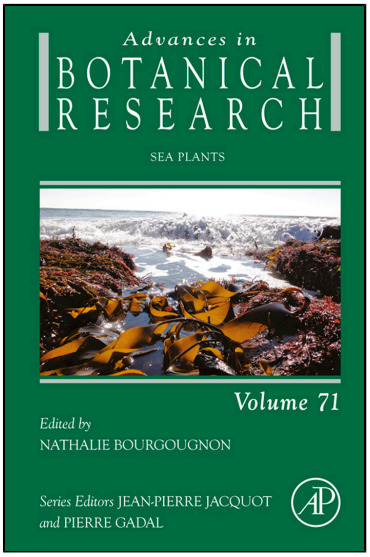

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CHAPTER NINE

Polysaccharides from *Codium* Species: Chemical Structure and Biological Activity. Their Role as Components of the Cell Wall

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Abstract

Polysaccharides from *Codium* species have drawn attention due to their high anticoagulant activity. However, many different mechanisms of action have been proposed and the structure of the active compounds was usually not elucidated. Recently, structural determination has been carried out using purified fractions showing the presence of at least three different polysaccharide types, namely: (1) 3-linked β-D-galactans with ramifications on C-6 and terminal β-D-galactopyranose units with pyruvic acid ketal

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linked to *O*-3 and *O*-4 (*S*-configuration). Sulphation occurs mainly on C-4 and some of the 3-linked galactose units are substituted by pyruvic acid ketals linked to *O*-4 and *O*-6 (*R*-isomer). (2) 3-linked linear pyranosic β-L-arabinans sulphated on C-2 and/or C-4. (3) 4-linked β-D-mannans, partially sulphated on C-2 or on C-6, but to a lesser extent. The backbone of this polysaccharide is similar to that of the fibrillar component of the cell wall. Antiherpetic activity and activity on the immune system were determined for sulphated galactans from *Codium fragile*, which do not have significant anticoagulant effect, while highly sulphated arabinans have an important anticoagulant activity by mechanisms different to those described for heparin. The utricle cell wall of *C. fragile* has a sandwich structure of two fibrillar-like layers delimiting a middle amorphous-like zone. Mannans and hydroxyproline-rich glycoprotein-like epitopes are located in two distinct cell-wall layers, whereas sulphated polysaccharides are distributed in the middle area of the wall. The overall cell-wall polymer arrangement in the utricles of different species proved to be somewhat different.

9.1 INTRODUCTION

The genus *Codium* (Codiaceae, Bryopsidales, Chlorophyta) currently comprises around 125 species widely distributed through the world's seas with the exception of the polar regions, being mainly found in temperate and subtropical areas (Oliveira-Carvalho, Oliveira, Barreto-Pereira, & Verbruggen, 2012). Seaweeds of this genus show a broad variation of forms and occur in various habitats, and, as the majority of those belonging to the suborder Bryopsidineae, they exhibit no calcification. *Codium* thalli can spread out over hard surfaces as mats, form spheres or grow upright, either unbranched and finger-like, or branched, with cylindrical or flattened branches. Anatomically, a *Codium* thallus is composed of a single, giant, branched tubular cell containing multiple nuclei, the branches are usually called siphons. The centre of the thallus consists of an entangled mesh of siphons, whereas in the surrounding cortex, siphons are closely adjoined and swollen into utricles. The utricles occur in a wide array of forms, varying in size, shape and composition, with gametangia and/or hairs borne along their sides (Figure 9.1). *Codium* is found in marine habitats ranging from rocky coasts exposed to full wave-forces to calm lagoons, from intertidal habitats to deep reefs, from arctic to tropical waters and from eutrophic estuaries to nutrient-depleted coral reefs (Verbruggen et al., 2007).

For the last two decades, *Codium* species have been of public and scientific concern because of the invasive, bloom-forming nature of some of them. *Codium fragile* subspecies *tomentosoides* is the most invasive seaweed in the world, and it is believed to be native to Japan and was then unintentionally spread around the world. Another species, *Codium isthmocladum*, forms

