al. 2013). Due to the negative charge of fucoidans, they can form water insoluble salts with cetavlon leading to precipitation, while other polysaccharides present in the supernatant such as laminaran are neutral and remain soluble in water. The salt complex of fucoidans-cetavlon was resolubilized by addition of sodium iodide in an ethanol solution, where fucoidans-cetavlon complexes were separated into fucoidan sodium salt (insoluble in ethanol) and cetavlon iodide (soluble in ethanol) (Thanh et al. 2013). The

precipitate was collected by centrifugation, dissolved in water and dialyzed to remove sodium and ethanol and dried (Thanh et al. 2013).

2.4.3 Novel techniques of fucoidan extraction

Ultrasound assisted extraction (UAE) has been applied to extract fucoidans from different brown macroalgae (Quitain et al. 2013; Song et al. 2015; Hmelkov et al. 2018). This method is based on the ultrasonic waves in solution, generating alternating high-pressure/low-pressure cycles, which results in the cavitation. The ultrasonic cavitation produces extreme high temperatures locally, differential pressure and high shear forces in solvent, which lead to disruption of the cell and cell wall. Ultrasonic-assisted extraction is a rapid, low solvent technique with potential for industrial up-scaling (Song et al. 2015). However, the ultrasound technique negatively affected the molecular weight of fucoidans during the extraction process. Fucoidans from *Undaria pinnatifida* extracted by an UAE method were reported having lower molecular weight (390 kDa) in comparison with fucoidans extracted by conventional (acid) method (528 kDa) (Song et al. 2015).

Microwave-assisted extraction (MAE) is another green method used for fucoidans extraction from macroalgae (Rodriguez-Jasso et al. 2011; Yuan and Macquarrie 2015; Ptak et al. 2019). In MAE microware induce vibrations of water molecules, increasing temperature of the intracellular liquids. This leads to water evaporation and increased pressure on the cell wall, disrupting the cell wall and the polysaccharides are released into the solution (Hahn et al. 2012). MAE is a thermal process, where the extraction time and temperature influence the monosaccharide composition and molecular weight of fucoidans. Fucoidans from *Ascophyllum nodosum* extracted by MAE at 90˚C contained fucose as the main sugar, while fucoidans extracted at 150 °C had glucuronic acid as the main component (Yuan and Macquarrie 2015). In the same study the molecular weight and sulfate content was also reported to be decreased depending on the extraction time (Yuan and Macquarrie 2015).

Enzyme-assisted methods were recently developed for fucoidans extraction. Enzymes are highly selective, and enzyme-assisted methods can be performed under mild extraction conditions and are furthermore environmentally friendly (Hahn et al. 2012). Since the cell wall of brown macroalgae is structurally complex, built up by different components (Deniaud-Bouët et al. 2014), a mixture of enzymes is required for degradation of the cell wall. The use of hydrolytic enzymes will selectively degrade the cell wall components, resulting in cell wall disruption and separation of the polysaccharides. So for fucoidan extraction, enzymes such as proteases, cellulases, and alginate lyases, can be used to hydrolyze proteins and polysaccharides, respectively, while not effecting the fucoidans. In most reported research papers different combinations of commercial enzymes were applied for fucoidan extraction: protease, cellulase, α-amylase, amyloglucosidase (Costa et al. 2010; Wijesinghe et al. 2011; Alboofetileh et al. 2018). The reported enzyme combinations used for fucoidan extraction from different macroalgae are shown in Table 2.1. However, the used enzymes have been used and developed for degradation of terrestrial plant materials, and are not specific for macroalgae polysaccharides. In our recent report, an alginate lyase was used in combination with cellulases for the first time for extraction of fucoidans from *F. evenescens* and *S. latissima* (Paper I: Nguyen et al. 2020). In this enzyme-assisted process, extraction time, pH and temperature were depended on the enzymes. The molecular weight of

fucoidans extracted by an enzyme-assisted method was higher than fucoidans from an acid extraction method (Sanjeewa et al. 2017; Nguyen et al. 2020).

To obtain fucoidans with higher purity for structure and bioactivity studies, crude fucoidans were further purified by different methods. Since fucoidans contain sulfate groups, having anionic charges, fucoidans were often purified by anion – exchange chromatography (Hahn et al. 2012). Other purification procedures such as membrane filtration, size exclusion or affinity chromatography have also been reported (Hahn et al. 2012).

Table 2. 1 Conventional and novel extraction methods of fucoidans from brown macroalgae. Some presented examples for different extraction methods.

Extraction method	Advantages	Disavantages	Macroalgae	Extraction procedure	References
Water extraction	Simplicity	-Low yield and selectivity -Long extraction time -High temperature - Using harmful chemicals solvent - Affect on the fucoidan fine structure	*Hizikia fusiforme*	Water, 70°C, 3 h, 3 times	(Li et al. 2006)
			Sargassum fusiforme	Water, 100°C, 4 h, 8 times	(Cong et al. 2014)
			Sargassum stenophyllum	Water, room temp., 12 h, 3 times	(Duarte et al. 2001)
Alkaline			*Fucus serratus*	2% aq $CaCl_2$, 85°C 5 h	(Bilan et al. 2006)
			U. pinnatifida	2% aq $CaCl_2$, 85°C, 5 h	(Mak et al. 2013)
			F. evanescens	2% $CaCl_2$, 60°C, 3 h, 2 times	(Hmelkov et al. 2018)
Acid method			*U. pinnatifida*	0.1 M HCl, room temp., 24h	(Song et al. 2015)
			F. evanescens	0.1 M HCl (pH 2–3), 60°C , 3 h	(Imbs et al. 2014)
			F. serratus, *Fucus vesiculosus*, *A. nodosum*	0.1 M HCl, 80 °C, 4 h	(Fletcher et al. 2017)
Ultrasound assisted	- Simplicity - Low cost - Effective mixing - Extraction at low temperature	- Noise pollution - Degradation of polysaccharides.	*U. pinnatifida*	0.8 g alga/10ml water, 5−120 min, 110−200°C	(Quitain et al. 2013)
			U. pinnatifida	0.1 M HCl, US at three different amplitude levels for 3, 6 and 24 h	(Song et al. 2015)
			F. evanescens	Water, 23°C, 15 min	(Hmelkov et al. 2018)

Microwave assisted	- Fast and uniform heating - Short extraction time - Less solvent requirement	- High energy consumption - Affect on chemical characters (monosaccharide composition and molecular weight) of polymer	*F. vesiculosus*	120 psi, 1 min, 1 g alga/25 ml water	(Rodriguez-Jasso et al. 2011)
			A. nodosum	90–150 °C, 5–30 min	(Yuan and Macquarrie 2015)
			F. vesiculosus, F. serratus, *F. evanescens*	10 mM H_2SO_4 and 100 mM HCl at 80°C, 100°C, 120°C	(Ptak et al. 2019)
Enzyme assisted	- Moderate temperature - High selective of enzyme - High yield of polysaccharide - Ecofriendly and nontoxic - Conserving the fucoidan structure	- Need to optimum the extraction condition depending on enzymes - Extraction efficiency depend on enzyme properties. - High cost of enzyme - Slow process - Limited enzyme recycle	*Chnoospora minima*	0.5% Celluclast, 50C°, pH 4.5, 24 h	(Fernando et al. 2017)
			Nizamuddinia zanardinii	Alcalase (protease) (pH 8, 50°C, 24 h), Celluclast (pH 4.5, 50°C, 24 h), Viscozyme (pH 4.5, 50°C, 24 h) or Flavourzyme (pH 7, 50°C, 24 h).	(Alboofetileh et al. 2018)
			Ecklonia cava	Amyloglucosidase (12h)	(Wijesinghe et al. 2011)
			Turbinaria turbinata	Cellulase, amyloglucosidase and viscozyme	(Hammed et al. 2017)
			Dictyota cervicornis, *Dictyopteris delicatula*, *Dictyota menstrualis*, *Dictyota mertensii* *Sargassum filipendula* and *Spatoglossum schroederi*	Protease (pH 7, 24 h at 60°C)	(Costa et al. 2010)
			F. evenescens and *S. lattisima*	Cellic®CTec2, Alginate lyase (pH 6, 24 h at 40°C)	(Nguyen et al. 2020)

2.5 Fucoidan degradation and low molecular weight fucoidan products

The complex and heterogeneity of native fucoidans limit structural studies (Wang and Zhang 2017). In addition, native fucoidans have currently limited use in biomedical applications due to their high molecular weight and viscosity (Morya et al. 2012). Therefore, degradation of the large polymer into shorter and more linear structures - low molecular weight products (Stefaniak-Vidarsson et al. 2020) is useful for structure investigations of fucoidans as well as for improving their application. Low molecular weight fucoidans (LMFs) can be obtained by different physical, chemical and enzymatic hydrolysis methods (Moon et al. 2011; Choi and Kim 2013; Vuillemin et al. 2020). The LMFs differ in molecular weight, composition, structure and bioactivities related to the source of fucoidans and preparation methods (Stefaniak-Vidarsson et al. 2020). Therefore, the LMFs preparation technique is very important for producing oligosaccharides. Some selected reports on LMFs preparation by different methods are shown in Table 2.2.

Autohydrolysis of fucoidans is a simple way to obtain LMFs. Fucoidans from *F. evanescens* were changed to the H^+ form using a cation-exchange minicolumn and left at 37°C for 40 hours resulted in oligosaccharides with polymerization degree of DP 2, 4 and 6 (Anastyuk et al. 2012, 2017). In another study, LMFs of 6 kDa was obtained after 72 hours autohydrolysis of fucoidans from *S. cichorioides* (Anastyuk et al. 2017). Yet another and simple method to depolymerize fucoidans is by hydrothermal treatment. The fucoidan samples were heated at 120–180 °C for 5–60 min resulting in mixtures of LMFs (Morimoto et al. 2014; Lahrsen et al. 2018). The molecular weight of the products depends on the temperature and treatment time. Conventional acid hydrolysis of fucoidans is commonly used for producing LMFs. This method is not specific, results in random degradation of the polymer into LMFs with different molecular weights and fine-chemical structures. Different acids have been used to degrade fucoidans such as HCl, H_2SO_4 (Pielesz et al. 2011), pyruvic and acetic acid (Moon et al. 2011). Radical methods of fucoidan degradation was also reported using chemical (hydrogen peroxide) or irradiation. The incubation time, H_2O_2 concentration and temperature effect the size of the LMFs. During the process, fucoidans from *F. vesiculosus* were gradually degraded to 4.9 kDa without removing the sulfate groups (Lahrsen et al. 2018). Degradation of fuciodans by H_2O_2 treatment appears more efficient compared to the hydrothermal treatment method (Lahrsen et al. 2018). In another study, when fucoidans from *F. vesiculosus* were treated by gamma-irradiation at intensities of 10 kGy/h, the LMFs were quickly produced, also without affecting the sulfate groups (Choi and Kim 2013).

Enzymatic hydrolysis of fucoidans is a promising method for preparing LMFs with many advantages such as highly specific cleavage site, mild reaction conditions and short treatment times. The type of enzymes used for preparing LMFs are named endo-fucoidanases. They have been found in marine bacteria, some fungi and invertebrates (Kusaykin et al. 2015). Endo – fucoidanases are able to cleave glycosidic bonds in fucoidans resulting in oligosaccharides. The enzymes have high selective bond cleaving specificity, therefore the obtained LMFs are homogenous and with a homogenous fine-chemical structure determined by the specificity of the fucoidanase used as well as the fucoidan substrate. Some studies of enzymatic degradation of fucoidans have been reported using partially purified enzymes from bacterial cultures (Bakunina et al. 2002; Kim et al. 2010b) and recombinant endo-fucoidanases (Colin et al. 2006; Silchenko et al. 2017a; Vuillemin et al. 2020; Tran et al. 2022; Trang et al. 2022). LMFs obtained from enzymatic hydrolysis have been separated, the chemical structure determined and the bioactivities in some

reports been investigated. After hydrolysis the LMFs were separated by ultrafiltration (Sakai et al. 2003b) or chromatography column such as DEAE Sepharose CL6B (Colin et al. 2006), Biogel P-6 (Silchenko et al. 2013), Q-Sepharose High performance (Silchenko et al. 2017a) to obtain pure oligosaccharides. LMFs differ in structure dependent on the source of fucoidans and the specificity of the endo-fucoidanases. Most of reported LMFs are sulfated oligosaccharides made up from 2 to 10 monosaccharides in linear structure. Recent reports on enzymatic hydrolysis of fucoidans are summarized in Table 2.2. NMR analysis on the oligosaccharides give information about the enzyme specificity and the fucoidan fine-chemical structure.

2.6 Bioactivities of low molecular weight fucoidans

LMFs with low viscosity, small molecular weight, high solubility and simple homogenous structure exhibit various interesting biological activities. The polymerization degree and sulfate groups of LMFs play an important role in the biological properties (Stefaniak-Vidarsson et al. 2020). Selected bioactivities of some LMFs prepared by different methods were showed in Table 2.2. Native fucoidans from *F. evanescens* exhibited anticancer activities in both SK-MEL-28 and SK-MEL-5 human malignant melanoma cell lines (Anastyuk et al. 2012). While the mixture of LMFs from *F. evanescens*, prepared by the auto-hydrolysis method, showed strong inhibition of colony formation only in the cell line SK-MEL-28 (Anastyuk et al. 2012). So here the effect of the presence of sulfates and (1→4)-linked α-L-Fuc*p* residues in the main chain of fucoidan/oligosaccharides on anticancer activity was suggested (Anastyuk et al. 2012). In another study LMF from *F. evanescens* prepared by enzymatic hydrolysis showed immunomodulating activity, inducing increase of the number of $CD3^+$-lymphocytes and $CD16^+$-cells (Silchenko et al., 2013). The native fucoidans and high molecular weight products from enzymatic hydrolysis of fucoidans from *S. horneri* showed activity against colony formation of colorectal carcinoma, while LMFs product lost the activity (Silchenko et al., 2017a). So in this case the degree of polymerization influences the anticancer activity (Silchenko et al., 2017a). Various bioactivities of LMFs were reported such as antihypertensive, hepatoprotective, renal protective, anti-inflammatory, antiviral, anticoagulant (Stefaniak-Vidarsson et al. 2020). In our recent study, LMFs from *F. evanesens* showed bone re-generation effects and enhanced implant fixation in a sheep model (Nielsen et al. 2021).

Table 2.2 Degradation of fucoidans by different methods and bioactivities of low molecular weight products

Source of fucoidans	Method of fucoidan degradation	Cleaving linkages	Molecular weight , structure of LMFs	Bioactivities	References
Fucus evanescens [3)-α-L-Fuc*p*-(2,4 OSO_3^-)- -1→4-α-L-Fuc*p*-(2 OSO_3^-)-(1→];	Autohydrolysis 5 mg/mL fucoidan was changed to the H^+-form and left for 40 h at 37°C.	(1→3)-type of linkages were cleaved faster than (1→4)-type of linkages	Monosulfated fucose and sulfated fucooligosaccharides with polymerization degree (DP) 2, 4 and 6, including galactose-containing sulfated oligosaccharides.	Anticancer in human malignant melanoma cell lines SK-MEL-28	(Anastyuk et al. 2012)
Brown macroalgae from Haewon Biotech, Korea	Acid hydrolysis Fucoidan (0.1 - 20%, w/v) were treated with 2 N pyruvic acid and 2 N acetic acid (1:4) for 5 h at 75°C		Average molecular mass 3 ± 0.5 kDa	LMWF enhances the regeneration of hair cells damaged by neomycin	(Moon et al. 2011)
Laminaria japonica	Radical method Hydrogen peroxide		Mixture of LMFs with different size	Antioxidant activities	(Hou et al. 2012)
Fucus vesiculosus →3)–α-L-Fuc*p*-(1→3)-α-L-Fuc*p*- (1→4)-α-L-Fuc*p* (2 OSO_3^-)-(1→ Branch*:* α-L-Fuc*p* (1→4)	Hydrothermal treatment 1 % (m/m) fucoidan solution was heated at 120 °C for 10, 20, 30, 40, 60 and 90 min Hydrogen peroxide 1 % (m/m) fucoidan solution in different hydrogen peroxide concentration was incubated at several temperature and time points		Mw was dropped from 38.2 kDa to 10.3 kDa after 90 min heating Mw was dropped to 4.9 kDa after 3% hydrogen peroxide treatment at 50 °C	Antioxidant and antiproliferative activities	(Lahrsen et al. 2018)
Fucus vesiculosus	Radical method Gamma-irradiation		7 kDa	Anticancer in Human breast cancer cells (MCF-7), human stomach cancer cells	(Choi and Kim 2013)

→3)–α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p* (2OSO_3^-)-(1→ Branch*:* α-L-Fuc*p* (1→4)	Fucoidan (10 mg/mL) was gamma-irradiated with a strength of 11.1 petabecquerel (pBq) at 22 ± 2 °C at a dose rate of 10 kGy/h.			(AGS), and human liver cancer cells (HepG-2)	
L. cichorioides [3)-α-L-Fuc*p*-(2,4 OSO_3^-)-1→3-α-L -Fuc*p*-(2,4OSO_3^-)-(1→]	Enzymatic hydrolysis Partially purified fucoidan from *Pseudoalteromonas citrea* KMM 3296, KMM 3297 and KMM 3298 strains	Endo-type hydrolysis	Sulfated α-L-fucooligosaccharides from 2.2 to 5.0 kDa for strain KMM 3297, From 1.7 to 5.0 kDa for strain KMM 3296 and from 1.3 to 5.0 kDa for strain KMM 3298:		(Bakunina et al. 2002)
Kjellmaniella crassifolia (-4-D-Glc*p*UAβ1-2(L-Fuc*p*(3-O-sulfate)α(1-3)D-Man*pα*1-)$_n$	Enzymatic hydrolysis Sulfated fucoglucuronomannan (SFGM) Lyase from marine bacterium *Fucobacter marina*	α-D-mannosyl linkage between D-Manp and D-GlcpUA residues	3 trisaccharides: $\Delta^{4,5}$Glc*p*UA1-2(L-Fuc*p*(3-O-sulfate)-α-(1-3) -Man*p*, $\Delta^{4,5}$Glc*p*UA1-2(L-Fuc*p*(3-O-sulfate) α-(1-3)-D -Man*p*(6-O-sulfate), and $\Delta^{4,5}$GlcpUA1-2(L-Fuc*p*(2,4-O-disulfate) α (1-3)-D-Man*p*(6-O-sulfate)		(Sakai et al. 2003b)
Cladosiphon okamuranus [3)-α- L-Fuc*p*-1→3-α-L-Fuc*p*-(4 OSO_3^-)-1→3-α-L -Fuc*p*-(4OSO_3^-) -1→3-(GlcUA-α-D-1→2)-α-L-Fuc*p*].	Enzymatic hydrolysis Intracellular enzymes of marine bacteria *Fucophilus fucoidanolyticus*	Endo fucoidanase And α-D glucuronidase SGUF-deacetylase	8 different oligosaccharides with size from 0.762 to 4.219 kDa The general structure formula: [(3)-α- L-Fuc*p*-1→3-α- L -Fuc*p*-(4OSO3$^-$)-1→3-α-L-Fuc*p*-(4OSO3$^-$)-1→3(GlcUA-α-D-1→2)-α- L -Fuc*p*)$_m$, −3)-α- L-Fuc*p*-1→3-α-L-Fuc*p*-(4OSO3$^-$)-1→3-α-L-Fuc*p*-(4OSO3$^-$)- 1→3-α-LFuc*p*-(1→]; (m = 0, 1, 2, or 3)		(Sakai et al. 2003a)

Fucus distichus [3)-α-L-Fuc*p*-(2,4OSO$_3^-$)-1→4-α-L-Fuc*p*-(2,3OSO$_3^-$)-1→]	Enzymatic hydrolysis Enzyme from marine mollusk *Littorina kurila*		Molecular weight of LMW product was ≥7 kD		(Bilan et al. 2005)
Pelvetia canaliculata [3)-α-L-Fuc*p*-(2OSO$_3^-$)-1→4-α-L-Fuc*p*-(2,3OSO$_3^-$)-(1→]	Enzymatic hydrolysis Recombinant fucoidanase from *Flavobacteriaceae* SW5 FcnA	α-(1→4) glycosidic linkages within the blocks of repeating motifs [→4)-α -L-Fuc*p*-(2,3OSO3$^-$)-(1→3)-α-L-Fuc*p*-(2OSO3$^-$)-(1→]n.	Tetra- and hexasaccharides: [3)-α-L-Fuc*p*-(2OSO3$^-$)-(1→4)-α-L-Fuc*p*-(2,3OSO3$^-$)-(1→]$_n$		(Colin et al. 2006)
Undaria pinnatifida Galactofucan	Enzymatic hydrolysis Partially purified fucoidanase from *Sphingomonas paucimobilis* PF-1.		Mixture of LMFOs from 0.305 to 3.749 kDa	Anticoagulating activities	(Kim et al. 2010b)
F. evanescens [3)-α-L-Fuc*p*-(2,4OSO$_3^-$) - -1→4-α-L-Fuc*p*-(2OSO$_3^-$)- (1→];	Enzymatic hydrolysis Purified fucoidanase from *Formosa algae* strain KMM 3553	α-(1→4) glycosidic linkages	A mixture of disaccharides with a ratio of 2:1 following structures α-L-Fuc*p*2OSO$_3^-$-(1→3)-α-L-Fuc*p*2OSO$_3^-$ and α-L-Fuc*p*2,3OSO$_3^-$-(1→3)-α-L-Fuc*p*2OSO$_3^-$	Immunomod-ulating: Induced a significant increase of CD3+-lymphocytes and CD16+-cells	(Silchenko et al. 2013)
F. evanescens [3)-α-L-Fuc*p*-(2,4OSO$_3^-$) -1→4-α-L-Fuc*p*-(2OSO$_3^-$)-(1→];	Enzymatic hydrolysis Fucoidanase from *Lambis sp*	α-(1→4) glycosidic linkages	Sulfated fucooligosaccharides contained α-1→3, α-1→4-linked fucose residues: mainly disaccharide, tetrasaccharide, sulfated branched pentasaccharide,		(Silchenko et al. 2014)
F. evanescens [3)-α-L-Fuc*p*-(2,4OSO$_3^-$)	Enzymatic hydrolysis	(1→4)-α-glycosidic			(Silchenko et al. 2017b)

-1→4-α-L-Fuc*p*-(2OSO$_3^-$)-(1→];	Recombinant fucoidanase FFA2 from *Formosa algae* strain KMM 3553	bonds within fragment (→3)-α-L-Fuc*p*2S-(1→4)-α-L-Fuc*p*2S-(1→)n			
Sargassum horneri →3-α-L-Fuc*p* -(2SO$_3^-$) -1→4-α-L-Fuc*p*(2,3SO$_3^-$)-1→ Branch: α-LFuc*p*-1→2-α-L-Fuc*p*-1→ or α-L-Fuc*p*-1→3-α-L-Fuc*p* -(4SO$_3^-$)-1→	Enzymatic hydrolysis Recombinant fucoidanase FFA1	α-(1→4) glycosidic linkages	5 sulfated oligosaccharides with polymerization degree from 4 to10. The basic motif of the LMP structure is sulfated tetrasaccharide and sulfated hexasaccharide.		(Silchenko et al. 2017a)
F. evanescens [3)-α-L-Fuc*p*-(2,4OSO$_3^-$)--1→4-α-L-Fuc*p*-(2OSO$_3^-$)-(1→];	Enzymatic hydrolysis Recombinant fucoidanase FFA1	α-(1→4) glycosidic linkages	Sulfated tetrasaccharide α-L-Fuc*p*2S-(1→3)-α-l-Fuc*p*2S-(1→4)-α-L-Fucp2S-(1→3)-α-l-Fuc*p*2S		(Silchenko et al. 2018)
F. evanescens [3)-α-L-Fuc*p*-(2,4OSO$_3^-$)--1→4-α-L-Fuc*p*-(2OSO$_3^-$) -(1→];	Enzymatic hydrolysis Fucoidanase Fhf1 from *Formosa haliotis*	α-(1,4)-glycosidic linkages in structural motif [→3)-α-L -Fucp2S-(1,4)-α- L -Fucp2S-(1→]	Sulfated tetrasaccharide α- L -Fuc*p*2S-(1→3)-α-L-Fuc*p*2S-(1→4)-α-L-Fuc*p*2S-(1→3)-α-L-Fuc*p*2S Octa- and decasaccharides	Effect on bone regeneration and enhance implant fixation	(Vuillemin et al. 2020; Nielsen et al. 2021)
Isostichopus badionotus	Enzymatic hydrolysis Endo-1,3-Fucanase from *Wenyingzhuangia fucanilytica*	α-1,3 glycosidic linkages	α-L-Fuc*p*-1→3-α-L-Fuc*p* -(2,4OSO$_3^-$)-1→3-α-L-Fuc*p* -(2SO$_3^-$)-1→3-α-L-Fuc*p* -(2OSO$_3^-$)		(Shen et al. 2020)
Laminaria japonica	Enzymatic hydrolysis Fucoidanase from *Flavobacteriaceae* RC2-3		LMW with different size from Lower then 5 to over 100 kDa	Tyrosinase inhibitory activity	(Wang et al. 2021c)
S. latissima	Enzymatic hydrolysis Fucoidanase from Marine Bacterium *Muricauda eckloniae*	α-1,3 glycosidic linkages	Sulfated octasaccharide		(Tran et al. 2022)

2.7 Alginates and alginate lyase

2.7.1 Alginate

Alginates are linear anionic polysaccharides found mainly in the cell wall and intercellular matrix of brown macroalgae (Kloareg and Quatrano 1988). In addition, some bacterial species of the genera *Pseudomonas* and *Azotobacter* also produce alginates as extracellular polysaccharides (Remminghorst and Rehm 2006). Alginates are built up from mannuronic acid (M) and glucuronic acid (G) residues which are linked by 1→4-O-glycosidic bonds, forming different patterns in blocks of M residues (polyM), G residues (Poly G) and alternating M- and G-residues (Poly MG or GM)(Haug et al. 1974). The alginate content and structure depends on many factors such as the macroalgae species (Chee et al. 2010), time of collection (Saraswathi et al. 2003; Bertagnolli et al. 2014) and location of the macroalgae (Venegas et al. 1993).

Alginates form gels with divalent ions, in particular calcium (Ca^{2+}), which interact with G blocks (Grant et al. 1973). Alginate is widely applied in food and pharmaceutical industries as a gelling, thickening, stabilizing and emulsifying agents (Puscaselu et al. 2020). Recently, alginate degradation products by enzymes were intensively studied for their application in agricultural and medical area (Mrudulakumari Vasudevan et al. 2021). Alginate oligosaccharides with low molecular weight have various bioactivities such as antioxidant, antitumor, immunomodulatory, antimicrobial, prebiotic, antihypertensive, antidiabetic (Liu et al. 2019b).

2.7.2 Alginate lyase

The enzymes degrading alginate, the alginate lyases, are a group of polysaccharide lyases (PL), cleaving the glycosidic bonds in alginate through a β-elimination mechanism to produce alginate oligosaccharides (Thiang et al. 2003). Based on the substrate specificities, alginate lyases are classified as polyM-specific lyase (EC4.2.2.3) - enzymes catalyze the cleavage of α-1→4-O-glycosidic bonds between β-D-mannuronic acid residues, polyG-specific lyase (EC 4.2.2.11) - enzymes catalyze the cleavage of α-1→4-O-glycosidic bonds between L-guluronic acid residues and bifuntional lyase, which are active on both polyM and polyG (EC 4.2.2.-). By sequence similarity alginate lyases are classified in different polysaccharide lyase families: PL5, PL6, PL7, PL14, PL15, PL17, PL18, PL31, PL32, PL34, PL36 and PL39 (Zhang et al. 2021)(Helbert et al. 2019; Ji et al. 2019). Alginate lyases are also divided into endolytic or exolytic type according to their catalytic reaction mechanism. The PL6 family of alginate lyases is multispecific and the lyases are further divided into three subfamilies (PL6_1-3). PL6_1 contain alginate lyases that have broad substrate specificities. PL6_2 and PL6_3 have been classified as endo-poly-MG-lyases (Mathieu et al. 2016).

Alginate lyases have been found in different organisms such as macroalgae, mollusks and bacteria and fungi (Zhu and Yin 2015; Pilgaard et al. 2021). Some representative alginate lyases are shown in Table 2.3. Most of the reported alginate lyases are endolytic lyases and belong to family PL6 and PL7 (Zhang et al. 2021). Bacteria often produce more than one alginate lyase (Table 2.3). Alginate lyases are a valuable tool in alginate oligosaccharide production and in the determination of alginate structure (Aarstad et al. 2012), isolation of protoplasts from brown macroalgae (Inoue et al. 2010), and for

utilization of macroalgae to release fucose (Manns et al. 2016). Recently, in our study alginate lyases were also used in fucoidan extraction from brown macroalgae (Chapter 4).

Table 2.3 Alginate lyases examples from different sources

Enzyme source	Bacterial source	PL	Alginate lyase	Substrate specificity	Mode of action	References
Bacteria	*Photobacterium* sp. FC615	PL6 PL15	AlyPB1 AlyPB2	polyM polyM, polyG	Endolytic Exolytic	(Lu et al. 2019)
	Formosa algae KMM 3553T	PL7 PL6	ALFA3 ALFA4	polyM, polyG polyM	Endolytic	(Belik et al. 2020)
	Pseudoalteromonas carrageenovora ASY5	PL7	Aly1281	polyM, polyG	Endolytic	(Zhang et al. 2020)
	Microbulbifer sp. Q7	PL7	AlyM	polyG	Endolytic	(Yang et al. 2018)
	Sphingomonas sp.	PL7	SALy	polyM	Endolytic	(Manns et al. 2016)
Fungi	*Paradendryphiella salina*	PL7	PsAlg7A, PsAlg7B, and -C	polyM polyM and polyG	Endolytic	(Pilgaard et al. 2021)
		PL8	PsMan8A	polyM	Exolytic	
Mollusk	*Haliotis discus hannai* *Haliotis iris* *Omphalius rusticus*	PL14 PL14 PL14	HdAly HiAly OrAly	polyM polyM polyM	Endolytic Endolytic Endolytic	(Hata et al. 2009)
	Lambis sp			polyG	Endolytic	(Sil'Chenko et al. 2013)
	Littorina brevicula	PL14	LbAly28	polyM	Endolytic	(Rahman et al. 2012)
Algae	*Saccharina japonica*		SjAly	polyM	Endolytic	(Inoue and Ojima 2019)

Chapter 3: Methodology

3.1 Materials

3.1.1 Brown macroalgae

Brown macroalgae from different orders were collected as presented in Table 3.1 *S. latissima* from Iceland was provided as dried flakes by Blaskel (Stykkisholmur, Iceland). Other macroalgae was washed with fresh water to remove impurities and dried. Then the dried macroalge were ground into powder and stored at room temperature for later fucoidan extraction.

Table 3.1 Brown macroalge that were studied in this thesis

Brown macroalge	**Order**	**Location**	**Collection time**	**Provided organization**
F. evanescens	*Fucales*	Kiel fjord, Germany	March 2017	Coastal Research & Management (Kiel, Germany)
S. mcclurei	*Fucales*	Nha Trang, Vietnam	April 2018	NhaTrang Institute of Research and Application (Vietnam)
S. latissima	*Saccharina*	Iceland	June 2017	Blaskel (Stykkisholmur, Iceland).
S. latissima (cultivated)	*Saccharina*	Faroe Islands	March 2016	Ocean Rainforest

3.1.2 Enzymes

Cellic®CTec2 is a commercially available cellulase prepared based on the *Trichoderma reesei* cellulolytic enzyme complex (Nguyen et al. 2020). The enzyme was provided from Novozymes (Bagsværd, Denmark). The optimal temperature and pH of Cellic®CTec2 are 45 – 50 °C and pH 5.0 – 5.5.

The alginate lyase SALy was expressed in autoinduction media as described in Manns et al., (2016) (Manns et al. 2016). The enzyme was purified by a Ni^{2+} Sepharose HisTrap HP column resin (GE Healthcare, Uppsala, Sweden) in elution buffer containing 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 100 mM imidazole as described in PAPER I (Nguyen et al. 2020). The imidazole was removed by using PD10 column (Sephadex G-25, GE Healthcare Uppsala, Sweden). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the purity of the enzyme and the enzyme concentration was determined by the Bradford method (Bradford 1976). SALy showed high activity at pH from 5.5 to 7 with maximum at pH 6.5 and temperature from 30 to 50 °C.

Alginate lyases from sea cucumber gut were expressed in *E. coli* and purified by the same method as described about for SALy. All the experiments related to enzyme characterization and activity on alginate from three different brown algae were descripted in Chapter 6.

3.2 Experimental flow

The PhD project was centered on the development of new enzymatic extraction method of fucoidans from different brown macroalgae and preparation of fucoidan oligosaccharides by enzymatic hydrolysis. Another part of the project was characterization of a new alginate lyase from sea cucumber gut bacteria and degradation of alginates from different brown macroalgae sources (Figure 3.1). The analytical methods of the macroalgae and fucoidans are presented in the following section. The detailed procedures of enzyme - assisted fucoidan extraction and oligosaccharide preparation are described in Chapter 4 and Chapter 5, respectively.

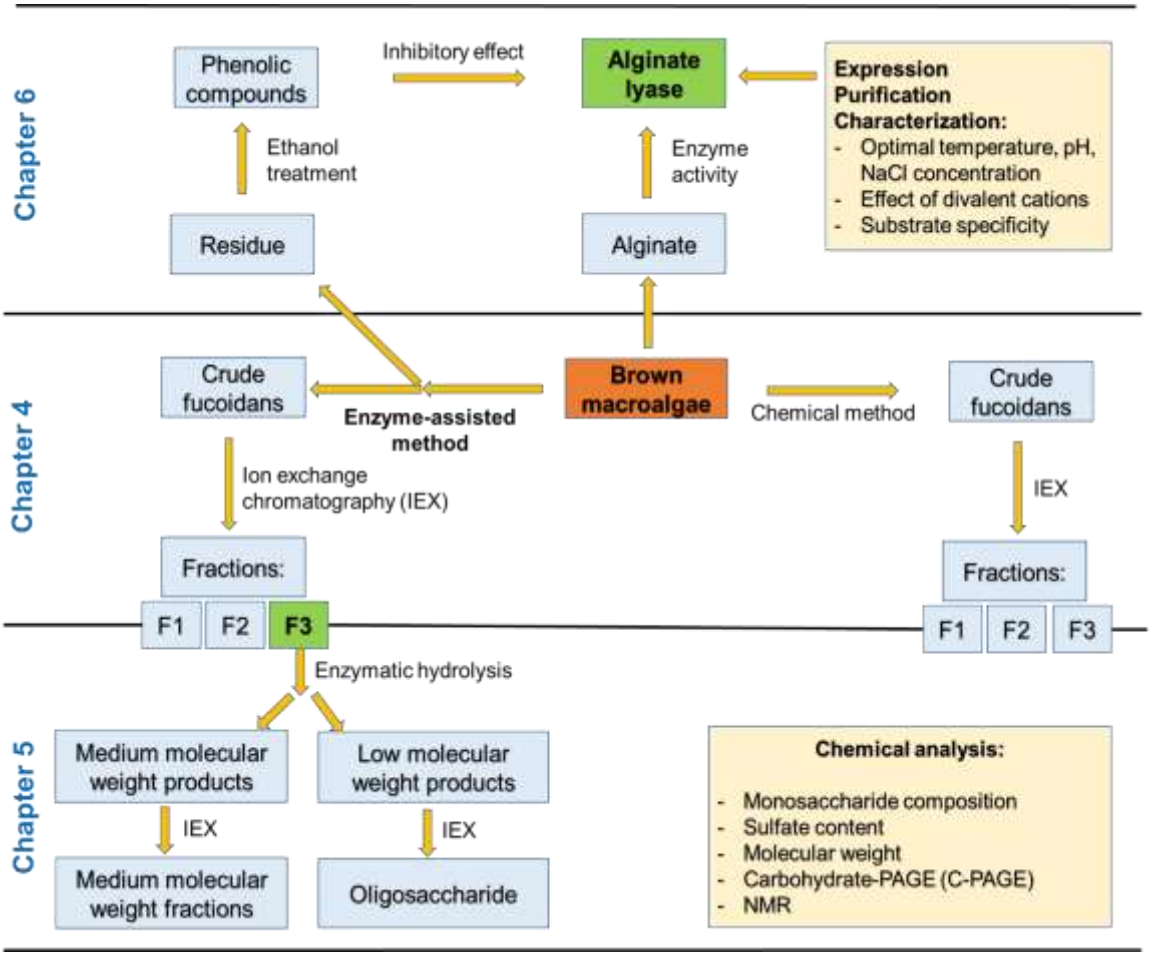


Figure 3.1 The overall experimental flow of the PhD project

3.3 Analysis methods of macroalge and fucoidans

3.3.1 Acid hydrolysis of samples

The macroalgae and fucoidan samples (5 mg) were hydrolyzed in acid by two step: First in 50µl 72% H_2SO_4 at 30 °C for 1 hour and then in 4% H_2SO_4 for 40 min at 120 °C in an autoclave (Manns et al. 2014). The hydrolysates were filtered through 0.22 µm syringe

filter and used for monosaccharide analysis (Figure 3.2a). The samples were hydrolyzed in 2M TFA (trifluoroacetic acid) (5-6 mg/mL) at 100°C for 6 hours. After hydrolysis, the TFA was removed by epovaration and the hydrolysates were used to determine the sulfate content (Figure 3.2b).

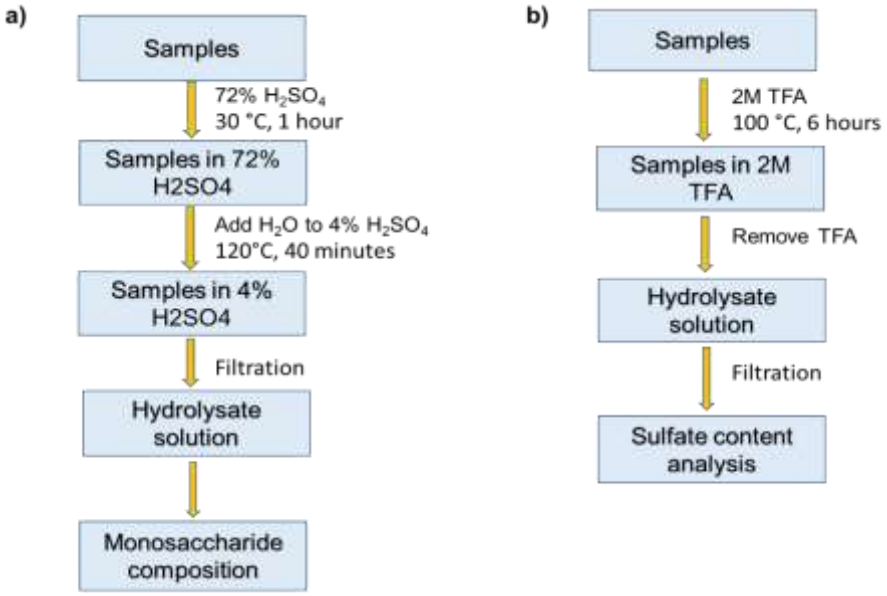


Figure 3.2 Acid hydrolysis of samples by sulfuric acid (a) for monosaccharide composition analysis and by trifluoroacetic acid (b) for sulfate content determination.

3.3.2 Chemical composition analysis

Monosaccharide composition and uronic acids of hydrolysates were analyzed on Dionex ICS-3000 HPAEC-PAD system with pulsed amperometric detector (PAD) and gold working electrode. The chromatographic separation process is presented in Figure 3.3. Data quantification was analyzed by Chromeleon™ 7.2 (Thermo Scientific). Recovery values of the monosaccharides and uronic acid were estimated from runs in triplicate at the same time (Manns et al. 2014).

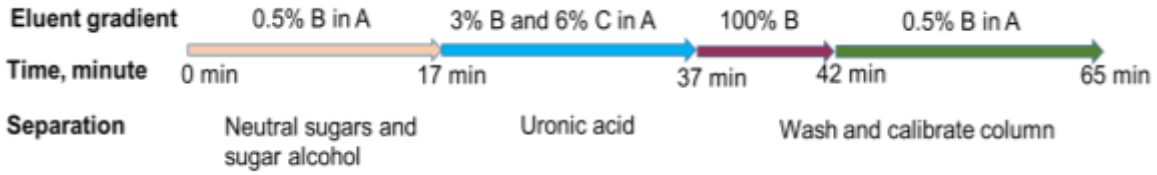


Figure 3.3 Chromatographic separation of sugar and uronic acids. A - deionized water, B - 200 mM NaOH and C - 200 mM NaOH, 1 M NaOAc.

3.3.3 Determination of sulfate content

The sulfate content was determined by following methods:
- The $BaCl_2$ gelatin method (Dodgson and Price 1962) (used in Chapter 4): 10 µl of hydrolysate solution was mixed 160 µl TCA and 100µl $BaCl_2$-gelatin reagent (0.5% $BaCl_2$ in 0.5% gelatin solution). The mixture was allowed to stand for 30 min. A blank was prepared with 170 µl TCA and 100µl $BaCl_2$-gelatin reagent. The released $BaSO_4$

suspension was measured at λ = 360 nm by UV–VIS spectrophotometry using potassium sulfate as standard.
- The turbidimetric method (Jackson and McCandless 1978) (used in Chapter 5): 110 µL hydrolysates were added to 120 µL 8% TCA. Then 60 µL 2 % $BaCl_2$ in 15 % PEG6000 reagent was added. The mixture was allowed to stand for 35 min. The released $BaSO_4$ suspension was measured at 500 nm in a microplate reader (TECAN Infinite 200, Salzburg, Austria). $BaSO_4$ was used as standard to generate a linear standard curve for the sulfate response.

3.3.4 Determination of molecular weight by size exclusion chromatography

The samples (fucoidans and fucoidan fractions) (3-5 mg) were dissolved in 1 mL elution buffer 100 mM sodium acetate, pH 6 and filtered through membrane filters with pore size 0.22 µm. The molecular weight (Mw) distribution of polysaccharides were determined by High Performance Size Exclusion Chromatography (HPSEC) using an Ultimate iso-3100SD pump with WPS-3000 sampler (Dionex, Sunnyvale, CA, USA) equipped with an RI-101 refractive index detector (Shodex, Showa Denko K.K., Tokyo, Japan). The samples were separated by Shodex SB-806 HQ GPC column (300×8 mm) connected with a Shodex SB-G guard column (50 mm×6 mm) (Showa Denko K.K., Tokyo, Japan) at 40 °C with flow rate of 0.5 mL/min. Pullulan of molecular weights of 1, 5, 12, 110, 400 and 800 kDa were used as reference standards (Cao et al. 2018).

3.3.5 Carbohydrate-Polyacrylamide Gel Electrophoresis (C-PAGE)

The method is based on the separation of polyanionic polymer and oligosaccharides in polyacrylamide gel according to their molecular weight and charge to mass ration (Min and Cowman 1986). This method was successfully used to evaluate the distribution of sulfated fucoidan oligosaccharides (Colin et al. 2006; Silchenko et al. 2013) and alginate oligosaccharide after enzymatic reaction (Belik et al. 2020). The samples were prepared in loading buffer containing 20% (v/v) glycerol and 0.02% (w/v) phenol red solution in water. The mixture was applied in 20% (w/v) polyacrylamide gel and electrophoresed for 90 min in 100 mM Tris-borate buffer pH 8.3. The gel was stained by mixture of 0.5% alcian blue 8 GX (Panreac, Barcelona, Spain) in 2% acetic acid and 0.02% O-toluidine (Sigma-Aldrich, Steinheim, Germany) in ethanol for 1 hour and then washed with distilled water several time until oligosaccharide bands were appeared (PAPER II, Tran et al. 2022).

3.3.6 NMR analysis

NMR spectra were recorded using 800 MHz Bruker Avance III HD instrument equipped with a 5 mm TCI cryoprobe and a SampleJet sample changer. The samples (10 mg) were prepared in 500 µl of $2H_2O$ and placed in 5 mm tubes. NMR spectra of were acquired at 25 °C for fucoidan oligosaccharides and 50°C for high molecular weight fucoidans. All NMR spectra were processed with ample zero filling in all dimensions and baseline correction and analyzed using Bruker Topspin 3.5 pl7 software (PAPER II, Tran et al. 2022).

3.3.7 Statistical analysis

The fucoidan extraction was performed in duplicate with triplicate in analytical experiments. All experiments for alginate lyase were done in triplicate. Statistical

differences were analyzed by one-way analysis followed by Tukey HSD comparison test (JPM 14 software, SAS). P values < 0.05 were considered statistically significant. The data were expressed as the average value of independent triplicates in all figures with standard deviations.