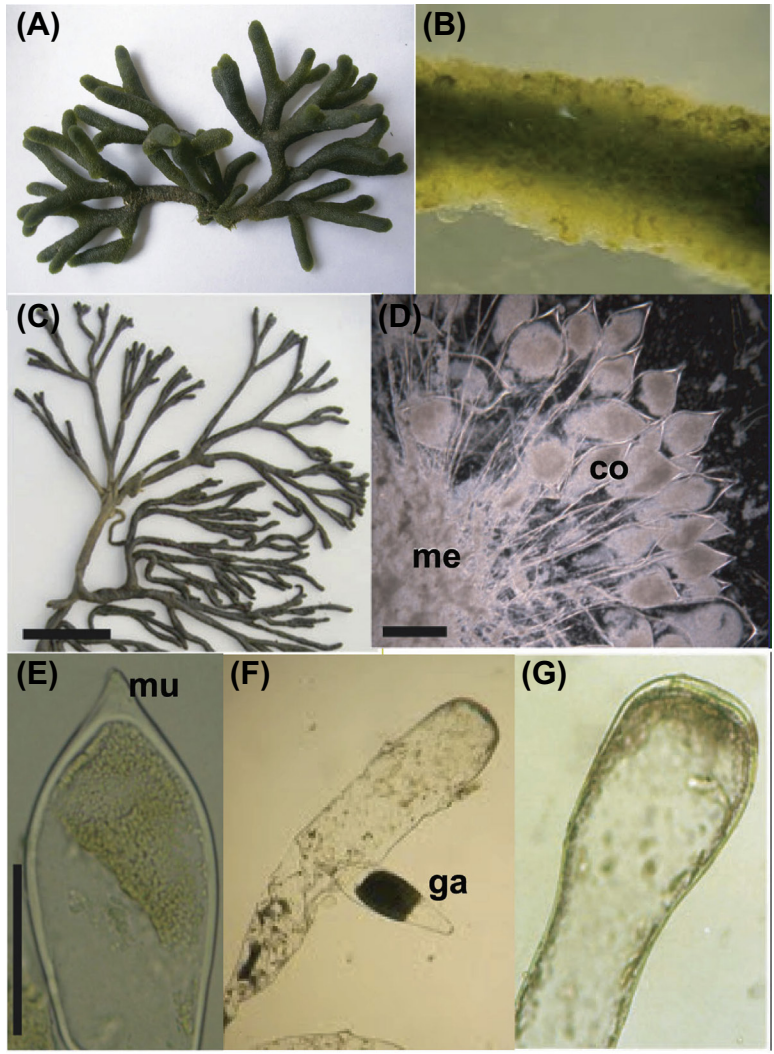

Figure 9.1 (A) General aspect of the Thallus of *Codium vermilara*. (B) Detail of the thallus of *Codium decorticatum*. (C) General aspect of the dry thallus of *Codium fragile*. Scale bar = 1 cm. (D) Inner structure of the thallus of *C. fragile*. Scale bar = 250 μm (me: medullar region; co: photosynthetic cortex). (E) Detail of an utricle of *C. fragile*. Scale bar = 250 μm (mu: mucron). (F) Detail of an utricle of *C. decorticatum* (ga: gametangium). (G) Detail of an utricle of *C. vermilara*. *(C), (D), and (E) from Estevez et al., 2009; (G) from Fernández et al., 2010; (B) and (F) courtesy from María Paula Raffo (Cenpat-Conicet, Puerto Madryn, Argentina)*. (See the colour plate.)

harmful blooms on reefs, consequence of increased eutrophication. On the other hand, *Codium* species are used as food for cultured abalone, they are consumed by humans, and they are a source of bioactive compounds (Verbruggen et al., 2007).

Cell walls from marine algae, like red and brown seaweeds, together with marine angiosperms, biosynthesise sulphated polysaccharides, mainly galactan sulphates (carrageenans, agarans) (Usov, 2011) or sulphated fucans (Usov & Bilan, 2009). Although less studied, cell walls from green seaweeds also comprise sulphated polysaccharides. The fact that seaweeds, as well as sea invertebrates, like sea urchins (Vilela-Silva, Hirohashi, & Mourão, 2008) and echinoderms (Pereira, Mulloy, & Mourão, 1999), possess structurally related sulphated polysaccharides in their cell walls-intercellular matrices might be the result of convergent and extreme directional selection forces in their marine environments to high saline conditions (Aquino & Landeira-Fernández, 2005). These sulphated polysaccharides in high ionic strength media such as seawater would remain strongly charged providing mechanical stress resistance, hydration by gel formation, and they would act as ionic and osmotic regulators (Kloareg & Quatrano, 1988).

Green seaweeds biosynthesise matrix-sulphated polysaccharides with a great variety of chemical structures. They have been classified into two main groups (Percival & McDowell, 1981): (1) sulphated glucuronoxylorhamnans and glucuronoxylorhamnogalactans, with negative optical rotation, and (2) sulphated xyloarabinogalactans, which have positive optical rotation. As examples of the first group, the major acidic polysaccharide structure of *Ulva* and *Enteromorpha* species (Ulvales) is a linear sulphated polymer comprising alternating 4-linked β-D-glucuronic acid and α-L-rhamnose units (Lahaye & Robic, 2007), while glycuronorhamnans/rhamnans from *Monostroma* and *Gayralia* (Ulotrichales) have a backbone consisting in 2- and 3-linked α-L-rhamnose units with different substitution patterns (Cassolato et al., 2008; Lee, Koizumi, Hayashi, & Hayashi, 2010; Li et al., 2011). On the other hand, seaweeds of the Bryopsidales (Chlorophyta) belong to the second group, and only small to trace amounts of uronic acids and rhamnose, if any, were detected in the polysaccharides.

Until the present century, the only detailed structural studies of sulphated polysaccharides from this order were those of Percival and coworkers (Percival & McDowell, 1981), including those carried out on the polysaccharides from *C. fragile* (Love & Percival, 1964a). From that time until a few years ago, most of the work published comprised the characterisation of some extracts or fractions derived from them with interesting anticoagulant

activity (Hayakawa et al., 2000; Matsubara et al., 2001; Matsubara, Matsuura, Hori, & Miyazawa, 2000; Siddhanta, Shanmugam, Mody, Goswami, & Ramabat, 1999; Uehara, Takeshita, & Maeda, 1992).

In addition, there is also information available about the fibrillar components of the cell walls of the Bryopsidales. These seaweeds lack cellulose, or at least this polymer is not the major fibrillar component of the cell wall, and have β-(1→4)-mannans (Chanzy, Grosrenaud, Vuong, & Mackie, 1984; Ciancia et al., 2007; Huizing & Rietema, 1975; Kaihou, Hayashi, Otsuru, & Maeda, 1993; Love & Percival, 1964b; Mackie, 1969) or β-(1→3)-xylans (Ciancia et al., 2012; Fukushi, Otsuru, & Maeda, 1988; Mackie, 1969; Mackie & Percival, 1959; Maeda, Fukushi-Fujikura, & Otsuru, 1990; Yamagaki, Maeda, Kanazawa, Ishizuka, & Nakanishi, 1997) instead. In addition, it has been shown that the major fibrillar component of the cell wall can vary in the different life stages (Huizing & Rietema, 1975; Huizing, Rietema, & Sietsma, 1979; Wutz & Zetsche, 1976). Thus, gametophyte microthalli of *Derbesia* sps. biosynthesise xylans, while sporophyte macrothalli of the same genus produce mannans. Similarly, gametophyte macrothalli from the genus *Bryopsis* biosynthesise xylans, while sporophyte microthalli produce mannans. However, considerable amounts of glucans were detected in some cases (Huizing et al., 1979; Wutz & Zetsche, 1976). Moreover, cellulose was found to be a minor fibrillar component of the cell walls from *Bryopsis maxima* and *Bryopsis plumosa* (Ciancia et al., 2012; Fukushi et al., 1988; Maeda et al., 1990).

9.2 STRUCTURE OF CELL WALL POLYSACCHARIDES FROM *CODIUM* SPECIES

The first structural study about the sulphated polysaccharides from *Codium* was carried out by Love and Percival (1964a), who found that the water extracts from *C. fragile* contained galactose and then arabinose as major monosaccharide constituents, but also small amounts of xylose, rhamnose, glucose, and mannose. This product contained 22% of sulphate (as SO_3Na), and minor amounts of uronic acids. It also contained 25% of protein and it was supposed to be a proteoglycan. Glucose and mannose were eliminated by periodate oxidation and were considered to arise from contaminant polymers. The resulting product was fractionated by anion exchange chromatography obtaining a fraction with similar quantities of galactose and arabinose, and important amounts of sulphate that would be in part, linked to C-2 or C-3 of the arabinose units. Partial acid hydrolysis

and fractionation of the oligosaccharides, allowed to isolate a neutral fraction containing 3-*O*-β-L-arabinopyranosyl-L-arabinose and 3-*O*-β-D-galactopyranosyl-D-galactose and an acidic fraction containing galactose 4-sulphate and galactose 6-sulphate. Results obtained from seaweeds collected in two distant locations were similar.

This pioneer study gave a first clear idea about the structure of these polysaccharides. At that stage, they were considered as arabinogalactans, being both sugars possibly in the same molecule.

9.2.1 Sulphated Galactans

During the following decades, no structural studies were published. The only reports correspond to very general characterisations of compounds with interesting biological properties, mostly anticoagulants, until the isolation and structural studies of the galactan obtained from *Codium yezoensis* (Bilan, Vinogradova, Shashkov, & Usov, 2006, 2007).

By fractionation of the water extracts of this seaweed a very complex sulphated galactan was isolated. Thus, its elucidation required several modifications of the polysaccharide, i.e. desulphation and depyruvylation, followed by methylation analysis and Smith degradations, these chemical data were supported by NMR spectroscopy. This galactan showed a highly ramified structure, which contained linear backbone segments of 3-linked β-D-galactopyranose residues connected by (1→6) linkages, about 40% of 3-linked residues being additionally substituted at C-6, possibly by short oligosaccharide residues also containing (1→3) and (1→6) linkages. Sulphate groups were found mainly at C-4 and in minor amounts at C-6.

The most unusual feature of this water-soluble galactans was the high pyruvate content (one pyruvate and two sulphate per every four galactose residues). Pyruvate was found to form mainly five-membered cyclic ketals with *O*-3 and *O*-4 of the nonreducing terminal galactose residues (*S*-isomer). However, a minor part of pyruvate formed six-membered cyclic ketals with *O*-4 and *O*-6 (*R*-configuration). This feature is common to red algal galactans, which often contain 3-linked 4,6-*O*-(1′-carboxy)ethylidene-D-galactopyranose units. Both six- and five-membered cyclic pyruvate ketals of different monosaccharide residues were detected in bacterial polysaccharides, but seldom in green algal polysaccharides. This galactan was the first algal polysaccharide containing five-membered cyclic pyruvate ketals studied. Its putative fragment containing all the main structural features is shown in Figure 9.2.

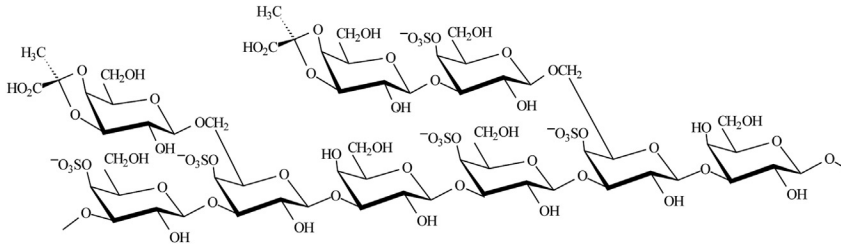

Figure 9.2 A possible sequence for the galactan from *Codium yesoense*.

Hence, the pyruvylated galactan sulphate isolated from *Codium yezoense* resembles red algal galactans in composition, but is quite different from these galactans in structure, having a ramified backbone similar to that of the 3,6-linked β-D-galactans of terrestrial plants, and bearing cyclic pyruvate ketals linked mainly to *O*-3 and *O*-4 of nonreducing terminal galactose residues, as in bacterial polysaccharides.

At the same time, the sulphated polysaccharides extracted with water from *C. fragile* and *Codium vermilara* from the Atlantic coast of Patagonia were studied (Ciancia et al., 2007). The first room temperature water extracts from both seaweeds (A1 and V1, respectively) showed galactose and arabinose as major sugar components. As no fractionation was carried out, it was not possible to deduce if this extracts contained arabinogalactans or a mixture of arabinans and galactans. However, in general terms, the galactan moieties in both extracts were similar, and similar to the galactan from *C. yezoense*. V1 was further fractionated by precipitation with potassium chloride and the product which remained soluble was a pure sulphated galactan (Fernández et al., 2013). This polysaccharide has a composition very similar to that of the galactan from *C. yezoense*; however, the structural units obtained by methylation and desulphation-methylation and NMR spectroscopy were somewhat different, mainly the presence of important amounts of 3-linked 4,6-disulphated units (~20%) and also the fact that the pyruvylated terminal units would form single stubs and not short chains. No appreciable pyruvylation was detected in the 3-linked β-D-galactose units of the backbone (Fernández, 2012).