Chapter 4: Enzymatic extraction of fucoidans from brown macroalgae

The hypothesis of this work is that the enzymatic treatment of brown macroalgae can extract fucoidans with conserved molecular weight and sulfate content, which will retain the bioactivity. Based on this hypothesis the objective of the work is the development of a new enzyme-assisted fucoidan extraction from brown algae *F. evanescens, S. latissima* and *S. mcclurei* using of a new enzyme combination with the alginate lyase SALy and glucanase enzymes.

This study includes following works:

- Enzymatic extraction of crude fucoidans from three different types of brown macroalgae (*F. evanescens*, *S. latissima* and *S. smcclurei*) by using glucan and alginate degrading enzymes
- Chemical extraction of crude fucoidans from *F. evanescens*, *S. latissima* and *S. mcclurei*
- Purification of fucoidans by anion - exchange chromatography.
- Analysis of monosaccharide composition, sulfate content and molecular weight of crude fucoidans and fractions.
- Elucidation of primary fucoidan structure by NMR analyses
- Optimization of enzymatic extraction parameters

4.1 Enzyme-assisted and chemical extraction of fucoidans from *F. evanescens* and *S. latissima*

The used brown macroalgae *F. evanescens* and *S. latissima* belonging to the two orders *Fucales* and *Laminaria*, were used to validate the enzyme-assisted extraction method. The macroalgae *F. evanescens* was from Kiel fjord, Germany and *S. latissima* was wild harvested from Iceland. For evaluation of fucoidan extraction efficacy, the monosaccharide composition of the macroalgae was determined. The macroalgae were hydrolyzed by a two-step acid hydrolysis method and their monosaccharide composition was analyzed by a Dionex ICS-3000 HPAEC-PAD system with pulsed amperometric detection (PAD) (Manns et al. 2014). The monosaccharide composition of the macroalgae is presented in Figure 4.1. Fucose, the main component of fucoidans, was significantly higher in the macroalgae *F. evanescens* (8.7%) than in *S. latissima* (4.7%). While the amount of guluronic acid (GuluA) and manuronic acid (ManA) (the main components of alginate) in both macroalgae were high as reported previously for brown macroalgae (Rhein-Knudsen et al. 2017). The content of glucose (from laminaran or cellulose) was found significantly higher in *S. latissima* (12.3%) than in *F. evanescens* macroalgae (6.7%).

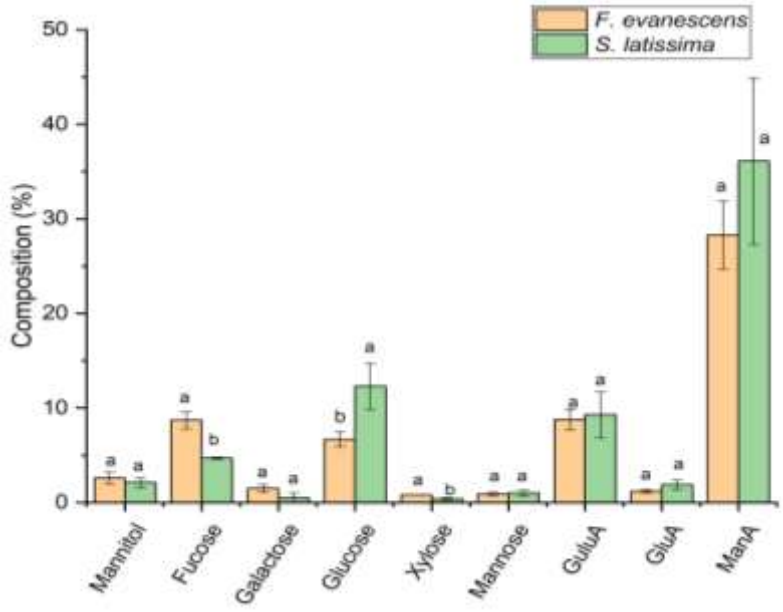


Figure 4.1 Monosaccharide composition of the brown macroalgae. The data are given as % weight (dehydrated monomers) of dry matter. Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b indicate statistically different values ($p < 0.05$) pairwise between values from each type of macroalgae.

In the enzymatic fucoidan extraction the macroalgae *F. evanescens* and *S. latissima* were firstly treated by enzyme mixture of alginate lyase SALy (0.35% (w/w) and the commercial Novozyme cellulase enzyme mix Cellic®CTec2 (5% (v/w) in 55 mM phosphate-38 mM citrate buffer pH 6 at 40 °C for 24 h on a horizontal mixer at 100 rpm (Figure 4.2a). The substrate concentration was 5 % (w/v). The reaction mixture was stopped by boiling for 10 minutes at 90°C and then cooling. The supernatant (Extract A in Figure 4.2a), obtained after removing macroalgae residue by centrifugation, was further treated by adding $CaCl_2$ to a final concentration of 2%, to remove residual alginate. After centrifugation, 96% ethanol was added to the supernatant with a ratio of 3:1 (v/v) to precipitate fucoidans. The crude fucoidans was isolated by centrifugation and lyophilized. For comparison of enzyme-assisted with a traditional extraction method, a mild acid isolation of fucoidans following Ale et al. 2012 methodology with slight modifications (Ale et al. 2012) was applied for both *F. evanescens* and *S. latissima* macroalgae (Figure 4.2b).

The crude fucoidans from the enzyme-assisted and chemical method differed in monosaccharide composition, sulfate content (Table 4.1) and molecular weight (Figure 4.3). The fucoidan extraction yield was calculated based on the total amount of fucose extracted in crude extract compared to the total fucose in the macroalgae. The fucoidan yields in both enzymatic and chemical extraction method were not significantly different for *F. evanescens* and *S. latissima* (Table 4.1). Crude fucoidans from enzymatic extraction contained low amount of fucose and high amount of alginate (especially ManA). During the enzymatic extraction process, the alginate was degraded into low molecular weight oligosaccharides, which was not precipitated by $CaCl_2$. Furthermore, the alginate lyase SALy has higher activity on polyG than on polyM (Manns et al. 2016), leaving oligo- and polyM in the extract. The glucose content was quiet different between the extraction

methods. The use of Cellic® CTec2 degraded cellulose and also laminarin to glucose (Manns et al. 2015), which was removed in ethanol precipitation step. Therefore, the enzymatic extracts contained low glucose content.

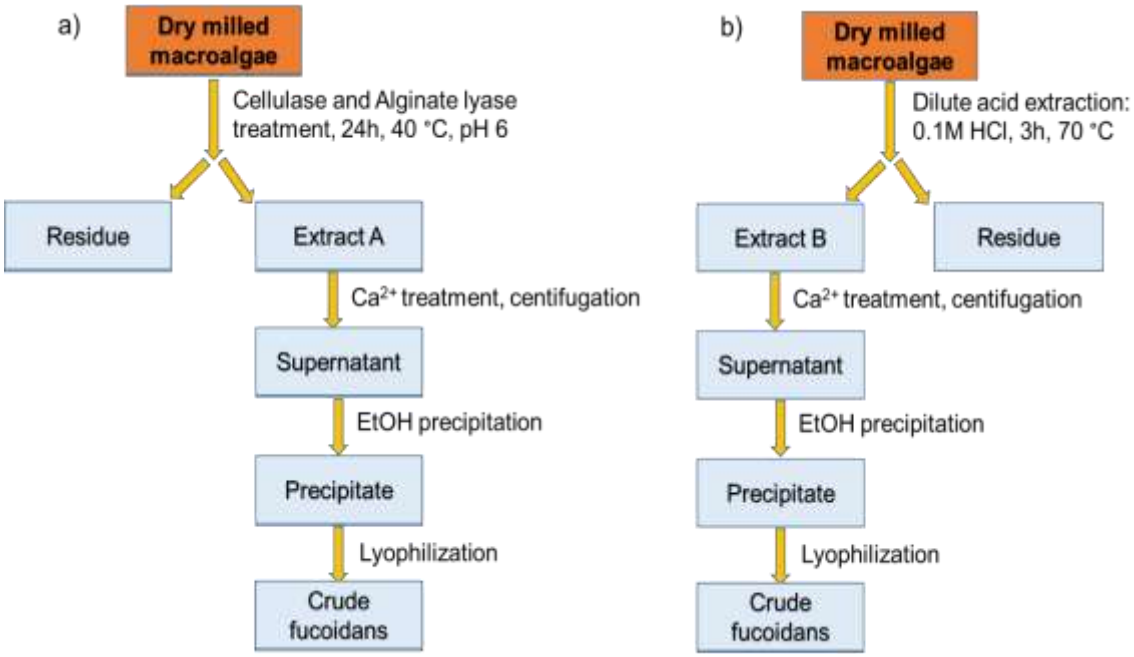


Figure 4.2 Flow-chart over extraction procedures. a) Enzyme-assisted extraction and b) Chemical extraction.

The bioactivity of fucoidans was reported to depend on the sulfate content (Haroun-Bouhedja et al. 2000; Koyanagi et al. 2003; Cho et al. 2011) and molecular weight (Hou et al. 2012), which was effected by fucoidan extraction method (Ale et al. 2011; Lyu et al. 2019). The sulfate content of crude fucoidans from both extraction methods determined by the barium chloride ($BaCl_2$) gelatin method (Dodgson & Price, 1962) as was described in Chapter 3. The enzyme-assisted extraction method has high substrate selective degradation, conserving the sulfate groups and also molecular weight of fucoidans. The sulfate content in crude fucoidans from enzymatic extraction was statistically significantly higher than from chemical extraction (Table 4.1). It was therefore expected that fucoidans from the enzyme-assisted method would result in higher bioactivity. The bioactivity of fucoidan fractions, purified from enzymatic extracts will be reported in the next chapter. Crude fucoidans from both extraction methods had wide range of mass distribution as displayed in the SEC chromatogram (Figure 4.3). For *F. evanescens*, the average size of enzymatic fucoidan extract was 100–800 kDa, which was larger than the size of the chemical extract (10–100 kDa). Similarly, the crude *S. latissima* fucoidan from the enzyme-assisted contained two populations of polymers, the first group was less than 10 kDa indicating the presense of low molecular weight alginates and the second group which has a size from 100 kDa to over 800 kDa. The chemical fucoidan extract of *S. latissima* was lower in size with one population less than 5 kDa and one from 50 to 100 kDa. Based on these results, fucoidans, from both macroalgae, extracted by the chemical method were partially degraded, resulting in lower molecular weight.

Table 4.1 Chemical composition of crude fucoidans from *F. evanescens* and *S. latissima*.

		F. evanescens		*S. latissima*	
		Enzymatic method	Chemical method	Enzymatic method	Chemical method
Neutral monosaccharides (%mol)	Mannitol	$0.2^{b} \pm 0.0$	$0.43^{a} \pm 0.0$	$2.1^{a} \pm 0.2$	$2.2^{a} \pm 0.3$
	Fucose	$24.8^{b} \pm 2.9$	$60.9^{a} \pm 0.9$	$12.6^{b} \pm 0.4$	$31.2^{a} \pm 4.2$
	Rhamnose	$0.2^{b} \pm 0.1$	$0.9^{a} \pm 0.2$	$0.2^{b} \pm 0.0$	$0.2^{a} \pm 0.0$
	Galactose	$0.9^{b} \pm 0.1$	$5.4^{a} \pm 0.1$	$2.3^{a} \pm 0.1$	$2.9^{a} \pm 2.4$
	Glucose	$0.7^{b} \pm 0.1$	$6.2^{a} \pm 0.1$	$1.6^{b} \pm 0.0$	$57.7^{a} \pm 3.1$
	Xylose	$0.8^{b} \pm 0.1$	$5.8^{a} \pm 0.1$	$0.8^{b} \pm 0.0$	$3.0^{a} \pm 0.0$
	Mannose	$0.4^{b} \pm 0.0$	$2.6^{a} \pm 0.1$	$0.9^{a} \pm 0.0$	$0.9^{a} \pm 0.1$
Uronic acid (%mol)	GuluA	$12.6^{a} \pm 1.8$	$0.9^{b} \pm 0.1$	$18.6^{a} \pm 0.9$	$0.2^{b} \pm 0.1$
	GluA	$1.0^{b} \pm 0.2$	$3.9^{a} \pm 0.1$	$1.3^{a} \pm 0.2$	$0.7^{b} \pm 0.1$
	ManA	$58.4^{a} \pm 2.6$	$13.1^{b} \pm 0.4$	$59.6^{a} \pm 1.9$	$1.0^{b} \pm 0.2$
Sulfate (SO_4^{2-}) (%)		$21.7^{b} \pm 0.5$	$38.7^{a} \pm 0.4$	$15.8^{b} \pm 1.7$	$32.1^{a} \pm 0.9$
Degree of sulfation (molar ratio SO_4^{2-}: Fuc)		$2.1^{a} \pm 0.1$	$1.9^{b} \pm 0.1$	$2.5^{a} \pm 0.2$	$2.2^{b} \pm 0.1$
Fucoidan yield (fucose extraction %)		$39.6^{a} \pm 1.9$	$43.1^{a} \pm 2.0$	$28.9^{a} \pm 2.1$	$29.3^{a} \pm 5.4$

Note: The monosaccharide and uronic acid data are given in %mol (relative level) of total carbohydrates analyzed in the extract, with total sulfate ($SO4^{2-}$) first calculated as %wt of total, then as degree of sulfation on dehydrated fucose moieties in the crude fucoidans extracted. The data are given as % weight (dehydrated monomers) of dry matter. Each data point represents the average value of independent triplicates. Different superscript roman letters a, b indicate statistically different values ($p < 0.05$) pairwise for the enzymatic method vs. the chemical method per macroalgae species.

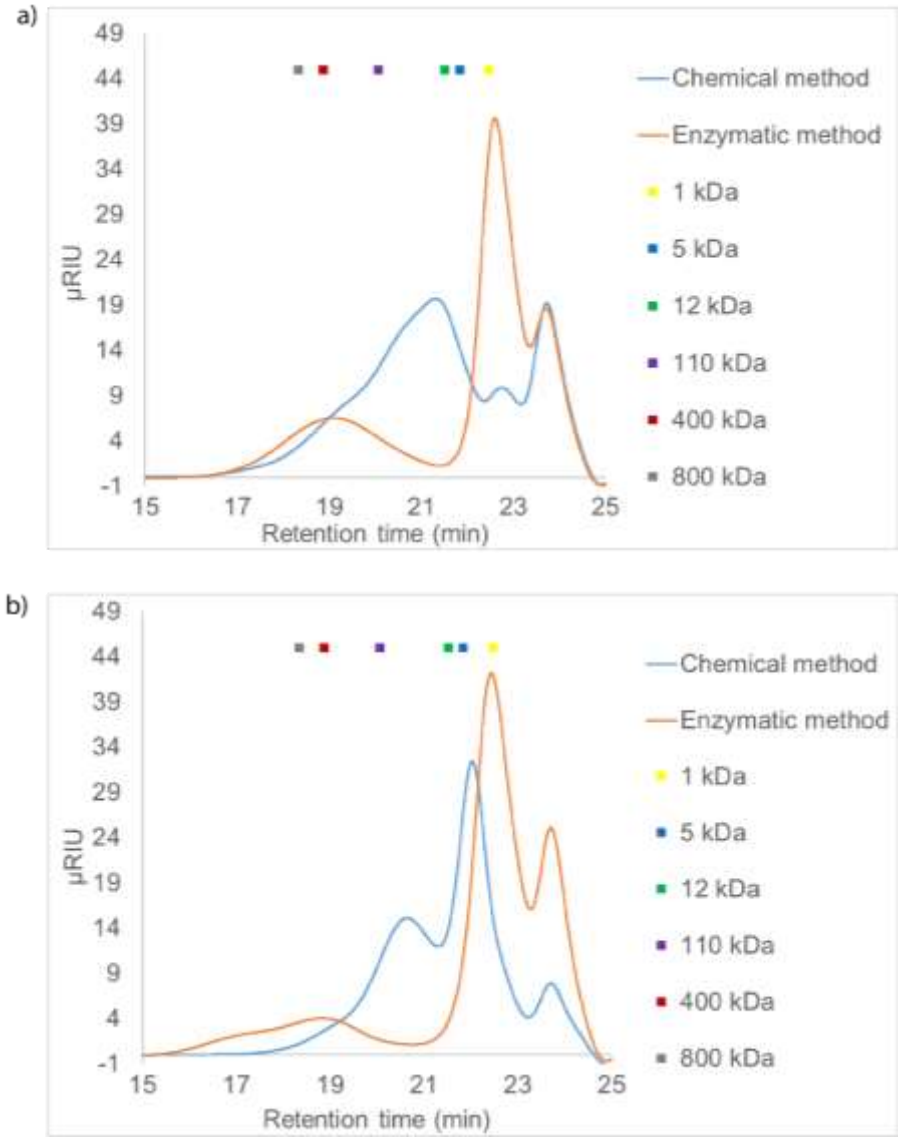


Figure 4.3 SEC chromatogram of crude fucoidans from chemical and enzyme-assisted purification. (a) *F. evanescens* and (b) *S. latissima*. Pullulan was used as standard.

4.2 Fucoidan purification and structure determination

Crude fucoidans from enzymatic extraction of both *F. evanescens*, *S. latissima* macroalgae and chemical extraction of *S. latissima* were further fractionated by anion-exchange chromatography with a NaCl gradient using DEAE - Macroprep resin. The polysaccharides were separated based on their negative charged intensity. The elutions were combined based on the total carbohydrate, which was determined by the phenol-sulfuric acid method (Dubois et al. 1956). In total, three different fucoidan fractions were obtained FeF1 – F3 from *F. evanescens*, SlF1 – F3 from *S. latissima* from enzyme-assisted method and SlChF1 – F3 for *S. latissima* from chemical method. These fractions differed in monosaccharide composition, sulfate content and molecular weight (Figure 4.4 and 4.6).

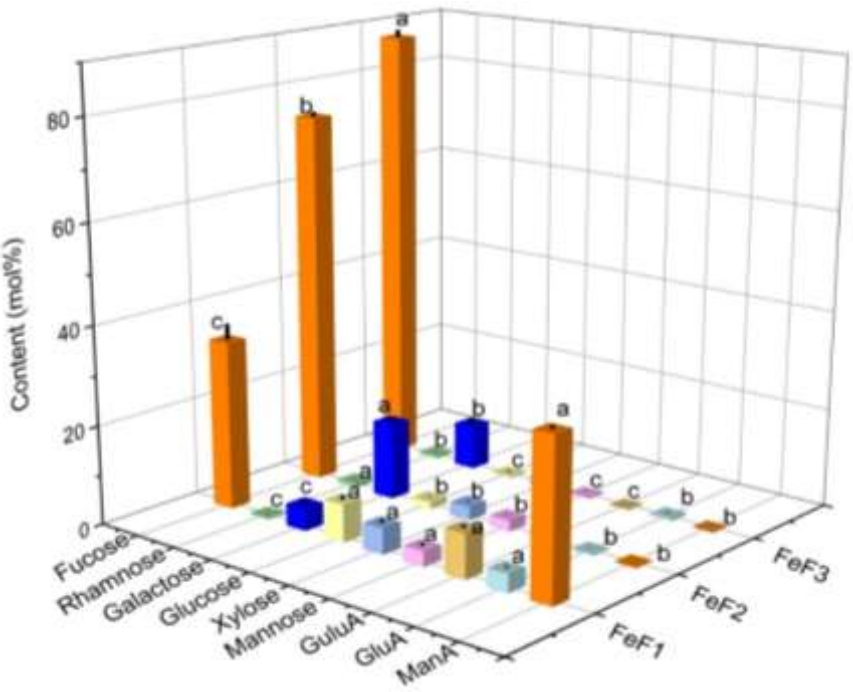


Figure 4.4 Composition of fucoidan fractions from *F. evanescens.* Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b, c indicate statistically different values ($p < 0.05$) for each sugar in the fucoidan fractions.

During the purification process the alginate was mostly eluted in fraction F1. Therefore, the alginate content was high in fraction F1 from both macroalgae and very low in fraction F2 and F3. This was confirmed by ^{1}H NMR where the signals characteristic for uronic acids appeared in the region of 5.8 ppm for fraction F1 and was absent in fraction F2 and F3. The FeF1 fraction contained 34% of fucose and low amounts of other monosaccharides, except the mannuronic acid as mentioned above. Since the FeF1 was heterogeneous in composition, it showed a wide range of mass distribution on SEC chromatogram (Figure 4.5a) with three main peaks at 2-3 kDa, 30-40 kDa and ~400 kDa. The two fractions FeF2 and FeF3 were considered pure sulfated fucans with high fucose (74.7 and 87.8%, respectively), and sulfate content (34.8 and 38.7%, respectively), while the alginate content was low (Figure 4.4). Fraction FeF2 contaned high molecular weight fucoidans with a broad mass distribution from 30 kDa to over 800 kDa, while FeF3 contained only one homogenous peak at 400-500 kDa (Figure 4.5a).

Fucoidan fractions from *S. latissima* purified by enzyme-assisted and chemical extraction differed in monosaccharide composition, sulfate content, molecular weight and also polysaccharide structure. Fraction SlF1 from enzymatic extraction mostly contained ManA (82.4%), which was presented by a homogeneous peak of less than 5 kDa in SEC (Figure 4.5c), while the fucose content was only 5.4%. SlChF1 from chemical extraction also contained high alginate, however the amount of fucose (17.1%) was higher than SlF1, resulting in heterogeneous molecular weight distribution of this fraction (Figure 4.5c). The enzymatic fucoidan fractions SlF2 and SlF3 obtained from *S. latissima* were sulfated and contained predominantly fucose residues and galactose residues with ratio of fucose: galactose was 1: 0.19 and 1:0.42, respectively. The content of other monosaccharides in

these fraction was negligible. The sulfate content of these two fractions SlF2 and SlF3 were high (35.6 and 46.4%, respectively). The fucoidan fractions SlF2 and SlF3 had similar molecular weight distribution pattern in range of 300 kDa to over 800 kDa (Figure 4.5b).

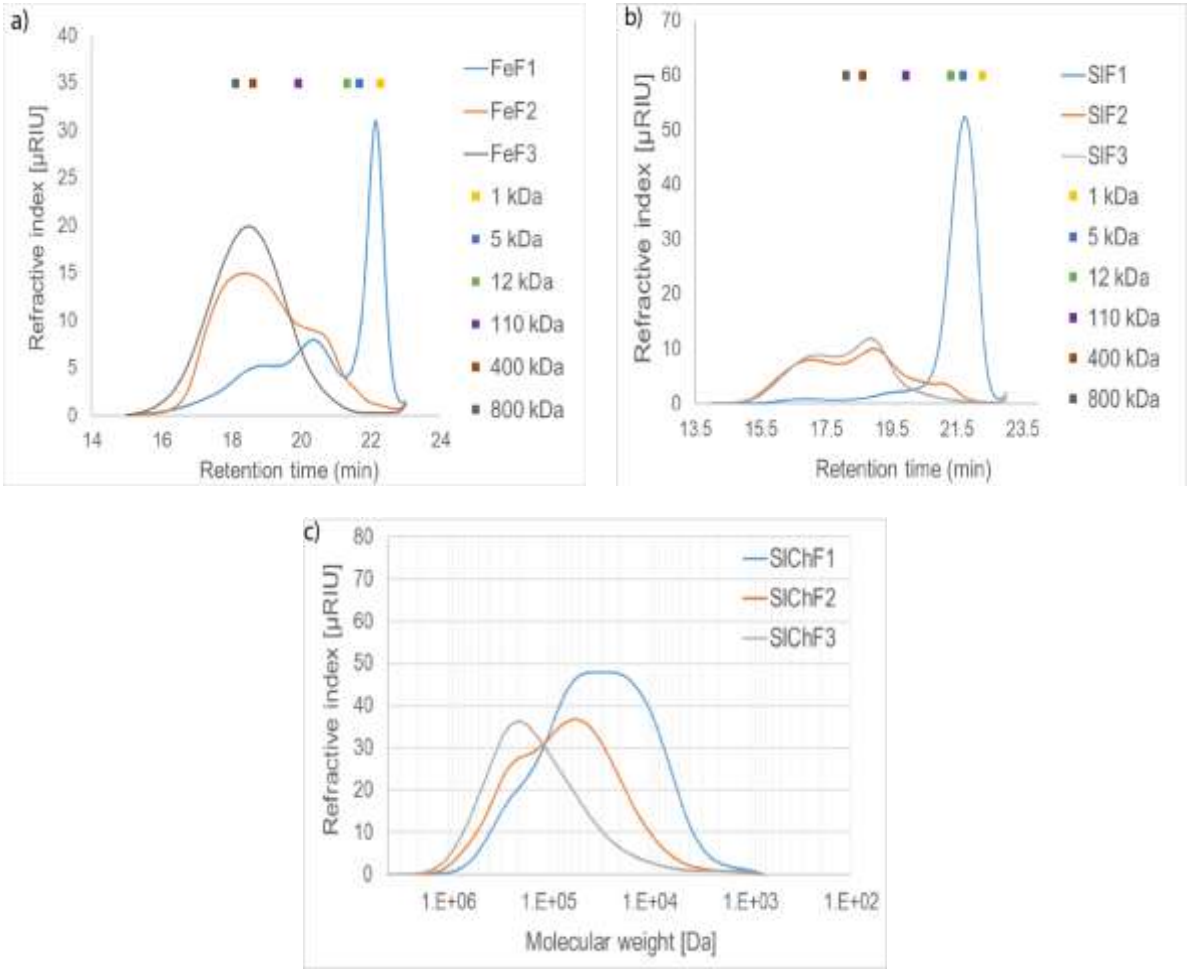


Figure 4.5 SEC chromatogram of fucoidan fractions after IEX purification: (a) of enzymatic crude extract from *F. evanescens*, (b) of chemical crude extracts and (c) of enzymatic crude extract from *S. latissima*.

Different from the fractions from the enzymatic-assisted method, the chemical fraction SlChF2 consisted of equal amounts of fucose and galactose, while SlChF3 mostly contained fucose with fucose: galactose ratio of 1: 0.1 (Figure 4.6). The sulfate content of SlChF2 and SlChF3 was lower (26.6 and 29.6%, respectively) compared to enzymatic fractions (35.6 and 46.4% for SlF2 and SlF3, respectively). The variation in monosaccharide composition of these fractions indicated the presence of different fucoidan structures, which were confirmed by NMR analysis in the following part of this section. The SEC profiles of SlChF2 and SlChF3 were displayed in Figure 4.5c where SlChF2 showed a heterogeneous peak ranging from 50 to 200 kDa and SlChF3 showed almost homogeneous peak around 200 kDa. The structure of fucoidan fractions F2 and F3 from *F. evanescens* and *S. latissima* were determined by NMR spectroscopy.

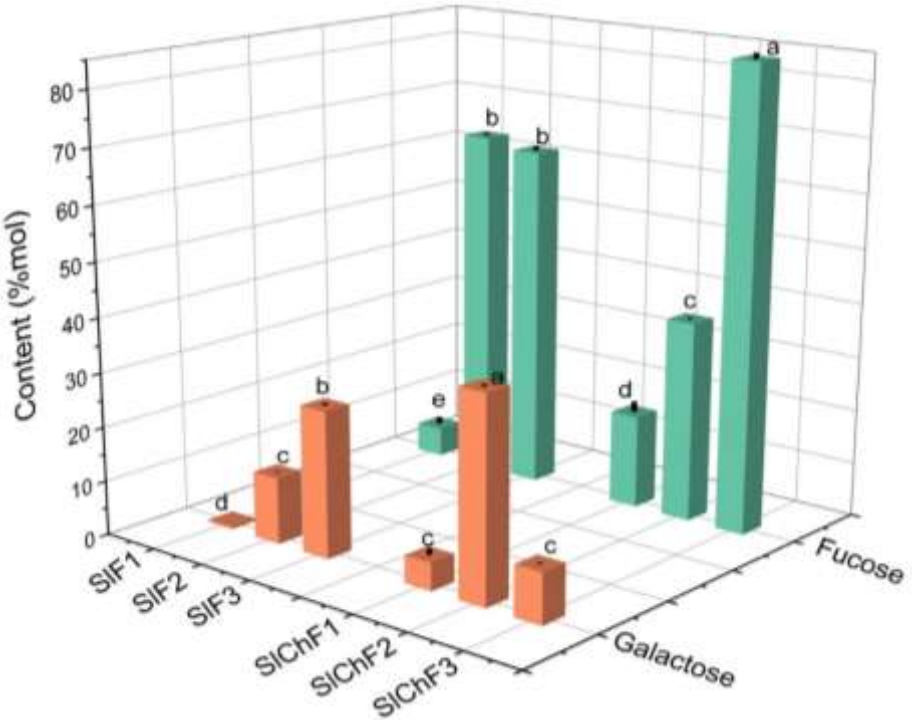


Figure 4.6 Composition of fucoidan fractions from enzyme-assisted (SlF1 – F3) and chemical extraction method (SlChF1 – F3) from *S. latissima.* Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b, c indicate statistically different values ($p < 0.05$) for each sugar in the fucoidan fractions.

Fucoidans from *F. evanescens* were treated by ammonia solution 12% overnight at 37°C to remove acetyl groups. The obtained spectra (Figure 4.7) were similar with the spectra of the polysaccharides described previously (Bilan et al., 2002; Menshova et al., 2016). The chemical shifts of carbon atoms were presented in Table 4.2. Four structure fragments A, A', B and B' were found where residue B is linked with residue A (→3)-a-L-Fuc*p*-(2,4SO_3^-)-(1→), residue B' is linked with residue A' (→3)-a-L-Fuc*p*-(2SO_3^-)-(1→). Thus, fucoidans from *F. evanescens* includes both structural fragments →3)-a-L-Fuc*p*-(2,4SO_3^-)-(1→4)a-L-Fuc*p*-(2SO_3^-)-(1→ and →3)-a-L-Fuc*p*-(2SO_3^-)-(1→4)a-L-Fuc*p*-(2SO_3-)-(1→. The backbone consisting of alternating 2-sulfated 1,3- and 1,4-linked α-L-fucose residues, sulfate groups occupied at C2 and less C4 positions.

The ^{13}C NMR spectra of enzymatic fractions SlF2 and SlF3 fucoidans (Figure 4.8a) and chemical fraction SlChF2 were comparable with the previously published data (Bilan et al. 2010). They were identified as mixtures of two polysaccharides. The main polysaccharide was a sulfated fucan with the backbone consisted of 1,3-linked α-L-fucopyranose residues, which was sulfated at C4 and/or at C2 and branched at C2 by single sulfated a-L-fucopyranose residues (about one branching point per 5–6 backbone residues). The second polysaccharide was a fucogalactan with a backbone of 6-linked β-D-galactopyranose residues (Bilan et al., 2010).

Table 4.2 Chemical shifts (ppm) of carbon atoms in the NMR spectrum of deacetylated fucoidans from *F. evanescens*

Fragment of structure	C1	C2	C3	C4	C5	C6
A →3)-α-L-Fuc*p*-(2,4SO_3^-)-(1→	99.8	76.4	74.4	81.2	68.8	17.1
B →4)-α-L-Fuc*p*-(2SO_3^-)-(1→	98.9	77.3	68.8	83.8	69.7	16.9
A* →3)-α-L-Fuc*p*-(2SO_3^-)-(1→	99.8	76.4	72.7	69.7	67.8	16.6
B* →4)-α-L-Fuc*p*-(2SO_3^-)-(1→	94.0	77.3	68.8	83.0	68.8	16.7

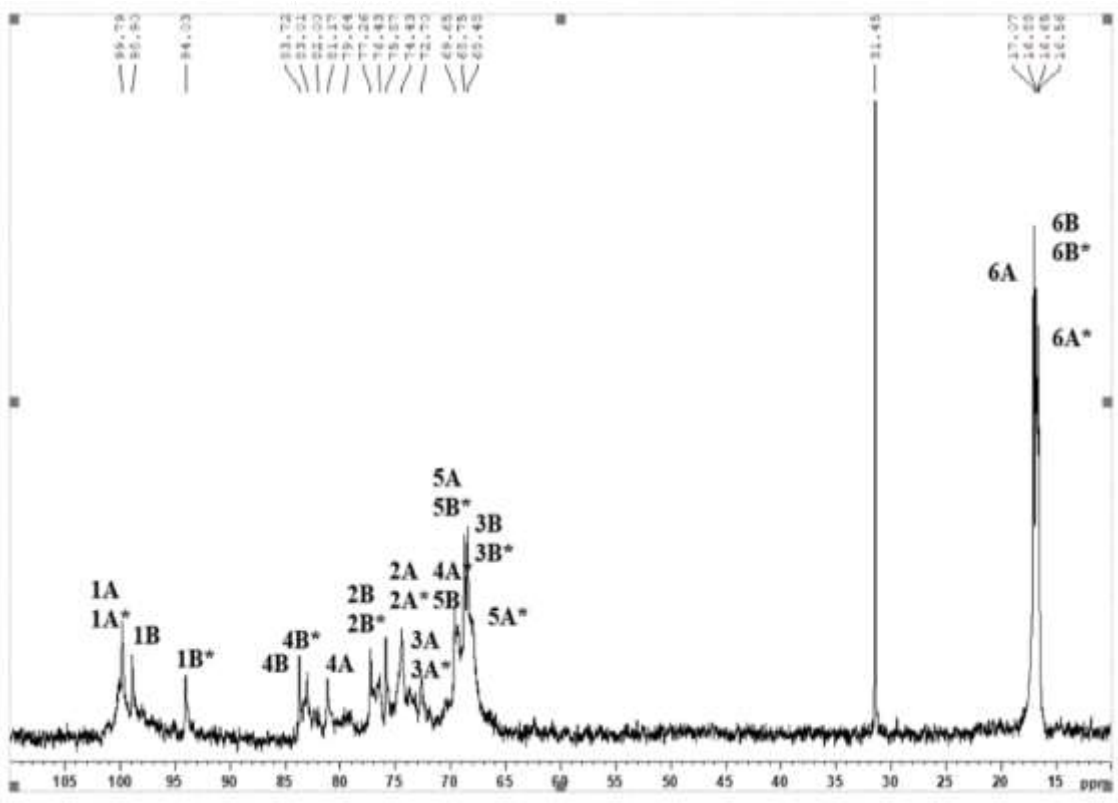


Figure 4.7 ^{13}C NMR spectrum of deacetylated fucoidans from *F. evanescens*

The characteristic signal of galactose in anomer carbon region 102 – 104 ppm was absent in ^{13}C NMR spectrum of SlChF3 (Figure 4.8c), indicating the main component of this fraction was sulfated fucan. Thus, the polysaccharides in chemical and enzymatic extract were separated differently, resulted in different polysaccharide components in the fractions. Fucoidan fractions purified from chemical extract from *S. latissima* displayed lower molecular weight as well as the sulfate content compared to the fucoidans extracted by the enzyme-assisted method.

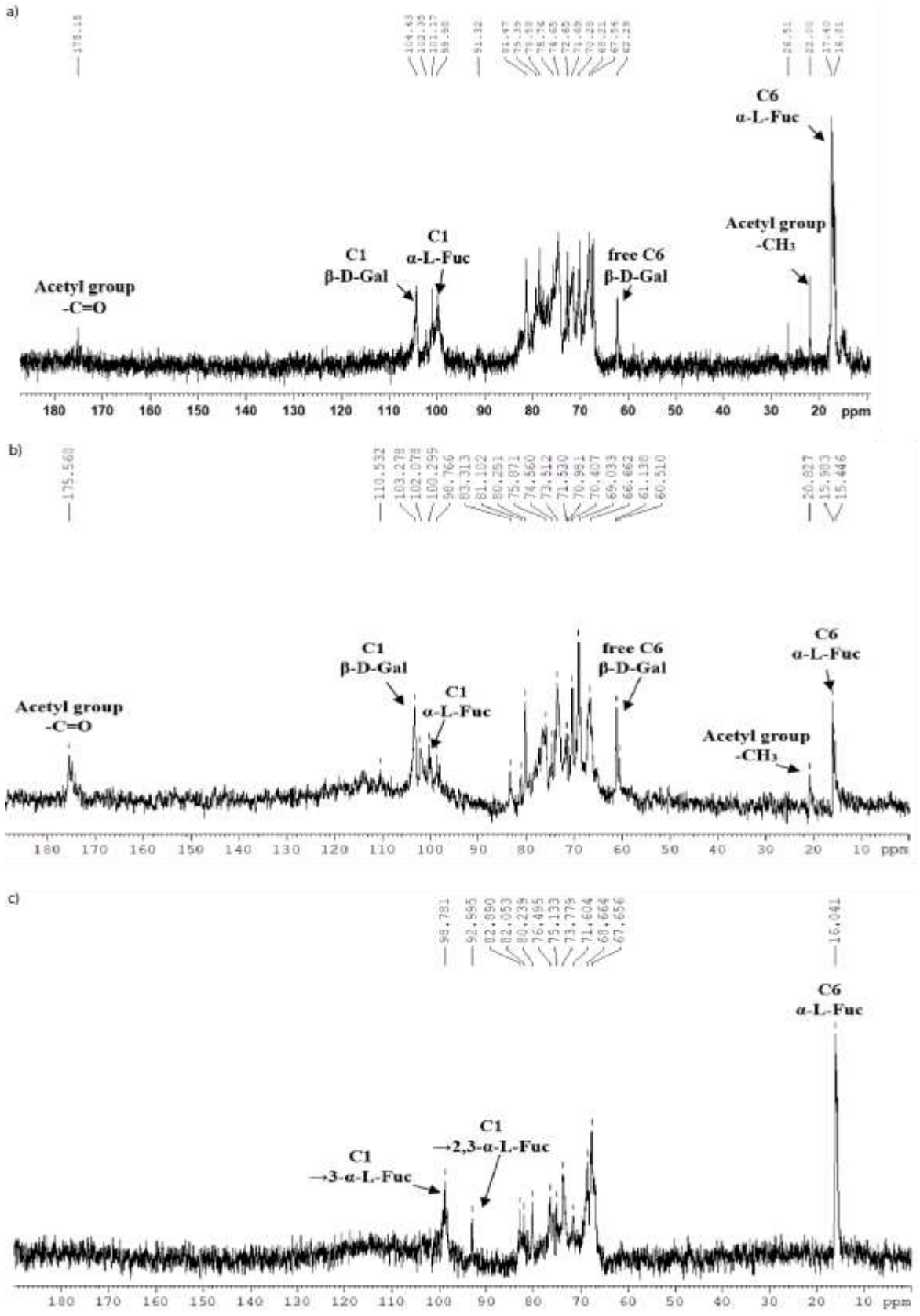


Figure 4.8 ^{13}C NMR spectrum of fucoidan fractions from *S. latissima* purified from (a) enzymatic extract SlF2 and SlF3 and chemical extract (b) SlChF2 and (c) SlChF3.

4.3 Optimization of fucoidan extraction from *S. latissima*

Fucoidans from *F. evanescens* and *S. latissima* were successfully extracted by the enzymatic method. However, extraction parameters such as temperature, extraction time, pH, enzyme concentration and extraction buffer may effect on the yield as well as the purity of fucoidans. In a previous work from our lab, the Cellic®CTec2 and alginate lyase SALy were combined to release glucose from brown macroalgae *Laminaria digitata* (Manns et al. 2016). In the same work, the temperature and enzyme concentration of macroalgae treatment process were optimized, therefore, we use these parameters to adapt to fucoidan extraction. In this part of study, the effects of extraction time, buffer, pretreatment of macroalgae, additional enzyme on the yield and purity of crude fucoidans from *S. latissima* macroalgae from Ocean Rainforest were investigated.

4.3.1 Effect of buffer and extraction time on the yield of fucoidans

The buffer plays an important role for normal activity of enzymes, also in the fucoidan extraction process. The effect of different buffers (universal buffer 4 (UB4), including 20 mM HEPES, 20 mM Bis-Tris and 20 mM sodium acetate), Bis-Tris, and Tris – HCl buffer) on alginate lyase SALy was investigated. As shown in Figure 4.9, alginate lyase SALy showed highest activity at pH 6.5 in UB4 buffer and Tris – HCl buffer. Therefore, UB4 buffer with pH 6.5, which is near the optimal pH for Cellic®CTec2 was chosen for optimization of fucoidan extraction experiments. The citrate – phosphate buffer, which was used in our previous study was used as standard extraction buffer.

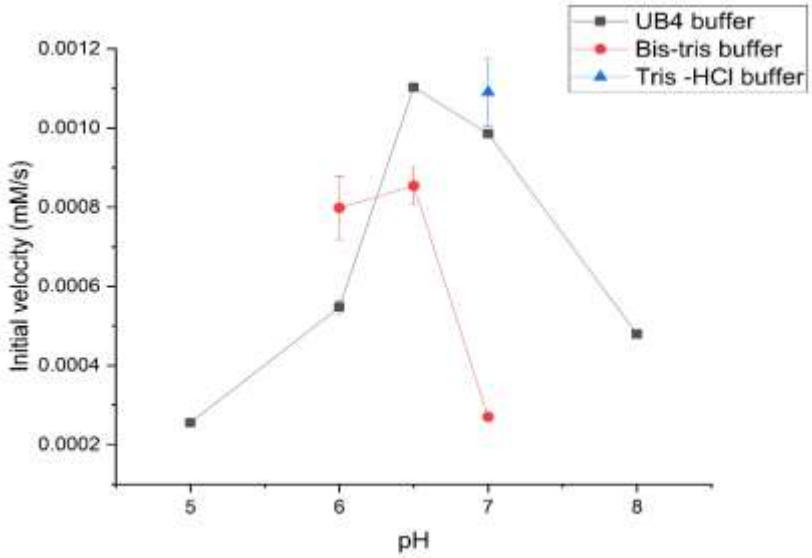


Figure 4.9 The effect of different buffers (a) and buffer concentration (b) on alginate lyase SALy activity.

The *S. latissima* macroalgae from Ocean Rainforest was cultivated in the sea near the Faroe Islands and grown at deep sea. The monosaccharide composition of this macroalgae was analyzed and presented in Table 4.3. This macroalgae contains 3.77% of fucose and high amounts of alginate.

Table 4.3 Monosaccharide composition of the brown macroalgae

Monomer Category		*S. latissima*	*S. mcclurei*
Neutral monosaccharides (%)	Fucose	$3.77^{a} \pm 0.99$	$1.69^{b} \pm 0.24$
	Rhamnose	$0.1^{a} \pm 0.01$	$0.03^{b} \pm 0.01$
	Galactose	$0.67^{a} \pm 0.06$	$0.76^{a} \pm 0.08$
	Glucose	$6.64^{a} \pm 1.34$	$3.42^{b} \pm 1.16$
	Xylose	$0.51^{a} \pm 0.07$	$0.5^{a} \pm 0.09$
	Mannose	$0.29^{a} \pm 0.07$	$0.15^{a} \pm 0.04$
Uronic acids (%)	Guluronic Acid	$8.52^{a} \pm 1.97$	$4.5^{a} \pm 2.26$
	Glucuronic Acid	$0.45^{a} \pm 0.14$	$0.19^{b} \pm 0.04$
	Mannuronic Acid	$50.46^{a} \pm 3.69$	$23.23^{b} \pm 4.51$

The data are given as % weight (dehydrated monomers) of dry matter. Each data point represents the average value of independent triplicates. Different superscript roman letters a, b indicate statistically different values ($p < 0.05$) pairwise between values from each type of macroalgae.