Galactan sulphates from *C. fragile* of two different locations, Puerto Deseado (Santa Cruz), Argentina (F1 and F6), and Kamakura, Japan (FG) were studied at the same time by Estevez, Fernández, Kasulin, Dupree, & Ciancia (2009) and Ohta, Lee, Hayashi, & Hayashi (2009), respectively. Table 9.1 gives the structural units deduced from data published in these papers. These galactans showed the same structural units found for other galactans from *Codium* species, with the exception of F1 where structural data suggests

Table 9.1 Structural Units Found in Sulphated Galactans from *Codium fragile* from Two Different Locations

	Codium fragile from Argentina[b]		*Codium fragile* from Japan
Structural Units[a]	**F1**	**F6**	**FG**
→3)β-D-Gal*p*(1→	25	26	19
→3) β-D-Gal*p* 4S(1→	3	6	8
3,4Pyr-β-D-Gal*p*(1→	28	36	40
→3,6)β-D-Gal*p*(1→	24	12	7
→3,6)β-D-Gal*p* 4S(1→	–	10	25
→3)β-D-Gal*p*4,6-pyr(1→	8	11	–
3,4Pyr-β-D-Gal*p* 6S(1→[c]	15	–	–

[a]Nomenclature used: Gal*p*: galactopyranose; S: sulphate; Pyr: pyruvate.
[b]Small amounts of other sugars were considered to derive from contaminant polymers.
[c]Speculative unit, deduced from the desulphation pattern.

the presence of terminal 3,4-pyruvylated β-D-galactose 6-sulphate units. In addition, there are important differences in the percentages of the structural units: (1) most of the 3,6-linked galactose units are sulphated in FG, while they are not sulphated in F1 and in F6 only half of these units are sulphated; (2) there are not only 3,4-pyruvylated galactose terminal units, but also significant amounts of 3-linked 4,6-pyruvilated β-D-galactose units in F1 and F6, while the latter units are absent in FG.

These results show that although similar, galactans from different *Codium* species have important interspecific differences (Bilan et al., 2007; Ciancia et al., 2007; Farias et al., 2008; Ohta et al., 2009); moreover, also important differences between galactans of the same species but from different locations were observed. Besides, galactans F1 and F6, fractions from the same seaweed and location are also distinct (Table 9.1).

9.2.2 Sulphated Arabinans

Room-temperature water extraction of *Codium latum* and further fractionation produced a sulphated arabinan with 20.7% sulphate (molar ratio arabinose/sulphate 1/0.3–0.4), which, based on IR and ^{1}H NMR spectroscopy (anomeric proton δ 5.240 (D_2O)), was considered a sulphated furanosic 5-linked α-L-arabinan (Uehara et al., 1992).

In addition, after extensive fractionation of the cold-water extract of *Codium dwarkense*, a sulphated arabinan (A2a) with 41.45% sulphate (molar ratio arabinose/sulphate 1/1) and a sulphated arabinogalactan (A2b) with 31.85% sulphate were obtained in ~0.3% and ~0.4% yield, respectively

(Matsubara et al., 2001). A2a gave after extensive fractionation a product (Jia) with the highest anticoagulant activity (molar ratio arabinose/sulphate 1/1). The structure of this product was deduced from IR and ^{1}H NMR spectra as a furanosic α-L-arabinan without any specification of the position of the sulphate groups. Only the anomeric proton in the original product δ 5.21 (D_2O) and desulphated derivative δ 4.93 (DMSO) were informed (Siddhanta et al., 1999). In a screening of inhibition of thrombin by sulphated polysaccharides isolated from green algae, arabinans were extracted from *Codium divaricatum*, *Codium adhaerens*, *C. latum*, and *C. fragile* (molar ratio arabinose:sulphate, 0.6, 0.8, 0.8 and 0.5, respectively). No structural details were determined (Hayakawa et al., 2000). These papers give an idea that sulphated arabinans are usual components of *Codium* cell walls. However, there was a lot of confusion due to reports with very different structures, but no detailed information.

The room temperature water extracts from *C. fragile* and *C. vermilara* showed important amounts of arabinose. Methylation and desulphation-methylation analyses showed that 3-linked 2,4-disulphated arabinopyranose was the major structural unit. No evidence of the presence of arabinose in the furanose form was found. From the NMR spectra, it was suggested that these residues were β-anomers (Ciancia et al., 2007). On the other hand, hot-water extracts from *C. vermilara* indicated high amounts of non-sulphated arabinopyranose units. Later, arabinans from the same seaweed (Ab1 and Ab2) were isolated in a pure form (96–97% of arabinose, molar ratio arabinose:sulphate 1:1.8 and 1:1.5, respectively) and their structure was determined in detail by chemical and spectroscopic methods (Fernández et al., 2013) (Table 9.2). Both arabinans were extracted with water and obtained after separation from other polymers, mainly sulphated galactans.

Table 9.2 Linkage Analysis of Pyranosic Arabinans from *Codium vermilara*[a]

Monosaccharide	Structural Unit[b]	Ab1	Ab2
2,3,4-Ara[c]	β-L-Ara*p*(1→	1	6
2,4-Ara	→3) β- L-Ara*p* (1→	13	35
2-Ara	→3) β-L-Ara*p*4S (1→[d]	26	30
4-Ara	→3) β-L-Ara*p*2S (1→	10	7
–	→3) β-L-Ara*p*2,4S (1→	50	22
Sulphate content (as SO_3Na)		47	38

[a]All the methylated monosaccharides were derivatives of L-arabinose.
[b]β-configuration of these units was deduced by optical rotation and NMR spectroscopy.
[c]2,3,4-Ara indicates 2,3,4-tri-*O*-methyl-L-arabinose, etc.
[d]Nomenclature used, S: sulphate.