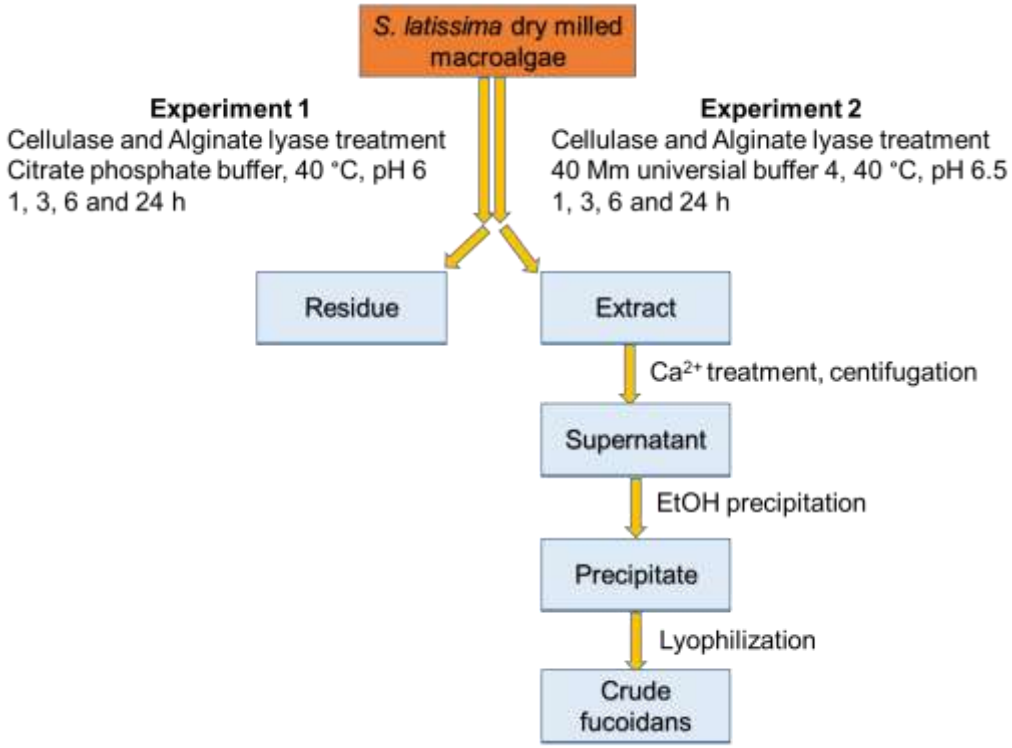
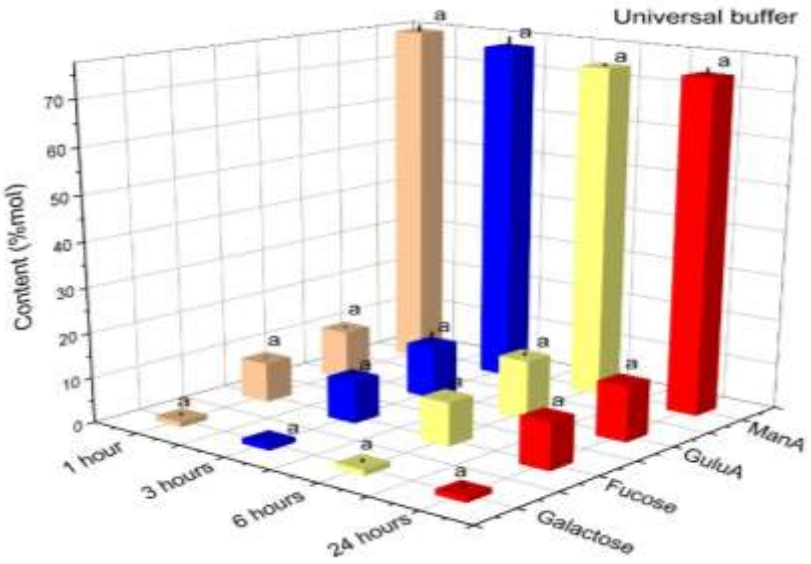


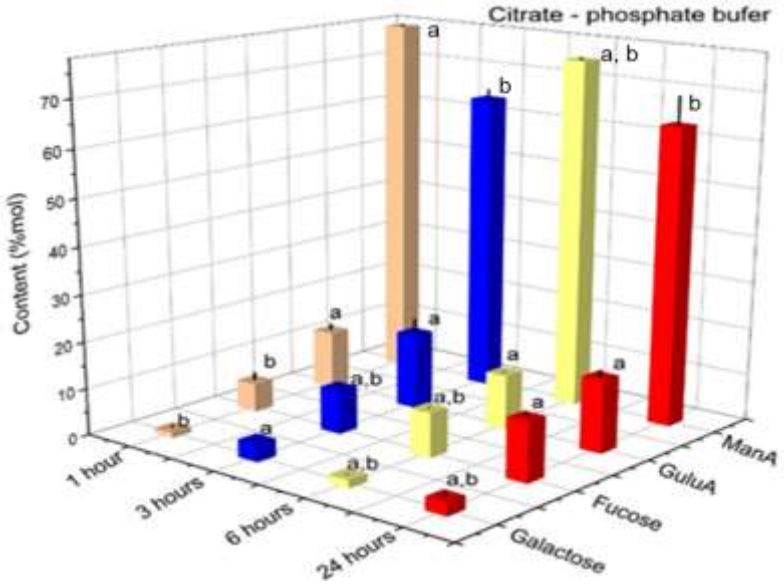
Figure 4.10 Flow-chart of extraction procedure in two different buffers: Citrate – phosphate buffer in experiment 1 and universal buffer 4 in experiment 2.

Fucoidans were extracted from *S. latissima* following the protocol shown in Figure 4.10. The monosaccharide composition of crude fucoidans from each extraction time point 1, 3, 6 and 24 hours was analyzed (Figure 4.11). In UB4 buffer extraction, the fucose content in crude fucoidans after 1 hour was 8.86%, then slowly increased to 10.49% after 24 hours extraction. The alginate content is not changed during extraction. However, in citrate – phosphate buffer, the fucose content in crude extract after 1 hour extraction was only 6.14%, then the number was increased to 12.75% after 24 hours. The effect of extraction buffer and time on the yield of fucoidans was evaluated based on the total amount of extracted fucose (Figure 4.11c). In the standard extraction procedure, using citrate-phosphate buffer, the fucose yield after 1 hour of extraction was very low (23.24%). Then the fucose yield was increased and unchanged after 3 hours extraction, which was not statistically significantly different with the fucose yield after only 1 hour extraction in UB4 buffer (42.59%). While in UB4 buffer the fucose yield was achieved high after only 1 hour extraction and unchanged during 3, 6 and 24 hours (Figure 4.11c). So the enzymes worked more slowly in citrate – phosphate buffer compared with UB4 buffer. Based on the obtained results, for further studies the enzymatic fucoidan extraction will be performed in UB4 buffer for 1 hour.

a)



b)



c)

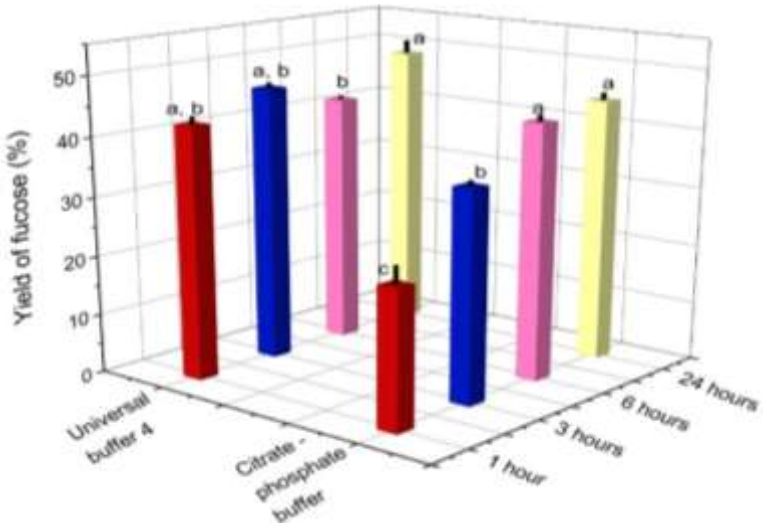


Figure 4.11 The effect of the buffers and extraction time on monosaccharide composition and yield of crude fucoidans from *S. latissima*. Monosaccharide composition of crude

fucoidans extracted in UB4 (a) and in citrate – phosphate buffers (b). The yield of fucose (c) at different extraction time in UB4 and citrate – phosphate buffers. Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b, c indicate statistically different values ($p < 0.05$) at different extraction time in each extraction buffer.

4.3.2 Pretreatment of macroalgae by ethanol

Brown macroalgae contain phenolic compounds that inhibit the activity of some enzymes (Shibata et al. 2002; Imbs et al. 2018). Therefore, to achieve high activity of the enzymes during the fucoidan extraction, these phenolic compounds could be removed before enzyme treatment of the macroalgae. With the aim to extract fucoidans in a green way, ethanol was chosen in this study, instead of formaldehyde or other toxic organic solvents. The dried *S. latissima* macroalgae was treated in ethanol from 30 to 80 % in 24 hours at room temperature and then dried. Fucoidan extraction was performed in 40 mM of UB4 buffer for 1 hour. The monosaccharide composition of crude fucoidans was analyzed. The effect of ethanol treatment on enzymatic fucoidan extraction was evaluated by total amount of extracted fucose (Figure 4.12). Although the treatment of macroalgae by 60% ethanol showed a higher extracted fucose yield among tested samples, the yield of fucose was still lower than in the standard conditions without ethanol pre-treatment. The enzymes seemed to work worse on pre-treated macroalgae, more time was required for the enzymes to act. This could be related to other factors, such as cell wall collapse during ethanol induced cell wall dehydration, leaving the enzyme sites inaccessible for the enzymes.

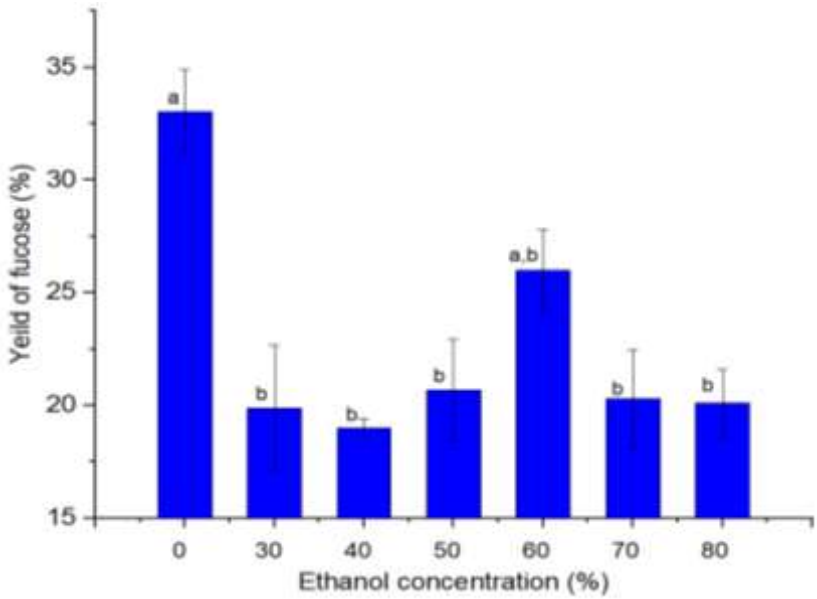


Figure 4.12 The yeild of extracted fucose from macroalgae *S. latissima*, which was pretreated by ethanol with different concentrations. Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b indicate statistically different values ($p < 0.05$) for each sugar from different ethanol concentration treatment.

4.3.3 Adding new secondary alginate lyase treatment step to the fucoidan extraction process

The crude fucoidans obtained from the enzyme-assisted extraction method contained high amounts of alginate as was mentioned above in section 4.1. To reduce the amount of alginate in the crude extracts, addition of alginate lyase to the supernatant after treatment by $CaCl_2$ was applied (Figure 4.13). Alginate lyase was added to a final 0.53, 0.83, 1.13 and 1.73 % of macroalgae dry-matter (w/w). The sample without adding more SALy (0.35% SALy) was used as standard. The monosaccharide composition of crude fucoidans was analyzed. The effect of adding more SALy was determined based on the ratio of alginate: fucose in the crude fucoidan extract. The ratio of alginate: fucose in the standard experiment (0.35% SALy) was decreased with the increasing of SALy concentration and significantly dropped when SALy was added to 1.73% (Figure 4.14). Based on these results, the second addition of SALy resulted in decreased alginate content in the crude fucoidans. However, to get pure fucoidans the crude extract still needs further purification by IEX. Alternatively, another alginate lyase could be used, with higher affinity for ManA.

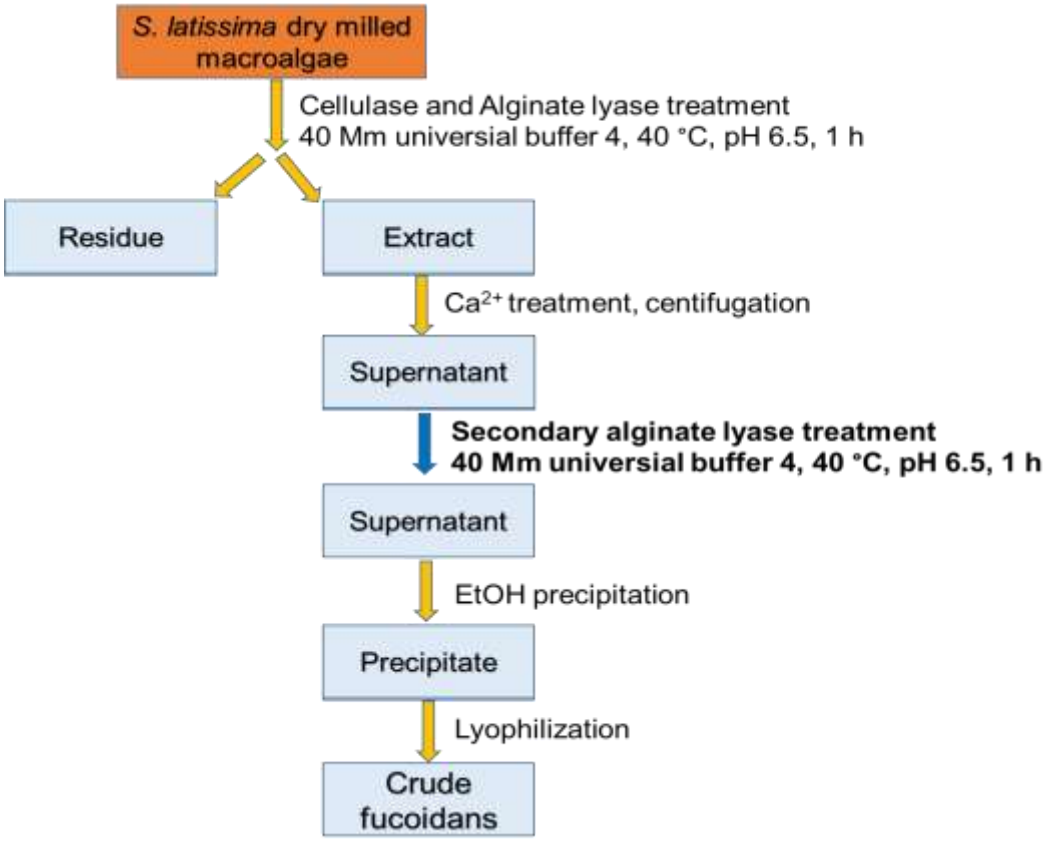


Figure 4.13 Flow – chart of enzymatic fucoidan extraction with adding new alginate lyase treatment step

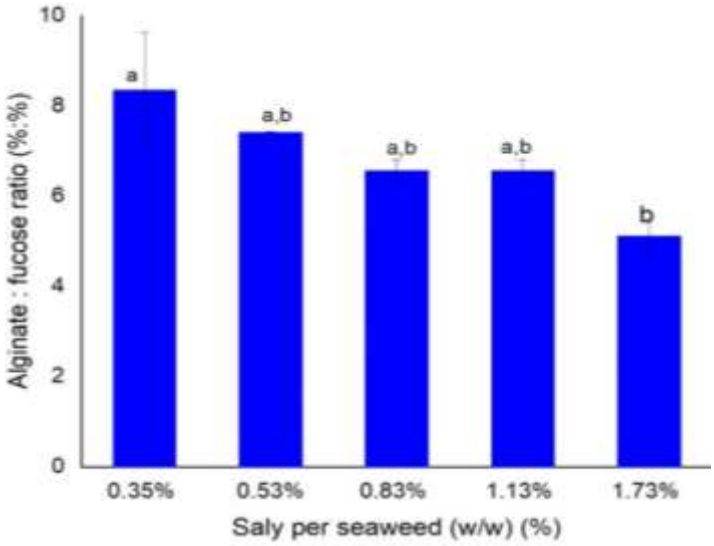


Figure 4.14 The alginate: fucose ratio in crude fucoidans from *S. latissima* after adding secondary alginate lyase SALy treatment step. Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b indicate statistically different values ($p < 0.05$) between from each alginate lyase SALy concentration.

4.4 Extraction of fucoidans from *S. mcclurei*

4.4.1 Enzymatic extraction of fucoidans from *S. mcclurei*

S. mcclurei is a typical macroalgae in the Vietnam Sea. Fucoidans from *S. mcclurei* are galactofucan and have only been extracted by chemical method previously (Thinh et al. 2013). Development of a green enzyme-assisted method for fucoidan extraction for this macroalgae would be valuable. Although, the combination of cellulase Cellic®CTec2 and alginate lyase was successfully used for *F. evanescens* and *S. latissima*, the method was unsuccessful for extraction of fucoidans from *S. mcclurei*, likely due to the very low fucoidans content in *S.mcclurei* (2.7%) (Thinh et al. 2013). Instead, another enzymatic-assisted extraction method was developed, using highly specific glucanases in combination with the alginate lyase SALy to obtain high efficacy of the extraction method. The specific glucanase enzymes are commercially available and their optimal conditions are closed to that of the alginate lyase, so the enzymatic procedure can be performed simultaneously. The optimal conditions for the cellulase from *Thermotoga maritima* and the endo-1,3-β-D-glucanase from *Trichoderma sp*. are pH 6 and 40°C and for the non-specific endo-1,3(4)-β-glucanase from *Clostridium thermocellum* the optimal conditions are pH 4.5 and 40°C. The experimental plan is displayed in Table 4.4. The fucoidan extraction was performed in 40 mM UB4 buffer, pH 6.5 at 40°C for 24 hours with the same procedure as described above for fucoidan extraction from *F. evanescens* and *S. latissima*. The macroalgae was treated with enzymes with an enzyme: macroalgae (w/w) ratio of 0.01 % for cellulase from *Thermotoga maritima*, 0.02% for endo-1,3-β-D-glucanase from *Trichoderma sp*., 0.002% for non-specific endo-1,3(4)-β-glucanase from *Clostridium thermocellum*, 0.5% for alginate lyase SALy and 10% (v/w) for Cellic®CTec2. All the experiments were performed in duplicates and the amount of macroalgae in each experiment was 2 g.

Table 4.4 Different combinations of enzymes used for fucoidan extraction from *S. mcclurei*

Experiments	Cellulase	Endo-1,3-β-D-glucanase	Non-specific endo-1,3(4)-β-glucanase	SALy	Ctech
1	+	+		+	
2	+		+	+	
3	+			+	
4		+	+	+	
5		+		+	
6			+	+	
7				+	+

The monosaccharide composition of all crude fucoidans was analysed. Fucoidans from *S. mcclurei* were reported as a galactofucan, so for the evaluation the fucoidan extraction yield both extracted yield of fucose and galactose were identified (Figure 4.15). In the result the yield of extracted fucose and galactose compared to their total amount in macroalgae was different from experiment 1 to 7 (Figure 4.15). Among the studied experiments the experiment 1 using the combination of cellulase, endo-1,3-β-D-glucanase and SALy showed highest fucose yield (18.59%), while the yield of galactose was not different between experiment 1 and 7 (Figure 4.15). In the chemical extraction method, the crude fucoidans had higher content of fucose compared to the enzymatic extract. However, the yield of crude fucoidans was low (2.7%). Therefore, the total extracted fucose in the chemical extract (18.21%) was not statistically different with extracted fucose in experiment 1. So the experiment 1 was chosen for optimization the extraction time and experiment 7 was set as the standard method.

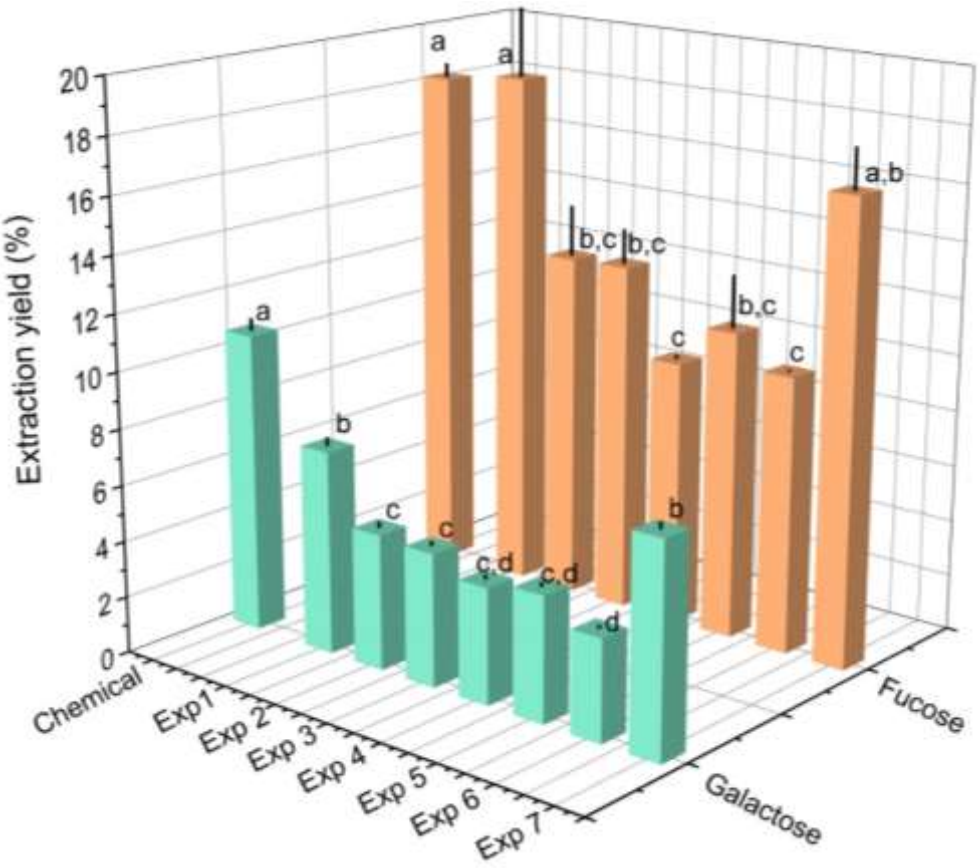


Figure 4.15 The extraction yield of fucoidans based on the amount of extracted fucose and galctose compared to the total fucose and galactose in macroalgae. Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b, c, d indicate statistically different values ($p < 0.05$) for each sugar from different experiments.

4.4.2 The effect of extraction time on the yield of fucoidans

The effect of extraction time on the fucoidan yield was evaluated for 1, 3, 6 and 24 hours extraction. The monosaccharide composition of all the crude extract were analysed. The yeild of crude fucoidans at each extraction time point was determined by amount of extracted fucose and galactose and presented in Figure 4.16. The results showed that the fucoidan yield was both experiment 1 and 7 was significantly increased from 1 to 24 hours extraction.

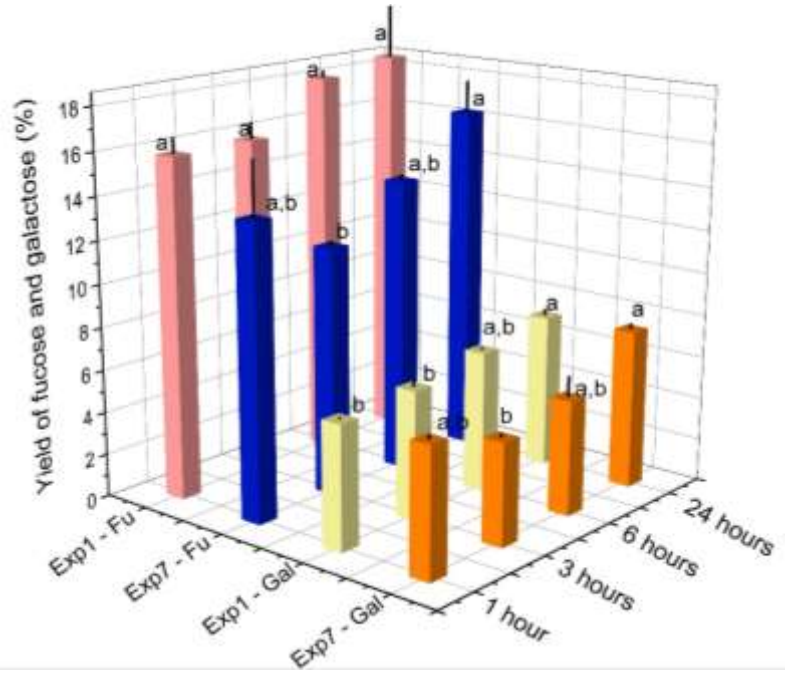


Figure 4.16 The effect of extraction time on fucoidan yield from *S. mcclurei.* The monosaccharide composition of crude fucoidans in experiment 1 (a) and experiment 7 (b). The yield of fucose and galactose in experiment and experiment 7 at different extraction time. Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation. Different superscript roman letters a, b indicate statistically different values ($p < 0.05$) for each sugar of fucoidans from each extraction time.

4.5 Conclusion and discussion

The main results of this work is to use enzymes with a combination of glucanases and an alginate lyase to successfully extract fucoidans from *F. evanescens*, *S. latissima* and *S. mcclurei*, without changing the molecular weight and sulfate groups - confirming Hypothesis 1. Although the fucose extraction yield in the enzyme-assisted extraction method was not statistically different with the chemical method, crude fucoidans from both methods showed different monosaccharide composition, sulfate content and mass distribution profiles. Since enzymes have high substrate specificity, they selectively degraded the target polysaccharides, releasing the fucoidans in intact form, while in the chemical extraction, fucoidans were partially degraded, resulting in lower molecular weight. Thus, based on these results, this work confirmed the influence of extraction method on the monosaccharide composition and molecular weight of fucoidans as was reported in earlier studies (Alboofetileh et al., 2019; Ale et al., 2012; Dong et al., 2016).

Since the crude fucoidans from enzyme-assisted method contained high amounts of alginate, to obtain pure fucoidans these crude extracts need to be further purified. The extracts were separated by anion exchanged chromatography into three different fractions F1, F2 and F3 with increased purity from F1 to F3. The high fucose and sulfate content in fucoidans, homogenous SEC profile and NMR results confirmed that fucoidan from *F. evanescens* are sulfated fucans with the backbone consisting of alternating 2-sulfated 1,3- and 1,4-linked α-L-fucose residues, sulfated mainly at C2 and less C4 positions as

previously reported (Bilan et al., 2002; Menshova et al., 2016). While fucoidans from *S. latissima* were more complex and identified as a mixture of two main polysaccharides, sulfated fucans with a backbone built of 1,3-linked α-L-fucopyranose residues and fucogalactan with a backbone of 6-linked β-D-galactopyranose residues, which are consistent with the monosaccharide composition and SEC analysis.

In this study, the effect of extraction parameters on fucoidan yield of *S. latissima* macroalgae was examined. The monosaccharide profile and extracted fucose varied in different extraction buffers during extraction time points since the enzyme activity was strongly effected by buffers. The extracted fucose yield reached a high value in 40 mM universal buffer after 1 hour of extraction. The ethanol pre-treatment of macroalgae using different concentrations, showed no increase in fucoidan yield in the framework of this study. So likely, after treatment by ethanol the enzymes had difficulty attacking and degrading the macroalgae cell wall. To remove the remaining alginate in the crude fucoidan extract, one more alginate lyase SALy treatment step was added to the extraction process. The addition of SALy significantly decreased the alginate content but did not remove all the alginate in the crude fucoidans. To get pure fucoidans the crude extract must be further purified. Thus, these results confirmed Hypothesis 2.

Fucoidans from *S. mcclurei* were extracted using highly specific glucanase compared to the standard method used for *F. evanescens* and *S. latissima*. The results showed that the used of more specific glucanase increased the fucoidan extraction yield - confirming Hypothesis 2. The same as enzymatic extraction from *F evanescens* and *S. latissima*, enzymatic extraction showed comparable fucoidan yield for *S. mcclurei*. However, it needed a longer reaction time for the enzymes to work on *S. mcclurei*, since the fucoidan yield reached the highest value only after 6 hours of extraction, compared to 1 hour for *S. latissima* and *F. evanescens*.

Fucoidans with high molecular weight and sulfate content have many useful bioactivities as reported above. However, for some medicinal applications, fucoidans must be degraded to lower moleculer weight fucoidans. The production of fucoidan oligosaccharides by enzymes is the new trend to obtain homogeneous bioactive products. In the next chapter, we will describe the preparation of fucoidan oligosaccahrides from *F. evanescens* and *S. latissima*, including product structure.

Chapter 5: Preparation of fucoidan oligosaccharides and structural elucidation

Fucoidans from *F. evanescens* and *S. latissima* were successfully extracted by the enzymatic method as were presented in Chapter 4. They were high molecular weight polymers. For some medical applications and to study the relationship between structure bioactivity, the fucoidans must be degraded into lower molecular weight fragments. Several methods of depolymerization of fucoidans were previously developed. However, the green, highly specific method using enzymes was chosen in our study for preparing fucoidan low molecular weight products.

Fucoidans from *F. evanescens* and *S. latissima* are known to differ in the backbone structures. Fucoidans from *F. evanescen*s contain 1→3 and 1→4 linked fucosyl residues, while fucoidans from *S. latissima* contain only (1→3) linked fucosyl residues and β(1→6) galactosyl in the backbone. The work is based on the following hypothesis: Fucoidans can be degraded by endo-fucoidanase enzymes that are able to cleave the glycosidic bonds between the fucose residues. It is possible to determine the fine-chemical structure of the hydrolysis products with low molecular weight. The structure of fucoidans from *S. latissima* are very heterogeneous, as previously mentioned, by the production of specific and homogeneous oligosaccharide products by enzymes new structures of the *S. latissima* fucoidans may be determined.

The objectives of the work include:

- Prepare fucoidan substrates from brown macroalgae *F. evanescens* and *S. latissima* by the enzymatic extraction method
- Hydrolyze fucoidans by using specific endo-fucoidanase enzymes into fucoidan fractions with different size.
- Analyze the monosaccharide composition and SEC of the hydrolysis products
- Elucidate the structure of hydrolysis products by NMR analysis

This part of the study is related to Paper II, III and V.

5.1 Enzymatic hydrolysis of fucoidans from *F. evanescens*

5.1.1 Degradation of fucoidans from *F. evanescens* by an endo-fucoidanase

Fucoidans from *F. evanescens* were extracted and separated into fractions by the method described in Chapter 4. The fucoidan fraction F3 (FeF3) with high purity was chosen for fucoidan oligosaccharide preparation. The method of enzymatic hydrolysis of fucoidans and separation of hydrolysis products are showed in Figure 5.1.

As was confirmed in Chapter 4 fucoidans from *F. evanescens* are sulfated fucans, made up from 1→3 and 1→4 linked fucose residues. So for degradation of these fucoidans, enzyme with endo-action specific to 1→3 or 1→4 glycosidic bonds can be used. Recently, in a previous study from our lab, the endo-fucoidanase Fhf1 from the marine bacterium *Formosa haliotis* was reported to be able to cleave 1→ 4 glycosidic linkages in fucoidans from *F. evanescens*, resulting in linear sulfated oligosaccharide with different polymerization degree (Vuillemin et al. 2020). However, the bioactivity of the hydrolysis products was not investigated. So in this study the Fhf1 fucoidanase was used for

degrading enzymatically extracted fucoidans from *F. evanescens*. The hydrolysis products were characterized and investigated for bioactivity (Paper V and VI). The endo-fucoidanase Fhf1 effectively degraded fucoidans from *F. evanescens* with a LMP yield of 44.44% (w/w).

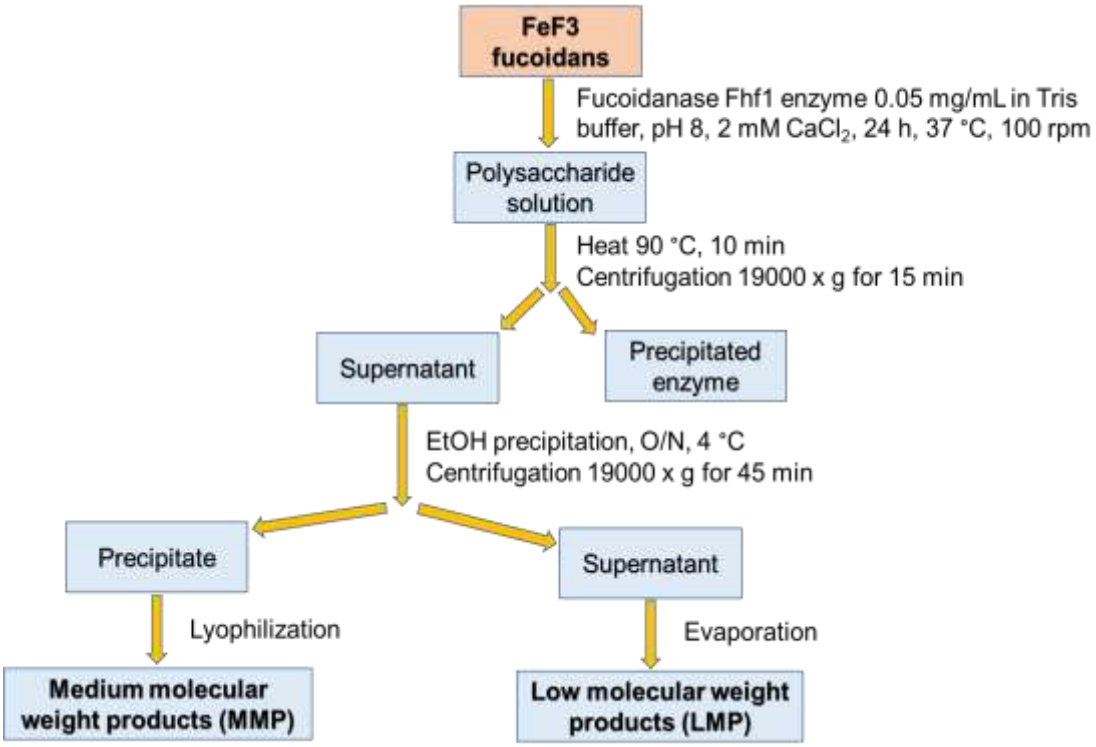


Figure 5.1 Flow-chart of enzymatic hydrolysis of fucoidans fraction F3 (FeF3) from *F. evanescens* and product separation

5.1.2 The chemical analysis of hydrolysis products

The monosaccharide, sulfate, total phenolic and protein content of the fucoidan fraction FeF3, MMP and LMP are shown in Table 5.1. These fucoidan fractions mostly contained fucose.

The average molecular weight of the FeF3 fucoidans and hydrolysis products were determined by SEC analysis (Figure 5.2). The fucoidans FeF3 contained one homogeneous peak with an average size of 400–500 kDa. After hydrolysis the molecular weight of the fucoidans was significantly decreased. The MMP were a heterogeneous mixture of fucoidans with molecular weight from 10 to 250 kDa, while the LMP was around 2 kDa. The enzymatic degradation of fucoidans is a controllable process, since the enzyme have high specificity on certain substrates. The endo-fucoidanase Fhf1 enzyme has been reported only to cleave α-(1→4) glycosidic bonds between 2-O-sulfated – L-fucopyranosyl residues in alternating motif [→3)-α-L-Fucp2S-(1,4)-α-L-Fucp2S-(1→] of fucoidans (Vuillemin et al. 2020). The low molecular products were identified as tetra-, octa- and decasaccharides with this basic structure (Vuillemin et al. 2020).

Table 5.1 Monosaccharide composition of fucoidan fraction FeF3 and enzymatic hydrolysis products from *F. evanescens*

		FeF3	MMW	LMW
	Yields [%]		53.56	46.44
Neutral sugar [% mol]	Fucose	$40.20^{a} \pm 3.97$	$43.7^{a} \pm 3.19$	$37.01^{a} \pm 1.2$
	Rhamnose	$0.22^{b} \pm 0.03$	$0.45^{a} \pm 0.02$	$0.05^{c} \pm 0.00$
	Galactose	$4.32^{a} \pm 0.46$	$4.61^{a} \pm 0.24$	$0.49^{b} \pm 0.00$
	Glucose	$0.16^{b} \pm 0.01$	$0.24^{a} \pm 0.02$	$0.03^{c} \pm 0.00$
	Xylose	$0.65^{b} \pm 0.09$	$1.17^{a} \pm 0.13$	$0.44^{b} \pm 0.00$
	Mannose	0.18 ± 0.03	nd	nd
Uronic acid [% mol]	GluA	$0.37^{a} \pm 0.05$	$0.26^{b} \pm 0.07$	nd
	ManA	0.46 ± 0.08	nd	nd
	GuluA	nd	0.49 ± 0.03	nd
Sulfate content	SO_4^{2-} [%]	$37.60^{b} \pm 1.60$	$49.60^{a} \pm 3.14$	$33.60^{b} \pm 3.37$
Weight ratio SO_4^{2-}:Fucose		0.9	1.1	0.9

Note: The monosaccharide and uronic acid data are given as %mol with total sulfate (SO_4^{2-}) first calculated as %wt of total, then as degree of sulfation on dehydrated fucose moieties in different fucoidan fractions. Each data point represents the average value of independent triplicates. Different superscript roman letters a, b, c indicate statistically different values ($p < 0.05$) of each sugar from different fucoidan fractions.

The bioactivity of fucoidans varies depending on monosaccharide composition, the molecular weight and the structure of fucoidans. Fucoidan crude and fractions FeF1, FeF2 and FeF3 from *F.evanescens* were studied for anti-angiogenic and -osteogenic effect on mono- and co-culture system mimicking bone tissue environments (Ohmes et al. 2020). From the tested fucoidans, the FeF3 with highest fucose and sulfate content show the best bioactivity. So here the anti-angiogenic and anti-osteogenic properties was likely related to the purity and sulfate content in the fucoidans (Ohmes et al. 2020). The fucoidans FeF3 and the enzymatic hydrolysis products of (MMP and LMP) differed in monosaccharide composition as well as molecular weight and was expected to have different bioactivities. In fact, in the recently research Ohmes and colleagues (Ohmes et al. 2022) found that the bioactivity of these enzymatic hydrolysis products in angiogenic and inflammatory processes was changed compared to native fucoidans. While the native fucoidans FeF3 showed inhibitory effect on angiogenesis-related mediators, this inhibitory effect was lost in MMP and LMP. In another hand, in the same study both native fucoidans and hydrolysis products were studied inflammatory effect and only the high doses of MMP causes a pro-inflammatory response in mesenchymal stem cells (MSC) and outgrowth endothelial cell (OEC) mono- and co-culture systems (Ohmes et al. 2022). In another study, the effect of the Fhf1 produced LMP from *F. evanescens* was studied *in vivo* on bone regeneration and implant fixation in 7 female Texel/Gotland/Romney sheep. Results showed that LMP fucoidans in combination with hydroxyapatite (HA/FUC) gave a nice effect on bone regeneration and enhanced implant fixation (Nielsen et al. 2021).

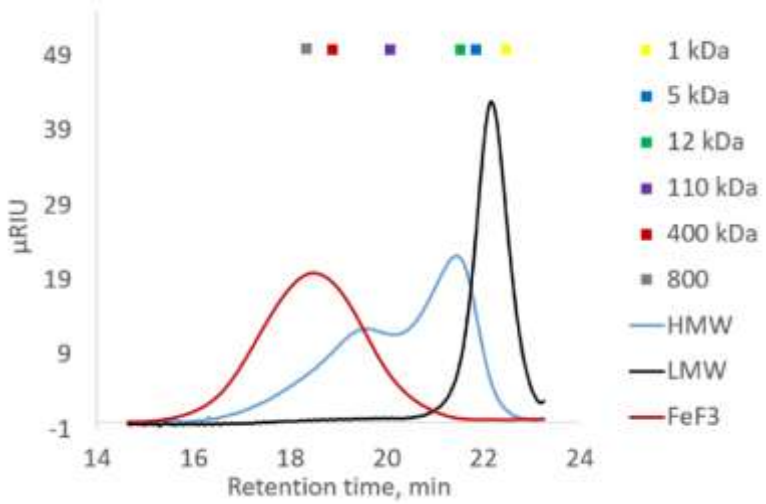


Figure 5.2 SEC chromatogram of fucoidans FeF3 from *F. evanescens* and enzymatic hydrolysis products

5.2 Enzymatic hydrolysis of fucoidans from cultivated *S. latissima* from Ocean Rainforest (SLOR)

5.2.1 Fucoidan degradation

Fucoidans from *S. latissima* macroalgae cultivated in the Faroe Islands by the company Ocean Rainforest, were extracted by the enzyme-assisted method (Nguyen et al. 2020) with slight modifications as described in Paper II. The crude fucoidans were separated by DEAE-Macroprep column and four fucoidan fractions SLORF1 – F4 were obtained. The monosaccharide composition of the four fractions were analyzed and presented in Table 5.2. These *S. latissima* fractions also showed a different molecular weight distribution (Figure 5.3). The SLORF3 fraction was pure with high content of fucose and galactose. Therefore, this SLORF3 fraction (1.24 g) was chosen for preparing fucoidan oligosaccharides. The structures of fucoidans from *S. latissima* were identified in Chapter 4. The two main polysaccharide structures were elucidated: one backbone consisted of 1,3-linked α-L-fucopyranose residues and one consisted of 6-linked β-D-galactopyranose residues. The novel endo-fucoidanase Mef2 enzyme from the marine bacterium *Muricauda ecklonia*e was the first enzyme capable of degrading the fucoidans from *S. latissima* and was identified in our lab (Paper II: Tran et al. 2022). The detailed method of the hydrolysis of fucoidans by endo-fucoidanase Mef2 was displayed in Figure 5.4. The enzymatic hydrolysis products were separated into MMP and LMP with the yield of 86% (1.07g) and 14% (0.17g) (w/w), respectively.

Table 5.2 Monosaccharide composition of fucoidan fractions purified from enzymatic extract of *S. latissima* from Ocean Rainforest.

Monomer Category		SLOR F1	SLOR F2	SLOR F3	SLOR F4
Neutral monosaccharides (mol%)	Fucose	$31.72^{c} \pm 2.23$	$69.03^{a} \pm 0.79$	$58.6^{b} \pm 0.25$	$63.07^{b} \pm 0.5$
	Rhamnose	$0.39^{c} \pm 0.05$	$0.96^{b} \pm 0.01$	$0.84^{b,c} \pm 0.03$	$1.95^{a} \pm 0.06$
	Galactose	$0.54^{d} \pm 0.06$	$17.44^{c} \pm 0.24$	$34.79^{a} \pm 0.44$	$22.06^{b} \pm 0.02$
	Glucose	$0.29^{d} \pm 0.03$	$0.4^{c} \pm 0.02$	$0.53^{b} \pm 0.01$	$0.88^{a} \pm 0.02$
	Xylose	$2.41^{c} \pm 0.31$	$5.05^{b} \pm 0.21$	$2.74^{c} \pm 0.11$	$7.26^{a} \pm 0.27$
	Mannose	$0.6^{d} \pm 0.09$	$2.1^{a} \pm 0.13$	$0.95^{c} \pm 0.03$	$1.76^{b} \pm 0.04$
Uronic acids (%)	GuluA	$2.49^{a} \pm 0.14$	$0.15^{b} \pm 0.09$	$0.06^{b} \pm 0.04$	$0.18^{b} \pm 0.00$
	GluA	$1.32^{b,c} \pm 0.27$	$3.68^{a} \pm 0.43$	$1.1^{c} \pm 0.04$	$2.33^{b} \pm 0.04$
	ManA	$60.24^{a} \pm 3.18$	$1.19^{b} \pm 0.14$	$0.39^{b} \pm 0.3$	$0.51^{b} \pm 0.13$
Sulfate (%)		$11.34^{c} \pm 1.01$	$28.44^{b} \pm 2.07$	$35.64^{a} \pm 0.43$	$32.11^{a} \pm 3.09$

Note: The monosaccharide and uronic acids data are given in %mol (relative level) of total carbohydrates analysed different fucoidan fractions. Total sulfate ($SO4^{2-}$) was calculated as %wt of total fucoidan fractions. Each data point represents the average value of independent triplicated. Different superscript roman letters a, b, c, d indicate statistically different values ($p < 0.05$) of each sugar from different fucoidan fractions.

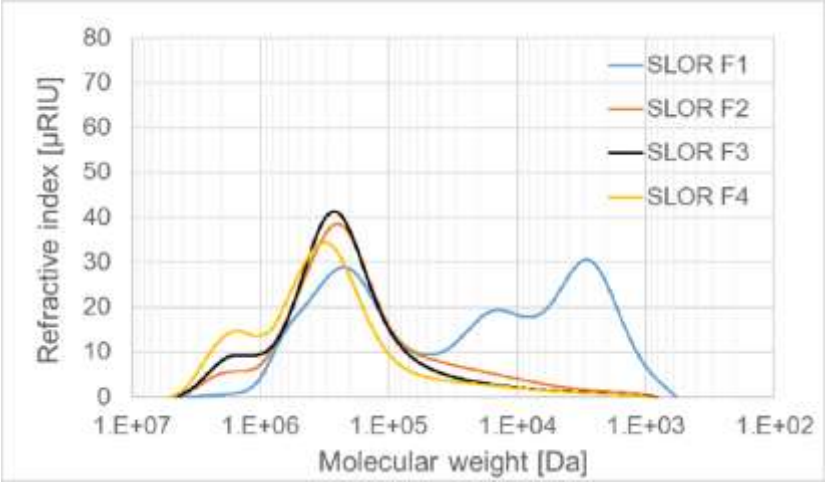


Figure 5.3. SEC chromatogram of fucoidan fractions from *S. latissima* from Ocean Rainforest after IEX purification. The SLORF1 fraction contains LMW compounds of around 2 - 12 kDa and a proportion of MMW compounds between ~200–600 kDa. The SLORF2 and SLORF3 contain HMW compounds ranging from ~250 kDa to over 800 kDa. The SLORF4 contain more HMW compounds ranging from ~300 kDa to over 800 kDa. Pullulan was used as standard.