Initially, the room-temperature water extract was fractionated by anion-exchange chromatography, but separation was difficult and its reproducibility was low (Fernández, 2012). Then, taking into account previous reports (Siddhantha et al., 1999), separation by potassium chloride precipitation, like that used frequently for carrageenans, was successfully applied to the room temperature water extract, obtaining Ab1 through a clean separation. However, it was only possible to isolate Ab2 from the hot-water extract after elimination of α-glucans by treatment with amylase and further precipitation of the treated product with potassium chloride (Fernández et al., 2013).

Ab1 was isolated through a sharp gelification at 0.115 M KCl. No precipitation occurred when using sodium chloride, indicating that the insolubilisation is K^+-specific as in the case of kappa-carrageenans. Lower or higher concentrations of potassium chloride (upper limit 2 M) did not show any other precipitation. The mechanism of gel formation in dilute potassium chloride solutions has been extensively studied for κ-carrageenan where junction zones in gels are formed by quaternary interactions at the superhelical level (Nilsson & Piculell, 1991) between ordered tertiary structures (double helices), promoted by potassium ions, through the one-stage domain mechanism of aggregation (coil→double helix→gel) (Robinson, Morris, & Rees, 1980). A similar mechanism was suggested for gel formation of other polysaccharides in which the primary mode of interchain association is through multistranded helices (Aspinall, 1982; Robinson et al., 1980). Ab2, less sulphated than Ab1, also precipitated with potassium chloride, but at a higher concentration (0.5 M).

9.2.3 Mannans

Linear β-(1→4)-D-mannans are the major fibrillar component of cell walls from *Codium* species (Chanzy et al., 1984; Ciancia et al., 2007; Estevez et al., 2009; Kahiou et al., 1993; Love & Percival, 1964b; Mackie & Sellen, 1969; Percival & McDowell, 1981). These polymers are the predominant skeletal wall polymers in several other genera of green algae, including *Derbesia* (Bryopsidales), *Acetabularia*, *Halicoryne*, *Dasycladus*, *Neomeris*, *Cymopolia*, and *Batophora* (Dasycladales) (Dunn et al., 2007; Percival & McDowell, 1981). Substitution to even a small extent would hinder their ability to form high tensile strength microfibrils, but might enhance their ability to form a flexible network (Dunn et al., 2007).

The insoluble mannan obtained from *C. fragile* after exhaustive water extraction was studied by degradation with a β-mannanase and analysis of the resulting oligosaccharides; confirming the linear nature of this structure and the β-(1→4)-linkages, no evidence of side chains was found (Estevez

et al., 2009). On the other hand, soluble mannan structures were found to be important components of the hot-water extracts from *C. fragile* and *C. vermilara*. The structure of the backbone was clearly established, being identical to that of the fibrillar component. The reason of the high solubility of these mannans in hot water was not clearly established. The number average molecular weight for those from *C. fragile* was 39–49 KDa and no side chains were detected. However, methylation analyses of mannans from *C. vermilara* suggested possible ramifications (Ciancia et al., 2007). Later, by anion exchange chromatography of the first hot-water extract from this seaweed, a fraction containing 94% of mannose and 12% of sulphate was isolated in low yield. The sulphate groups were linked to C-2 of 23% of the mannose units, while most of these units were not substituted. This degree of sulphation would explain the higher solubility of this β-(1→4)-D-mannans, compared to that of the nonsulphated fibrillar polymer. Other fractions also contain mannose in different proportions, but structures of these components were not studied (Fernández, Estevez, Cerezo, & Ciancia, 2012).

It has been shown that the unrelated green seaweed *Codiolum pusillum* (Chlorococcales, Endosphaeraceae) synthesizes a β-(1→4)-D-mannan branched at C-6 and carrying sulphate groups on C-2, although this is not the major sulphated polysaccharide biosynthesised by this unicellular seaweed (Carlberg & Percival, 1977).

Moreover, a β-(1→4)-D-mannan with similar substitution pattern to that of *C. pusillum* was isolated from red seaweeds *Chondrophycus papillosus* and *Chondrophycus flagelliferus* (Ceramiales) (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007). This mannan was partially sulphated on C-2 of the mannose units and also substituted on C-6 with single stubs of β-D-xylose, as well as with β-D-mannose 2-sulphate units. On the other hand, the xylomannans from red seaweeds of the order Nemaliales have a linear backbone of α-(1→3)-D-mannopyranose units with different sulphation patterns (Pérez Recalde, Noseda, Pujol, Carlucci, & Matulewicz, 2009).

Linear β-D-mannans fulfill primarily structural functions not only in some green seaweeds, but also in the seeds of many plants, such as ivory nut, green coffee and coconut kernel. A low degree of α-(1→6)-D-galactose substitution results in (1) linear mannan chains with a degree of polymerisation of ~15 compacted into dense granular and crystalline structures (mannan I), or in (2) microfibrils, similar to those of cellulose that are less crystalline and with a higher degree of polymerisation of ~80 (mannan II). Both forms are insoluble and provide rigidity and protection to the endosperm. Once germination takes place, these mannans can be mobilised as

nonstarch storage polysaccharides. Water retention by galactomannans is particularly important to the long-term survival of legume seeds in arid areas (Van Zyl, Rose, Trollope, & Gorgens, 2010).

Recently, a very different structure was proposed for a mannan isolated from a water extract of *C. fragile* collected from the coast of Sokcho, Gangwon province, Korea, comprising 3-linked α-D-mannose units (Tabarsa, Karnjanapratum, Cho, Kim, & You, 2013). This structure is completely different to those isolated and characterised by many other researches for the same species from various locations (Chanzy et al., 1984; Ciancia et al., 2007; Estevez et al.,2009; Love & Percival, 1964b, Mackie & Sellen, 1969). Evidence presented by the authors could be subject of different interpretations.