5.2.2 Chemical analysis of fucoidan fraction SLORF3 and hydrolysis products

The DeSLORF3 and enzymatic hydrolysis products were analyzed by SEC. DeSLORF3 are high molecular weight polysaccharides with the mass ranging from 250 to over 800 kDa. After treatment of fucoidans by Mef2 fucoidanase, the molecular weight of fucoidans was decreased with the size around 150 to over 800 kDa in MMP and 3 kDa in LMP (Figure 5.5). The fucoidan fraction SLORF3 contained high amounts of fucose and galactose. The

ratio of fucose: galactose in the substrate SLORF3 was 1:0.65 (%w: %w). While the fucose: galactose ratio was increased in the MMP to 1: 1.01, indicating that part of the polymer containing fucose was degraded into smaller fragments and separated out in the LMP.

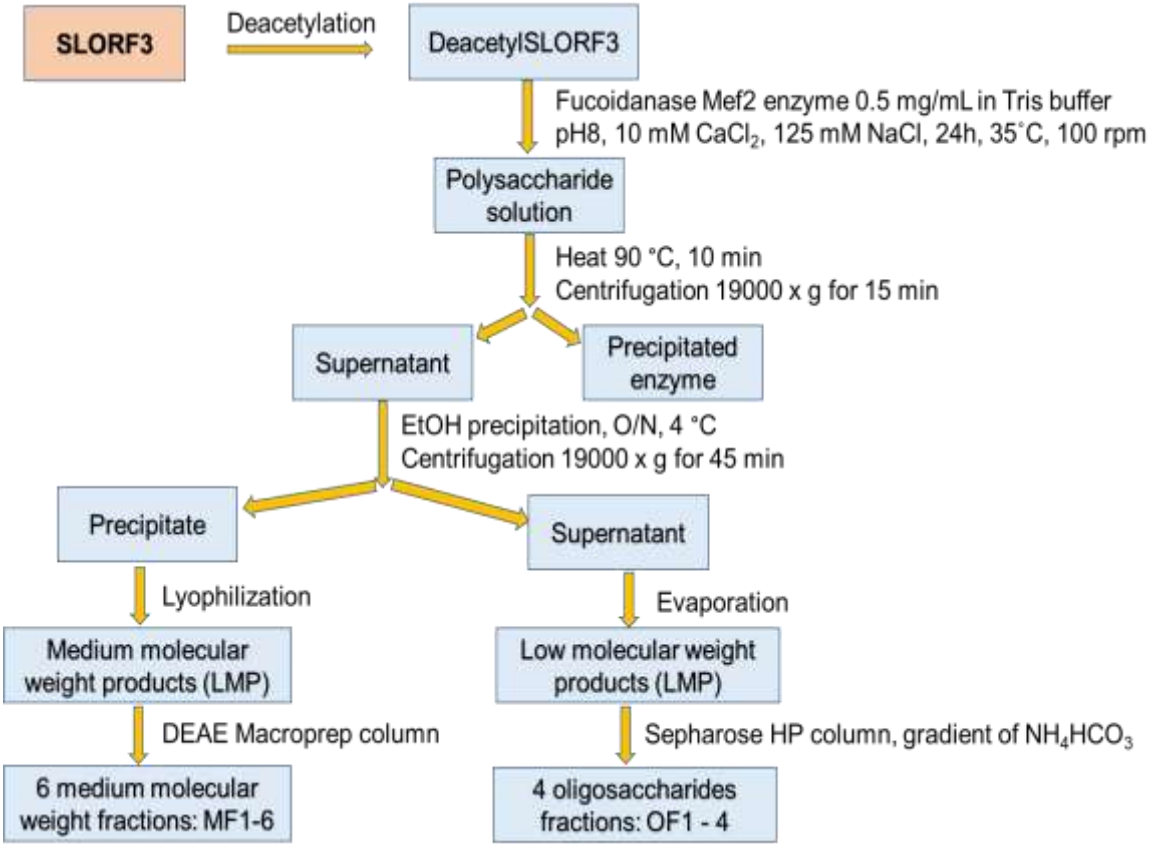


Figure 5.4 Flow-chart of enzymatic hydrolysis of fucoidan and product separation

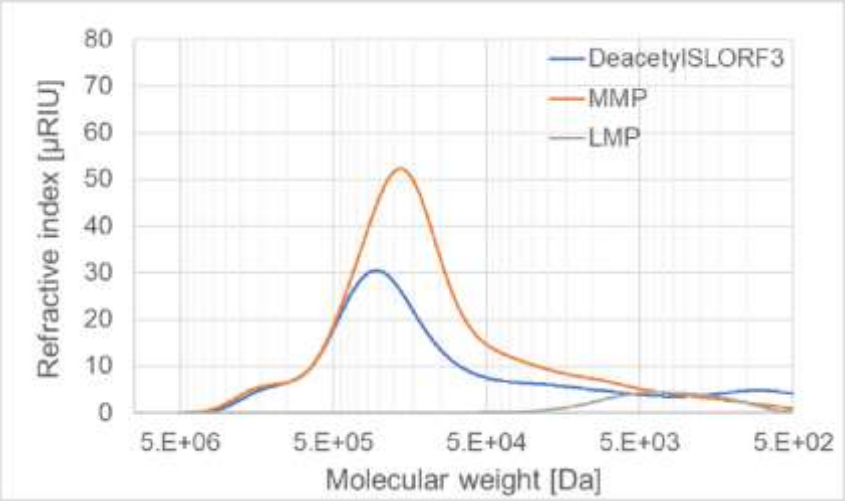


Figure 5.5 SEC chromatogram of deacetylated fucoidans SLORF3 from *S. latissima* and enzymatic hydrolysis products.

5.2.3 Separation of specific oligosaccharides from LMP and structure determination

The LMP was further separated using anion exchange-chromatography with a High performance Q sepharose column (1x20cm). The total carbohydrate content was analyzed in all obtained fractions by the phenol-sulfuric acid method. Fractions containing carbohydrates were run on C-PAGE (Figure 5.6). The fucoidan oligosaccharides showed bands on the C-PAGE gel after staining with alcian blue. Based on the purity, fractions with one and the same band were combined, resulting in four oligosaccharides (OF1-4) with different molecular weights. However, the amount of each oligosaccharide was low (1 – 3 mg), only giving enough oligosaccharide for NMR analysis.

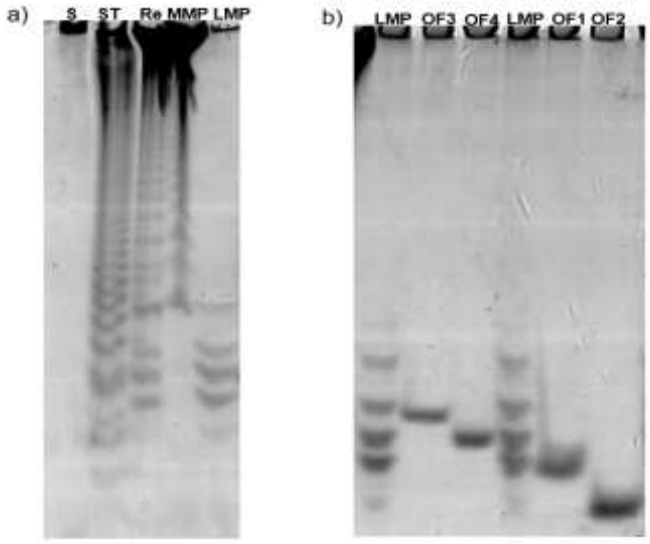


Figure 5.6 C-PAGE analysis of products from enzymatic treatment of fucoidans from *S. latissima*. a) Enzymatic reaction and separation of medium and low molecular weight products (MMP and LMP). S stands for control substrate DeSLORF3 with high molecular weight therefore it did not migrate and standed on the top of the gel. ST stands for standard reaction of FFA2 fucoidanase treatment of fucoidans from *F. evanescens*, where oligosaccharide products with different size migrated along the gel. Re stands for reaction of Mef2 on DeSLORF3, where different oligosaccharides were produced and migrated in the gel. b) Purified oligosaccharide fractions OF1 to OF4, which showed only one band in the gel.

Homogeneous fucoidan oligosaccharides of small size are suitable for structure analysis. The assignment of oligosaccharides was achieved by 2D NMR spectroscopy analysis. Fraction OF1, with highest purity, gave the best NMR spectra. The other oligosaccharide fractions OF2, OF3 and OF4 provided signals similar to those of OF1. Therefore, all purified oligosaccharides were suggested to have the same core structure as the OF1 oligosaccharide. The results obtained were assigned with TOCSY and heteronuclear NMR ^{1}H-^{13}C HMBC, ^{1}H-^{13}C H2BC and ^{1}H-^{13}C HSQC. Based on the results, ten spin systems were investigated. However, the reducing end residue was observed in the spectra as two of the spin systems Fα and Fβ. Similar the adjacent residue also yielded two distinct spin systems Eα and Eβ. Therefore, fraction OF1 contain eight fucosyl residues. The details of ^{1}H and ^{13}C NMR data for the OF1 are summarized in Table 5.3. The sequence analyses of the OF1 fraction were performed using ^{1}H-^{13}C-HMBC and ^{1}H-^{1}H NOESY experiments

to detect $^{3}J_{CH}$ correlations and NOES across the glycosidic bonds. The presence of sulfate groups was determined based on the deshielding of ^{1}H and corresponding ^{13}C chemical shifts. From the data six out of the eight fucopyranose residues carry sulfates. From the ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and NOESY spectra, a new branch point at C4 positions of the oligosaccharide backbone was identified, which has not previously been found in *S. latissima* fucoidans. Figure 5.8 shows the evidence of the 1→4 linkage between G and B residues. It is very interesting since only branch points at C2 positions had previously been reported, but not C4 linked branches (Bilan et al. 2010).

The ^{1}H-^{13}C spectra of the OF2 fraction were similar to the spectra of the OF1 fraction. However, there are also some differences in NMR data between the two oligosaccharides. In the OF2 structure a new reducing end was observed with signals for residue F vanishing. The reducing end residue E was observed as two spin systems Eα and Eβ. Similar the next residues D and H also yielded two sets of signals due to their vicinity Eα and Eβ. The fraction OF2 contain seven fucosyl residues. The structures of OF1 and OF2 are presented in Figure 5.7.

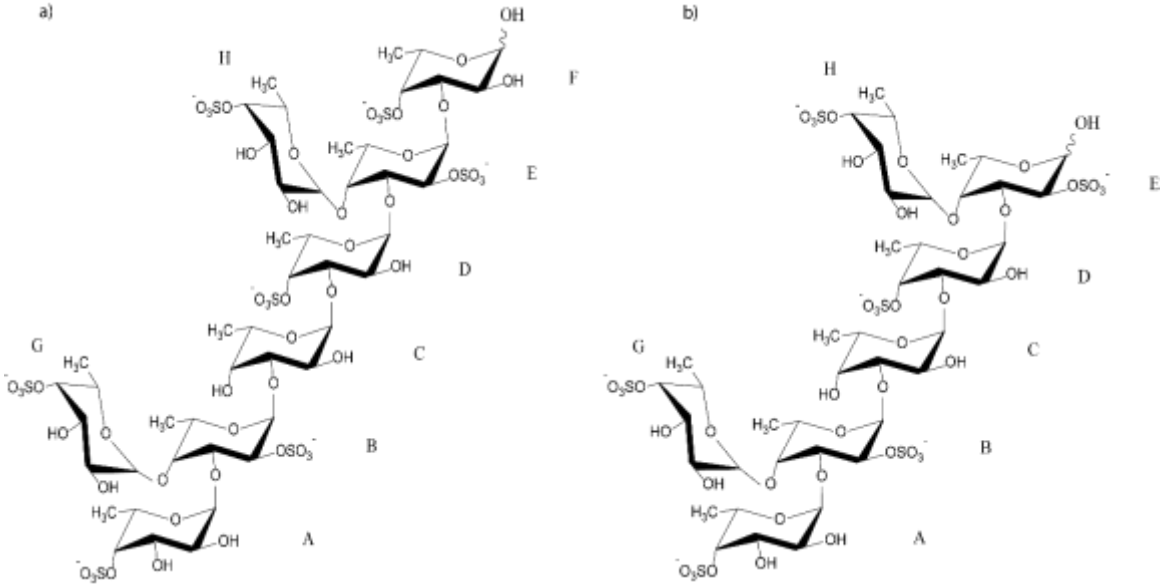


Figure 5.7 Molecular structure of the purified octasaccharide OF1 (a) and heptasaccharide OF2 (b) from *S. latissima* fucoidans showing the α-1,3 backbone, sulfation, and unique substitutions.

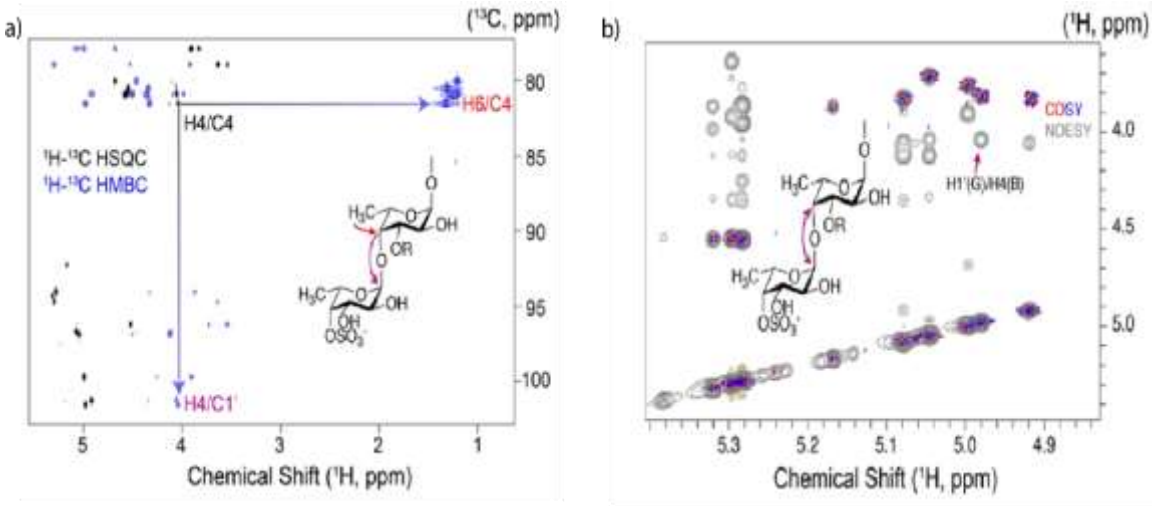


Figure 5.8 NMR analysis of the OF1 oligosaccharide. The evidence of the 1→4 linkage between G and H residues: a) Overlay of ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectrum showing the correlation across the glycosidic bond for the well-resolved signals of the 1-4 linked residues G and B in OF1 fraction. b) Overlay of ^{1}H-^{1}H COSY and ^{1}H-^{1}H NOESY spectra, showing the NOE across the glycosidic bond for hydrogens 1 and 4 of residues G and B, respectively in OF1 fraction.

Table 5.3 ^{1}H and ^{13}C NMR data for the OF1 oligosaccharide purified after cleavage of SLORF3 from *S. latissima*

Residue\Atom		Chemical shifts (ppm)					
		H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	*α*-L-Fuc*p*(4SO_3^-)-(1-	5.036	3.71	3.982	4.561	4.458	1.211
		96.87	68.5	69.06	80.87	66.6	15.84
B	→3,4)-*α*-L-Fuc*p*(2SO_3^-)-(1-	5.283	4.556	4.122	4.037	4.331	1.325
		94.05	73.2	73.2	81.46	67.96	15.47
C	→3)-*α*-D-Fuc*p*-(1-	4.995	3.764	3.858	3.954	4.25	1.181
		99.81	66.96	75.67	68.62	66.7	15.59
D	→3)-*α*- D-Fuc*p*(4SO_3^-)-(1-	5.076	3.833	3.905	4.68	4.475	1.211
		96.74	67.53	77.9	80.08	66.54	15.84
Eα	→3,4)-*α*- D-Fuc*p*(2SO_3^-)-(1-	5.32	4.552	4.12	4.05	4.34	1.29
		94.28	73.2	73.2	80.85	67.95	15.65
Eβ	→3,4)-β- D-Fuc*p*(2SO_3^-)-(1-	5.297	4.545	4.114	4.054	4.35	1.29
		94.69	73.2	72.8	80.85	67.95	15.65

Fα	→3)-α- D-Fuc*p*	5.161	3.872	3.871	3.986	4.118	1.165
		92.25	66.56	75.36	68.41	66.07	15.61
Fβ	→3)-β- D-Fuc*p*	4.517	3.539	3.633	3.92	3.72	1.203
		96.25	70.12	78.93	68.1	70.67	15.583
G	α- D-Fuc*p*(4SO_3^-)-*(1*→	4.974	3.824	4.103	4.584	4.37	1.254
		101.6	69.05	68.03	80.95	67.172	16.14
H	α- D-Fuc*p*(4SO_3^-)-*(1*→	4.918	3.831	4.074	4.546	4.338	1.307
		101.34	69.38	68.37	80.55	67.33	16.38

5.2.4 Separation of medium molecular weight products and structure determination

More Mef2 enzyme was added to MMP and no further degradation was observed. The mixture of medium molecular weight fucoidans were separated by DEAE-Macroprep column. Based on the results of all eluted fractions by total carbohydrate and C-PAGE analysis (Figure 5.9) six fucoidan fractions (MF1-6) with different average of molecular weight were collected. The monosaccharide composition of all these fucoidan fractions vary, especially the galactose and sulfate content (Table 5.4). MF1 and MF2 fractions contain lower molecular weight polysaccharides (Figure 5.10) and also lower amounts of galactose compared with the other MF fractions. All fractions MF2-MF6 were further analyzed by NMR.

Table 5.4 The structure characteristics of high molecular weight fractions

Fractions	Monosaccharide composition, Fuc:Gal:Rha:Glu:Xyl:Man (mol:mol)	Sulfate, %
MMP	1 : 0.02 : 0.92 :0 :0.04 :0.05	34.28 ± 1.8
MF1	1 : 0.29 : 0.07: 0.02 : 0.14 : 0.02	52.05 ± 0.79
MF2	1 : 0.42 : 0.06: 0.02 : 0.12 : 0.02	48.17 ± 1.9
MF3	1 : 0.56 : 0.04: 0.02 : 0.07 : 0.01	52.05 ± 0.79
MF4	1 : 0.52 : 0.03: 0.02 : 0.06 : 0.01	54.8 ± 2.89
MF5	1 : 0.73 : 0.03: 0.02 : 0.07 : 0.01	27.77 ± 1.55
MF6	1 : 0.65 : 0.03: 0.02 : 0.06 : 0.01	28.67 ± 3.27

The NMR data showed that fraction MF2 was a mixture of fucan oligosaccharides, mainly containing 1→3 linked α-fucosyl units. The larger sized fucoidans MF3 – MF6 are mainly fucogalactans from *S. latissima*, since they have high galactose content. They are made of β-1→4 as well as β-1→6 linked galactosyl units with NMR spectral characteristics similar as reported previously (Bilan et al. 2010). The main backbone of fucogalactan consists of 1→6 linked galactopyranose residues. The fucogalactan chain branched at C-4 by the disaccharide substituent α-L-Fuc*p*(3Ac, 4SO_3^-)-(1→3)-β-D-Gal*p*(2Ac, 4SO_3^-)-(1→.

The ^{1}H and ^{13}C NMR data of fractions MF3 – MF6 are shown in Table 5.5. Additionally, the NMR data analysis on the native, non-deacetylated fucoidan SLORF3 showed a strong

and characteristic deshielding of ^{1}H3 in unit I (Table 5.5 and Figure 5.11) (to chemical shifts of 5.260 ppm/71.70 ppm for the C3H3 group) and an equivalent deshielding of ^{1}H2 in unit J (to 5.218 ppm/70.68 ppm for the C2H2 group) (Table 5.5 and Figure 5.11). Hence, the native fucoidans was predominantly acetylated at the O3 of unit I and O2 of unit J. Here the NMR data confirmed that fucoidans from *S. latissima* are basically galactofucans, carrying terminal sulfated and acetylated α-fucosepyranose residue at non-reducing branch point ends.

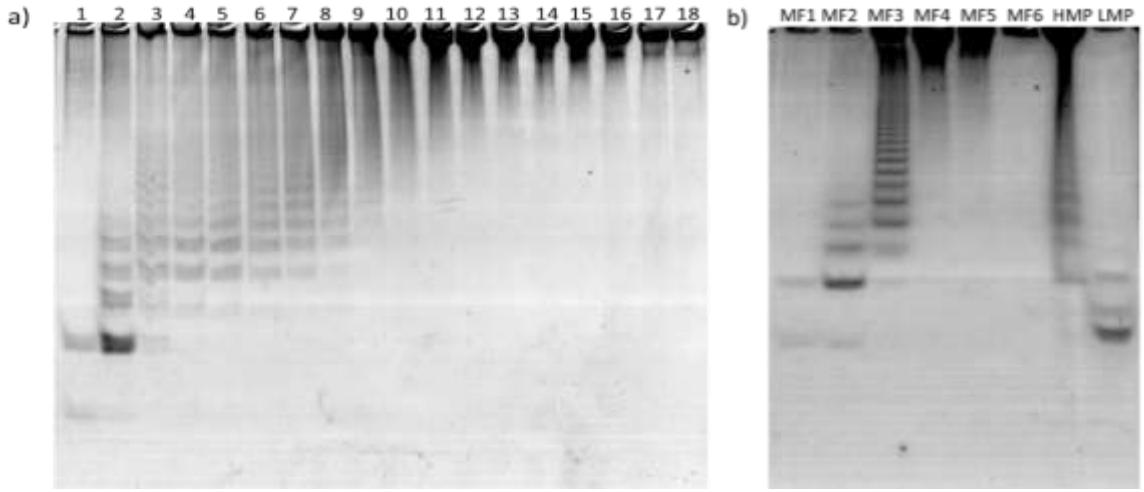


Figure 5.9 C-PAGE analysis of medium molecular weight products from Mef2 enzymatic treatment of fucoidans from *S. latissima*. a) DEAE eluted fractions. b) Purified high molecular weight fractions MF1, MF2, MF3, MF4, MF5 and MF6. MF fractions are heterogeneous and contained more than one band. MF1 – 2 contained oligosaccharides with low molecular weight which migrated in the gel and high molecular weigh fucoidan which did not migrate. MF3 was mixture of oligosaccharides weight which migrated in the gel and high molecular weight fucoidans which slightly migrated into the gel as MF4 and 5. While MF6 stayed on the top of the gel and did not migrate.

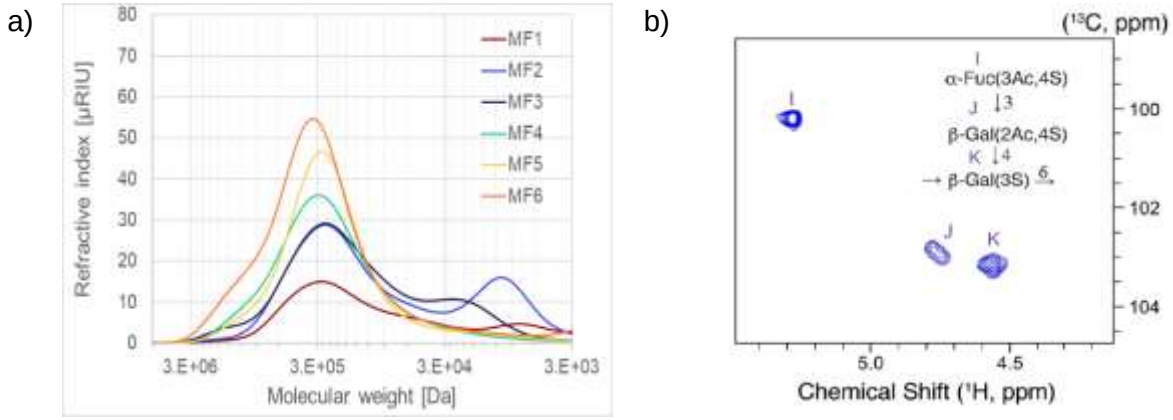


Figure 5.10 Analysis of MF fractions. a) SEC chromatogram: HF1- 3 fractions contain fucoidans in the range of 6 to 200 kDa. MF4 – 5 fractions contain compounds of around 220 - 230 kDa. MF6 fractions is high molecular weight polysaccharide ranging from 260 to over 800 kDa. Pullulan was used as standard. b) NMR analysis of MF3-6 fractions. Anomeric region of the ^{1}H-^{13}C HSQC spectrum.

Table 5.5 ^{1}H and ^{13}C NMR data for the medium molecular weight fractions purified after cleavage of SLORF3 from *S. latissima*.

	Residue	Chemical shifts (ppm)					
		H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
I	α-L-Fuc*p*(3Ac, 4SO_3^-)-(1→	5.282	3.785	4.099	4.589	4.51	1.258
		100.31	69.01	68.58	81.11	66.46	15.8
J	-3)-β-D-Gal*p*(2Ac, 4SO_3^-)-(1→	4.773	3.731	3.908	4.692	3.798	3.802
		102.84	71.6	76.47	77.28	74.39	61
K	-4,6)-β-D-Gal*p*(3SO_3^-)-(1→	4.58	3.815	4.426	4.54	3.93	4.188/3.924
		103.1	68.85	79.95	73.76	73.22	70.3

5.3 Conclusion and discussion

In this part of the study the endo-fucoidanase enzymes succesfully degraded fucoidans from *F. evenescens* and *S. latissima*, although different enzymes with different substrate specificities are necessary for oligosaccharide production from the different fucoidan substrates – confirming Hypothesis 4. The Fhf1 fucoidanase cleaved fucoidans from *F. evanescens* with high yeild of LMP, indicating the more available cleaving site for the enzyme in this substrate . Both the native *F. evenescens* fucoidans and their LMP derivatives showed bioactivity (Ohmes et al. 2020; Nielsen et al. 2021).

This is the first report of an enzyme capable of catalysing the hydrolysis of fucoidans from *S. latissima*. The new endo-fucoidanase Mef2 enzyme was used for degradation of fucoidans from *S. latissima*. However, the yield of LMP was much lower than of the degradation of fucoidans from *F. evanescens*. Based on NMR assigments of MMP and LMP, two main structures of fucoidans from *S. latissima* macroalgae were confirmed. The first is a sulfated fucan made up of 1→3 linkages of fucose residues. The endo-fucoidanase enzyme Mef2 attack only on this structure in fucoidans, cleaving the α-(1→3) linkages of fucopyranose residues, producing sulfated octosaccharide and heptasaccharide with 2 single fucose branches at C4 position. The second structure is a sulfated fucogalactan, containing 1→6 linked galactosyl residues in the backbone, branched at C4 by a disaccharide, with a terminal fucose residue. This structure was resistant to Mef2 hydrolysis. It is interesting that the new observed brached oligosaccharides gave new information about the structure of fucoidans from *S. latissima* macroalgae.

Beside fucoidans, brown macroalgae are rich in alginates, which have broad applications in food, cosmetics and medicinal industries. The availability of alginate active enzymes can enable the production of alginate oligosaccharides, which have more bioactivity diversity. In the next chapter, new findings of an alginate lyase enzyme isolated from sea

cucumber gut bacteria that can degrade alginates from *F. evanecens*, *S. latissima* and *S. mcclurei* will be presented.

Chapter 6: A new alginate lyase enzyme PALy1 from the marine bacterium *Pseudoalteromonas sp.* MB47

Marine derived oligosaccharides, including alginate oligosaccharides has gained interest, since they not only can retain the bioactivity of the polysaccharides but also have better solubility and bioavailability (Trincone 2015). Alginate as a hydrocolloids has been reported to be useful as a drug carrier, wound healing material, a therapeutic adjuvant (Fan et al. 2019) as well as applied in food and cosmetic (Lee and Mooney 2012). However, the high molecular weight, high viscosity and low solubility limit the use of alginate as a potential bioactive component itself. Brown macroalgae belonging to the *Laminariales* and *Fucales* orders are the main sources of alginate (Zhu et al. 2021). Sea cucumbers are algae feeders and their associated bacteria (mainly from their gut) may produce enzymes that able to degrade algal polysaccharides. Hence, the hypothesis of this study is sea cucumber gut bacteria may harbor genes that encode hitherto uncharacterized alginate-degrading enzymes. Based on this, the objective is finding new alginate lyases, which are active on alginates isolated from *F. evanescens*, *S. latissima* and *S. mcclurei*.

Thus, the work is based on the objectives:

- Expression, purification and characterization of a new alginate lyase enzyme
- Investigation of the effects of phenolic compounds in enzymatic ethanol extracts from *F. evanescens*, *S. latissima* and *S. mcclurei* macroalgae on alginate lyase activity
- Extraction of alginate from *F. evanescens*, *S. latissima* and *S. mcclurei* macroalgae, determine the molecular weight and M/G ratio of these alginates
- Determination of alginate lyase activity on extracted alginates of *F. evanescens*, *S. latissima* and *S. mcclurei*

6.1 Cloning of alginate lyase genes and expression of enzymes

Aerobic bacteria were isolated from sea cucumber guts from the Vietnamese sea and screened for enzymes able to modify brown algal polysaccharides including fucoidanase, alginate lyase and fucoidan sulfatase. Alginate lyase activity was determined by Gram's iodine plate method (Sawant et al. 2015). Among the tested bacteria, strain MB47 showed highest alginate lyase activity. This strain was also selected for fucoidan sulfatase study in our previous work (Mikkelsen et al. 2021). Based on 16S ribosomal DNA comparisons, MB47 was identified as *Pseudoalteromonas* sp., closely related to *Pseudoalteromonas shioyasakiensis* (Mikkelsen et al. 2021).

Four putative alginate lyases from different CAZy families PL6, PL7 and PL17 were found in the genome of MB47 (Mikkelsen et al. 2021). The constructs containing the gene encoding these four alginate lyases named PALy1 (NCBI Reference Sequence: WP_166338612.1), PALy2 (NCBI Reference Sequence: WP_166338614.1), PALy3 (NCBI Reference Sequence: WP_166338624.1) and PALy4 (NCBI Reference Sequence: WP_166338626.1) were designed to harbor C-terminal 6xhistidine tags. The synthetic codon-optimized genes (optimized for *Escherichia coli* expression) without signal peptides, were synthesized by GenScript (Piscataway, NJ, USA) and inserted in the pET-

28b+ vector between the NdeI and XhoI restriction sites. The *E. coli* strain DH5α (Thermo Fisher Scientific, Waltham, MA, USA) was used as plasmid amplification host and BL21 (DE3) with the Pch2 (pGro7) chaperone was used as protein expression host.

Expression of all four alginate lyases was performed in *E. coli* BL21 (DE3) with the pGro7 chaperone. The overnight cultures of *E. coli* hosting alginate lyases from *Pseudoalteromonas sp.* were grown at 37 °C, 180 rpm in lysogeny broth (LB) medium containing 100 µg mL^{-1} kanamycin and 34 µg mL^{-1} chloramphenicol. The expression was performed in 500 ml LB media with 100 µg mL^{-1} kanamycin and 34 µg mL^{-1} chloramphenicol and 0.05 % arabinose. When the cultures reached OD_{600} 0.6–0.8, gene expression was induced by 1 mM IPTG and carried out for overnight at 20°C, 180 rpm. The cells were collected by centrifugation, re-suspended in cold extraction-buffer (20 mM Tris-HCl buffer, pH 7.4, 250 mM NaCl, 0.2 mg/mL lysozyme), and sonicated. The cell debris was removed by centrifugation. The supernatant (crude enzyme) was collected. The crude enzymes were applied to SDS-gel and western blot for determination the homogeneity, molecular weights and expression level (Figure 6.1). Based on the electrophoresis gel, the alginate lyase PALy1 belonging to the PL6 family giving best expression, was chosen for further studies. The crude enzyme of PALy1 was further purified using Ni^{2+}-Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden) in 20 mM Tris-HCl buffer pH 7.4, 250 mM NaCl, 100 mM imidazole. Imidazole was then removed by using PD-10 columns (GE Healthcare, Chicago, IL, USA). The purified PALy1 enzyme showed a clear and unique band on SDS-PAGE and western blot.

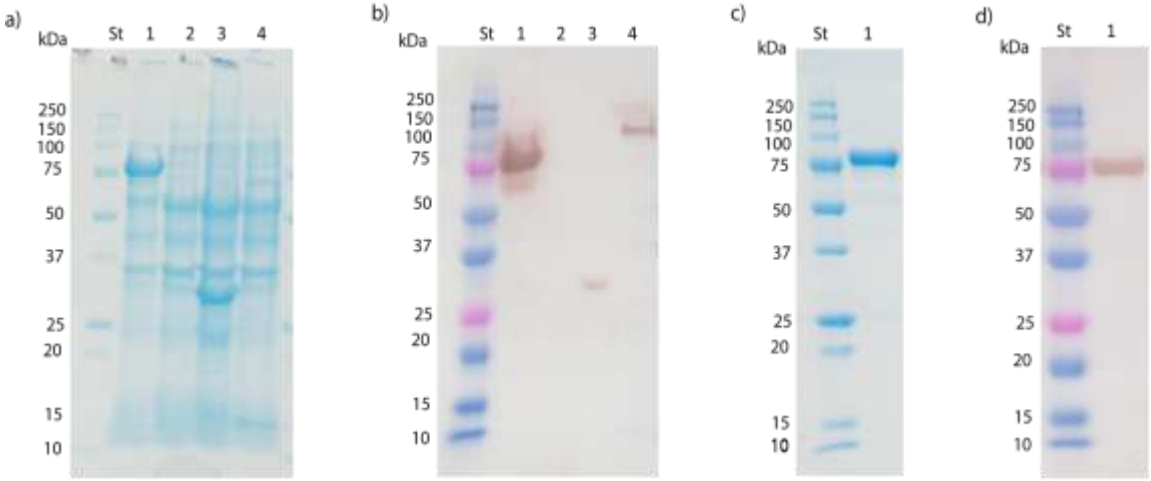


Figure 6.1 Alginate lyase enzymes. (a) SDS-PAGE and (b) Western blot of crude enzymes: St stands for standard – Protein marker, 1 – alginate lyase PALy1, 2 – alginate lyase PALy2, 3 – alginate lyase PALy3, 4 – alginate lyase PALy4. The molecular weight of PALy1, PALy2, PALy3 and PALy4 were 82, 81, 35, 95 kDa. (c) SDS-PAGE and (d) Western blot of purified PALy1.

6.2 Enzyme Characterization

Alginate lyases catalyze the degradation of alginate by β-elimination of glycosidic bonds with formation of unsaturated double bonds in the oligosaccharide products at the non-reducing end. Experiments were performed in a 200 µl reaction mixture, containing 0.025

mg/ml enzyme and 2 mg/ml alginate from Sigma in 20 mM UB4 buffer. Alginate lyase activity was determined by monitoring the formation of unsaturated products, measuring the absorbance at 235 nm every 30 seconds for first 15 minutes. The initial velocities (linear range over 5 minute runs) were convered to mM per seconds using the extinction coefficient of 6150 M^{-1} cm^{-1} (Stender et al. 2019).

The optimum pH and temperature values for alginate lyase PALy1 were determined in 20 mM universal buffer 4 (UB4) (20 mM HEPES, 20 mM Bis-Tris, and 20 mM sodium acetate) with pH ranged from 4 to 8 and temperature from 25 to 45 °C, 150 mM NaCl. Thermal stability of enzyme was studied at 25, 30, 35, 40, 50 °C. The PALy1 enzyme exhibited the highest activity at 40 °C and pH 7. The melting temperature (T_m) of PALy1 was determined by differential scanning calorimetry. As shown in Figure 4.2D, the T_m of enzyme was 41 °C, in the absence of substrate.

The enzyme activity was not only influenced by pH but also on the type of the buffers. Three buffer systems Tris- HCl, UB4 and citrate phosphate were evaluated their effect on the alginate lyase activity. The experiments were carried out in 20 mM of buffers at optimal pH and temperature. The enzyme showed different activity in the different buffers used (Figure 6.2b). A low initial rate was observed in citrate phosphate buffer and the rate increased in UB4 and Tris-HCl buffer. So the different enzyme activity in the same buffer concentration indicated that buffer ions influence the enzyme activity.

6.3 Effect of NaCl and divalent ions on alginate lyase activity

The alginate lyase PALy1 originated from a marine bacteria and therefore likely needed NaCl for activity. The effect, on alginate lyase activity, of different NaCl concentrations from 6.5 to 506.5 mM was investigated. The results showed that the enzyme had high salt tolerance with optimal salt concentrations in the range of 256.5 - 356.5 mM. The enzyme activity was found to be NaCl dependent.

The effect of divalent metal ions was investigated. The enzyme was treated with 2 mM EDTA for 20 minutes at room temperature. Then the mixture was passed through PD10 column to remove EDTA, the enzyme activity was performed by standard assay. As shown in Figure 6.3, the activity of PALy1 was activated by various divalent ions at 2 mM concentration Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and inhibited by Cu^{2+}, Zn^{2+}. These results are similar with the reported data on another alginate lyase from PL6 family (Li et al. 2016, 2019).

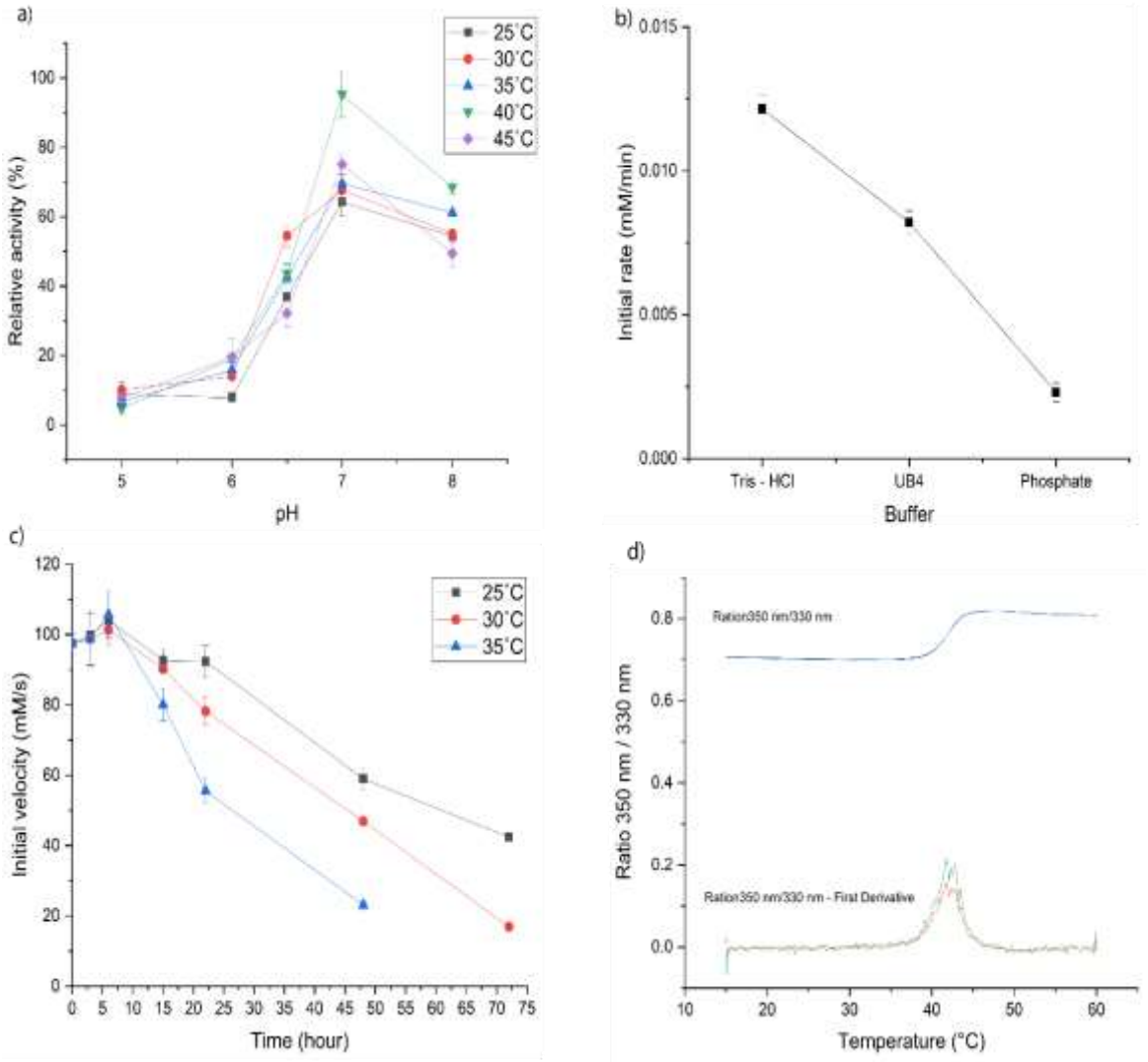


Figure 6.2 The biochemical characteristics of the alginate lyase PALy1. (a) The optimal temperature and pH of alginate lyase. The enzyme activity was determined at temperature from 25 to 45 °C and pH from 5 to 8 in UB4 buffer. (b) The effect of different buffers on alginate lyase activity. The enzyme activity was determined in 3 different buffers at the same reaction conditions. (c) The thermal stability of alginate lyase. The enzyme was firstly incubated at 25, 30, 35, 40, 50 °C and then the activity was measured on alginate from sigma. The activity of PALy1 was retained 80% after 15 hours incubation at 35 °C. (d) The melting temperature (T_m) of PALy1. Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation.

6.4 Substrate specificity of the alginate lyase

The alginate lyase activity was tested on three different substrates: alginate, PolyM and PolyG at two enzyme concentration to determine the substrate specificity (Figure 6.4). The enzyme showed low activity on alginate. The alginate lyase PALy1 was inhibited by different substrates: alginate, polyM and polyG at high substrate concentrations. Especially, polyG showed more inhibitory effects on PALy1. Therefore, at low enzyme concentrations the enzyme was strongly inhibited by polyG, resulting in lower initial

velocity than on polyM substrate. However, when the enzyme concentration increased, the enzyme showed more activity on polyG (Figure 6.4a).

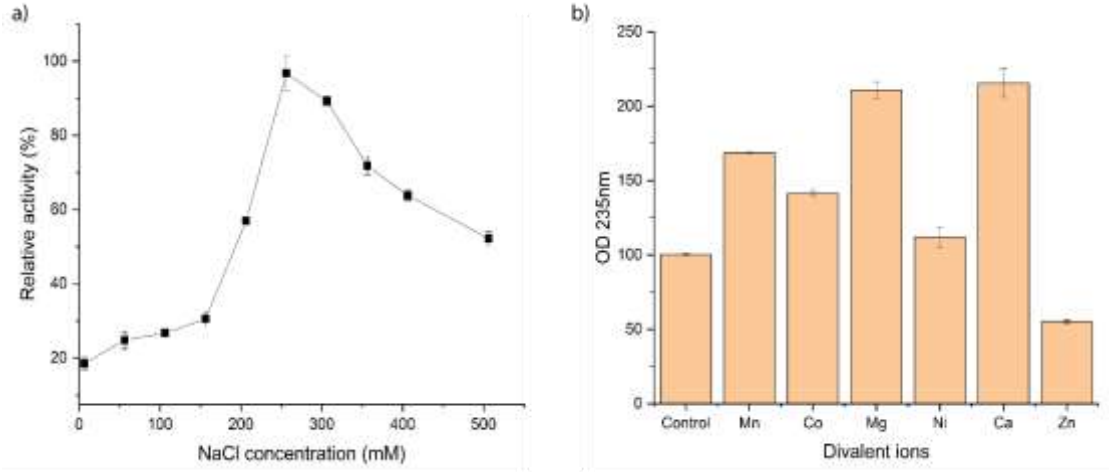


Figure 6.3 Effect of sodium chloride (a) and divalent ions (b) on PALy1 activity. Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation.

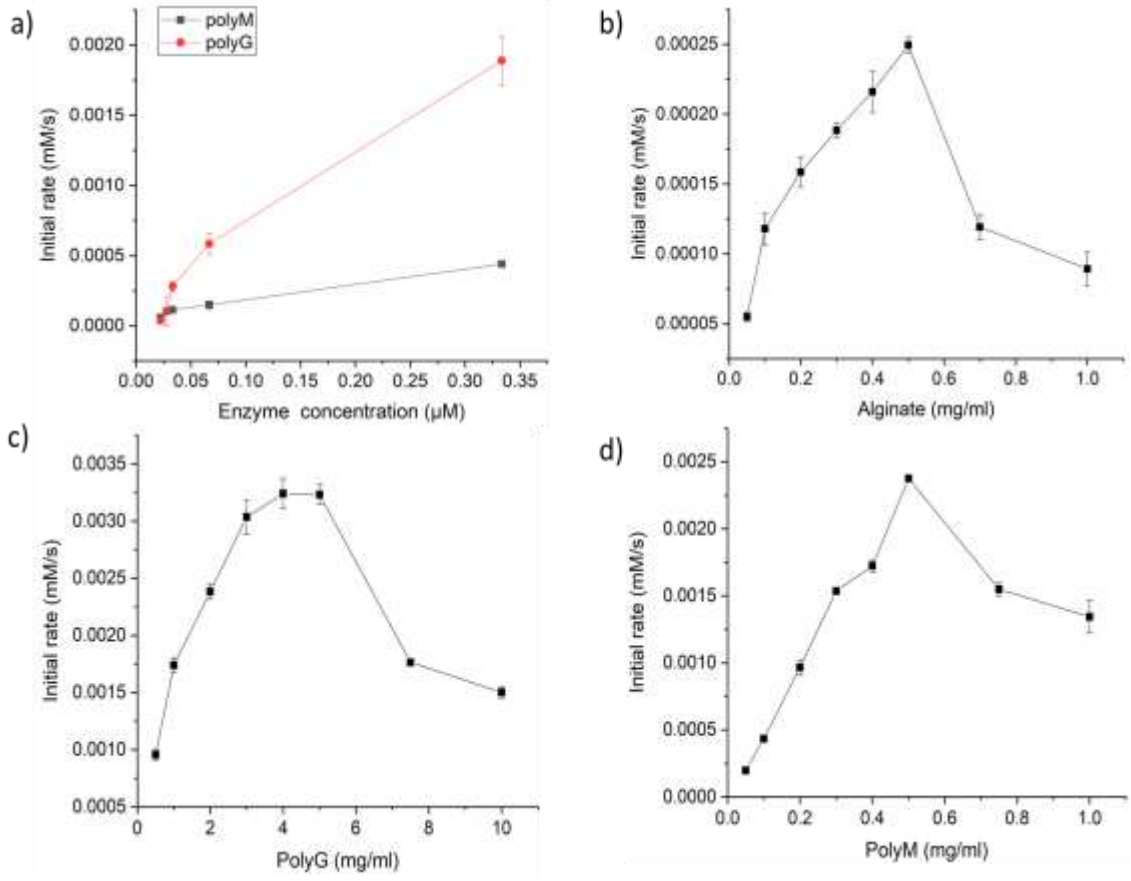


Figure 6.4 Substrate specificity of PALy1. The enzyme activity on 2 mg/ml polyM and polyG at different enzyme concentrations. At low enzyme concentration, the enzyme

activity on polyG was inhibited but at high enzyme concentration the activity on polyG was much higher than on polyM. Alginate lyase activity on alginates from Sigma (b), polyM (c) and polyG (d) at 0.33 µM enzyme, 2 mg/ml substrate. The enzyme activity was inhibited at high substrate concentration. Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation.

6.5 Effect of enzymatic ethanol extracts of *F. evanescens*, *S. latissima* and *S. mcclurei* on alginate lyase activity

The macroalgae pellets of *F. evanescens*, *S. latissima*, *S. mcclurei* after enzymatic fucoidan extraction were further treated with 80% ethanol at room temperature for 24 hours (Figure 6.5). The ethanol extracts were evaporated and lyophilized. The phenolic content in ethanol extracts were determined by Folin-Ciocalteu method in a microplate and expressed as gallic acid equivalents (mg GAE/g dry ethanol extract) with gallic acid used as standard (Schneider et al. 2015). The yield of ethanol extracts from *F. evanescens*, *S. latissima*, *S. mcclurei* was 4.24%, 2.32% and 26.6 %, respectively. The total phenolic content in ethanol extracts of *F. evanescens*, *S. latissima*, *S. mcclurei* were 42.11, 17.85 and 166.47 mgGAE/g, respectively. The ethanol extracts were investigated for inhibitory effects on alginate lyase activity. The enzyme was incubated with the extracts for 20 min, then the activity of enzyme was determined. The inhibitory effect on alginate lyase was related to the phenolic content in the extract. Ethanol extract from *S. mcclurei* with high phenolic content strongly inhibited alginate lyase activity, while extracts from *F. evanescens* showed lower inhibition level (Figure 6.6). The phenolic compound – phlorotannins from brown alga *Eisenia bicyclis* were previously found to inhibit α-fucosidase, β-galactosidase and β-mannosidase partially purified from turban shell *T. cornutus* (Shibata et al. 2002). In another study, the water-ethanol extract from *F. evanescens* was reported to contain the substances inhibiting the fucoidanases FFA from the marine bacterium *F. algae* KMM 3553^T (Imbs et al. 2018).

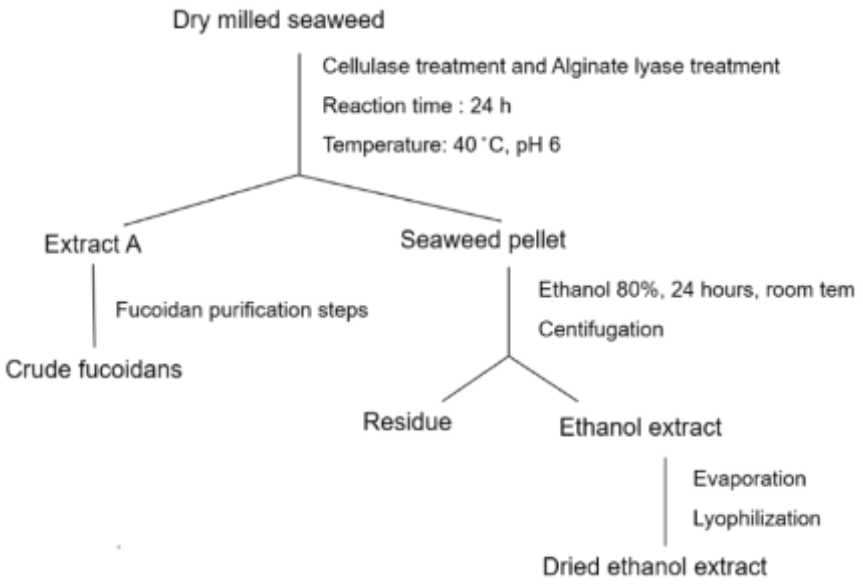


Figure 6.5 Flow chart of extraction of phenolic compounds from brown macroalgae pellets after enzyme-assisted fucoidan extraction.

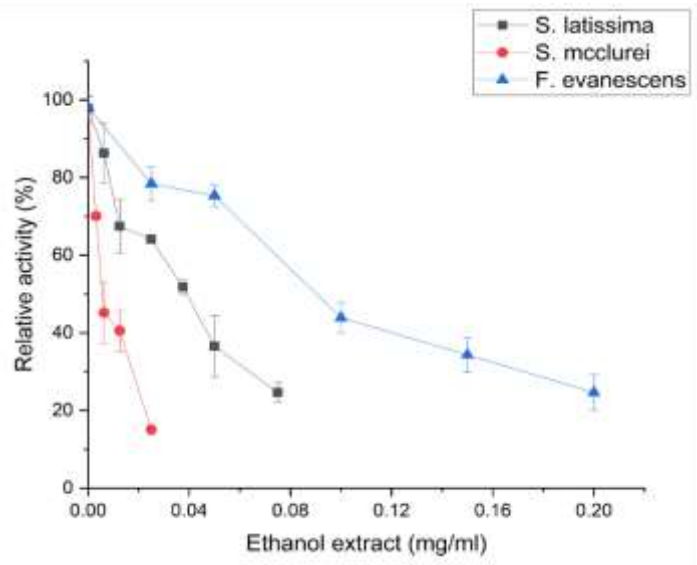


Figure 6.6 Inhibitory effects on alginate lyase activity of different ethanol extracts from the pellet after fucoidan extraction of *F. evanescens*, *S.latissima* and *S. mcclurei* on alginate lyase activity. The alginate substrate was 2 mg/ml. Each data point represents the average value of independent triplicates; vertical bars indicate the standard deviation.

6.6 Alginate lyase activity on alginates from *F. evanescens*, *S. latissima* and *S. mcclurei*

Alginates from brown macroalgae were extracted from *F. evanescens*, *S. latissima* from Iceland, *S. latissima* Ocean Rain Forest and *S. mcclurei* followed Rhein-Knudsen et al. (2017) (Rhein-Knudsen et al. 2017). The alginate extraction yields were presented in Table 6.1. The alginate contents in studied macroalgae were high as for most brown macroalgae (Rhein-Knudsen et al. 2017). As was showed here the alginate content vary depending on macroalgae species and environmental conditions. Alginate content in cultivated *S. latissima* was higher than in wild macroalgae collected from Iceland. The ability of PALy1 degrading these alginates was determined at optimal conditions with 1.98 µM enzyme, 0.4% substrate. The hydrolysis products were analyzed by C-PAGE. As shown in Figure 6.7 products contained a mixture of low molecular weight alginate oligosaccharides, indicating the alginate lyase was a endo-lytic enzyme.

Table 6.1 Extraction yield and M/G ratio of alginate from *F. evanescens*, *S. latissima* from Iceland, *S. latissima* Ocean Rrainforest and *S. mcclurei.*

Brown algae	Yield of alginate (%)	Brown algae	Yield of alginate (%)
F. evanescens	27	*S.mcclurei*	26
S. latissima from Iceland	21.5	*S. latissima* Oceanrain Forest	38

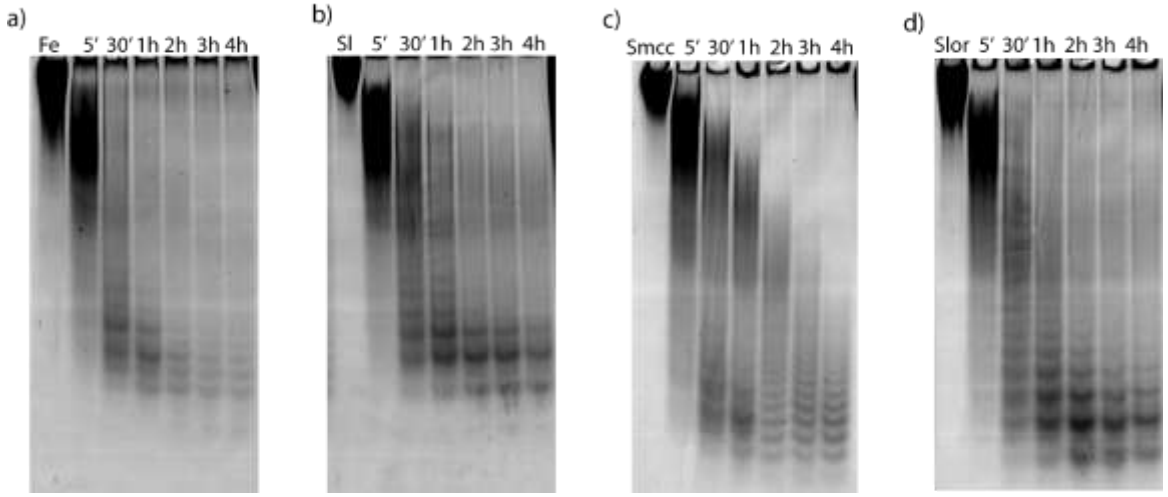


Figure 6.7 C-PAGE of digestion of alginates from *F. evanescens* (a), *S. latissima* from Iceland (b), *S. mcclurei* (c) and *S. latissima* from Ocean Rainforest (d) at different reaction time (5 and 30 minute,1, 2, 3 and 4 hours) by alginate lyase PALY1. The Fe, Sl, Smcc, Slor are stand for alginate substrate. The alginate substrates showed high molecular weight and could not migrate into the gel. After 5 minute of reaction, alginates were cleaved into lower products and started migrate to the gel. After 30 minutes, 1 – 4 hours the alginate substrates were gradually degraded into oligosaccharides, which well migrated in the gel.

6.7 Conclusion and discussion

The new PL6_1 alginate lyase PALy1 from the sea cucumber gut bacteria *Pseudoalteromonas sp.* was successfully cloned, expressed and characterized. The PALy1 alginate lyase showed highest activity at 40 °C and pH 7 in Tris-HCl buffer. Most of the alginate lyase PL6 have optimum at neutral and alkaline values (Xu et al. 2017; Belik et al. 2020; Li et al. 2020). Likely because the enzyme was found in the marine environment, it showed a high salt tolerance with optimal activity at 256 mM NaCl. The buffer system is important to maintain the enzyme activity since the pH in reaction may be changed during reaction time. In this study, the enzyme activity varied in different buffers. Among the studied buffers, Tris-HCl was the best for enzyme activity. The effect of buffers were also reported for other alginate lyase (Yang et al. 2018).

The PALy1 enzyme is more polyG specific enzyme, although the enzyme showed activity on both polyG and polyM. The activity of the alginate lyase PALy1 on alginate from Sigma was lower than on polyG and polyM. On another hand, the presence of phenolic compounds as contaminants in the substrates may also inhibit the enzyme activity. Therefore, in this study the inhibitory effect of enzymatic ethanol extracts, containing phenolic compounds from *F. evanescens*, *S. latissima* and *S. mcclurei* were examined. The results showed that the alginate lyase was inhibited by ethanol extracts from three tested macroalgae, especially by extracts from *S. mcclurei* with highest total phenolic content.

The alginate lyase PALy1 degraded alginate from *F. evanescens*, *S. latissima* and *S. mcclurei* to oligosaccharides by an endo-lytic mechanism. As were shown on C-PAGE, all alginates were completely degraded after 4 hours. So the alginate lyase PALy1 is a

promising tool for preparation of oligo-alginates from *F. evanescens*, *S. latissima* and *S. mcclurei* macroalgae. The oligosaccharide profile and their bioactivity however, needs further analysis.

Chapter 7: Discussion and Perspectives

7.1 Enzymatic extraction of fucoidans from brown macroalgae

Up to date, only few enzyme-assisted extraction methods of fucoidans have been reported with a wide range of carbohydrate degrading enzymes were used (Wijesinghe et al. 2011; Hammed et al. 2017; Alboofetileh et al. 2018). The cell wall of brown macroalgae are built of polysaccharides, which are likely arranged in two main networks. The first network is fucoidans interlocking the cellulose microfibers. The second network is alginates associated with polyphenolic compounds. The first network is embedded in the second and covalent interactions between them may exist. Therefore, to release fucoidans from the polysaccharide networks in the cell wall, the used enzymes must be selective hydrolyze non-fucoidan targets. It was reported that the treatment of the cell wall by protease and cellulase released large amount of compounds while the treatment by alginate lyase only released a minor fraction of the fucose-containing sulfated polysaccharides (Deniaud-Bouët et al. 2014). So the selection of enzymes used for extraction is an important factor in the extraction of fucoidans from brown macroalgae, as this may affect the yield and composition of polysaccharides. Four different enzymes Alcalase, Flavourzyme, Celluclast and Viscozyme were used to extract fucoidans from *N. zanardinii* compared to conventional method (hot water) (Alboofetileh et al. 2018). From the used enzymes only Alcalase increased the extraction yield of sulfated polysaccharides compared to hot water extraction method (Alboofetileh et al. 2018). However, the molecular weight and sulfate content of fucoidans extracted by enzyme-assisted method were higher compared to water extraction (Alboofetileh et al. 2018). The present study focused on the use of combination of glucanases of alginate lyases for extraction of fucoidans from brown macroalgae *F. evanescens*, *S. latissima* and *S. mcclurei.* The differences between chemical and enzymatic extracts was also observed although the fucose extraction yield was comparable between the two methods (Paper I).

The chemical features of fucoidans were affected by extraction conditions (Ale et al. 2012). The low sulfate content and degradation of fucoidans from *Sargassum fusiforme* in acid extraction methods were observed (Liu et al. 2019a). The novel methods such as UAE and MAE were also reported decrease the molecular weight of fucoidans (Song et al. 2015; Yuan and Macquarrie 2015). The enzyme-assisted extraction of fucoidans was performed under mild conditions that conserve the molecular weight and sulfate content, which related to bioactivity of fucoidans. Fucoidans from *F. evanescens* has been previously reported to have various molecular weight depending on extraction method: from 150 to 500 kDa by acid extraction (Khil'chenko et al. 2011), 181 kDa by aqueous $CaCl_2$ (Neupane et al. 2020) and 240-280 kDa by ultrasound-assisted extraction (Hmelkov et al. 2018). While in this present study fucoidans from *F. evanescens* extracted by enzyme-assisted method showed higher range of size distribution from 400 to over 800 kDa. Similar the fucoidans from *S. latissima* obtained by enzyme-assisted extraction were high molecular weight polysaccharides with the size in range 300 to over 800 kDa while fucoidans obtained by chemical method had a size from 50 to 200 kDa. This result indicated the high selectivity of enzyme in degradation only non-fucoidans targets in the cell wall of brown macroalgae. Moreover, the molecular weight was important factor in bioactivity of fucoidans (Dörschmann and Klettner 2020). Fucoidan fractions from enzymatic extract F2

and F3 of *S. latissima* showed high vascular endothelial growth factor (VEGF) inhibition and oxidative stress protection (only fraction F2), potential for treatment of age-related macular degeneration (Dörschmann et al. 2020). Among the tested fucoidans extracted by different methods, our enzymatic extract from *S. latissima* with high molecular weight and purity was one of promising agents for further investigation and development for the treatment of age-related macular degeneration (Dörschmann and Klettner 2020).

The extraction parameters including enzyme concentration, extraction time, pH and temperature are crucial factors affecting the extraction process. Most reported enzymes for fucoidan extraction have optimal activity at pH 4.5 -8 and optimal temperatures of 50-60˚C (Wijesinghe et al. 2011; Hammed et al. 2017). Since the alginate lyase plays an important role in destroying the alginate barrier, opening for cellulase attack on cellulose microfibers, we focused on alginate lyase activity. SALy showed optimum activity at pH 6.5 in citrate – phosphate buffer as was published (Manns et al. 2016). However, in our study we found that the activity of SALy was inhibited in citrate – phosphate buffer. The extracted fucose yield in UB4 buffer reached high only after 1 hour extraction while there needed 6 hours to get the same yield in citrate – phosphate buffer. This result is in agreement of the activity of SALy in two different buffers. The prolonged extraction time may increase the degradation of cell walls, the optimal extraction time have been commonly reported at 12-24 hours (Wijesinghe et al. 2011; Fernando et al. 2017; Alboofetileh et al. 2018). Though, in some case the polysaccharides may be partially degraded after a long extraction time (Alboofetileh et al. 2018).

Alginates in the cell wall of brown macroalgae are tightly contact with polyphenolic compounds (Deniaud-Bouët et al. 2014), which were reported to inhibit enzyme activity (Shibata et al. 2002). Therefore, the removal of phenolic compounds will increase the enzyme activity. However, this study indicated that the removal of phenolic compounds by ethanol treatment did not increase the fucose yield after 1 hour extraction. The ethanol might collaps the cell wall by removing the water in the cell wall matrix, which in turn could make it more difficult to rehydrate it and that could therefore be the reason why the enzymes have a hard time degrading the cell wall polymers after ethanol treatment. Longer extraction time seemed needed to reach high fucose extraction yields.

During extraction process, other polysaccharides (alginate, laminarin) may co-extracted with fucoidans, resulting in low purity of the crude fucoidan extract. Alginate can be removed by $CaCl_2$ (Domozych 2019) . However, in enzyme-assisted method the used of polyG specific alginate lyase SALy led to release large amount of oligoalginate which were not precipitate by $CaCl_2$ and leaved in the crude extract. Therefore, to get pure fucoidan further purification of fucoidans is required. In this study, a second alginate lyase SALy treatment step was applied in order to remove residual alginate in the extract after $CaCl_2$ treatment. However, the amount of alginate in the crude extract was decreased but still high. So for future work, the use of an exo-acting alginate lyase might improve the purity of fucoidans.

Sargassum is one of the most complex genus in order *Fucales*, primary found in tropical and subtropical marine water (Rushdi et al. 2020). In the other hand, the fucoidan content in *Sargassum* is very low (Zvyagintseva et al. 2021). So more specific enzymes are

required for obtaining high yeild of extracted fucoidans from *Sargassum* macroalgae. Fucoidans from *Sargassum sp*. that were extracted by papain enzyme solution contained low fucose level than that extracted by conventional method (Baba et al. 2016). *S. mcclurei* is an abundant brown macroalgae in Vietnam. The monosaccharide composition of macroalgae showed that the fucoidan content in this macroalgae is low. So for extraction of fucoidans from *S. mcclurei* the glucanase enzymes with more specific were used compare to standard enzymes which used for *F. evanescens* and *S. latissima* macroalgae. The use of a combination of cellulase, endo-1,3-β-D-glucanase, non-specific endo-1,3(4)-β-glucanase and alginate lyase, which selectively degraded all the main polysaccharides cellulose, laminarin and alginate, extracted fucoidans from *S. mcclurei* with highest fucose yield. As in *F. evanescens* and *S. latissima*, the fucose yield in enzyme-assisted and chemical extract from *S. latissima* was comparable, confirmed the workability of our enzyme-assisted extraction of fucoidans from different macroalgae species. However, enzymes needed more time (6 hours) to release fucoidans from *S. mcclurei* than from *F. evanescens* and *S. latissima*. It might be related to the presense of enzyme inhibitors in *S. mcclurei*, including phenolic compounds, which were high in this macroalgae (Van et al. 2013). In addition, high phenolic content in *S. mcclurei* results in high rigidity of the cell wall, which proved by alginate and phenolic compounds network. For increasing the efficiency extraction of fucoidans from *S. mcclurei* the macroalgae pre-treatment to remove enzyme inhibitors, the use of more specific alginate lyase must be considered.

7.2 Fucoidan oligosaccharide preparation

Fucoidans from *F. evanescens* and *S. latissima* extracted by the enzyme-assisted method showed interesting bioactivity. However, for application as pharmaceuticals, high molecular weight fucoidans must be hydrolyzed to oligosaccharides, which more easily transported through cell membranes (Flórez-Fernández et al. 2018). From different methods of oligosaccharide preparation, the hydrolysis of fucoidans by enzymes was chosen in this study due to high specific cleavage site, mild reaction condition. Only few reports on fucoidan oligosaccharide preparation using endo-fucoidanase enzyme were reported. Since endo-fucoidanase are highly specific with regards to cleaving sites, the hydrolysis products are specific for certain substrate. Few oligosaccharide, prepared from fucoidans of *F. evanescens* by using fucoidanase were reported such as disaccharides (Silchenko et al. 2013), disaccharide, tetrasaccharide (Silchenko et al. 2014), disaccharide, tetrasaccharide, sulfated branched pentasaccharide (Silchenko et al. 2014), sulfated tetrasaccharide (Silchenko et al. 2018). In the present study, the treatment of fucoidans from *F. evanescens* by the Fhf1 fucoidanase produced the low molecular weight products including sulfated tetra-, octa- and decasaccharides (Vuillemin et al. 2020), which showed effect on bone regeneration and enhance implant fixation in combination with hydroxyapatite (Nielsen et al. 2021). Both native fucoidans extracted by enzyme-assisted method from *F. evanescens* and their oligosaccharides showed bioactivity. In addition, the fucoidans content in *F. evanescens* is high. Therefore, *F. evanescens* is potential source for commercial production of fucoidans and fucoidan oligosaccharides in industrial medicine in future.

This study is the first report on oligosaccharide preparation from cultivated *S. latissima* using the new α-(1-3)-linked fucoidanase Mef2. Different from *F. evanescens,* the yield of

low molecular weight products of *S. latissima* was low. This is likely related to the complex structure of fucoidans from *S. latissima* and the specificity of Mef2. The fucoidanase Mef2 only cleaved the α-(1→3) linkages of fucopyranose residues in sulfated fucan structure, leaving fucogalactan in the high molecular weight products. Most of reported fucoidan oligosaccharide production used fucoidanase with α-(1-4)-linked specific, degrading fucoidans from *F. evanescens* (Silchenko *et al.*, 2014, Silchenko *et al.*, 2018), *P. canaliculata* (Colin et al. 2006)*, S. horneri* (Silchenko et al. 2017a)*.* The remarkable of this study is the finding new branched fucoidan oligosaccharides from *S. latissima.* These were the first branch oligosaccharides of α-(1→3) linked fucose residues. In another study a sulfated branched fucoidan oligosaccharide of α-(1→4) and α-(1→3) linked fucose residues from *F. evenescens* was reported with branch point at C2 (Silchenko et al. 2014). The sulfate fucan structure with branched point at C2 of fucose residues was determined in fucoidans from wild macroalgae *S. latissima* (as was mentioned in Chapter 3 and reported (Bilan et al. 2010)). While the new branch point at C4 of fucose residues was found in cultivated *S. latissima.* The hydrolysis products of fucoidans from *S. latissima* displayed differences in molecular weight, monosaccharide composition as well as structure. Therefore, it is interesting to compare the bioactivity of these products to evaluate the relationship between the molecular weight and structure with bioactivity for future application of fucoidans from *S. latissima*. Since *S. latissima* is now commercialy cultivated, the examination of fucoidans from this macroalgae is of particular value for economic exploitation of this macroalgae.

7.3 Alginate lyase

The finding of alginate lyase, which have broad activity on alginates from different brown macroalgae species is required for production of specific alginate oligosaccharides. In this study, the new PL6 alginate lyase PALy1 was isolated from sea cucumber gut bacteria *Pseudoalteromonas sp.* MB47. To date only few alginate lyase from PL6 were characterized. The reported PL6 alginate lyase showed highest activity at wide range of temperature (30 - 55 °C) and pH (7.0–10.0) as presented in Table 6.1. The PALy1 has optimal activity at pH 7 and 40 °C. Since the PAL1 alginate lyase was isolated from a marine source, this enzyme was high salt tolerant as marine alginate lyase ALFA4, which showed highest activity at 0.6M NaCl (Belik et al. 2020). While alginate lyase from human gut BcelPL6 showed very low level of salt tolerance (Stender et al. 2019).

Alginate lyase from PL6 showed various structures and catalytic mechanism, reflexing the adaption to living environment (Wang et al. 2021a). PALy1 activity was activated by the divalent ions Mn^{2+}, Mg^{2+}, Ca^{2+} and inhibited by Cu^{2+}, Zn^{2+}, similar to other reported alginate lyases (Li et al. 2016, 2019). Among characterized PL6 enzyme, AlyGC (Xu et al. 2017) and Alg823 (Zeng et al. 2019) from PL6 catalyze the lysis with Ca^{2+}-assisted mechanism as opposed to AlyF (Lyu et al. 2019) that showed endo activity with Ca^{2+}-independent mechanism.

PL6 enzyme showed different substrate specific and type degradation as shown in Table 6.1. PALy1 showed endo type action that release unsaturated oligosaccharide with different DP. Enzyme PL6 reported produced oligosaccharides with DP 2 (Belik et al. 2020), DP 2 – 3 (Stender et al. 2019), DP 2-5 (Li et al. 2019). In present study, PALy1 showed potential in using for preparing oligosaccharides from *F. evanescens*, *S. latissima*

and *S. mcclurei*. Therefore, to understand the pattern of action of PALy1 enzyme, alginate oligosaccharides must be further analyzed. And the assess of bioactivity of alginate oligosaccharides is valuable for exploitation of brown macroalgae polysaccharides.

Table 6.1 Characteristics of some PL-6 alginate lyases.

Alginate lyase	Source	Optimum conditions	Substrate specific	Action mode	References
AlyPL6	Marine bacterium *Pedobacter hainanensis* NJ-02	45 °C /10	polyMG	endo	(Li et al. 2020)
ALFA4	Marine bacterium *Formosa algae* KMM 3553T	30 °C /8	polyM	endo	(Belik et al. 2020)
AlyGC	Marine bacterium *Glaciecola chathamensis* S18K6T	30 °C/7.0	polyG	exo	(Xu et al. 2017)
OalS6	Marine bacterium *Shewanella sp*.Kz7	40 °C/7.2	polyG	exo	(Li et al. 2016)
BcelPL6	Human gut microbe *Bacteroides cellulosilyticus*	30 °C/7.5	polyM	endo	(Stender et al. 2019)
AlyF	*Vibrio splendidus* OU02	30 °C/7.5	polyG	endo	(Lyu et al. 2019)
Alg823	Marine bacterium *Pseudoalteromonas carrageenovora* ASY5	55 °C/8.0	polyM	endo	(Zeng et al. 2019)

7.4 Perspectives

Fucoidans from brown macroalgae have been studied intensively in recently years. There is still area that need to further investigation including the extraction technique and bioactivity assessment. A conventional method for isolating fucoidans from brown macroalgae involves the use of chemicals. In this PhD study the new enzyme-assisted method has been developed to alternate the conventional methods, conserving the bioactivity of the fucoidans. However, this study was made only on laboratory level. Further investigations should be considered in order to improve the purity of crude fucoidans. High polyM specific alginate lyase enzymes are proposed to remove all oligoM from extract, increasing the purity of crude fucoidans. In the other hand, for large scale fucoidan extraction, the large alginate lyase production is also required.

The finding of new enzyme-assisted extraction method of fucoidans in this PhD is relevant to the hot trend for research and development of sustainable extraction method of marine products in general. The new enzyme-assisted extraction method works well on both the chosen macroalgae *F. evanescens* and *S. latissima*. As known *F. evanescens* is high in fucoidans, however the commercial use of these polysaccharides is still not available. Therefore, the results of fucoidan extraction from *F. evanescens* in PhD study are potential for utilize this macroalgae for commercial use while reducing the negative impact of extraction process on environment and and give safe end - products. *S. latissima* macroalgae is successfully cultivated by Nordic companies in large scale. The sustainable method for extraction of fucoidans from this species is meaningful for effective macroalgae exploitation. The *S. mcclurei* macroalgae is abundant in Vietnam and chemically extracted *S. mcclurei* fucoidans are currently commercially available. However, the extraction yield of the fucoidans is low. Therefore, the development of a green method as an alternative to the traditional chemical method is not only environmental friendly but also economically useful. Besides the environmental benefits, in the new extraction process the products the native and highly active fucoidans are kept intact, increasing the sustainability of the method. There is possibility of combination of fucoidans extraction with recovery other value products such as glucose, oligoalginate, phenolic compounds…, saving the exploitation cost. The enzyme – assisted extraction of fucoidans has potential to be applied for other brown macroalgae species and in large scale.

Although fucoidans have broad bioactivity, they are limited to use as therapeutic agent due to high molecular weight. Therefore, the research on production of LMW fucoidans with high bioactivities is an important topic. The PhD work was highlighted by using a green method to prepare fucoidan oligosaccharides, finding the new structural characteristic of fucoidans from *S. latissima*, contributing to the understanding of the diversity of fucoidans in general. Similar to enzyme - assisted extraction process, the use of enzymes in hydrolysis of polymers is not only environmental friendly, but also conserve the sulfate groups, which are important for bioactivity (Haroun-Bouhedja et al. 2000; Koyanagi et al. 2003; Cho et al. 2011). In addition, enzymatic hydrolysis of fucoidans is controllable, therefore this is a potential method for preparation of specific oligosaccharides for medical use as well as to study the relationship between structure and bioactivity. However, the separation of all hydrolysis products into pure state is not always easy. For further study to improve the yield of separation process we need to increase the started matter and control the separation parameters (resin, elution buffer, flow rate). The bioactivity of these enzymatic products must be investigated to see the effect of structure and molecular weight of oligosaccharides on bioactivity.

Chapter 8: Conclusions

In this study, a new enzyme-assisted extraction of fucoidans from brown macroalgae *F. evanescens*, *S. latissima* and *S. mcclurei* was introduced. The use of enzymes successfully extracted fucoidans achieving yields comparable to fucoidan yields obtained with a mild chemical extraction method. The enzymatic fucoidan extract showed higher molecular weight and sulfate content then the chemical extraction method. The results obtained confirmed the validity of Hypothesis 1 and Hypothesis 2.

The further anion exchange chromatography resulted in highly pure fucoidan fractions, which showed interesting bioactivities. Therefore, the enzyme-assisted extraction method is promising for extraction of fucoidans from brown macroalgae.

Fucoidan oligosaccharides prepared by enzymatic hydrolysis of fucoidans also showed bioactivity. The oligosaccharides obtained were a result of enzyme specific cleavage of linkages in the backbone of the fucoidan substrate. Notably, in this work, a unique type of fucoidan oligosaccharides from cultivated *S. latissima* were produced by enzymatic treatment using a new fucoidanase Mef2, from the marine bacterium *Muricauda eckloniae* (discovered as part of this work), and well separated. The NMR data provided a first new branched oligosaccharide from fucoidans of *S. latissima*. In addition, the new branch point at C4 of fucose residues in the sulfated fucan structure was identified compared to previous reported branch point at C2 in fucoidans from cultivated *S. latissima*. The data confirmed the validity of Hypothesis 4 (bioactivity assessment of this oligomer is outside the scope of this PhD work).

A new alginate lyase from sea cucumber gut was also described in this study. The enzyme showed endo-lytic activity on alginates from brown macroalgae *F. evanescens*, *S. latissima* and *S. mcclurei*. The identification of this enzyme confirmed Hypothesis 3. The enzyme is not as suitable as SALy for enzymatic fucoidan extraction, but may be a promising tool for obtaining specific alginate oligosaccharides from macroalgae.

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Appendix: Published papers

marine drugs

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Article

Enzyme-Assisted Fucoidan Extraction from Brown Macroalgae *Fucus distichus* subsp. *evanescens* and *Saccharina latissima*

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Abstract: Fucoidans from brown macroalgae (brown seaweeds) have different structures and many interesting bioactivities. Fucoidans are classically extracted from brown seaweeds by hot acidic extraction. Here, we report a new targeted enzyme-assisted methodology for fucoidan extraction from brown seaweeds. This enzyme-assisted extraction protocol involves a one-step combined use of a commercial cellulase preparation (Cellic®CTec2) and an alginate lyase from *Sphingomonas* sp. (SALy), reaction at pH 6.0, 40 °C, removal of non-fucoidan polysaccharides by Ca^{2+} precipitation, and ethanol-precipitation of crude fucoidan. The workability of this method is demonstrated for fucoidan extraction from *Fucus distichus* subsp. *evanescens* (basionym *Fucus evanescens*) and *Saccharina latissima* as compared with mild acidic extraction. The crude fucoidans resulting directly from the enzyme-assisted method contained considerable amounts of low molecular weight alginate, but this residual alginate was effectively removed by an additional ion-exchange chromatographic step to yield pure fucoidans (as confirmed by ^{1}H NMR). The fucoidan yields that were obtained by the enzymatic method were comparable to the chemically extracted yields for both *F. evanescens* and *S. latissima*, but the molecular sizes of the fucoidans were significantly larger with enzyme-assisted extraction. The molecular weight distribution of the fucoidan fractions was 400 to 800 kDa for *F. evanescens* and 300 to 800 kDa for *S. latissima*, whereas the molecular weights of the corresponding chemically extracted fucoidans from these seaweeds were 10–100 kDa and 50–100 kDa, respectively. Enzyme-assisted extraction represents a new gentle strategy for fucoidan extraction and it provides new opportunities for obtaining high yields of native fucoidan structures from brown macroalgae.

Keywords: fucose-containing sulfated polysaccharides; *Fucus evanescens*; enzymatic extraction; chemical extraction; cellulase; alginate lyase

1. Introduction

Fucoidans are a group of sulfated polysaccharides mainly found in the cell walls of brown seaweeds. Polysaccharides are intensively studied due to their useful bioactivities, such as antioxidant [1],

antitumor [2], neuroprotective [3], anti-inflammatory, and anticoagulant [4]. Fucoidan content and monosaccharide composition depend on many factors, such as seaweed species, environmental conditions, collecting time, and methods used for polysaccharide extraction and purification [5–7]. Fucoidans are heteropolymers that are primarily composed of fucose, but may include other sugars, i.e. glucose, galactose, xylose, mannose, and uronic acids in various proportions. Based on the sugar composition, fucoidans from brown seaweeds were previously reported as sulfated fucans, mainly containing fucose and sulfate groups, galactofucans mainly consisting of sulfated fucose and galactose in different ratios, and urono-fucoidans with different monosaccharide units, less sulfate, and a large amount of uronic acid [8]. Fucoidans differ in their backbone structure and branching patterns and in presence and numbers of functional groups, such as sulfates and acetylations. The backbone consists most commonly of α-(1–3)-L-fucopyranose units or alternating α-(1–3)- and α-(1–4)-linked L-fucopyranose units with a sulfate group at C2 or C4 [9]. In general, the backbone of fucoidans from *Fucus distichus* subsp. *evanescens* (a species that we in this report refer to as *F. evanescens*) is made up of alternating 2-sulfated 1,3- and 1,4-linked α-L-fucose residues [10,11], although on some of the fucose residues sulfate groups may be found at both C2 and C4 [12]. Fucoidans from *Saccharina latissima* are very diverse. Four partial structures of fucoidans from *S. latissima* have been reported: fucan sulfate, fucogalactan, fucoglucuronomannan, and fucoglucuronan [13].

The bioactivities of fucoidans from different species of brown seaweeds are closely related to their monosaccharide composition, molecular weight, and fine structure [14–18]. The cell wall of brown seaweeds is a complex matrix of compounds, primarily consisting of the polysaccharides alginate, fucoidans, cellulose, and hemicellulose, as well as proteins, polyphenols, and other components [19].

The main goal when extracting fucoidans is to isolate the fucoidans as intact as possible while eliminating interfering molecules. Traditional fucoidan extraction techniques (acidic, hot water, organic solvents) are based on the solubility of cell wall polysaccharides under various conditions. But these methods are time consuming, toxic, have low efficiency and may affect the fucoidan fine structure, which potentially could have a detrimental effect on fucoidan bioactivity. Novel green fucoidan extraction techniques have been developed to overcome these disadvantages, including supercritical water extraction [20], microwave-assisted extraction [21], ultrasound-assisted extraction [22], and enzyme-assisted extraction [23]. Enzyme-assisted fucoidan extraction should preferably be based on selective removal of all non-fucoidan polysaccharides from the cell wall matrix and also target depolymerization of the storage polysaccharide laminarin, and leave the sulfated fucoidan intact. However, until now, the reported enzyme-assisted extraction methods for sulfated fucoidan polysaccharides have involved use of various mixed commercial enzyme preparations, such as carbohydrase mixtures (Viscozyme, Celluclast, Amyloglucosidase), known to attack a wide range of plant polysaccharides (even including starch, which is not present in brown seaweeds) and broadly acting proteases (Flavourzyme, Alcalase) [23–25]. Hence, these enzyme mixtures do not selectively target alginate, which is a particularly abundant polysaccharide in the cell walls of brown macroalgae. Alginate is particularly important to remove enzymatically to release fucoidan, because fucoidans are believed to be involved in the cross-linkage of alginate and cellulose [26]. Therefore, it is important to judiciously select specific substrate-targeted enzymes, i.e. alginate lyase and cellulases, which specifically catalyze the degradation of the fucoidan cross-linking polysaccharides in order to selectively extract fucoidans from the cross-linked network of brown algae cell walls in a controlled and gentle way by enzymes. This concept, i.e. the combined use of alginate lyase and cellulases, is the foundation of the new, targeted enzyme-assisted method that we report here. In a previous study, cell wall treatment with alginate lyase only released a minor fraction of the fucose-containing sulfated polysaccharides [27].

In this study, we report the comparative isolation of fucoidans from two different brown seaweeds, *F. evanescens* and *S. latissima*, by acid extraction and enzymatic extraction while using Cellic®CTec2 from Novozymes and the alginate lyase SALy from *Sphingomonas sp*. [28]. The reason for choosing these species for study is that they are both available in the Northern hemisphere zone, and *S. latissima* is moreover commercially cultivated by Nordic companies, which is why it is particularly useful to assess

fucoidan extraction from this species for value addition. *F. evanescens* is collected for investigating potential commercial use of the fucoidan and known as a species that is quite rich in fucoidan. In addition, since *S. latissima* is of the order Kelp and *F. evanescens* belongs to the Fucales, these two species would serve to demonstrate the workability of the enzyme-assisted extraction method for two significantly different, relevant types of brown algae to provide a robust case. The fucose extraction yield, monosaccharide composition, and molecular size of crude fucoidans from these two seaweeds were analyzed. Furthermore, the crude fucoidans from *F. evanescens* and *S. latissima* were fractionated by anion-exchange chromatography into three fractions. We also studied the structural and molecular properties of these fucoidan fractions.

2. Results

2.1. Monosaccharide Composition of the Brown Seaweeds F. evanescens and S. latissima

The monosaccharide composition of *F. evanescens* and *S. latissima* was analyzed in order to evaluate the effect of using enzymes to extract fucoidans from brown seaweeds (Table 1).

Table 1. Monosaccharide composition of the brown seaweeds. The data are given as % weight (dehydrated monomers) of dry matter. Different superscript roman letters a,b indicate statistically different values ($p < 0.05$) pairwise between values from each type of seaweed.

Monomer Category	Monomer	*F. evanescens*	*S. latissima*
Neutral monosaccharides (%)	Mannitol	2.6 ^{a} ± 0.6	2.1 ^{a} ± 0.5
	Fucose	8.7 ^{a} ± 0.9	4.7 ^{b} ± 0.1
	Rhamnose	0.1 ^{a} ± 0.0	0.1 ^{a} ± 0.0
	Galactose	1.5 ^{a} ± 0.4	0.5 ^{b} ± 0.5
	Glucose	6.7 ^{b} ± 0.8	12.3 ^{a} ± 2.4
	Xylose	0.8 ^{a} ± 0.0	0.4 ^{b} ± 0.1
	Mannose	0.9 ^{a} ± 0.2	1.0 ^{a} ± 0.3
Uronic acids (%)	Guluronic Acid	8.8 ^{a} ± 1.1	9.3 ^{a} ± 2.4
	Glucuronic Acid	1.2 ^{a} ± 0.2	1.9 ^{a} ± 0.5
	Mannuronic Acid	28.3 ^{a} ± 3.6	36.1 ^{a} ± 8.8

The predicted monosaccharides were found in different proportions in the seaweeds. The neutral monosaccharides found were mannitol, fucose, rhamnose, galactose, glucose, xylose and mannose, and uronic acids included mannuronic acid (ManA), guluronic acid (GuluA), and glucuronic acid (GluA). Fucose is the major component of fucoidans, while glucose is a component of laminarin or cellulose, and ManA and GuluA are components of alginic acid (alginate). Hence, the data presented in Table 1 are in agreement with fucoidans, laminarin, cellulose, and alginate, being the main polysaccharides present in *F. evanescens* and *S. latissima* constituting about 60% and 68%, respectively, of the dry matter (sum of data in Table 1). The content of fucose in *F. evanescens* and *S. latissima* differs, and as expected, *F. evanescens* contained the highest levels: Hence, in *F. evanescens*, fucose comprised 8.7% of the dry weight, which is considerably higher than the 4.7% found in *S. latissima* (Table 1). Furthermore, fucoidans extracted from these two species of seaweeds have previously been reported to have different chemical composition and structure [10,13,29].

The content of alginate in *F. evanescens* and *S. latissima* was very high at 37.1% and 45.4% of the dry weight in the two seaweeds, respectively, (calculated as the sum of guluronic acid and mannuronic acid levels in each seaweed, Table 1), which was anticipated [30]. Lower amounts of other neutral sugars found in fucoidan were also present in *F. evanescens* and *S. latissima*: galactose (1.5% and 0.5%), xylose (0.8% and 0.4%), and mannose (0.9% and 1%). Additionally, some glucuronic acid, which is also a component of fucoidans, such as the fucoglucuronomannan and fucoglucuronan fucoidans reported in *S. latissima* [13], was also present in lower amounts in *F. evanescens* (1.2%) and *S. latissima* (1.9%) (Table 1).

In a previous study from our lab, we found that the alginate lyase SALy and the commercial cellulase enzyme preparation Cellic®CTec2 could be combined to release glucose from the biomass of the brown seaweed *Laminaria digitata* [28]. In the same study, it was suggested that the fucoidans were released from the cell wall unharmed, although this possibility was not tested further; in another study, Cellic®CTec2 was shown to be able to catalyze the degradation of laminarin from seaweeds [31].

2.2. Enzyme-Assisted Fucoidan Extraction from *F. evanescens* and *S. latissima*

In the first step of the enzyme-assisted fucoidan extraction method, SALy and Cellic®CTec2 were combined to trigger fucoidan extraction from *F. evanescens* and *S. latissima*. A mild chemical acidic extraction was used for comparison in order to evaluate the effectiveness of the enzyme-assisted method.

2.2.1. Yields of Crude Fucoidans Extracted from *F. evanescens* and *S. latissima*

The amount of fucose was used to determine the extraction efficiency of fucoidans by comparing the ratio of total fucose in crude fucoidans and the content in the starting material. The chemical composition of crude fucoidans obtained by the chemical and enzymatic extraction methods from *F. evanescens* and *S. latissima* was analyzed by high performance anion chromatography with pulsed amperometric detection (HPAEC-PAD) (Table 2).

Table 2. Chemical composition of crude fucoidans from *F. evanescens* and *S. latissima*. The monosaccharide and uronide data are given in %mol (relative level) of total carbohydrates analysed in the extract, with total sulfate (SO_4^{2-}) first calculated as %wt of total, then as degree of sulfation on dehydrated fucose moieties in the crude fucoidans extracted. Fucoidan yield is calculated as amount of fucose extracted compared to the total fucose (theoretical maximum) in the starting-material. Different superscript roman letters a,b indicate statistically different values ($p < 0.05$) pairwise for the enzymatic method vs. the chemical method per seaweed species.