9.2.4 Hydroxyproline-Rich Glycoproteins

Arabinogalactan proteins (AGPs) and extensins are hydroxyproline-rich glycoproteins (HRGPs) usually present in plant cell walls (Albersheim, Darvill, Roberts, Sederoff, & Staehelin, 2011).

There are contradictory data about the exact functions of AGPs in a broad variety of processes as plant growth and development, plant defence, cell proliferation, cell expansion, cell differentiation, cell extension and somatic embryogenesis. The group of 'classical' AGPs consists of hyperglycosylated polypeptides with a C-terminal hydrophobic sequence that directs the addition of a glycosylphosphatidylinositol (GPI) anchors and tethers them to the plasma membrane. Soluble AGP monomers are released through or into the middle lamella and the intercellular space. The continuous release of AGPs is characteristic of the rapid growing plant cells.

On the other hand, extensins are secreted as rod-like monomers with limited flexibility in an extended helical conformation. Extensin peroxidase catalyses polymerisation of extensin monomers via diisodityrosine and pulcherosine (tetramer and trimer of tyrosine, respectively) to yield cross-linked networks. Recently, it has been postulated that a positively charged extensin scaffold reacts with acidic pectin to form extensin pectate, which further templates orderly assembly of a nascent cell wall, changing the focus on extensin, from its role exclusively in the cessation of growth to an essential role in its initiation (Cannon et al., 2008).

Although they have also been detected in some Chlorophycean and Charophyceae green algae (Domozych et al., 2012), the first seaweed that was found to contain HRGPs was *C. fragile* (Estevez et al., 2009) by immunolabeling using antibodies against specific cell wall HRGP epitopes (AGPs and extensins) and by reaction with the β-glucosyl Yariv reagent.

In addition, a unique furanosic α-arabinosyl structure was detected in an arabinose-rich fraction obtained by fractionation of a room temperature water extract of the same seaweed, consisting in a 5-linked arabinan branched at C-3. 3-Linked and 3,6-linked β-D-galactose units were also detected. Although the latter units could be part of the major galactan biosynthesised by this seaweed, altogether, these results showed the presence in this fraction of AGP-like structures. Cell walls from *C. vermilara* also showed to have similar HRGPs by immunolabeling and by reaction with the Yariv reagent (Fernández, Ciancia, Miravalles, & Estevez, 2010); however, no furanosic arabinan structures were detected.

9.2.5 The Cell Wall Polysaccharides from *Codium*

Cell walls in *C. vermilara* are composed of ~32% (w/w) β-(1→4)-D-mannans, ~12% sulphated polysaccharides, and small amounts of hydroxyproline-rich glycoproteins (Fernández et al., 2010). Similar quantities of mannans and sulphated polysaccharides were reported previously in the related seaweed *C. fragile* (Estevez et al., 2009). Overall, both seaweed cell walls comprise ~40–44% of their dry weights. No other polysaccharide system from *Codium* was studied as a whole, although most of the data published until now suggest the possibility of similar polysaccharide systems. Within the sulphated polysaccharides group, a variety of polysaccharide structures were isolated and characterised, comprising (1) highly ramified pyruvylated sulphated galactans, with 3,6-, and 3,6-linkages, (2) linear sulphated pyranosic 3-linked arabinans and (3) sulphated 4-linked β-mannans. In spite of the fact that certain other structures were proposed (Kahiou et al., 1993; Tabarsa et al., 2013), it appears that this outline would be repeated for all the systems of sulphated polysaccharides from *Codium* species, with small differences. Results obtained so far for the cell wall components of *Codium decorticatum* (Fernández, Raffo, Alberghina, Ciancia, unpublished results) supports this generalisation.

9.3 POLYSACCHARIDE DISTRIBUTION ON THE CELL WALL

Despite the extensive knowledge of the chemical structure of most of the cell-wall polymers, the ways in which they interact to assemble into the cell-wall architecture remain elusive for most groups of plants. There are different analytical techniques available to achieve the understanding of the spatial arrangement of the cell-wall components. The use of *in situ* methods, such as the current library of monoclonal antibodies and carbohydrate

binding modules (CBMs) against several cell-wall carbohydrate epitopes (Moller et al., 2007), together with noninvasive Fourier transform infrared spectroscopy coupled to a microscope (FTIR microspectroscopy), allows us to perform chemical imaging in plant cells and reveals intrinsic heterogeneities in a single cell wall or in many different cell-wall types within a tissue (Carpita et al., 2001; McCartney et al., 2006).

In green algae, spatial arrangement of the cell-wall polymers was characterised only in simple models, such as volvocacean and desmid algae (Domozych & Domozych, 2003; Domozych, Lambiasse, Kiemle, & Gretz, 2009; Ender, Godl, Wenzl, & Sumper, 2002; Hallman, 2006) and also in the more complex wall of *Ulva* (Bobin-Dubigeon, Lahaye, Guillon, Barry, & Gallant, 1997). Lately, *in situ* methods were applied to study spatial arrangement of well characterised cell-wall polymers in *Codium* species (Estevez et al., 2009; Fernández et al., 2010).

In situ distribution of structural β-(1→4)-D-mannans in cell walls at the utricle tip from *C. fragile* was analysed using SR-FTIR microspectroscopy, Calcofluor White staining, birefringence detected by phase contrast and antibody labelling with anti-mannan mAb. These techniques showed a similar localisation pattern, with a central layer of the cell wall rich in fibrillar mannans. In addition, after an *in situ* enzymatic treatment with mannanase performed on cross-sections, cell-wall integrity was severely damaged, showing the important role of this polymer in cell-wall assembly and stability. Distribution of sulphated polysaccharides was determined using Toluidine Blue *O* (at pH = 1) and ruthenium red staining. The latter reacts not only with sulphate groups but also with the carboxylate groups of pyruvic acid. These polymers localise mostly in the tip of the mucron area and close to the plasma membrane. Finally, the *in situ* distribution of AGPs and extensin carbohydrate epitopes was assayed by several probes. AGP epitopes were localised in the inner part of the cell wall, close to the plasma membrane, and also in the outer faces of the utricle cell wall. Extensin epitopes were detectable mostly in the tip of the mucron, showing that the distribution of both types of glycoproteins only overlaps in the apical region of the utricle cells, but not in the cell-wall region close to the plasma membrane. Coomasie Blue staining and characteristic FTIR protein absorbance bands in SR-FTIR map are in good agreement with these results.