Content	Monomer	*F. evanescens*		*S. latissima*	
		Enzymatic Method	**Chemical Method**	**Enzymatic Method**	**Chemical Method**
Neutral monosaccharides (%mol)	Mannitol	0.2 ^{b} ± 0.0	0.43 ^{a} ± 0.0	2.1 ^{a} ± 0.2	2.2 ^{a} ± 0.3
	Fucose	24.8 ^{b} ± 2.9	60.9 ^{a} ± 0.9	12.6 ^{b} ± 0.4	31.2 ^{a} ± 4.2
	Rhamnose	0.2 ^{b} ± 0.1	0.9 ^{a} ± 0.2	0.2 ^{a} ± 0.0	0.2 ^{a} ± 0.0
	Galactose	0.9 ^{b} ± 0.1	5.4 ^{a} ± 0.1	2.3 ^{a} ± 0.1	2.9 ^{a} ± 2.4
	Glucose	0.7 ^{b} ± 0.1	6.2 ^{a} ± 0.1	1.6 ^{b} ± 0.0	57.7 ^{a} ± 3.1
	Xylose	0.8 ^{b} ± 0.1	5.8 ^{a} ± 0.1	0.8 ^{b} ± 0.0	3.0 ^{a} ± 0.0
	Mannose	0.4 ^{b} ± 0.0	2.6 ^{a} ± 0.1	0.9 ^{a} ± 0.0	0.9 ^{a} ± 0.1
Uronic acid (%mol)	GuluA	12.6 ^{a} ± 1.8	0.9 ^{b} ± 0.1	18.6 ^{a} ± 0.9	0.2 ^{b} ± 0.1
	GluA	1.0 ^{b} ± 0.2	3.9 ^{a} ± 0.1	1.3 ^{a} ± 0.2	0.7 ^{b} ± 0.1
	ManA	58.4 ^{a} ± 2.6	13.1 ^{b} ± 0.4	59.6 ^{a} ± 1.9	1.0 ^{b} ± 0.2
Sulfate (SO_4^{2-}) (%wt)		21.4 ^{b} ± 0.5	38.0 ^{a} ± 0.4	15.5 ^{b} ± 1.7	31.6 ^{a} ± 0.9
Degree of sulfation (molar ratio SO_4^{2-}: Fucose)		2.1	1.9	2.5	2.1
Fucoidan yield (fucose extraction %wt)		40	43	29	29

The crude fucoidan extracts from *F. evanescens* and *S. latissima* were mainly composed of four types of neutral sugars, fucose, galactose, glucose, and xylose, and a small amount of mannose and rhamnose and we also consider the low levels of the uronic acid glucuronic acid to be a part of the fucoidan (Table 2). A major part of the glucose in the original seaweed materials (Table 1) is presumed to originate from cellulose and laminarin, and the crude fucoidan data show that both are effectively degraded because glucose is removed by the enzymatic treatment. Thus, glucose levels were considerably lower

in the enzyme-assisted extracts when compared to the chemical extractions, at 0.7% as compared to 6.2% in *F. evanescens* and 1.6% as compared to 57.7% in *S. latissima* (Table 2); this result was in complete accord with the expected effect of the Cellic®CTec2 acting on both cellulose and laminarin [31]. We interpret the presence of GuluA and ManA as being indicative of alginate contamination. Indeed, the relative levels of both GuluA and ManA were considerably higher for the enzymatic extraction when compared to the chemical extraction, namely 58.4% ManA as compared to 13.1% in *F. evanescens* and 59.6 % ManA when compared to 1.0% in *S. latissima* (Table 2). This difference led to the *relative* fucose contents being higher with the chemical extraction than with the enzyme-assisted extraction, at 60.9% as compared to 24.8% in *F. evanescens* and 31.2% when compared to 12.6% in *S. latissima* (Table 2). Yet, the total yields of fucoidan were comparable between the two methods being 29% with both methods for *S. latissima* and 40% and 43% for *F. evanescens* (Table 2).

The sulfation degree tended to be slightly higher in the enzymatically-assisted extracted fucoidans than in the chemically extracted at 2.1% as compared to 1.9% in the crude *F. evanescens* fucoidan and 2.5% compared to 2.1% in the *S. latissima* fucoidan extracts (Table 2). The finding that crude fucoidans from *F. evanescens* and *S. latissima* were highly sulfated polysaccharides is in complete accordance with what has been reported previously [10,13].

2.2.2. Yields and Chemical Compositions of Fucoidan IEX Fractions of *F. evanescens* and *S. latissima*

Crude fucoidans from the enzyme-assisted purifications were further purified and separated by ion-exchange chromatography (IEX). This method allows for different populations of fucoidans to be separated according to differences in size and charge. The polysaccharides were eluted depending on negative charge intensity by increasing salt concentrations. Based on the total carbohydrate content, determined by the phenol-sulfuric acid method [32] of each eluate, the eluates of *F. evanescens* and *S. latissima* were combined into three different fractions FeF1–F3 and SlF1–F3, respectively (Figure 1). The yield and composition of these fucoidan fractions were different with regards to monosaccharide distribution and sulfate content (Table 3).

Table 3. Yields and composition of fucoidan fractions from *F. evanescens* and *S. latissima*. Different superscript roman letters a,b,c indicate statistically different values ($p < 0.05$) between the values in the fractions per seaweed species.

Content	Monomer	*F. evanescens*			*S. latissima*		
		FeF1	**FeF2**	**FeF3**	**SlF1**	**SlF2**	**SlF3**
Yield, % of crude extract		4.2	7.9	18.2	3.4	6.2	3.8
Neutral monosaccharides (%mol)	Mannitol	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$0.0^{a} \pm 0.0$	$0.1^{a} \pm 0.0$	$0.0^{a} \pm 0.0$
	Fucose	$34^{c} \pm 3.1$	$74.7^{b} \pm 0.8$	$87.8^{a} \pm 1.4$	$5.4^{b} \pm 1.2$	$64.7^{a} \pm 0.3$	$63.3^{a} \pm 0.7$
	Rhamnose	$0.3^{c} \pm 0.1$	$0.8^{a} \pm 0.1$	$0.5^{b} \pm 0.1$	$0.1^{b} \pm 0.0$	$0.3^{a} \pm 0.0$	$0.3^{a} \pm 0.0$
	Galactose	$4.6^{c} \pm 0.4$	$15.4^{a} \pm 0.4$	$9.0^{b} \pm 0.9$	$0.5^{c} \pm 0.0$	$12.2^{b} \pm 0.1$	$26.9^{a} \pm 0.3$
	Glucose	$7.7^{a} \pm 0.7$	$1.4^{b} \pm 0.1$	$0.3^{c} \pm 0.1$	$0.4^{b} \pm 0.0$	$0.6^{a} \pm 0.1$	$0.4^{b} \pm 0.1$
	Xylose	$5.3^{a} \pm 0.5$	$2.8^{b} \pm 0.1$	$1.5^{c} \pm 0.3$	$0.8^{c} \pm 0.1$	$4.8^{a} \pm 0.0$	$3.4^{b} \pm 0.2$
	Mannose	$3.2^{a} \pm 0.5$	$2.3^{b} \pm 0.1$	$0.3^{c} \pm 0.1$	$0.8^{c} \pm 0.1$	$3.5^{a} \pm 0.2$	$2.1^{b} \pm 0.1$
Uronic acid (%mol)	GuluA	$9.1^{a} \pm 0.5$	$2.2^{b} \pm 0.2$	$0.0^{c} \pm 0.0$	$1.1^{c} \pm 0.1$	$6.9^{a} \pm 0.3$	$2.8^{b} \pm 0.2$
	GluA	$3.8^{a} \pm 0.3$	$0.3^{b} \pm 0.0$	$0.5^{b} \pm 0.1$	$8.5^{a} \pm 4.7$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$
	ManA	$32.2^{a} \pm 0.6$	$0.2^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$82.4^{a} \pm 4.3$	$6.9^{b} \pm 0.1$	$0.8^{c} \pm 0.1$
Sulfate (SO_4^{2-}) (wt%)		$20.4^{b} \pm 3.4$	$34.8^{ab} \pm 2.0$	$38.7^{a} \pm 1.0$	$6.6^{c} \pm 3.6$	$35.6^{b} \pm 2.5$	$46.4^{a} \pm 3.5$
Degree of sulfation (molar ratio SO_4^{2-}: Fuc)		1.3	1.7	1.6	1.8	2.4	3.0

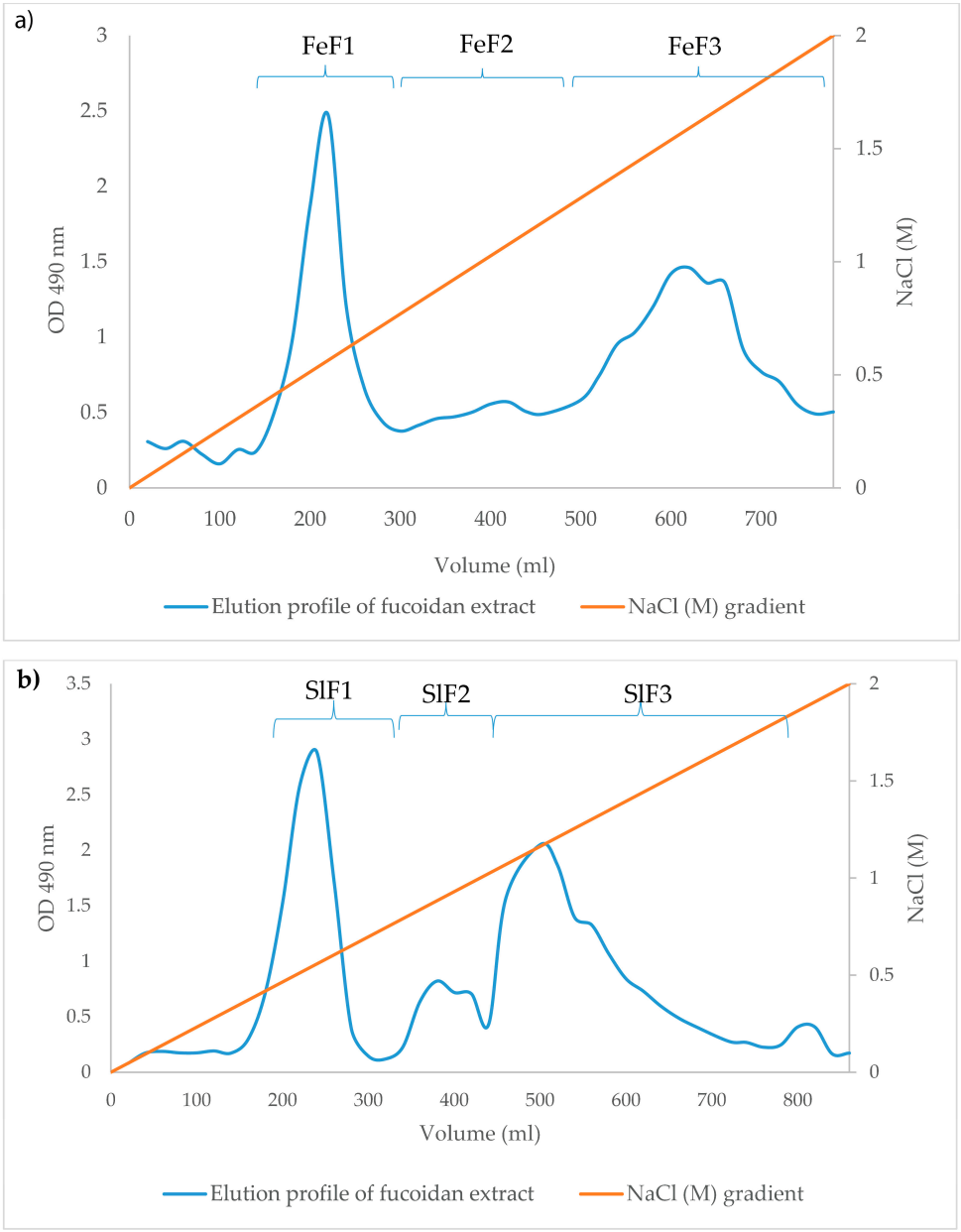

Figure 1. The elution profile of ion-exchange chromatography (IEX) fractionated fucoidan. (**a**) Elution profile for the *F. evanescens* chromatography purificatonand. (**b**) Elution profile for the *S. latissima* fucoidan during IEX. Based on the elution profile, three extracts FeF1–F3 and SlF1–F3 were collected from the *F. evanescens* and *S. latissima* purifications, respectively.

FeF1 consisted of fucose (34%), galactose, glucose, xylose, mannose, traces of rhamnose, and a high level of ManA (32.2%) (Table 3). Most uronic acids from alginate contamination eluted in FeF1. Fucose was the major component found in FeF2 and FeF3 at 74.7% and 87.8%, while the galactose content was 15.4% and 9.0% in these two fractions, respectively. Furthermore, the amount of glucose was very low, 1.4% and 0.3% in FeF2 and FeF3 (Table 3), respectively, suggesting that laminarin and cellulose had been successfully removed. Likewise, because alginate had been eluted in FeF1, its content was very low in FeF2 and FeF3 at 2.4% and 0%, respectively. The content of sulfate was also high with 34.8% in FeF2 and 38.7% in FeF3.

Three fucoidan fractions SlF1–F3 were obtained based on the elution profile of extracts from *S. latissima* (Figure 1b) (Table 3). Fraction SlF1 almost exclusively consisted of ManA (82.4%) and the content of neutral sugars was low and included only 5.4% fucose. Fractions SlF2 and SlF3 mainly

consisted, respectively, of fucose at 64.7% and 63.3% and galactose at 12.2% and 26.9%. Alginic acid content was low with a slightly higher concentration in SlF2 (13.8%) in comparison to SlF3 (3.6%), while the glucose content was very low in both SlF2 (0.6%) and SlF3 (0.4%). Sulfate content was also high in SlF2 (35.6%) and SlF3 (46.4%) when compared to only 6.6% in SlF1, which was consistent with the amount of fucoidan in the samples.

The current analysis indicates that FeF2, FeF3, SlF2, and SlF3 can be considered pure fucoidans due to high levels of fucose, galactose, and sulfates and low amounts of glucose and alginate.

2.3. Size Exclusion Chromatography (SEC) Analysis

2.3.1. SEC of Crude Fucoidans

Extraction procedures can affect the molecular weight of fucoidans and molecular weight is an important factor that can influence fucoidan bioactivity [6]. The enzyme-assisted extraction procedure was expected to result in intact fucoidans with larger molecular weight than that obtained by chemical extraction. Thus, in this study, the mass distribution of enzymatic and chemically extracted crude fucoidans from *F. evanescens* and *S. latissima* were compared. The molecular weight of crude fucoidans normally ranges from 21 to 1600 kDa [33]. All crude extracts showed heterogeneous molecular weight profiles based on High Performance Size Exclusion Chromatography (HP-SEC) analysis (Figure 2). The enzymatic extracts from *F. evanescens* consisted of two different populations of polymers, one with a molecular weight less than 5 kDa and a second with a broad distribution with molecular weights from approximately 50 kDa to more than 800 kDa and averaging 100–800 kDa. A more heterogeneous distribution in the range of less than 1 kDa to approximately 800 kDa was observed for the chemical extract, and an average molecular weight of 10–100 kDa, i.e. considerably lower than for the enzyme-assisted purification (Figure 2a), and the very low molecular weight species (<21 kDa) may, in a strict definition, not be defined as fucoidan molecules. The same pattern was observed for the enzymatic extract of *S. latissima*, which gave two different populations, one with a molecular mass less than 10 kDa and one from ~100 kDa to over 800 kDa, with the latter population being larger and more broadly distributed compared to *F. evanescens* (Figure 2b). The chemical extract of *S. latissima* was better defined than the *F. evanescens* extract, with two defined populations, one of less than 5 kDa and the second with approximately 50−100 kDa (Figure 2b).

The difference in molecular weights between fucoidans from *F. evanescens* and *S. latissima* shows the size diversity of native fucoidans in brown seaweeds. The HP-SEC results thus showed that the enzyme-assisted technique resulted in the extraction of fucoidans with a generally higher molecular weight as compared to the chemical technique for these two seaweed species.

2.3.2. SEC of Fucoidan Fractions from *F. evanescens* and *S. latissima*

The IEX fractionated extracts were also analyzed by HP-SEC and they showed different molecular weight distributions of the different fucoidan fractions from *F. evanescens* and *S. latissima*.

The *F. evanescens* FeF1 consisted of poly- and oligosaccharides with a very wide range of molecular weight distribution and three main peaks at 2–3 kDa, 30–40 kDa, and ~400 kDa (Figure 3a). This suggests the presence of many different components, which was also evident in the monosaccharide composition analysis. A high content of ManA was observed for FeF1, therefore the peak at 2–3 kDa is suggested to be oligo-alginate and poly-ManA contaminants from the SALy catalyzed alginate degradation. FeF2 contained a broad molecular weight distribution from approximately 30 kDa to over 800 kDa, with the largest peak around 400–800 kDa and a small shoulder around 30–40 kDa, as was observed in FeF1. The shoulder might represent smaller sized fucoidans, since FeF2 only contains very low amounts of alginate (2.4%). FeF3 contained one homogeneous peak around 400–500 kDa (Figure 3a), and monosaccharide analysis of FeF3 (Table 3) confirmed the presence of highly pure fucoidans.

S. latissima fractions showed a different molecular weight distribution than that observed for *F. evanescens* and the size distribution of the populations of fucoidan molecules seemed more complex

than those from *F. evanescens* (Figure 3b). The major component of SlF1 was a highly homogeneous peak of less than 5 kDa, and monosaccharide analysis showed the main carbohydrate content to be ManA (Table 3). SlF2 and SlF3 had similar SEC profiles, with a molecular weight distribution in the range of approximately 300 kDa to over 800 kDa (Figure 3b). In addition, SlF2 had a small shoulder of around 10 kDa, likely due to the presence of minor alginate impurities and confirmed by the monosaccharide composition (13.8%). The monosaccharide composition of SlF2 and SlF3 implied pure fucoidans (Table 3), although the mass distribution of the fractions appeared to be rather heterogeneous. This could indicate that the fucoidans from *S. latissima* have very complex structures.

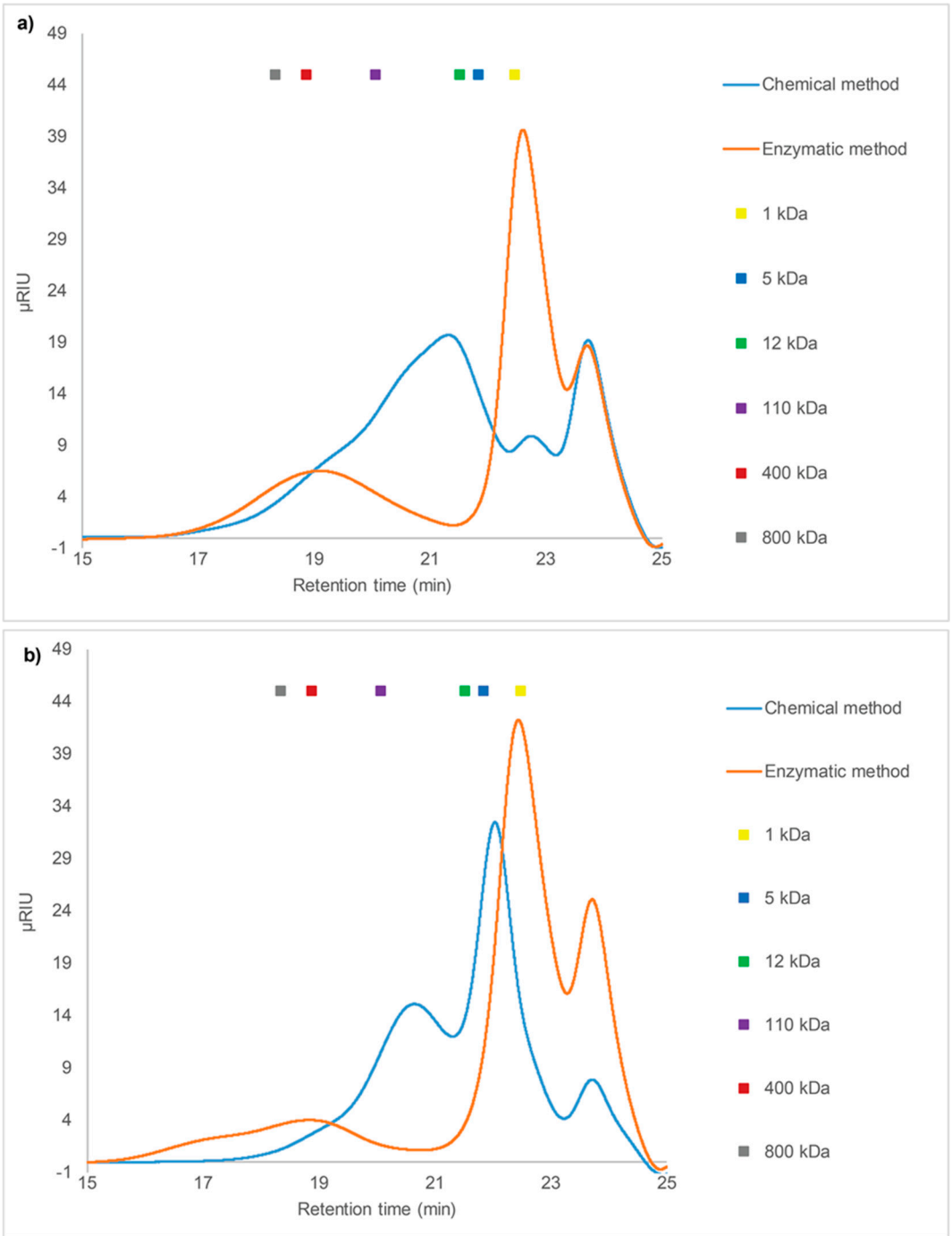

Figure 2. SEC chromatogram of crude fucoidans from chemical and enzyme-assisted purification. (**a**) *F. evanescens* and (**b**) *S. latissima*. Pullulan was used as standard.

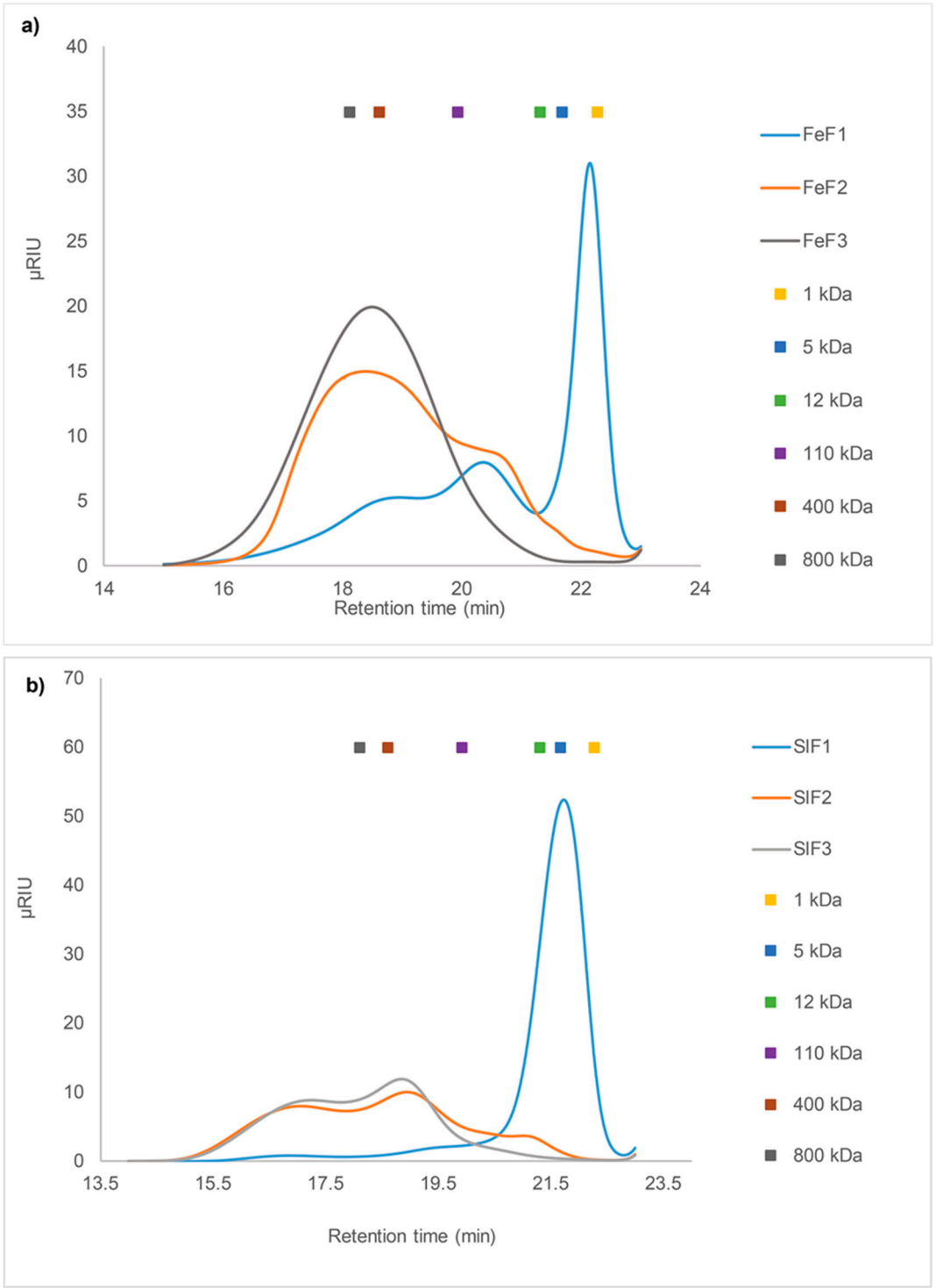

Figure 3. SEC chromatogram of fucoidan fractions after IEX purification. (**a**) *F. evanescens*: the FeF1 fraction contains LMW compounds of around 2 kDa and a smaller proportion of HMW compounds between ~100–500 kDa, while FeF2 and FeF3 contain primarily HMW compounds ranging from ~400 kDa to ~800 kDa; (**b**) *S. latissima*: the SlF1 fraction contains almost exclusively LMW compounds of around 5 kDa, while SlF2 and SlF3 contain more HMW compounds ranging from ~300 kDa to over 800 kDa. Pullulan was used as standard.

2.4. ^{1}H NMR Spectrum of Fucoidan Fractions from F. evanescens and S. latissima

^{1}H NMR spectroscopy was used for the preliminary determination of fucoidans from *F. evanescens* and *S. latissima*. The spectra displayed several signals that are indicative of the diversity and complexity of the fucoidans. However, several specific signals for fucose were observed in the ^{1}H NMR spectra from fucoidan fractions of both seaweeds (Figure 4), such as signals of anomeric protons (5–5.6 ppm), ring protons (3.6–4.8 ppm), and methyl protons (1.2–1.5 ppm) regions [34].

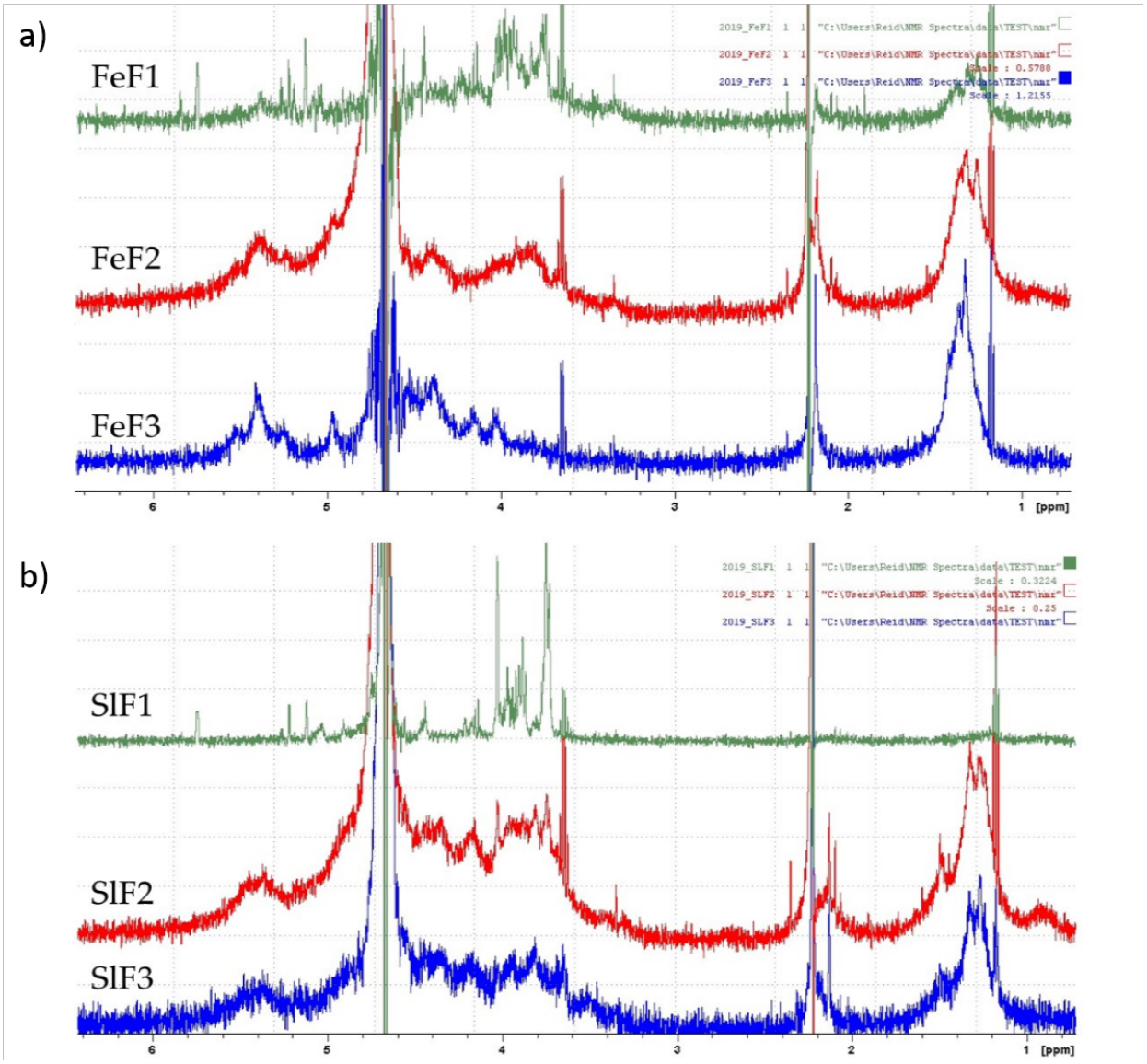

Figure 4. ^{1}H NMR spectra of fucoidan fractions. (**a**) *F. evanescens* (FeF1, FeF2, FeF3) and (**b**) *S. latissima* (SlF1, SlF2, SlF3) in D_2O.

The spectra of all three *F. evanescens* fractions contained specific chemical shifts characteristic of fucoidans (Figure 4a). Only the FeF1 fraction gave signals in the region of 5.8 ppm, characteristic for uronic acids, confirming the presence of alginate impurities that are consistent with the monosaccharide and SEC analysis. Fraction FeF2 and FeF3 were purer than FeF1 and the characteristic peak of uronic acids was not observed. Indications of 1→3 linked L-fucose were detected in the high-field signals at 1.2–1.3 ppm with high intensity. In addition, signals with low intensity at around 1.4 ppm were also observed, which confirmed the presence of 1→4 linked L-fucose, as anticipated [35,36].

The ^{1}H NMR spectra of fucoidan fractions from *S. latissima* (Figure 4b) were similar to the spectra of *F. evanescens*, though the methyl proton signal was absent in the SlF1 fraction. The specific resonances of uronic acid at 5.74 ppm (~5.8 ppm) were detected in SlF1, which confirmed the presence of alginate in this fraction. In the fractions SlF2 and SlF3, methyl signals appeared at around 1.3 ppm, with high intensity indicating the 1→3 linked L-fucose in the fucoidan structure, as anticipated [35,36].

3. Discussion

The use of a combination of the alginate lyase SALy and the cellulase preparation Cellic®CTec2 was effective in extracting fucoidans from both the brown algae, *F. evanescens* and *S. latissima*, which were used here as prototype fucoidan sources to demonstrate the validity of enzyme-assisted extraction. The yields of fucoidans extracted by the enzymatic method were comparable to those that were obtained by mild chemical extraction. The monosaccharide composition of the crude fucoidan extracts differed between the two methods. The chemical extract contained a relatively higher percentage of fucose than the crude enzyme-assisted extract, but that was mainly due to the lower levels of alginate and, as expected, the fucose content in the enzyme-assisted extracts after IEX purification was indeed considerably higher than in the crude chemical extracts. In addition, the galactose content, which is a minor component of fucoidan, was considerably higher in the enzyme-assisted IEX extracts as compared to the chemical extracts. Importantly, the sulfate content was slightly higher in the crude enzyme-assisted extracts than in the chemical extracts. However, the glucose contents of the crude fucoidans that were extracted by the chemical method were higher than the levels obtained by the enzymatic method, and notably much higher in the fucoidan extracted from *S. latissima* as compared to that from *F. evanescens* (Table 2). The analyzed glucose in the fucoidan extracts implies the presence of residual components of laminarin. As mentioned, the difference in glucose levels between the chemical and enzyme-assisted extracts indicates that laminarin, as expected, is readily degraded by Cellic®CTec2 in the enzyme-assisted method. The glucose liberated by this treatment is then removed by the ethanol precipitation. This result is in complete agreement with a previous report, where Cellic®CTec2 was shown to catalyze the release of glucose and other monosaccharides from *Laminaria digitata* and shown to catalyze the degradation of pure laminarin [31].

The alginate was not fully degraded in the crude extract from the enzyme-assisted purification. The $CaCl_2$ precipitation removed higher molecular weight alginates remaining after the enzyme treatment, but low molecular weight alginates were still present in the extracts. These alginates did not precipitate with $CaCl_2$ addition, most likely due to their low molecular weight, but presumably also due to the high amount of relative ManA as compared to GuluA, where notably the latter forms gels with $CaCl_2$ [37]. The results correlate with the substrate specificity of the alginate lyase used in the extraction process. According to Manns et al [28], the alginate lyase SALy has higher specificity towards poly-(G) than poly-(M) blocks in alginate, consequently leaving behind poly- and oligo-(M) blocks [28]. The alginate lyase was added in excess and it was not an apparent limiting factor, although enzyme inhibitory compounds, like polyphenols, might be present in the seaweed. In the future, the use of bifunctional alginate lyase, or other alginate lyases with higher M-block specificity might improve the purity of the crude fucoidans. Although the low molecular weight alginates were easily removed by IEX, the elimination of this step would be preferable. In literature, many bifunctional recombinant alginate lyases with higher M-block specificity are found, but they all have apparent high pH optima around 8–8.5 [38–41], which would not work in concert with the Cellic®CTec2, which has a pH optimum of 5.0–5.5. Recently, a fungal alginate lyase from *Paradendryphiella salina* with preference for mannuronate was discovered [42], this enzyme and other new alginate lyase enzymes may be developed for large scale use in a protocol in which the IEX procedure could be omitted.

As previously reported, the sulfate content is decreased by acid extraction [43]. According to published data, sulfate plays an important role in the bioactivity of fucoidan, such as anti-angiogenic and antitumor [44], anticoagulant and antiproliferative effects [45], reduction of cancer cell viability *in vitro*, and immunostimulatory effects, such as activation of natural killer cells [46]. The sulfate

content was slightly higher in the enzymatically extracted crude fucoidans, even though a gentle method with only 3 h extraction at 70 °C was used for the chemical extraction. An expected higher bioactivity of fucoidan following the enzymatic method on account of higher sulfate content needs further investigation.

The molecular sizes of crude fucoidans and fractions were analyzed in order to evaluate fucoidan degradation during chemical vs. enzyme-assisted extraction. As expected, the molecular weights of crude fucoidans from *F. evanescens* and *S. latissima* were quite high after the enzymatic extraction as compared to the chemical extraction, which is likely related to the extraction conditions. In the chemical procedure, hot acid treatment probably contributed to partial degradation of the fucoidans and resulted in polysaccharides with lower molecular weight. In contrast, in the enzyme-assisted extraction, the selectivity of the enzymes was exploited in order to specifically degrade only the target non-fucoidan polysaccharides in the cell wall of seaweed as well as laminarin, avoiding fucoidan depolymerization and desulfatation.

The molecular weight of crude fucoidans from *F. evanescens* has been previously reported to have various sizes from 181 to 400 kDa after extraction with aqueous $CaCl_2$ [47] and HCl purification [48]. In this study, the average size of crude fucoidans from *F. evanescens* was 200–400 kDa, which is a high molecular size when compared with fractions that were extracted by acid (from 150 to 500 kDa) [49] and by ultrasound-assisted extraction (280 and 240 kDa) [50]. Fucoidans from *F. evanescens* were previously reported as a fucansulfate [10]. This interpretation of the *F. evanescens* fucoidans being fucansulfate molecules was confirmed by the high amount of fucose and the monomodal profile of SEC curves of the pure fractions FeF2 and FeF3. Characteristic signals confirmed the presence of fucose and also indicated 1→4 and 1→3 linked L-fucose in the ^{1}H NMR spectra of fractions from *F. evanescens* (Figure 4). This is in agreement with the data reported by Bilan et al. [10].

The fucoidans from *S. latissima* were more complex based on their monosaccharide composition. In addition, the SEC profiles for fraction SlF2 and SlF3 showed broad double peaks, suggesting that they might be large heterogeneous polysaccharides [47]. Three types of sulfated fucan-polysaccharides have been reported in *S. latissima*: fucogalactan, fucoglucuronomannan, and fucoglucuronan [13]. The average molecular weights of crude fucoidans and fractions from this seaweed were very high. Recently, fucoidan fractions of *S. latissima* extracted by an aqueous $CaCl_2$ method with sizes up to 543 kDa were reported [51]. With the enzyme-assisted extraction method fucoidans with even higher molecular weights were obtained. ^{1}H NMR spectra of fucoidan fractions of *S. latissima* confirmed the presence of fucose 1→3 linked L-fucose, similar to published data [10,13].

4. Material and Methods

4.1. Materials

S. latissima from Iceland, harvested in June 2017, was provided as dried flakes by Blaskel (Stykkisholmur, Iceland). *F. evanescens*, from Kiel fjord, Germany, was collected in March 2017 and provided as fresh frozen by Coastal Research & Management (Kiel, Germany). The *F. evanescens* seaweed was washed with fresh water to remove impurities and lyophilized. The dried *F. evanescens* seaweed was ground into powder (~0.5 mm) and stored at room temperature.

Cellic®CTec2 was obtained from Novozymes (Bagsværd, Denmark). This is a commercial cellulase preparation based on the *Trichoderma reesei* cellulolytic enzyme complex; this preparation had an activity of 142 FPU/mL (filter paper activity units). Apart from the cellulolytic enzyme base from *T. reesei* RUT C-30 containing at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), several different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel45A), β-glucosidase EC 3.2.1.21, and a GH3 β-xylosidase EC 3.2.1.37; this preparation includes extra β-glucosidase and lytic cellulose monooxygenases (1.14.99.54, 1.14.99.56, AA9) and other proprietary proteins.

4.2. Alginate Lyase Expression and purification

The overnight culture of *E. coli* hosting the *Sphingomonas* sp. alginate lyase SALy was prepared, as described in Manns et al. 2016 [28]. The expression was performed in 5 L fermenters in auto-induction media (6 g Na_2HPO_4, 3 g KH_2PO_4, 20 g tryptone, 5 g yeast extract, 5 g NaCl, 0.06% v/v glycerol, 0.05% w/v glucose, and 0.04% w/v α-lactose, pH 7.2) at 20 °C. The cells were collected by centrifugation, re-suspended in cold extraction-buffer (20 mM Tris-HCl buffer, pH 7.4, 250 mM NaCl, 2 mg/mL lysozyme), sonicated to destroy the cell wall, and then centrifuged to remove debris. The supernatant was collected and passed through a 0.22 µm filter, and then loaded onto a Ni^{2+}-Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden). Unbound material was washed off the column with 10 column volumes of wash buffer 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 20 mM imidazole. The enzyme was eluted with elution buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 100 mM imidazole). The enzyme activity of the alginate lyase was ~18 units/mg enzyme quantified as %substrate consumption/(min·mg enzyme) calculated from formation of double bonds at 235 nm on sodium alginate at 40 °C, pH 7 [28]. SDS-PAGE confirmed protein purity and the protein concentration was determined by the Bradford method [52].

4.3. Fucoidan Extraction from Brown Seaweeds

4.3.1. Chemical Extraction Method

The chemical extraction of crude fucoidans was performed in 0.1 M HCl solution with a ratio of seaweed to extracting liquid of 1:20 and treatment for 3 h at 70 °C (methodology modified from Ale et al. 2012 [53]). The supernatant (Extract A, Figure 5a)) was collected by centrifugation for 10 min at 19,000 rpm. Next, 2% $CaCl_2$ solution was added to remove alginate as a precipitate after centrifugation (Figure 5). The crude fucoidans were isolated from the supernatant by 72% ethanol (EtOH), recovered by centrifugation at 19,000 rpm for 30 min, and lyophilized. The latter steps were comparable to the enzyme-assisted extraction method (Figure 5).

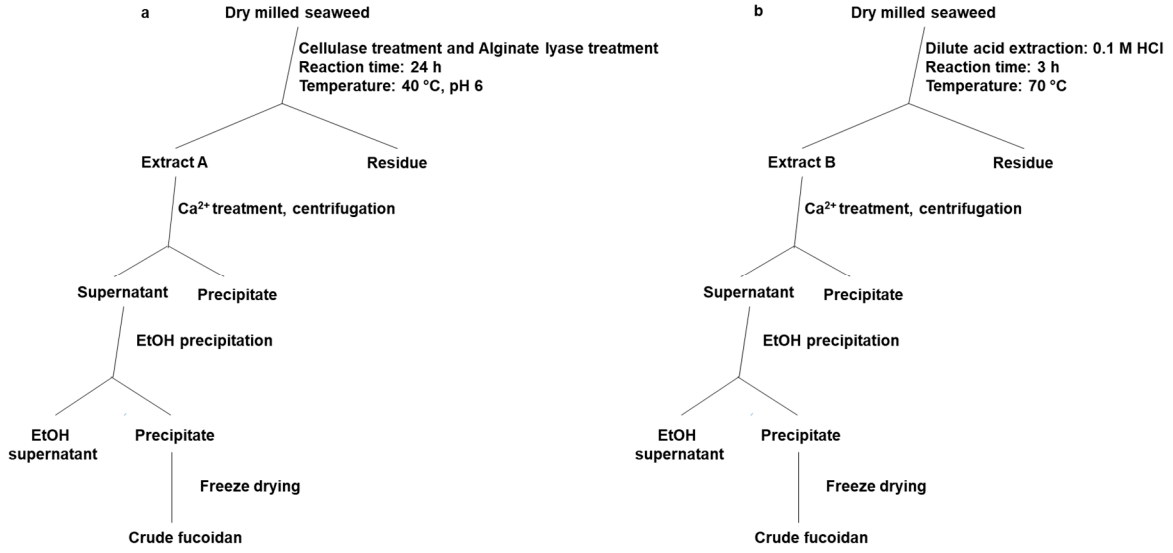

Figure 5. Flow-chart of extraction procedures. (**a**) Enzyme-assisted extraction and (**b**) Chemical extraction (mild acid extraction). (EtOH is ethanol).

4.3.2. Enzyme-Assisted Extraction Method

The enzymatic extraction of fucoidans was initiated by a combined cellulase and alginate treatment (Figure 5): in practice, the first step included simultaneous addition of the enzymes at a level of 5% (v/w) for Cellic®CTec2, and 0.35% (w/w) for alginate lyase. The treatment was conducted at 40 °C for 24 h on a horizontal mixer at 100 rpm performed in 55 mM phosphate-38 mM citrate buffer pH 6 with

5% (w/v) substrate concentration. The reaction was stopped by boiling at 90 °C for 10 min and then cooling on ice. The supernatant (Extract B, Figure 5b) was collected after centrifugation for 10 min at 19,000 rpm. To remove residual alginate, 2% $CaCl_2$ was added and the mixture was centrifuged again. The fucoidans were precipitated from the supernatant after alginate removal and isolated by the addition of EtOH to a final concentration of 72%, recovered by centrifugation at 19,000 rpm for 30 min, and lyophilized.

4.4. Fucoidan Fractionation by Anion-Exchange Chromatography

Crude fucoidan samples in aqueous solution (5 g in 100 mL) were applied to a column (2.6 cm × 40 cm) that had been manually packed with DEAE-Macroprep resin material (Bio-Rad, CA, USA) and then equilibrated with acidic NaCl (0.04 N HCl in 0.1 M NaCl) (packing was done according to the instruction manual (Lit271 Rev D) from Bio-Rad). The unbound materials were washed from the column with 0.1 M NaCl. Fucoidans were eluted at a flow rate of 5 mL/min in a sequential concentration gradient of NaCl from 0.1 to 2M. The eluted fractions were combined based on the results of total carbohydrate analysis (reducing ends analysis) by the phenol-sulfuric acid method [32]. The fractions were passed through a 10 kDa membrane in order to concentrate the fucoidan and remove salt and then lyophilized.

4.5. Chemical Composition Analysis

4.5.1. Two-Step Acid Hydrolysis

Seaweed and fucoidan samples (5 mg) were hydrolyzed in 50 µL 72% H_2SO_4 at 30 °C for 1 h and the mixture was then diluted to 4% H_2SO_4 and hydrolysis continued for an additional 40 min at 120 °C in an autoclave. The hydrolysates were neutralized, filtered through a 0.22 µm syringe filter, and used for monosaccharide analysis [54].

4.5.2. Chemical Composition Analysis

Monosaccharide composition, mannitol, and uronic acids of the hydrolysates were analyzed on a Dionex ICS-3000 HPAEC-PAD system with pulsed amperometric detection (PAD). Three eluents were used: A—deionized water, B—200 mM NaOH, and C—200 mM NaOH, 1 M NaOAc. Chromatographic separation was performed with a flow rate 0.4 mL/min, using 0.5% B in A for the first 17 min for elution of neutral sugars and sugar alcohol. Next, 3% B and 6% C in A were applied for 20 min for the separation of uronic acid. The process was completed with 100% B in 6 min, after which 0.5% B in A was applied to calibrate the column. Data quantification was analyzed by Chromeleon™ 7.2 (Thermo Scientific). Recovery values of the monosaccharides and uronic acid were estimated from runs at the same time [54].

4.5.3. TFA Hydrolysis and Sulfate Content Analysis

Fucoidan samples (5–6 mg) were hydrolyzed in 1 mL 2 M trifluoroacetic acid (TFA) at 100 °C for six hours (in closed vials in a thermostatted water bath). After hydrolysis, the TFA was evaporated and the residual TFA was removed by the addition of 2.5% ammonium hydroxide (NH_4OH). The hydrolysates were used to determine the sulfate content by the barium chloride ($BaCl_2$) gelatin method [55]. 0.5% gelatin solution was prepared in warm water (60–70 °C). 0.5 % $BaCl_2$ was dissolved in gelatin solution and then allowed to stand for 2 h at 25 °C, then centrifuged at 10,000× g for 10 min 10 µL of hydrolysate solution was added to 160 µL trichloroacetic acid (TCA) and 100 µL $BaCl_2$-gelatin reagent. The mixture was allowed to stand for 30 min. A blank was prepared with 170 µL TCA and 100 µL $BaCl_2$-gelatin reagent. The released $BaSO_4$ suspension was measured at λ = 360 nm in a microplate reader (TECAN Infinite 200, Salzburg, Austria) while using UV-transparent 96-well microplates (Corning®, Tewksbury, MA, USA). Potassium sulfate was used as standard to generate a (linear) standard curve for the sulfate response at 360 nm.

4.6. Determination of Molecular Weight by Size Exclusion Chromatography

The molecular weights (MW) of fucoidan fractions were determined by High Performance Size Exclusion Chromatography (HP-SEC) using an Ultimate iso-3100SD pump with WPS-3000 sampler (Dionex, Sunnyvale, CA, USA) connected to an RI-101 refractive index detector (Shodex, Showa Denko K.K., Tokyo, Japan). The samples were dissolved in 1 ml HP-SEC buffer (100 mM sodium acetate, pH 6) and filtered through 0.22 µm filters. 100 µL of fucoidan samples (3 mg/mL) were injected into a Shodex SB-806 HQ GPC column (300 × 8 mm) coupled with a Shodex SB-G guard column (50 mm × 6 mm) (Showa Denko K.K., Tokyo, Japan) [56]. Elution was carried out at a flow rate of 0.5 mL/min at 40 °C [56]. Pullulan samples of molecular weight 1, 5, 12, 110, 400, and 800 kDa were used as standards [56].

4.7. ^{1}H NMR Analysis

NMR spectra were recorded using an Avance III-700 NMR spectrometer (BrukerBiospin AG, Switzerland) and Avance III-500 HD NMR spectrometer (Bruker, Germany). The samples (3 mg) were prepared by dissolving freeze-dried fucoidan fractions in 550 µL of deuterated water (D_2O) and then placing them in 5 mm tubes. The ^{1}Hspectra were recorded at 35 °C with acetone as the internal standard (2.225 ppm).

5. Conclusions

An enzyme-assisted extraction method for fucoidans from brown seaweeds was introduced and compared to mild chemical extraction of fucoidan from *F. evanescens* and *S. latissima*. The enzyme-assisted procedure, including IEX purification, resulted in highly satisfactory yields of pure fucoidans having similar or slightly higher sulfate, and a higher molecular weight than the fucoidans obtained with the chemical acidic extraction method. Therefore, the enzyme-assisted extraction method appears to be promising for obtaining authentic brown seaweed fucoidans of high bioactivity and, in particular, offers a new approach that can continue to be improved to further advance the understanding of the structure-bioactivity effects of fucoidans and promote further sustainable explorations of fucoidans.

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The Endo-α(1,3)-Fucoidanase Mef2 Releases Uniquely Branched Oligosaccharides from *Saccharina latissima* Fucoidans

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Abstract: Fucoidans are complex bioactive sulfated fucosyl-polysaccharides primarily found in brown macroalgae. Endo-fucoidanases catalyze the specific hydrolysis of α-L-fucosyl linkages in fucoidans and can be utilized to tailor-make fucoidan oligosaccharides and elucidate new structural details of fucoidans. In this study, an endo-α(1,3)-fucoidanase encoding gene, *Mef2*, from the marine bacterium *Muricauda eckloniae*, was cloned, and the Mef2 protein was functionally characterized. Based on the primary sequence, Mef2 was suggested to belong to the glycosyl hydrolase family 107 (GH107) in the Carbohydrate Active enZyme database (CAZy). The Mef2 fucoidanase showed maximal activity at pH 8 and 35 °C, although it could tolerate temperatures up to 50 °C. Ca^{2+} was shown to increase the melting temperature from 38 to 44 °C and was furthermore required for optimal activity of Mef2. The substrate specificity of Mef2 was investigated, and Fourier transform infrared spectroscopy (FTIR) was used to determine the enzymatic activity (Units per μM enzyme: U_f/μM) of Mef2 on two structurally different fucoidans, showing an activity of 1.2 × 10^{-3} U_f/μM and 3.6 × 10^{-3} U_f/μM on fucoidans from *Fucus evanescens* and *Saccharina latissima*, respectively. Interestingly, Mef2 was identified as the first described fucoidanase active on fucoidans from *S. latissima*. The fucoidan oligosaccharides released by Mef2 consisted of a backbone of α(1,3)-linked fucosyl residues with unique and novel α(1,4)-linked fucosyl branches, not previously identified in fucoidans from *S. latissima*.

Keywords: endo-α-1,3 fucoidanase; Fourier transform infrared spectroscopy (FTIR); fucoidan; sulfated oligosaccharide; *Saccharina latissima*; GH107; glycosyl hydrolase

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1. Introduction

Fucoidans are present in the cell walls of brown macroalgae and constitute the most complex class of marine polysaccharides found to date. Fucoidans are mainly composed of a backbone of sulfated fucosyl residue in addition to normally minor amounts of other sugars such as galactose, xylose, glucose, mannose, rhamnose, and uronic (mostly glucuronic) acids [1]. Fucoidans can be classified by their monosaccharide composition and the backbone linkage type [2], which includes α(1,3)-L-fucose linkages (group 1), repeating α(1,3)- and α(1,4)-L-linked fucose residues (group 2), galactofucans/fucogalactans (group 3), or fucoidans with higher amounts of mannose and/or uronic acid residues (group 4).

Finally, there are more complex fucoidans with a composition that can include five or more different sugars (group 5) [2]. Fucoidans may furthermore be randomly acetylated and branched. The structure of fucoidans may differ within the same alga, resulting in a mixture of polyanionic molecules that are difficult to separate and structurally characterize [3–5].

S. latissima, or sugar kelp, is gaining ground as a new commercially cultivated brown macroalgae due to its ability to grow in the Northern hemisphere, notably in the North Atlantic [6]. Fucoidans from *S. latissima* are particularly complex. However, the following four different partial structures have been reported to date: sulfated fucan, galactofucan, fucoglucuronomannan, and fucoglucuronan [3].

Fucoidans show promising applications in disease treatment with biological activities such as antioxidants, anticancer [7], anti-inflammatory [8], anticoagulant [9,10], and immune-modular properties [11]. Newer results have identified fucoidans as a promising drug against age-related macular degeneration (AMD), including an effective inhibition of the vascular endothelial growth factor (VEGF) [12]. In addition, certain fucoidan oligosaccharides can induce bone-regeneration in osteoporotic sheep when coated on implants [13,14]. Together, these functional studies show that fucoidans have many potential drug applications that are largely influenced by fucoidan structure and size. Endo-fucoidanases can be used as tools to design particular and uniform fucoidan oligosaccharide structures, to pave the way for understanding structure–bioactivity relationships, and in turn, to help tailor-make specialized fucoidans for distinct bioactivity functions.

Fucoidan-degrading organisms are mainly found in marine bacteria such as *Flavobacteriaceae* [15] and *Alteromonadales* [16]. These bacteria produce extracellular fucoidanases (EC 3.2.1.-) with specific substrate and linkage preferences that have been classified based on sequence similarities in the Carbohydrate Active enZyme database (CAZy) [17] families GH107 (EC 3.2.1.212) and GH168 (EC 3.2.1.211). The first GH107 member MfFcnA, an endo-α(1,4)-fucoidanase from *Mariniflexile fucanivorans* SW5, was characterized in 2006 [18]. GH107 contains 28 sequences, all bacterial, of which only six have been characterized, and the linkage specificity has been determined for a few members. In addition, the linkage specificity has been described for a few fucoidanases not yet added to CAZy GH107. The α(1,4)-specific fucoidanases include MfFcnA [18], FFA1, and FFA2 from *Formosa algae KM3553* [19–21], Fhf1 [22], and Fhf2 from *Formosa haliotis* [23], and FWf1 and FWf2 from *Wenyingzhuangia fucanilytica* CZ1127 [24]. To date, the only characterized GH107 members with α(1,3) specificity are Fda1 and Fda2 from *Alteromonas* sp. SN-1009 [25]. The characterization of the GH107 fucoidanases has demonstrated varying selectivity for not only the glycosidic linkage but also for sulfation, acetylation, and branching patterns. Because of the wide variety of fucoidan structures in nature, many fucoidanases specific for different structures are thought to exist, though only a few have been discovered to date.

The mesophilic marine bacterium *Muricauda eckloniae* (previously known as *Flagellimonas eckloniae*) from the *Flavobacteriaceae* family was originally isolated from the rhizosphere of the brown seaweed *Ecklonia kurome* [26]. In the present study, we functionally characterized a novel GH107 fucoidanase, Mef2, from *M. eckloniae.* Mef2 was shown to be the first fucoidanase acting on fucoidans from *S. latissima* and furthermore, the first fucoidanase releasing a novel branched fucoidan oligosaccharide from *S. latissima* fucoidans.

2. Results

2.1. Sequence Analysis of the Mef2 Fucoidanase from M. eckloniae

Through BLAST analysis using known GH107 fucoidanase sequences, the putative fucoidanase Mef2 was identified (RefSeq: WP_055392200.1). Mef2 was categorized as a discoidin domain-containing protein in NCBI. The predicted Mef2 protein was 1067 amino acids long and was found to contain an 18 amino acid long N-terminal signal peptide according to SignalP [27]. Modular architecture analysis of Mef2 by InterProScan predicted various domain types, some of which overlap (Figure 1a).

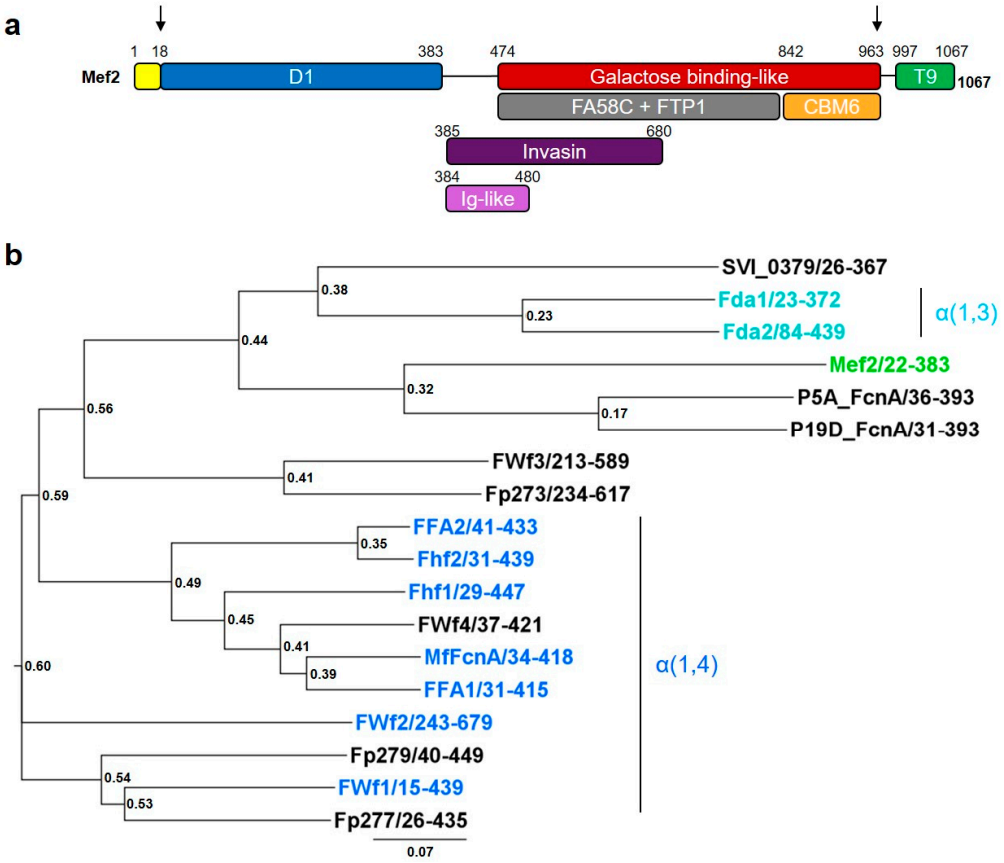

Figure 1. Modular structure of the native Mef2 and recombinant Mef2 protein and phylogenetic overview. (**a**) The native Mef2 protein sequence, arrows indicate the recombinantly expressed part of the Mef2 protein. Yellow: signal peptide, blue: D1 catalytic domain, purple: invasin/intimin cell-adhesion domains (IPR008964), pink: Ig-like domain (IPR003343), red: galactose binding-like domain (IPR000421), grey: FA58C domain (IPR000421) and FTP1 domain (IPR006585), orange: CBM6 domain (IPR005084), and green: secretion system C-terminal sorting domain (T9SS domain (T9)) (IPR026444). Domains were predicted using SignalP (signal peptide), InterProScan, and sequence alignment (D1) with P5AFcnA and MfFcnA. (**b**) phylogenetic analysis of the D1 domain of selected GH107 fucoidanases (numbers indicate the used sequence span), blue: α(1,4)-linkage specific fucoidanases, Turquoise: α(1,3)-linkage specific fucoidanases, green: Mef2. Accession numbers can be found in Figure S1.

As expected from the discoidin categorization, a coagulation factor F5/8 C-terminal (FA58C) domain (IPR000421 from 464–834) was predicted in Mef2. *Dictyostelium discoideum* (slime mold) has a related domain in the cell adhesion protein discoidin. The FA58C domain in Mef2 is likely the reason why Mef2 was categorized as a discoidin domain-containing protein when deposited in NCBI.

A Secretion system C-terminal sorting domain (IPR026444 from 997–1067) or Type IX Secretion System (T9SS) domain was predicted in the C-terminus of Mef2. T9SS domains are normally associated with protein sorting to the outer membrane [28]. These domains have not been associated with catalytic activity in fucoidanases and can readily be removed for heterologous expression [18–20,22,23,29].

An invasin/intimin cell-adhesion fragment (IPR008964 from 385-680) and a bacterial Ig-like group 2 domain (IPR003343, from 387–480) were also predicted in Mef2. The superfamily comprising the Ig-like domain type, including invasin/intimin cell-adhesion domains, is common in GH107 fucoidanases [18–20,22,23]. These Ig-like domains are thought to be calcium-binding, which was verified in the crystal structure of the fucoidanase MfFcnA [30]. Ig-like domains have been hypothesized to play a role in substrate recognition

and/or enzyme activity of fucoidanases, although this needs further investigations [18,30]. However, these domains might not be crucial for function in in vitro experiments, since at least one fucoidanase, Fhf2, where the domains had been removed, retained activity [23].

Several domains likely involved in carbohydrate binding were furthermore predicted in Mef2, a fucolectin tachylectin-4 pentraxin-1 (FTP1) domain (IPR006585 from 475–845), a galactose-binding-like superfamily domain (IPR000421 from 474–963) and a carbohydrate-binding module CBM6 domain (IPR005084 from 842–963). None of these domain types have previously been identified in fucoidanases, and although they might be involved in carbohydrate binding in fucoidanases, their function remains to be investigated.

The previously identified fucoidanase catalytic D1 domain, comprising a $(\beta/\alpha)_8$-barrel fold [30], has not yet been added to any domain prediction programs but was identified in Mef2 through sequence alignment with MfFcnA and P5AFcnA, from which D1 was determined through crystal structure analysis [30]. Sequence alignments of the catalytic D1 domain of Mef2 and the characterized GH107 fucoidanases revealed identity values ranging from 15–52% (Table S1), with the highest identity to the P5AFcnA (39.2%) and the α(1,3)-linkage specific Fda1 (21%) and Fda2 (22.5%) fucoidanases [25]. A phylogenetic tree (Figure 1b) constructed based on D1 multiple alignments (Figure S1) showed that Mef2 clusters closely with the fucoidanases with which it shares a high identity, while α(1,4)-linkage specific fucoidanases cluster more distantly, giving indications of a likely α(1,3)-linkage specificity of Mef2.

The conserved GH107 fucoidanase motifs 'RxxxxxDxxxxD' and 'DxxxGH', including the conserved catalytic site amino acids aspartate and histidine, were both identified within the D1 domain of Mef2 (Table 1); however, in the Mef2 sequence, the conserved 'RxxxxxDxxxxD' motif was replaced by 'VxxxxxDxxxxD'. The catalytic aspartate and histidine residues that are predicted to function as the catalytic nucleophile and acid/base and that are likely responsible for the cleavage of the glycosidic linkages by GH107 fucoidanases [30] were identified as Asp182 and His260 in Mef2 (Table 1 and Figure S1).

Table 1. Alignment of the catalytic amino acids in Mef2 and other GH107 members. Red: the active site aspartate (D), blue: the active site histidine (H), and orange: the change in the conserved arginine to valine in Mef2. Protein accession numbers are listed in Figure S1.

Conserved Domains around the Catalytic Amino Acids				
GH107		**I.**		**II.**
MfFcnA	214	QRYGDLIDAWCF**D**SA	288	DDYTFG**H**PFG
FFA2	225	MRYGDLIDAWCF**D**AA	302	EDYKFG**H**PFG
Fhf1	213	ERYGDLIDAWCF**D**SA	287	DDYTFG**H**PFG
Fhf2	215	MRYGDLIDAWCF**D**AA	291	EDYKFG**H**PFG
P19D_FcnA	186	KRFKGLVDGFWL**D**NS	270	MDFTNG**H**VTP
P5A_FcnA	189	ERFDGLVDGYWL**D**NS	270	MDFTNG**H**VTP
Mef2	170	E**V**LKDYADGYWL**D**TV	254	QDFTNG**H**VTS
Fda1	214	LRYGSTIDGWWF**D**HS	267	DDYTFG**H**PTP
Fda2	283	LRYGTLIDGWWF**D**HS	336	EDFTGG**H**PTP
		:. .*.: :* :		*.: **:

Below the alignment, an * indicates positions in the alignment which have a fully conserved residue. A colon (":") indicates conservation between amino acids of strongly similar properties, and a period (".") indicates conservation between groups of amino acids of weakly similar properties. I and II indicates the investigated protein domains.

The alignment also revealed conserved amino acids in Mef2 (Tyr127, Asn207 and Trp307) (Table 2 and Figure S1), previously predicted to be involved in coordinating the −1 subsite important for fucoidan hydrolysis [30]. However, another asparagine (Asn149 in MfFcnA) showing conservation in many GH107 fucoidanases was a serine (Ser129) in Mef2. Two other characterized fucoidanases, the α(1,3) linkage specific fucoidanases Fda1 and Fda2, also show differences to the canonical Ser129, having an alanine in this site.

Table 2. Alignment of the −1 subsite in Mef2 and other GH107 members. Purple: conserved amino acids in the −1 subsite and orange: the change in the conserved asparagine to serine in Mef2. Protein accession numbers are listed in Figure S1.

Conserved Domains around the Amino Acid of the −1 Subsite							
GH107	**1.**		**2.**		**3.**		**Specificity**
MfFcnA	141	GLRTEI**Y**VNSYNL	260	AGNPNAAIAF**N**NS	342	FFPKQSTTS**W**NAG	α-(1,4)
FFA2	148	GLKTEV**Y**VNSANL	270	AGNPDAAITF**N**NG	356	FFPKQSTTS**W**NDG	α-(1,4)
Fhf1	138	GLKTEI**Y**VNSYNL	258	AGNPNAAISF**N**NS	357	FFPKQSATS**W**NAG	α-(1,4)
Fhf2	138	GLKTEV**Y**VNSANL	260	AGNPNAAITF**N**NG	346	FFPKQSTTS**W**NDG	α-(1,4)
P19D_FcnA	134	GKKVLL**Y**LNTAGP	219	DIDPSFAIGV**N**YE	318	WFPIRF--S**W**SGS	n.d
P5A_FcnA	137	GKKVIL**Y**LNSAGP	219	SVDPELTIAV**N**YD	318	WFPIRN--S**W**SGS	n.d
Mef2	121	DKKIIL**Y**I**S**TQYF	197	EVDPTAVVTT**N**KG	300	WFPVRY--R**W**HTS	study
Fda1	129	GIRVVA**Y**IATQGP	241	AGNNDAAVAF**N**EG	314	FMPLQ--ES**W**NGG	α-(1,3)
Fda2	182	GIKVVA**Y**IATQGP	310	AGNSNAAVSL**N**LE	383	FLPLQ--ET**W**NGG	α-(1,3)
		:.:. * . .		... .. .*		.:* .*. .	

Below the alignment, an * indicates positions in the alignment which have a fully conserved residue. A colon (":") indicates conservation between amino acids of strongly similar properties, and a period (".") indicates conservation between groups of amino acids of weakly similar properties. 1, 2 and 3 indicates the investigated protein domains.

2.2. Functional Characterization of the Recombinant Mef2 Fucoidanase

The recombinant Mef2 encoding gene was constructed without the predicted signal peptide and T9SS domain but with a C-terminal 10×his tag, giving a predicted molecular weight of 105 kDa (GenBank: ON099398). Mef2 was co-expressed in *E. coli* BL21 (DE3) with the pGro7 chaperone. Expression of Mef2 at 20 °C overnight resulted in a partially degraded protein (Figure S2a,b), which was however resolved by an expression for 4 h at 37 °C (Figure S2c,d), resulting in one protein band at the expected size of approximately 105 kDa. This might indicate that fast expression of Mef2 at 37 °C in the presence of a chaperone results in better folding and less degradation than slower expression over a longer time at 20 °C. In previous expressions of several fucoidanases with extended C-terminal domains, expression was optimized by C-terminal deletions [22,23,29], which were, however, not necessary for successful Mef2 expression.

The substrate specificity of Mef2 was investigated on fucoidans with different structures isolated from 11 different species of brown seaweeds (Figure 2). Mef2 efficiently catalyzed the hydrolysis of fucoidans isolated from the brown alga *F. evanescens* that consist of alternating α(1,3)- and α(1,4)-linked fucose residues [1], releasing fucoidan oligosaccharides with varying degrees of polymerization (DP), including low molecular weight products. Furthermore, Mef2 also catalyzed hydrolysis of the galactofucans isolated from *Sargassum mcclurei*, *Sargassum polycystum*, *Saccharina cichorioides,* and *S. latissima*, the latter three of which are known to contain α(1,3)- linked α-L-fucose residues [3,31]. Interestingly, Mef2 was the first enzyme found able to efficiently catalyze the hydrolysis of fucoidans from *S. latissima*. Together, results indicate that Mef2 catalyzes hydrolysis of α(1,3) glycosidic bonds in fucoidans, consistent with the phylogenetic clustering.

The hydrolysis of fucoidans from *F. evanescens* and *S. latissima* by Mef2 was monitored from 0 to 48 h by Carbohydrate-Polyacrylamide Gel Electrophoresis (C-PAGE) and High Performance Size Exclusion Chromatography (HP-SEC) (Figure 3). The small fucoidan oligosaccharides were visible in the C-PAGE after 5 and 30 min reactions on fucoidans from *F. evanescens* and *S. latissima*, respectively, while complete degradation was not achieved before 48 h on *F. evanescens* and 5 h on fucoidans from *S. latissima*.

The HP-SEC analysis showed that both fucoidan substrates decreased substantially in size already after 5 min of reaction with Mef2. The resulting molecular weight distribution of fucoidans from *F. evanescens* changed from a single broad peak of approximately 350 kDa (100 to >800 kDa) to a polydisperse peak of approximately 5–200 kDa (Figure 3b). Mef2 HP-SEC analysis on *S. latissima* fucoidans revealed a reduction of the most dominant molec-

ular weight from approximately 300 kDa (70 to >800 kDa) to two fucoidan populations, one with the most dominant molecular weight at 200 kDa (70–500 kDa) and a low molecular weight population with a most dominant molecular weight of around 1 kDa (0.6–3 kDa) (Figure 3d). These results showed that the Mef2 catalyzed hydrolysis of fucoidans was initiated by the hydrolysis of larger polymers, as visible by HP-SEC analysis, followed by further hydrolysis into smaller oligosaccharides, as visible by C-PAGE analysis.

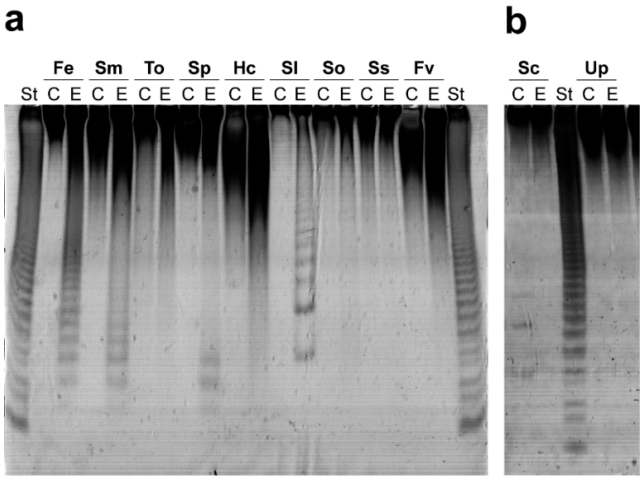

Figure 2. Substrate specificity of Mef2 on the fucoidans from different brown seaweeds. C-PAGE of (C) substrate control and (E) Mef2 reaction on different fucoidans from brown seaweeds (**a**) *F. evanescens* (Fe), *S. mcclurei* (Sm), *Turbinaria ornata* (To), *S. polycystum* (Sp), *Hormophysa cuneiformis* (Hc), *S. latissima* (Sl), *Sargassum oligocystum* (So), *Sargassum serratum* (Ss), *Fucus vesiculosus* (Fv), (**b**) *S. cichorioides* (Sc), and *Undaria pinnatifida* (Up). St) reaction of FFA2 on fucoidans from *F. evanescens*.

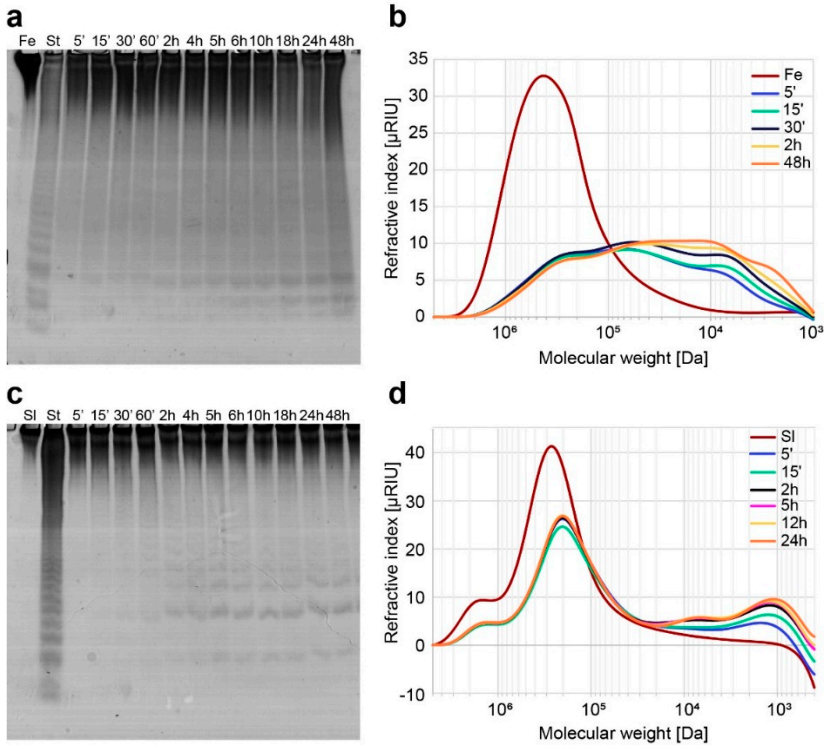

Figure 3. Mef2 catalyzed hydrolysis of fucoidans by C-PAGE and HP-SEC analysis. C-PAGE analysis of Mef2 on fucoidans from (**a**) *F. evanescens* (Fe) and (**c**) *S. latissima* (Sl), from 5 min to 48 h of reaction. HP-SEC chromatograms of Mef2 hydrolysis on the fucoidans from (**b**) *F. evanescens* and (**d**) *S. latissima*, from 5 min to 48 h. (St) *F. evanescens* fucoidans hydrolyzed by FFA2.

The lowest band in C-PAGE resulting from FFA2 catalyzed hydrolysis of fucoidans from *F. evanescens* corresponds to a tetra-saccharide of (1,4)- and (1,3)-linked α-L-fucosyls with each fucosyl residue sulfated at C2 (DP4) [20]. However, the *S. latissima* and *F. evanescens* fucoidan-derived oligosaccharides migrating furthest in the gel after Mef2 catalyzed fucoidan hydrolysis did not co-migrate with the tetra-saccharide of the standard but migrated slightly slower in the gel, suggesting slightly larger and/or less charged oligosaccharides.

The influence of pH on Mef2 activity using buffers with overlapping pH values (UB4 buffer pH 2–8 and borate buffer pH 8–11) showed that Mef2 was active at pH values from 6 to 8 in the UB4 buffer but not in the borate buffer, while the optimal pH was found at 7–8 (Figure 4a). Mef2 was found active at temperatures ranging from 15–50 °C, while the optimal temperature was between 30 and 37 °C (Figure 4b). Consistent with the C-PAGE results, the melting temperature (Tm) of Mef2 was determined to be 38 °C when Ca^{2+} had been removed by EDTA (Figure S3 and Table S2). For further experiments, 35 °C was used.

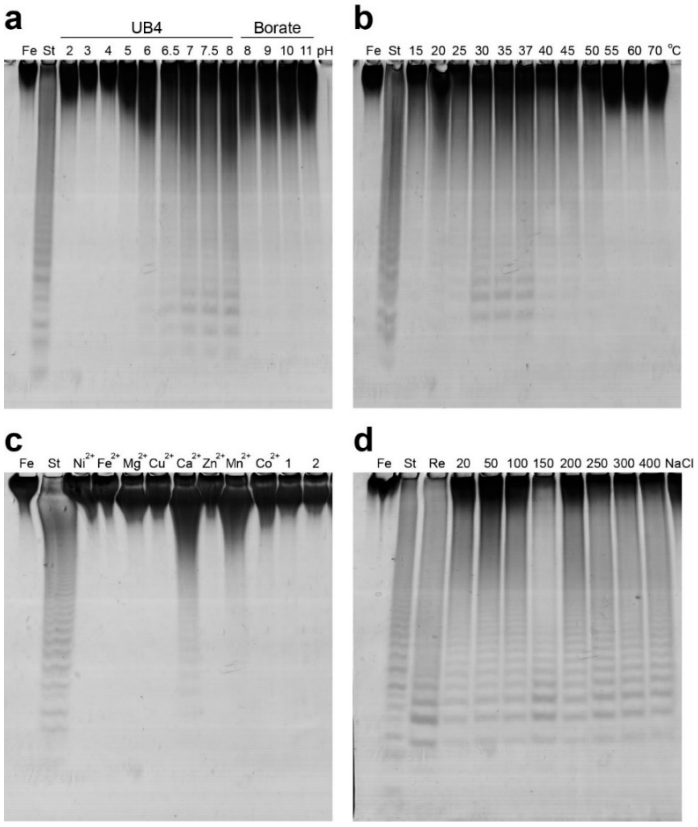

Figure 4. Mef2 optimal conditions on fucoidans from *F. evanescens*. C-PAGE analysis of Mef2 activity at different pH (**a**), temperature (**b**), divalent cations (**c**), and NaCl concentrations (**d**). (St) Reaction of FFA2 on fucoidans from *F. evanescens*. (Fe) *F. evanescens* fucoidan substrate.

Most fucoidanases are divalent cation-dependent enzymes [19,20,22–24], which has been supported by fucoidanase crystal structures, where Ca^{2+} was found in the catalytic D1 domain [30]. The metal ion-dependency was therefore investigated for Mef2. The Mef2 enzyme was stripped of divalent cations by EDTA, resulting in a complete loss of function (Figure 4c). Different divalent cations at 10 mM concentration showed varying effects on the Mef2 enzyme, where Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} did not reactivate the Mef2 fucoidanase, while Mn^{2+} resulted in a slight re-activation, and Ca^{2+} showed the best re-activation of Mef2. The effect of Ca^{2+} on the stability of the protein (Tm) showed an increase in Tm of 6 °C in the presence of Ca^{2+}, resulting in a Tm of 44 °C compared to 38 °C without Ca^{2+} (Figure S3).

The influence of NaCl on Mef2 activity showed that Mef2 was not affected by NaCl as the activity did not differ significantly at concentrations from 25 to 400 mM (Figure 4d).

Together, the optimal conditions for Mef2 were 0.9% fucoidan, 20 mM Tris-HCl pH 8, 10 mM $CaCl_2$, 100 mM NaCl, and 35 °C.

2.3. Determination of the Mef2 Fucoidanase Unit by Fourier Transform Infrared Spectroscopy (FTIR)

A Fourier transform infrared spectroscopy was previously used to determine the activity of four different α(1,4) specific GH107 endo-fucoidanases (MfFcnA, Fhf1, Fhf2, and FFA2) on fucoidans from *F. evanescens* [23,32] and three (MfFcnA, Fhf1, and FFA2) as well on fucoidans from *F. vesiculosus* [32]. The enzyme dose-related changes in the FTIR spectrum were followed using the multivariant platform PARAFAC, where the enzyme dose increase is linearly correlated to the PARAFAC score. In this manner, a fucoidanase unit was established, where one enzymatic unit was defined as the amount of enzyme able to increase the PARAFAC value score by 0.01 [32]. A linear equation results from each calibration curve as follows: PARAFAC score = a × concentration of enzyme + b [32].

To determine the fucoidanase unit of Mef2, *F. evanescens* fucoidan degradation was monitored by FTIR (Figure 5). The Mef2 concentration-dependent enzymatic hydrolysis of *F. evanescens* fucoidans (FeF4, Table S3) showed an increase in absorption for wavenumbers 1150–1200 cm^{-1} (score increasing from 0 to 2) and 1300–1350 cm^{-1} (from 0 to 1), which indicated changes in vibrations of C–O–C stretching of the glycosidic bonds and unidentified bonds, respectively. Furthermore, a decrease in wavenumbers 1200–1250 cm^{-1} (from 0 to −0.5) and 1400–1500 cm^{-1} (from 0 to −3) was observed, indicating changes in vibrations of S=O of the sulfate group and O–C–O of carboxylate group bonds, respectively. Changes in these wavenumbers were also previously observed for the fucoidanases on fucoidans from *F. evanescens* [23,32]. PARAFAC analysis on Mef2 FTIR data resulted in a linear curve with the equation 0.01 = −0.001 × conc Mef2 + 0.0019 and an R^2 value of 0.97 (Figure S4a), which led to the specific activity ($U_f/\mu M$) of 1.2 × 10^{-3} $U_f/\mu M$.

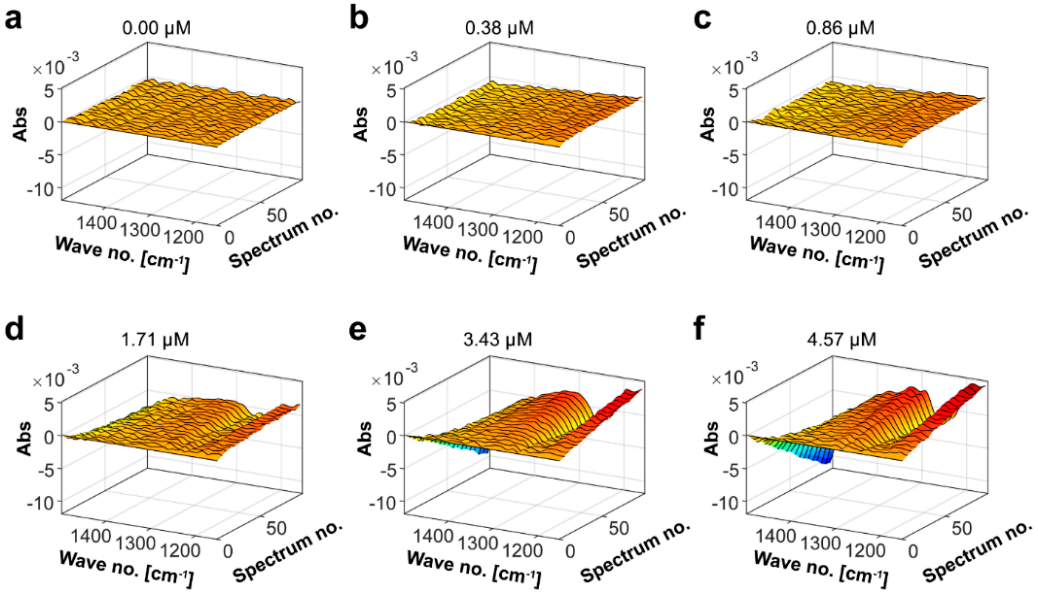

Figure 5. Mef2 kinetics on fucoidans from *F. evanescens* using FTIR. Spectral evolution profiles for the Mef2 endo-fucoidanase using 2% *w*/*v* of *F. evanescens* fucoidans (FeF4) using different enzyme dosages: (**a**) 0.00 µM, (**b**) 0.38 µM, (**c**) 0.86 M, (**d**) 1.71 µM, (**e**) 3.43 µM and (**f**) 4.57 µM. The spectral evolution depends on the enzyme concentration. The spectral changes of buffer alone and substrate alone were subtracted. Time per spectrum was 16.6 s [32]. Chemical composition of FeF4 is shown in Table S3.

$$\text{Mef2}: \ 0.01 = -0.001 \times conc\text{Mef2} + 0.0019 \Rightarrow conc\text{Mef2} = 8.10\ \mu M \quad (1)$$

Hence, the specific activity of Mef2 (F. evanescens) = 1.2 × 10−3 $U_f/\mu M$.

Compared to the units of the previously characterized α(1,4)-specific fucoidanases Fhf1 (1.2×10^{-3} $U_f/\mu M$), MfFcnA (2×10^{-3} $U_f/\mu M$), Fhf2 (2.4×10^{-4} $U_f/\mu M$) and FFA2 (4×10^{-3} $U_f/\mu M$) on fucoidans from *F. evanescens*, the Mef2 enzyme presumably works slower than FFA2 and MfFcnA, to a comparable level as Fhf1, while faster than Fhf2 [23,32].

Since the Mef2 was active on fucoidans from *S. latissima* and this substrate has not previously been subjected to FTIR analysis using fucoidanases, FTIR kinetics was also performed with *S. latissima* fucoidans (SlF4) (Table S4, Figure 6). The spectral evolution profile resulted in changes in the same wavenumbers as observed for *F. evanescens* fucoidans, but the changes were larger for the *S. latissima* fucoidans. An increase in absorption in wavenumbers 1150–1200 (from 0 to 5) and 1300–1350 (from 0 to 2.5), and decreases in wavenumbers 1200–1250 (from 0 to −6) and 1400–1500 (from 0 to −9), indicated that the overall change in the substrate-product solution was larger for *S. latissima* than for *F. evanescens* fucoidans. PARAFAC analysis on the Mef2 FTIR data resulted in a linear curve with the equation 0.01 = 0.0129 × conc Mef2–0.0257 and an R^2 value of 0.98 (Figure S4b), which led to the specific activity ($U_f/\mu M$) of 3.6×10^{-3} $U_f/\mu M$.

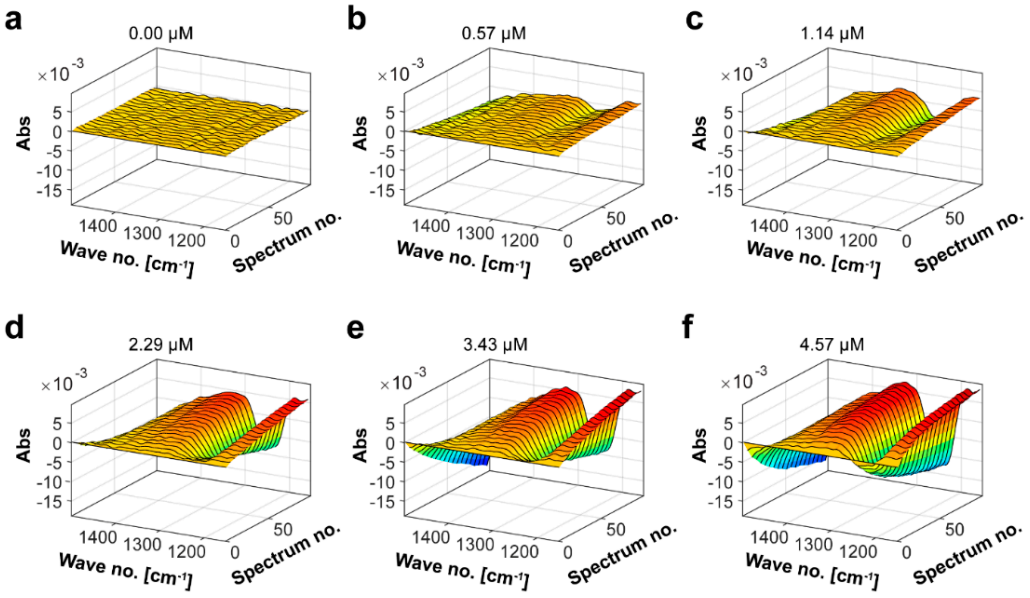

Figure 6. FTIR spectral evolution profiles for Mef2 activity on fucoidans from *S. latissima*. SlF4 hydrolysis using different Mef2 enzyme dosages: (**a**) 0.00 µM, (**b**) 0.57 µM, (**c**) 1.14 µM, (**d**) 2.29 µM, (**e**) 3.43 µM and (**f**) 4.57 µM. The spectral evolution depends on the enzyme concentration. The spectral changes of buffer and substrate were subtracted. Time per spectrum was 16.6 s [32]. Chemical composition of SlF4 is shown in Table S4.

$$\text{Mef2 } (S.latissima) : 0.01 = 0.0129 \times conc\text{Mef2} - 0.0257 \Rightarrow conc\text{Mef2} = 2.80\ \mu M \quad (2)$$

Hence, the specific activity of Mef2 (*S. latissima*) was 3.6×10^{-3} $U_f/\mu M$.

2.4. Determination of the Mef2 Linkage Specificity and Structural Elucidation of the S. latissima Fucoidan Products by NMR

Due to the large structural complexity of the fucoidans from *S. latissima*, the fucoidans were deacetylated and verified by NMR analysis (Figure S5) before Mef2 catalyzed hydrolysis. The Mef2 reaction products were separated into the following two fractions: low molecular weight fucoidan products (LMP) and medium molecular weight products (MMP) by ethanol precipitation (Figure S6). The yields of MMP and LMP were 86% and 14%, respectively. While MMP was composed of different monosaccharides, with fucose and galactose in almost comparable amounts (Table S5), the only monosaccharide detectable in the LMP was fucose.