A sandwich structure was proposed for the utricle cell walls from *C. fragile*, with two boundary zones of HRGPs and a central layer rich in β-(1→4)-D-mannans and sulphated polysaccharides. The presence of fibrillar mannans and charged polysaccharides in the same region suggests

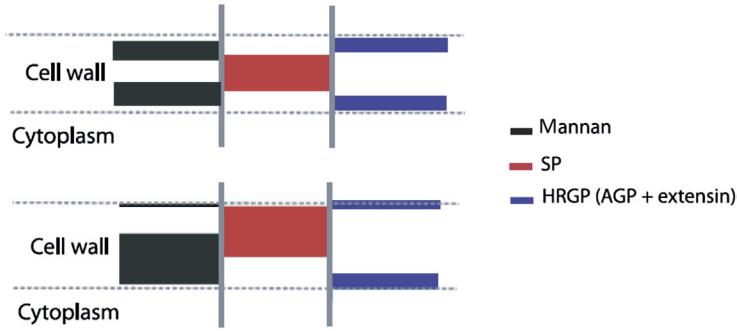

Figure 9.3 Proposed models for the utricule cell wall of *Codium vermilara* (above) and *Codium fragile* (below), including the *in situ* distribution of fibrillar mannan, sulphated polysaccharides (SP) and hydroxyproline-rich glycoproteins (HRGP), including arabinogalactan proteins (AGP) and extensins.

an important degree of interaction between both macromolecular types (Figure 9.3).

In *C. vermilara*, the utricle cell wall shows an external cuticle and two fibrillar-like layers delimiting a middle amorphous layer. *In situ* localisation of the main polymers was assayed by immunolabelling and chemical imaging. β-(1→4)-D-mannans are localised in two well-defined layers with similar development. *In situ* enzymatic treatment with mannanase caused not only an important loss of CW staining and birefringence, but also severe damage in some cell-wall areas, as it was shown previously for *C. fragile*. SR-FTIR microspectroscopy showed that sulphated polysaccharides concentrate in the amorphous central layer of the cell wall. Toluidine Blue *O* staining showed a similar pattern. AGPs labelling with β-glucosyl Yariv reagent showed that these glycoproteins are present in two marginal cell-wall layers. This two-layered distribution of AGPs was confirmed by immunolabelling, while extensin epitopes (also detected with specific antibodies) followed a similar pattern.

Based on these results, cell wall models for *C. fragile* and *C. vermilara* were proposed. The overall cell-wall polymer arrangement is different in both species, in spite of being phylogenetically very close. In *C. fragile*, mannans and HRGPs are distributed in a very wide internal layer, and only a narrow external layer was observed. On the other hand, in *C. vermilara,* two boundary zones of fibrillar mannans and HRGPs were detected, whereas in both seaweeds sulphated polysaccharides form an amorphous middle layer. The apical high concentration of AGP epitopes in *C. fragile* and *C. vermilara* suggests a relationship with apical expansion

in the tip-growing cells, as it was shown in the moss protonemal growth and in the tip growth of pollen tubes. Extensins have been proposed to contribute to wall architecture, increased tensile strength, and to participate in a cell-plate formation interpenetrating cross-linked networks in the wall. Since *Codium* and all Bryopsidales are composed of continuous siphons lacking cross-cell walls and cell-plate formation, it is possible that the apical extensin scaffold would provide mechanical support just after or during apical expansion.

9.4 BIOLOGICAL ACTIVITY

Sulphated polysaccharides are known to have a number of biological activities including anticoagulant, antiviral, and immuno–inflammatory activities that could be used in nutraceutical/functional food, cosmetic/cosmeceutical and pharmaceutical applications.

Marine environment offers a great biodiversity and original sulphated polysaccharides, which have different chemical structures that are species-specific. The study of the biological properties of these polysaccharides could provide a valid alternative to traditional polysaccharides, such as glycosaminoglycans. Marine polysaccharides have a real potential for natural product drug discovery and for the delivery of new marine-derived products for therapeutic applications (Jiao, Yu, Zhan, & Ewart, 2011).

The fine structure of these sulphated polysaccharides allows them to behave in a similar way to the biologically active and informative oligosaccharide motifs of human glycoproteins, proteoglycans and glycolipids. Overall, the specific bindings of sulphated polysaccharides with basic proteins, or domains in viral particles, and thus binding affinities in molecular interactions account in conjunction to the resultant pharmacological actions and effectiveness of these glycans. The nature of these interactions has proved to be not a mere consequence of the net charge originated from sulphate content, but mostly driven by stereospecific features like monosaccharide composition, sulphation and glycosylation sites, anomericity and conformational preference (Mulloy, Ribeiro, Alves, & Mourão, 1994).

Although most of the research was carried out on sulphated polysaccharides from red and brown seaweeds, in the last few years promising results in this field were also found about sulphated rhamnans or glucuronorhamnans from green seaweeds of the first group (see Introduction). Besides, there are many reports about different biological activities of sulphated

polysaccharides from *Codium*. However, only a few have been carried out with well purified and defined structures, and we will focus on these studies.