The fucoidan digestion using the Mef2 endo-fucoidanase was completed after the first reaction since both the MMP and LMP were not further degraded by the second step of Mef2 hydrolysis (Figure S7a). The Mef2 HP-SEC analysis on the *S. latissima* fucoidans revealed that the LMP had an average molecular weight of about 4 kDa, and the MMP had an average molecular weight of about 200 kDa (Figure S7b).

For structure investigations, MMP was separated by ion-exchange chromatography (IEX) and pooled according to total carbohydrate content and C-PAGE analysis, resulting in six fractions (MF1-6) (Figure 7a,b and Figure S8). The MF fractions were heterogeneous and contained a mixture of fucoidan oligosaccharides of different sizes (Figure 7d). MF1-6 were additionally analyzed by C-PAGE and did not represent homogenous samples but rather fractions with more than one band, as well as high molecular weight fucoidans not migrating in the gel, indicating that the fractions contained different fucoidan poly- and oligosaccharides (Figure 7c). In particular, the MF fractions contained fucoidans of high molecular weight (around 200 kDa), visual as a band in the top of the C-PAGE gel, too large to migrate, and low molecular weight fucoidans (around 4–10 kDa), migrating as bands in the C-PAGE gel. For MF4 and 5, no low molecular weight fucoidans were observed, but in contrast to MF6 (and the high molecular weight fucoidans in MF1), where the high molecular weight fucoidans could not migrate into the C-PAGE gel, MF4 and 5 contained high molecular weight fucoidans able to migrate slightly into the gel, resulting in a smear at the top of the C-PAGE gel. The slight migration of MF4 and MF5 fucoidans could be discerned in the HP-SEC, where a slight shift to the right (lower molecular weight) could be observed for MF4 and 5 compared to MF6. The monosaccharide composition of the MF fractions was determined (Table S6). The ratio of fucose:galactose varied but correlated with the average molecular weight of each fraction. The galactose content was higher in the larger-sized fucoidan fractions.

The structure of fractions MF2 to MF6 was further investigated by NMR spectroscopy. The NMR spectra resembled a mixture of 1-3 linked α-fucosyl units (the small fraction MF2) and β-1,4 as well as β-1,6 linked galactosyl units predominating in the MMP with NMR spectral characteristics corresponding to structures previously described [3].

In the higher molecular weight fractions (MF3-MF6), three separate spin systems were observed that resembled spin systems present in the substrate fraction of *S. latissima* (Figure 7d). Two of those spin systems could be assigned to galactose based on the distinct coupling pattern yielding efficient magnetization transfer between H1 and H4, but not between H4 and H5 (see Supplementary Figure S9). The third spin system was identified as fucose. Chemical shifts and sequential assignments are collected for the deacetylated medium molecular weight samples in Table 3. Linkage patterns were established based on HMBC and NOE correlations, i.e., proximity between pairs of nuclear spins in chemical structure and space across the glycosidic bonds. The structure is consistent with a previously reported structural motif detected in the reinvestigation of desulfated *S. latissima* fractions [3] and with the presence of galactose and fucose in the polysaccharide as determined by monosaccharide analysis. The NMR on the deacetylated sample showed the sulfation pattern displayed in Figure 7d. The NMR spectroscopy on the native, non-deacetylated high molecular weight fraction SlF3 showed a strong and characteristic deshielding of ^{1}H3 in unit I (to chemical shifts of 5.260 ppm/71.70 ppm for the C3H3 group) and an equivalent deshielding of ^{1}H2 in unit J (to 5.218 ppm/70.68 ppm for the C2H2 group; not shown). Hence, the preparation was predominantly acetylated at O3 of unit I and O2 of unit J.

The LMP were further separated by IEX to obtain pure oligosaccharides. In total, four fractions (OF1-4) were obtained, as determined by total carbohydrate analysis via the phenol-sulfuric acid method [33] (Figure S10) and C-PAGE (Figure 8a). OF1 showed the highest NMR spectral quality (Table 4) and provided sufficient material for a full NMR structure determination. OF2-4 proved to provide some signals resembling OF1 and thus indicating that these oligosaccharides share the same core as the OF1 oligosaccharide. The signals within each spin system of OF1 were assigned primarily based upon ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C H2BC, and ^{1}H-^{13}C HSQC correla-

tions. As fucose is a 6-deoxygalactose, it shares the coupling pattern of galactose between hydrogens one and five, with a small coupling of approximately 1 Hz between the hydrogen atoms four and five. Spin systems were thus primarily assigned with TOCSY and heteronuclear NMR to identify CH groups for atoms 1-4 in each fucose residue. C4 could then be correlated to the CH groups five and six through HMBC correlations from the methyl group. A total of ten spin systems were identified and designated A-H, as shown in Table 4. Reducing end signals constituted two of the spin systems, while the adjacent residue likewise yielded spin systems that were distinct for the reducing end anomer on a high-field (800 MHz) NMR instrument. Overall, the oligosaccharide of fraction OF1 was thus a reducing oligosaccharide constituted of eight carbohydrate residues, with the structure α-L-Fucp-(4OSO_3^-)-(1,3)-α-L-Fucp-(2OSO_3^-)-(1,3)-α-L-Fucp-(1,3)-α-L-Fucp-(4OSO_3^-)-(1,3)-α-L-Fucp-(2OSO_3^-)-(1,3)-α-L-Fucp-(OSO_3^-) with branches α-L-Fucp-(4OSO_3^-)-(1→ connected to α-L-Fucp-(2OSO_3^-) at C4 of two of the units (second unit from the reducing end and the non-reducing end, respectively).

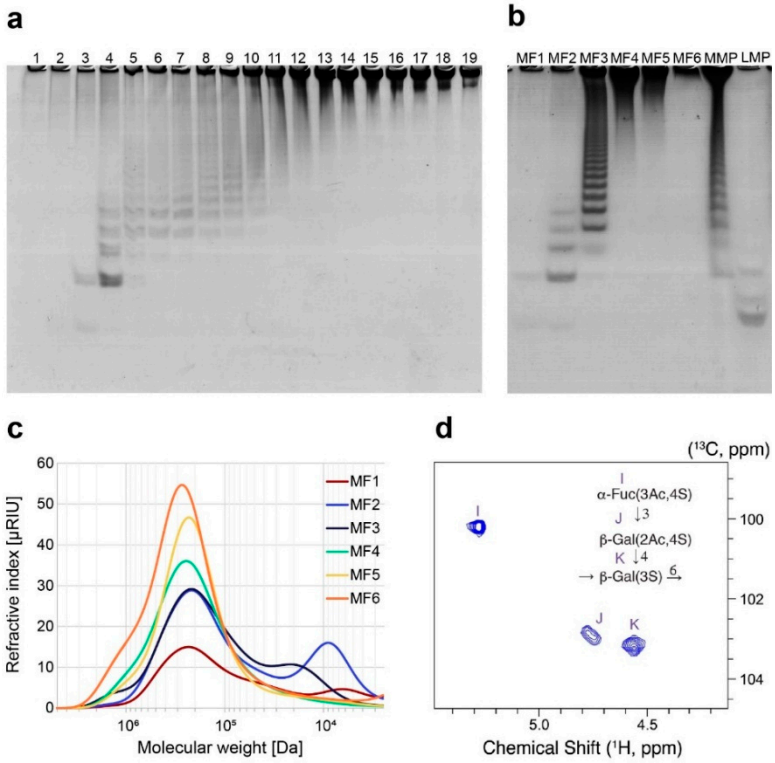

Figure 7. Analysis of Mef2 released *S. latissima* medium molecular weight products (MMP) and further separated fractions (MF). Medium molecular weight products (MMP) after Mef2 hydrolysis was further separated into (**a**) 19 fractions (1–19), which were pooled when the same oligosaccharides were present in C-PAGE. (**b**) C-PAGE of pooled and purified medium molecular weight fractions MF1-MF6 compared to LMP and MMP. (**c**) HP-SEC chromatogram of MF1-6. Pullulan was used as standard. (**d**) Anomeric region of the ^{1}H-^{13}C HSQC spectrum for the acetylated MMP. The basic structure of the MMP as determined by NMR yields the substitutions shown schematically and is consistent with the high galactose content in this fraction and with previous structural analyses of *S. latissima* fucoidans [3]. The ^{1}H-^{1}H TOCSY NMR spectrum for the deacetylated MMP is shown in Figure S9.

A sequential assignment of the spin systems was achieved primarily using ^{1}H-^{13}C-HMBC and ^{1}H-^{1}H NOESY spectra to detect $^3J_{CH}$ correlations and NOES across the glycosidic bonds, yielding the sequential assignment shown in Table 4. The structural assignment is consistent with chemical shift data insofar as glycosydically linked positions show a deshielding of their ^{13}C chemical shifts but less so with their ^{1}H chemical shifts. Six

of the eight residues additionally showed a characteristic deshielding of ^{1}H positions (to approximately 4.5 ppm) and the corresponding ^{13}C signals at these positions, strongly indicative of the attachment of sulfate groups at these secondary alcohol sites. In contrast, acetyl groups were not present, as witnessed by the absence of acetyl ^{1}H NMR signals near 2 ppm and of attachment sites at secondary alcohol CH groups with strongly deshielded ^{1}H.

Table 3. ^{1}H and ^{13}C NMR data for the deacetylated medium molecular weight products (MMP) purified after cleavage of *S. latissima* fucoidans with endo-fucoidanase Mef2. Acetylations as determined on native samples are indicated in italic font.

	Residue	Chemical Shifts (ppm)					
		H1/C1	**H2/C2**	**H3/C3**	**H4/C4**	**H5/C5**	**H6/C6**
I	α-L-Fuc*p*(*3Ac*, 4SO_3^-)-(1→	5.282 100.3	3.785 69.0	4.099 68.6	4.589 81.1	4.510 66.5	1.258 15.8
J	-3)-β-D-Gal*p*(*2Ac*, 4SO_3^-)-(1→	4.773 102.8	3.731 71.6	3.908 76.5	4.692 77.3	3.798 74.4	3.802 61.0
K	-4,6)-β-D-Gal*p*(3SO_3^-)-(1→	4.580 103.1	3.815 68.9	4.426 79.9	4.540 73.8	3.930 73.2	4.188/3.924 70.3

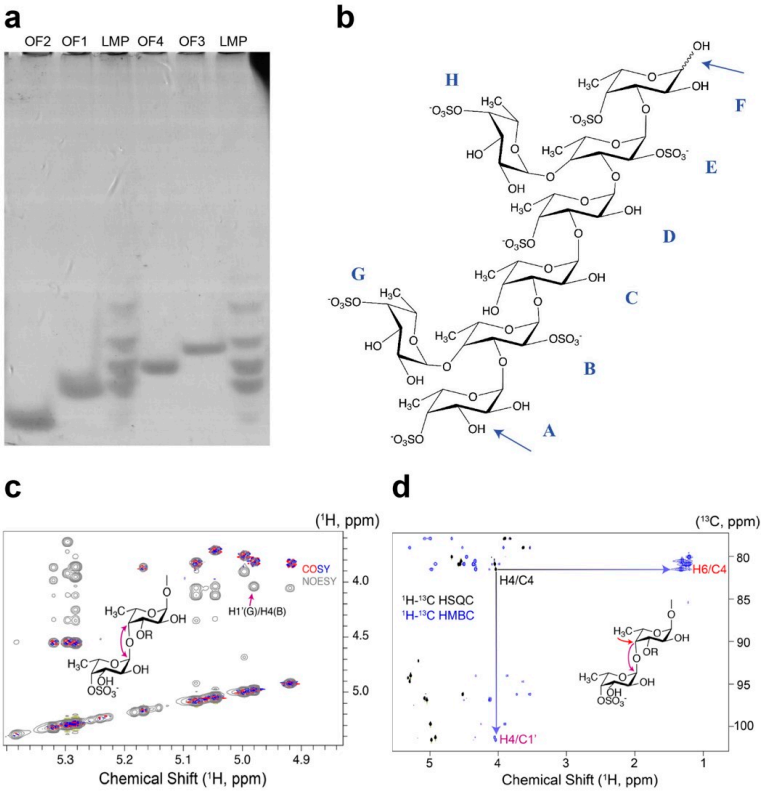

Figure 8. Analysis of Mef2 released LMP from *S. latissima* fucoidans. (**a**) C-PAGE of purified oligosaccharide fractions OF1 to OF4. (**b**) Molecular structure of the purified octa-saccharide OF1. Units have the same identifier as in Table 4; blue arrows indicate Mef2 cleavage sites. Structure determination of the α(1,3)-linked fucoidan backbone with the 1-4 linked sulfated fucosyl substitution of the OF1 oligosaccharide. (**c**) Overlay of ^{1}H-^{1}H COSY and ^{1}H-^{1}H NOESY spectra, showing the NOE across the glycosidic bond for hydrogens 1 and 4 of residues G and B. (**d**) Overlay of ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectrum showing the correlation across the glycosidic bond for the well-resolved signals of the 1-4 linked residues G and B.

Table 4. ^{1}H and ^{13}C NMR data for the purified low molecular weight product fraction OF1 after Mef2 cleavage of *S. latissima* fucoidans. Both the reducing end F and the adjacent residue E exhibit two sets of signals due to the anomeric forms of the reducing end.

	Residue\Atom	**Chemical Shifts (ppm)**					
		H1/C1	**H2/C2**	**H3/C3**	**H4/C4**	**H5/C5**	**H6/C6**
A	α-L-Fuc*p*(4SO_3^-)-(1-	5.036 96.87	3.71 68.5	3.982 69.06	4.561 80.87	4.458 66.6	1.211 15.84
B	→3,4)-α-L-Fuc*p*(2SO_3^-)-(1-	5.283 94.05	4.556 73.2	4.122 73.2	4.037 81.46	4.331 67.96	1.325 15.47
C	→3)-α-D-Fuc*p*-(1-	4.995 99.81	3.764 66.96	3.858 75.67	3.954 68.62	4.25 66.7	1.181 15.59
D	→3)-α- D-Fuc*p*(4SO_3^-)-(1-	5.076 96.74	3.833 67.53	3.905 77.9	4.68 80.08	4.475 66.54	1.211 15.84
Eα	→3,4)-α- D-Fuc*p*(2SO_3^-)-(1-	5.32 94.28	4.552 73.2	4.12 73.2	4.05 80.85	4.34 67.95	1.29 15.65
Eβ	→3,4)-β- D-Fuc*p*(2SO_3^-)-(1-	5.297 94.69	4.545 73.2	4.114 72.8	4.054 80.85	4.35 67.95	1.29 15.65
Fα	→3)-α- D-Fuc*p*	5.161 92.25	3.872 66.56	3.871 75.36	3.986 68.41	4.118 66.07	1.165 15.61
Fβ	→3)-β- D-Fuc*p*	4.517 96.25	3.539 70.12	3.633 78.93	3.92 68.1	3.72 70.67	1.203 15.583
G	α- D-Fuc*p*(4SO_3^-)-(1→	4.974 101.6	3.824 69.05	4.103 68.03	4.584 80.95	4.37 67.172	1.254 16.14
H	α- D-Fuc*p*(4SO_3^-)-(1→	4.918 101.34	3.831 69.38	4.074 68.37	4.546 80.55	4.338 67.33	1.307 16.38

Surprisingly, the oligosaccharide structure showed the branching of the expected α(1,3)-linked backbone at the C4 position, in contrast to previous findings of only C2 branchings in fragments of *S. latissima* fucoidans [3]. Figure 8 exemplifies the identification of the α(1,4) linked branch from the ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectrum. Beyond correlating to C5 atoms with characteristic chemical shifts near 67 ppm, the methyl protons at C6 also correlate to C4 in the ^{1}H-^{13}C HMBC spectrums. Two characteristic C4 positions can be identified with deshielded ^{13}C nuclei (80.85–81.46 ppm) but non-deshielded ^{1}H, indicative of glycosidically linked C4 positions. These C4 positions accordingly show $^{3}J_{CH}$ correlations in the ^{1}H-^{13}C HMBC spectrum to anomeric CH groups of other spin systems (highlighted for one of the residues with branching at C4 in Figure 8c,d). Both H1-C4′ and C1-H4′ correlations across the glycosidic bond are detected for the two C4-branched residues. The C4 branching is further corroborated by the observation of ^{1}H-^{1}H NOEs between the corresponding H1′ and H4, where the apostrophe designates the terminal residue, as shown in the NOESY spectrum of Figure 8c. Specifically, the 1,4 branchpoint highlighted in Figure 8c is the linkage between residues B and G.

The ^{1}H-^{13}C spectra from another fraction (OF2) resembled the spectra for OF1 with some differences; however, a new reducing end was formed in this structure, with signals for the OF1 glycosidically linked residue E vanishing, the reducing end signal shifting due to the presence of a new reducing end, and signals for residues D and H exhibiting two sets of signals, indicative of their vicinity to the new OF2 α- and β-reducing end residue E (Figure S11). A possible structure of the OF2 fraction was suggested as a branched sulfated hepta-saccharide (Figure S11), but further experiments are necessary for a full structure determination.

Together, the results verify that Mef2 hydrolyzes fucoidans by endo-α(1,3)-specificity, supported by NMR analysis as well as substrate specificity and phylogenetic clustering. Mef2 is the first characterized fucoidanase shown to be able to hydrolyze the very com-

plex fucoidans from *S. latissima*, thus releasing fucoidan oligosaccharides with a new branch linkage while the galactose containing oligo- and poly-saccharides were retained in the MMP.

Additionally, the low molecular weight products released by Mef2 from fucoidans from *F. evanescens* were subjected to NMR spectroscopy. The results indicated a backbone of α(1-3)- and α(1-4)-linked fucosyl units at comparable amounts, as follows: the chemical shifts in these fragments were consistent with the linear polymer fraction that was previously isolated as a minor component from fucoidans from *F. evanescens* with enzyme preparations from a marine mollusk [34]. The fucose residues were partly acetylated and devoid of 2,4 disulfations (Figure S12).

3. Discussion

The Mef2 endo-fucoidanase was found to have optimal activity in the neutral or slightly alkaline pH range and an optimal temperature of about 35 °C. Mef2 had optimal activity at salt concentrations of 100–400 mM and was Ca^{2+} dependent. Previous findings showed that the endo-α(1,3) fucoidanases Fda1 and Fda2 have a slightly lower optimal temperature of 30 and 32 °C, respectively [35], whereas most characterized endo-α(1,4) fucoidanases have an optimal temperature of 35–37 °C [18,20,22,23]. Interestingly, Mef2 even showed activity at 50 °C, indicating that Mef2 is more heat stable than Fda1 and 2. Interestingly, the melting temperature of Mef2 was largely affected by the presence of Ca^{2+}, resulting in an increase of Tm from 38 °C to 44 °C in the presence of Ca^{2+}, consistent with the general Ca^{2+} dependency of known GH107 fucoidanases and the hypothesized function in stabilization of the enzymes [30].

The activity of Mef2 was analyzed by FTIR on fucoidans from *F. evanescens* and showed that the spectral changes upon increasing concentrations of enzyme resembled the results obtained from the Fhf1 fucoidanase, giving a specific activity of 1.2×10^{-3} $U_f/\mu M$ and a lower activity than the endo-fucoidanases MfFcnA and FFA2, while a higher activity than Fhf2 [23,32]. In addition, the FTIR assay was evaluated on fucoidans from *S. latissima* for the first time and indicated that Mef2 activity results in larger spectral changes on fucoidans from *S. latissima* than on fucoidans from *F. evanescens*. These spectral changes could be related to changes in vibrations in both the substrate and products. Due to the complex structure of *S. latissima* fucoidans, the large spectral changes might be related to changes in the substrate/product rather than being the direct result of glycosidic cleavage.

Mef2 interestingly showed activity on both branched and unbranched fucoidans from *S. latissima* and *F. evanescens*, respectively, and was highly selective for fucose, not allowing galactose in the oligosaccharide products, as supported by NMR. NMR analysis showed that MMP were a mixture of galactofucans, resembling previously reported structural motifs from *S. latissima* fucoidans [3]. Together, these findings indicate that Mef2 is selective for fucosyl residues in the backbone and only for α(1,3) linkages, but that the active-site region, likely at the +2 and −2 subsite, would allow for fucosyl branchings. The amino acids in these subsites have not yet been identified in fucoidanases since no crystal structures have been published with substrate bound in the active site. Only the active site and the −1 subsite have been proposed [30]. In the active site, a conserved amino acid has been changed from an arginine to a valine in Mef2, while in the −1 subsite, a conserved asparagine that shows conservation in many other GH107 fucoidanases, was an alanine in the α(1,3) linkage-specific Fda1 and 2 and a serine in Mef2. Whether or not these amino acid changes contribute to the unique specificity of Mef2 requires further investigation.

Further selectivity of Mef2 is supported by the substrate specificity since no activity was detected on other substrates with α(1,3)-linkages, including *F. vesiculosus* or *U. pinnatifida*, indicating that sulfate position and degree of sulfation influence Mef2 activity. In *F. evanescens* C2 and C4, sulfations predominate [36], while fucoidans from *F. vesiculosus* have been found with many different sulfation patterns, including sulfates on C2, C2/C3, C2/C4 or C4 [37,38]. The fucoidans from *U. pinnatifida* are, moreover, assumed to be rich in 2,4-disulfate substitutions [39], while *S. latissima* fucoidans are sulfated at C2 and/or at

C4 and C3 [3]. These observations indicate that Mef2 prefers C2 and C4 mono-sulfations and few or no disulfations. Indeed, NMR analysis showed that all the fucosyl residues close to the cleavage site in the oligosaccharides deriving from Mef2 cleavage of *S. latissima* fucoidans are C2 or C4 mono-sulfated.

Some other branched fucoidan oligosaccharides that can be released by fucoidanase activity have been described previously, including the *F. evanescens* fucoidan product of an endo-α(1,4)-fucoidanase from the marine mollusk *Lambis sp*. with the backbone α-L-Fucp-(2OSO_3^-)-(1,3)-α-L-Fucp-(2OSO_3^-)-(1,4)-α-L-Fucp-(2OSO_3^-)-(1,3)-α-L-Fucp-(2OSO_3^-) with an α-L-Fucp-(1,4)-branch [40]. In another study, the treatment of fucoidans from *S. horneri* by the FFA1 fucoidanase released branched oligosaccharides with the backbone structure α-L-Fucp-(1,3)-α-L-Fucp-(1,4)-α-L-Fucp-(1,3)-α-L-Fucp and the side chain of α-L-Fucp-(1,2)-α-L-Fucp-1→ at C4 of one main fucosyl residue [19]. Both of these fucoidan oligosaccharides have α(1,3)- and α(1,4)-linked backbones, whereas the Mef2 released oligosaccharides from *S. latissima* have exclusively α(1,3)-linked backbones.

The current study is the first report of fucoidanase activity on fucoidans from *S. latissima* and reports new structural features for fucoidans from *S. latissima*. The LMP were sulfated oligosaccharides of α(1,3)-linked fucose residues and branched at the new C4 position by single mono-sulfated fucose residues. In contrast, the sulfated fucan portion of fucoidans from *S. latissima* was previously only reported to be branched at C2 by single fucose residues [3]. The differences in branching linkage might be related to the cultivated (in this study) versus wild-harvested (in previous studies) *S. latissima* seaweed, or simply the different geographic areas from which they grew, since fine-structure differences are common in fucoidans from different locations [2]. Thus, the use of enzyme hydrolysis provided evidence of the presence of a new branch point as well as branch structure in fucoidans from *S. latissima*. The obtained data indicate that Mef2 can selectively hydrolyze the α-(1,3)-glycosidic bonds in the sulfated fucan part of fucoidans from *S. latissima*.

Only a few of the fucoidanases discovered to date share the exact specificity with regards to glycosidic linkages, branching, and sulfate pattern, while most fucoidanases are unique. This might be expected due to the large structural complexity of fucoidan molecules within and between species of brown seaweed. For a complete degradation of all the different linkages, with different sulfation patterns and branching in fucoidans, a whole battery of hydrolytic enzymes is hence likely necessary, and only a few of these enzymes have been characterized to date.

4. Materials and Methods

4.1. Fucoidan Substrates

Fucoidans from the brown alga *F. vesiculosus* was purchased from Sigma-Aldrich (Steinheim, Germany). Fucoidans from *S. mcclurei*, *T. ornata*, *S. polycystum*, *H. cuneiformis*, *S. oligocystum*, and *S. serratum* were extracted by a chemical method according to Bilan et al. (2002). Briefly, seaweeds were treated with 0.1 N HCl for 3 h at 70–85 °C to obtain water-soluble polysaccharides. The soluble extract was then treated with 2% $CaCl_2$ to remove alginates. The fucoidans were then precipitated from the supernatant by 1% hexadecyltrimethylammonium bromide (Cetavlon, Sigma-Aldrich, Steinheim, Germany). The fucoidan-cetavlon precipitate was isolated by centrifugation and washed with water stirred with 20% ethanolic NaI solution for 2–3 days at room temperature, washed with ethanol, and dissolved in water. The solution was dialyzed, concentrated, and lyophilized [36] and then fractionated by IEX as described previously [33]. For all fucoidan extracts, the polysaccharide fraction eluting the latest, fucoidan fraction 3 (F3), considered the purest fucoidan fraction [33], was used for enzyme experiments. The chemical composition of the individual fucoidans used in the experiments, analyzed as described in [29] with sulfate content determined according to [30], see Section 2.2, is presented in Table S7.

Fucoidans from *F. evanescens* were extracted using an enzyme-assisted method and further fractionated according to the method previously described [33], with an extra later eluted fraction FeF4 (Table S3), used for FTIR analysis. In addition, fucoidans from

cultivated *S. latissima* seaweeds from Ocean Rainforest were extracted by the same enzyme-assisted method with slight modifications. In short, seaweed was treated by a mixture of the commercial cellulase blend Cellic®CTec2 (5% *v*/*w*) and the alginate lyase SALy (0.5% *w*/*w*) (the latter prepared by heterologous expression in *E. coli* as described previously in [41]) in 40 mM Tris-HCl buffer pH 7 at 40 °C for 24 h. The reaction was stopped by incubating at 90 °C for 10 min. The alginate was precipitated by 2% $CaCl_2$ and removed by centrifugation. The fucoidans were precipitated by the addition of 96% ethanol (EtOH) in a fucoidan:EtOH (*v*/*v*) ratio of 1:3 and lyophilized. The fucoidan extracts were further separated by DEAE-Macroprep resin (Bio-Rad, Hercules, CA, USA) column [33] into four fractions SlF1-4. Fucoidan fractions F3 (SlF3) and F4 (SlF4) contained the highest amounts of fucose and galactose compared to fractions 1 (SlF1) and 2 (SlF2) (Table S4). SlF3 and 4 were high molecular weight polysaccharides with average mass distribution ranging from 250 kDa to over 800 kDa (Figure S13).

4.2. Chemical Analysis of Fucoidans

Monosaccharide compositions of the fucoidans were analyzed as described previously [42]. Fucoidan fractions were hydrolyzed in 72% H_2SO_4 (5 mg/mL) at 30 °C for 1 h in water bath and then the mixture was diluted to 4% H_2SO_4 by adding water. The hydrolysis was continued for 40 min at 120 °C in autoclave. The hydrolysates were filtered through a 0.22 µm syringe filter and used for monosaccharide analysis [42]. Chromatographic separation was carried out at a flow rate 0.4 mL/min using the following three eluents: A-deionized water, B-200 mM NaOH and C-200 mM NaOH, 1 M NaOAc. The elution of neutral sugars was performed at 0.5% B in A for the first 17 min. Next, elution of uronic acid was performed by 3% B and 6% C in A for 20 min and completed with 100% B in 6 min. To calibrate the column, the 0.5% B in A was applied. The Dionex software Chromeleon™ 7.2 (Thermo Scientific, Waltham, MA, USA) was used for data quantification. Recovery values of the monosaccharides and uronic acid were estimated from runs at the same time.

The sulfate content was determined by the turbidimetric method [43]. In total, 110 µL hydrolysates after TFA hydrolysis were mixed with 120 µL 8% TCA. Then 60 µL 2% $BaCl_2$ in 15% PEG6000 reagent was added. The mixture was allowed to stand for 35 min. The released $BaSO_4$ suspension was measured at 500 nm in a microplate reader (TECAN Infinite 200, Salzburg, Austria). $BaSO_4$ was used as standard to generate a linear standard curve for the sulfate response.

The molecular weights (MW) of fucoidan fractions were determined by HP-SEC using an Ultimate iso-3100SD pump with WPS-3000 sampler (Dionex, Sunnyvale, CA, USA) connected to an ERC RefractoMax 520 refractive index detector (Thermo Scientific, Waltham, MA, USA) [23]. The fucoidan samples were prepared in 100 mM sodium acetate, pH 6 (3 mg/mL) and filtered through 0.22 µm filters. In total, 100 µL of samples were injected into a Shodex SB-806 HQ GPC column (300 × 8 mm) coupled with a Shodex SB-G guard column (50 mm × 6 mm) (Showa Denko K.K., Tokyo, Japan). Elution was carried out at a flow rate of 0.5 mL/min at 40 °C. External pullulan standards in the range of 305–805,000 Da (PSS Polymer Standards Service GmbH, Mainz, Germany) were applied to establish a polynomial relationship between the logarithmic molecular weight and the corresponding retention time in order to convert the retention times of the samples to molecular weights. Molecular weights above and below the applied standard range are estimates based on extrapolation of the polynomial model.

4.3. Sequence Analysis of the Mef2 Gene

The amino acid sequence of Mef2 (GenBank: ON099398; RefSeq: WP_055392200.1) was identified in the genome of *M. eckloniae* by BLAST using known GH107 fucoidanase encoding genes. The Clustal Omega service using HHalign algorithm (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 23 March 2022) [44] was used for multiple sequence alignments of the GH107 endo-fucoidanases. The α(1,4)-specific endo-fucoidanases MfFcnA (GenBank: CAI47003.1), FFA1 (RefSeq: WP_057784217.1), FFA2 (RefSeq: WP_057784219.1),

Fp273 (GenBank: AYC81238.1), Fp277 (GenBank: AYC81239.1), Fp279 (GenBank: AYC81240.1) Fhf1 (RefSeq: WP_066217780) and Fhf2 (RefSeq: WP_066217784.1); the α(1,3)-specific endo-fucoidanses Fda1 (GenBank: AAO00508.1), Fda2 (GenBank: AAO00509.1) and L- fucoidanases P5AFcnA (GenBank: AYF59291.1), P19DFcnA (GenBank: AYF59292.1), SVI_0379 (GenBank: BAJ00350.1) were used for amino acid sequence comparisons.

The signal peptide sequence and the protein domain predictions were performed using the SignalP5.0 server (http://www.cbs.dtu.dk/services/SignalP/, accessed on 17 June 2020) and the InterProScan V5 (https://www.ebi.ac.uk/interpro/, accessed on 29 March 2022), respectively. Pairwise alignments of Mef2 were performed using the MAFFT algorithm (EMBL_EBI, MAFFT < Multiple Sequence Alignment < EMBL-EBI) and Jalview software [45].

4.4. Construction and Cloning of the Expression Vectors

The construct containing the gene encoding Mef2 was designed to harbor a C-terminal 10×his tag. The synthetic gene, codon-optimized for *E. coli* expression, devoid of the predicted signal peptide and T9SS domain [28], was synthesized by GenScript (Piscataway, NJ, USA) and inserted into the pET-28b(+) vector between the NdeI and XhoI restriction sites.

4.5. Recombinant Enzyme Expression and Purification

The expression of the Mef2 fucoidanase was performed in *E. coli* BL21 (DE3) harboring the Pch2 (pGro7) plasmid (Takara Biolabs, Göteborg, Sweden). The LB broth contained 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol, while 0.05% (*w*/*v*) arabinose was used to induce expression of the pGro7 chaperone. The cells were cultured to reach OD_{600} 0.6–0.8 at 37 °C and 180 rpm before enzyme expression was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactoside (IPTG). After 20 hours at 20 °C or for optimal expression at 4 h induction at 37 °C and 180 rpm, the cells were harvested by centrifugation at 8000× *g* for 15 min and 4 °C and were re-suspended in buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 20 mM imidazole) at the ratio 1:3 (*m*/*v*). The enzyme was purified using a Ni^{2+} Sepharose HisTrap HP column resin (GE Healthcare, Uppsala, Sweden) as previously described [22]. A PD10 column (Sephadex G-25, GE Healthcare Uppsala, Sweden) was used to remove imidazole from the enzyme solution. Protein content was measured by the Bradford (Bio-Rad, Hercules, CA, USA) assay with bovine serum albumin as standard [46]. The enzymes were stored at −80 °C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12%) and western blotting, performed as previously described [22], were used to analyze the purity and size of the enzyme.

4.6. Endo-Fucoidanase Activity Assays

C-PAGE, using small quantities of fucoidan substrate (approximately 7 mg) and enzyme, was used to determine the optimal assay conditions for Mef2, while kinetic studies were conducted using FTIR analysis, using high amounts of fucoidans (approximately 700 mg, assay conditions are described in the FTIR section).

The Mef2 optimal reaction conditions were 0.12 mg/mL Mef2 in 20 mM Tris-HCl pH 8, 100 mM NaCl, 0.9% fucoidan, and 10 mM $CaCl_2$. For full fucoidan hydrolysis, the reaction was performed at 35 °C for 24 h, while for investigating optimal reaction conditions the reactions were incubated for 2 h at 35 °C. The pH optimum determination was performed in reactions containing 20 mM buffer (UB4 buffer pH 2–8 or borate buffer pH 8–11). The temperature optimum was determined by running reactions at 20, 25, 30, 35, 37, 40, 45, 50, 55, 60, and 70 °C. Influence of divalent cations was investigated by first removing divalent cations by addition of 2 mM Ethylene DiamineteTetra acetic Acid (EDTA), desalting by PD10 followed by addition of 10 mM of different divalent cations ($CaCl_2$, $CuSO_4$, $FeCl_2$, $MgCl_2$, $CoCl_2$, $MnCl_2$, $NiSO_4$, and $ZnCl_2$). The influence of NaCl was investigated at different concentrations of 25, 50, 100, 150, 200, 250, 300, 350, 400, and 500 mM. Time-course experiments were performed from 0 min to 48 h. All reactions were stopped by heating at 80 °C for 10 min, and protein debris was pelleted by centrifugation (10,000× *g*, 15 min at 20 °C).

Before loading on the C-PAGE gel, the reaction was mixed with loading buffer (ratio reaction:buffer = 1:1) containing a 20% (*v*/*v*) solution of glycerol in water and 0.02% (*w*/*v*) phenol red. The samples (6–8 µL) were electrophoresed through a 20% (*w*/*v*) 1 mm thick resolving polyacrylamide gel in 100 mM Tris-borate buffer pH 8.3 at 30 mA for 90 min. Gel staining was performed with a solution containing 0.5% alcian blue 8 GX (Panreac, Barcelona, Spain) in 2% acetic acid and 0.02% O-toluidine (Sigma-Aldrich, Steinheim, Germany) in ethanol, for 1 h at room temperature. The gel was washed with distilled water until bands were visible.

4.7. Thermal Stability of Recombinant Fucoidanase Mef2

The thermal stability of the Mef2 protein was determined using dynamic light scattering (DLS). Mef2 (5 µM) was transferred to a 10 µL capillary tube and analyzed with a nanoDSF Prometheus NT.Plex instrument (Nanotemper-technologies, Munich, Germany). To obtain denaturation profiles, a temperature gradient of 25–80 °C with an increase of 1 °C min^{-1} was used to monitor thermal stability. The raw data were exported into datasets containing fluorescence between 330 and 350 nm (F330 and F350), as well as the ratio of these values (F330/F350) and absorbance at 350 nm (A350). Denaturation was visualized by plotting the first derivatives of F330/F350. The peak of the first derivative corresponds to the melting temperature (Tm), which is the transition midpoint of protein unfolding. Mef2 was treated with 2 mM EDTA followed by PD10 desalting to remove all EDTA. In total, 10 mM Ca^{2+} was afterward added for Tm assessments with Ca^{2+}. The concentration of Mef2 (5 µM) was determined by measuring the absorbance at 280 nm using the calculated molar extinction coefficient computed by the ProtParam tool at ExPASy (https://web.expasy.org/protparam/, accessed on 10 December 2019).

4.8. Mef2 Kinetics by Fourier Transform InfraRed (FTIR) Spectroscopy Measurement and Parallel Factor (PARAFAC) Analysis

FTIR spectroscopy was used to monitor the degradation of fucoidans from *F. evanescens* and *S. latissima* [32]. Mef2 was dosed at (0.00), 0.38, 0.86, 1.71, 3.43, and 4.57 µM for reactions on fucoidans from *F. vesiculosus*, and at (0.00), 0.57, 1.14, 2.29, 3.43, and 4.57 µM for reactions on fucoidans from *S. latissima.*

All IR spectra were scanned using a MilkoScanTM FT2 FTIR instrument (Foss Analytical, Hillerød, Denmark) in the range from 1000–2000 cm^{-1}. The cuvette was kept at 42 °C and had a path length of 50 m. A 1 mL reaction mixture containing 2% weight/volume (*w*/*v*) fucoidan in 0.02 M Tris-HCl buffer pH 7.4 and 100 mM NaCl and 20 mM $CaCl_2$. Following the addition of the enzyme, each reaction mixture was injected directly into the cuvette, and 100 spectra were acquired in a row for the reactions with *F. vesiculosus* and *S. latissima* SlF4. To export the acquired spectral data, the Foss integrator (version 1.5.3, Foss Analytical, Hillerød, Denmark) was used. The data was then analyzed as described previously [32].

Parallel factor analysis (PARAFAC) was used to estimate calibration curves for all fucoidanase reactions. PARAFAC decomposition of the tensor X into three different matrices using one component. The spectral signal received from each enzyme reaction is represented by Matrix A (spectral mode loadings). The number of spectra received in a continuous period of time is represented by Matrix B, the distance between spectra is 16.6 s (time mode loadings), and Matrix C represents the relationship between the change in spectra in A and the different enzyme dosages in B. (the scores of PARAFAC). In this study, FTIR-PARAFAC was used to quantify endo-fucoidanase activity, as previously described [32].

4.9. Enzymatic Hydrolysis of Fucoidans and Product Separation

The *S. latissima* fucoidan fraction 3 (SlF3) was deacetylated by dissolving 1.5 g of fucoidans in 150 mL 12% ammonia solution as previously described for fucoidan from *F. evanescens* [22]. The yield of deacetylated SlF3 (deSlF3) was 89.3%. The Mef2 enzymatic

hydrolysis of deSlF3 was performed with 10 g/L substrate, 10 mM $CaCl_2$, 0.5 mg/mL Mef2 enzyme in 20 mM Tris-HCl buffer pH 8, and 100 mM NaCl at 35 °C for 24 h. The reaction was stopped by heating at 80 °C for 10 min and the precipitated enzyme was removed by centrifugation at 19,000× *g* for 15 min. The products were separated into two fractions, the medium molecular weight products (MMP) and the low molecular weight products (LMP) by adding cold ethanol 96% with a ratio of 3:1 (*v*/*v*) and collected by centrifugation at 15,000× *g* for 45 min. The ethanol in the supernatant was evaporated, thus isolating the low molecular weight products (LMP).

Further separation of the LMP was performed by applying 0.6 g/L LMP in water to a Q sepharose high-performance resin column (1 cm × 20 cm) equilibrated with water. The oligosaccharides were eluted at a flow rate 0.7 mL/min by a linear gradient of ammonium bicarbonate salt in water from 0 to 2 M. The total carbohydrate content in fractions was analyzed by the phenol-sulfuric acid method [47]. In total, 20 µL aqueous phenol solution 5% (*w*/*v*) was added to 20 µL fucoidan fraction followed by addition of 200 µL concentrated sulfuric acid. The absorbance was measured at 490 nm. Fractions containing carbohydrates were further analyzed by C-PAGE. The fractions containing the same oligosaccharides on C-PAGE were combined and ammonium bicarbonate salts were removed by evaporation and the samples were lyophilized.

Further separation of the MMP was performed by DEAE-Macroprep resin (Bio-Rad, CA, USA) column using a linear gradient of NaCl from 0.1 to 2 M. The fractions were combined based on total carbohydrate content [47] and C-PAGE analysis. The MMP-derived fractions were dialyzed over water using a 3.5 kDa dialysis tube (Thermo Fisher Scientific, Waltham, MA, USA), to remove salts, and lyophilize.

4.10. NMR Spectroscopy

Native *S. latissima* fucoidans, as well as LMP, MMP, and all the further separated oligosaccharides were analyzed by NMR spectroscopy. The samples (approximately 10 mg) were dissolved in 500 µL ^2H_2O, and NMR spectra were collected on an 800 MHz Bruker Avance III HD instrument equipped with a 5 mm TCI cryoprobe and a SampleJet sample changer. Oligosaccharide spectra were acquired at 25 °C. The ^{1}H 1D NMR spectra (of 16,384 complex data point sampling 1.7 s) were acquired by summing up 16 transients. The ^{1}H-^{1}H TOCSY (2048 × 400 complex data points sampling 128 ms and 25 ms in the direct and indirect dimensions, respectively) was acquired by using a 10 kHz spin lock field during a mixing time of 80 ms. The ^{1}H-^{1}H COSY was acquired by sampling 2048 × 512 complex data points for 213 ms and 53 ms in the direct and indirect dimensions, respectively, while ^{1}H-^{1}H NOESY was acquired as 2048 × 256 complex data points by sampling the FID for 213 ms and 53 ms in the direct and indirect dimensions, respectively, and using a mixing time of 600 ms. The ^{1}H-^{13}C HMBC (2048 × 128 complex data points sampling 256 ms and 6.3 ms, respectively) and multiplicity-edited ^{1}H-^{13}C HSQC using adiabatic decoupling (2048 × 512 complex data points sampling 213 ms and 15.5 ms) were acquired alongside ^{1}H-^{13}C HMBC (1024 × 100 complex data points sampling 128 ms and 3 ms). Assignment spectra of the high molecular weight fractions were acquired at 50 °C with an 800 MHz Bruker Avance III instrument equipped with a 5 mm TCI cryoprobe and an Oxford magnet. All NMR spectra were processed with ample zero filling in all dimensions and baseline correction using Bruker Topspin 3.5 pl7 software. All spectra were subsequently analyzed with the same Bruker Topspin software.

5. Conclusions

In the present study, the novel endo-fucoidanase Mef2, from the marine bacterium *M. eckloniae*, was characterized. Mef2 is the first enzyme of the GH107 family to show activity on the highly complex fucoidans of *S. latissima*. In *S. latissima* fucoidans, Mef2 specifically catalyzed hydrolysis of sulfated α(1,3)-linked fucosyl residues with α(1,4)-linked fucosyl branches. These fucoidan oligosaccharide structures with α(1,4) branches

have not previously been described for fucoidans from *S. latissima*, and they are the first fucoidan oligosaccharides from *S. latissima* released by a fucoidanase.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md20050305/s1, Table S1: Percent identity matrix of the catalytic D1 domain of Mef2 and the characterized members of the GH107 family; Table S2: Thermostability by DLS of Mef2 with Ca^{2+} and without Ca^{2+}; Table S3: Monosaccharide composition of fucoidan fraction 4 (FeF4) purified from enzymatic extracts of *F. evanescence*; Table S4: Monosaccharide composition of fucoidan fractions purified from enzymatic extracts of *S. latissimi* from Ocean Rainforest; Table S5: Monosaccharide composition of *S. latissima* MMP after Mef2 hydrolysis; Table S6: Monosaccharide composition and sulfate content of fractionated *S. latissima* MMP; Table S7: Monosaccharide composition of fucoidan fraction 3 from different brown seaweed species; Figure S1: Multiple alignment of the catalytic domain (D1) of the fucoidanases Mef2 with other GH107 family members; Figure S2: Recombinant expression and purification of the Mef2 fucoidanase; Figure S3: Thermostability assessments of Mef2 by DLS; Figure S4: PARAFAC first component scores versus enzyme dosage were plotted to build calibrations; Figure S5: NMR analysis of deacetylation of *S. latissima* fucoidans; Figure S6: Flow-chart of enzymatic hydrolysis of fucoidans from *S. latissima* and further product separation; Figure S7: Medium and low molecular weight fucoidans after Mef2 hydrolysis of fucoidans from *S. latissimi*; Figure S8: Separation of *S. latissima* MMP products on DEAE macroprep column; Figure S9: ^{1}H-^{1}H TOCSY NMR spectrum for the deacetylated *S. latissima* MMP; Figure S10: Separation of *S. latissima* LMP products on Q sepharose High Performance column; Figure S11: The proposed structure of the *S. latissima* OF2 fraction; Figure S12: ^{1}H-^{13}C NMR spectra of LMP from *F. evanescens* fucoidan after Mef2 hydrolysis; Figure S13: HP-SEC chromatogram of fucoidan fractions from *S. latissima* from Ocean Rainforest after IEX purification.

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