9.4.1 Anticoagulant Activity

Antithrombotic agents have been extensively used as a systemic therapy in cardiovascular diseases and heparin is the initial choice; nevertheless, heparin can induce several side effects, such as development of thrombocytopenia, arterial embolism, bleeding complications and so on. Furthermore, the incidence of prion-related diseases in mammals and the increasing requirements of anticoagulant therapy increase the need to look for alternative sources of anticoagulant and antithrombotic compounds.

The anticoagulant activity of sulphated polysaccharides is mainly attributed to thrombin inhibition mediated by antithrombin and/or heparin cofactor II, with different effectiveness depending of the compound. Other mechanisms were also proposed and these differences could be attributed to the diversity of structures of the polysaccharides evaluated and to the fact that one compound may have more than one target protease. There are some previous reviews about anticoagulant activity of sulphated polysaccharides from seaweeds (Ciancia, Quintana, & Cerezo, 2010; Matsubara, 2004; Shanmugam & Mody, 2000).

An exhaustive revision of the work carried out on the anticoagulant properties of sulphated polysaccharides from seaweeds concluded that the driving force for the formation of the sulphated polysaccharide/protein complex is the nonspecific polar interaction between the negatively and positively charged groups in the polysaccharide and protein, respectively and that the complex is further stabilised by short-range interactions. The polysaccharide binding site should be able to go through the following conformational steps in the formation of the complex: random coil→ordered conformation→low distortion of this conformation to form a complementary fitting structure with the protein backbone. The sulphated monosaccharide units with the highest potential for anticoagulant activity should have two sulphate groups and a glycosidic linkage on the pyranose ring with C-2, C-3 and C-4 in 2S, 3R, 4R or 2R, 3S, 4S configurations for galactose, fucose and arabinose and 2S, 3S, 4R, for rhamnose. Three distributions of these substituents appear: 3-linked 2,4-disulphated units, 4-linked 2,3-disulphated units and 2-linked 3,4-disulphated residues. These types of units have the possibility, through the equilibrium of the chair conformations, to place their sulphate groups in adequate spatial positions to interact with basic groups of the protein (Ciancia et al., 2010).

These structural conditions are in agreement with the fact that pure sulphated galactans from *Codium* usually did not show high anticoagulant activity. Although there are some reports of samples rich in galactose with important anticoagulant activity, this activity could be due to other structures present in the sample (Ciancia et al., 2010; Ciancia et al., 2007). However, a sulphated polysaccharide was obtained from *Codium cylindricum* by an extraction and purification procedure (Matsubara, 2004; Matsubara et al., 2001); it was composed by galactose as major monosaccharide, but also glucose (11%) and sulphate (13.1%). The galactan structure would be similar to the other galactans obtained from *Codium* species, although the presence of pyruvic acid was not investigated (Matsubara, 2004). It prolonged thrombin time (TT) and activated partial thromboplastin time (APTT), but did not affect prothrombin time (PT) (APtt and TT ratios >10 for a concentration of the sample of 15 μg/ml). It did not exert an indirect effect on thrombin by potentiating antithrombin (AT) or heparin cofactor II (HCII), and thus it would inhibit thrombin directly or fibrin polymerisation. It also showed direct inhibition of factor Xa, but at high concentrations (Matsubara et al., 2001).

Highly sulphated arabinans from *C. dwarkense* showed very high anticoagulant activity by global coagulation tests, i.e. a TT ratio >10 was achieved with a 5 μg/ml solution with respect to the control (Siddhanta et al., 1999).

In a screening of inhibition of thrombin by sulphated polysaccharides isolated from green algae, the arabinans from several *Codium* species were analysed. Anticoagulant mechanism proved to be by an HCII-dependent pathway with higher effectiveness than that of heparin or dermatan sulphate. It was suggested that binding of sulphated polysaccharides to HCII mainly induces conformational changes in the reactive site of this serpin, thereby optimising its interaction with thrombin, its target protease (Hayakawa et al., 2000).

On the contrary, proteoglycan from *Codium pugniformis* containing major amounts of glucose (72.4%), but also arabinose (17.3%) and galactose (10.0%), showed both direct and antithrombin mediated inhibition for thrombin activity, but it did not potentiate HCII (Matsubara et al., 2000). We speculate that the activity would be due to the arabinogalactan or to the arabinan moiety and that possibly glucose derives from contaminant glucans that were not eliminated by the fractionation procedure carried out.

The anticoagulant properties of extracts from *C. fragile* and *C. vermilara* were evaluated by general coagulation assays. All the fractions significantly prolonged APTT and TT regards to control saline solution, in dose-dependent

manner, being this effect higher in those with higher arabinose and sulphate content. As fibrinogen levels were in normal range and no effect was demonstrated by Reptilase® time, neither the fibrinogen molecule, nor the polymerisation process would be altered. Anticoagulant activity was intermediate between that of DS and heparin. Besides, several fractions induced platelet aggregation by dose-dependent manner. In order to understand a possible aggregation mechanism, the extracts were tested on thrombastenic platelets that have GPIIbIIIa deficiency. This glycoprotein is the main receptor necessary to provide adequate support for platelet aggregation. The GPIIbIIIa receptors bind fibrinogen and other adhesive proteins to form cross-bridges between adjacent platelets. As no induction of aggregation was observed, it was concluded that GPIIbIIIa was involved in the aggregation mechanism when the seaweed extracts were used as agonists (Ciancia et al., 2007).

The highly sulphated 3-linked β-arabinan (Ab1) obtained from green seaweed *C. vermilara* (Bryopsidales), comprising major amounts of units sulphated on C-2 and C-4, showed anticoagulant activity by global coagulation tests. Less sulphated arabinans with similar backbones have less or no activity. Ab1 exerts its activity through direct and indirect (antithrombin- and heparin cofactor II-mediated) inhibition of thrombin. Direct thrombin inhibition was studied in detail. By native PAGE, it was possible to detect formation of a complex between Ab1 and human thrombin (HT). Ab1 binding to HT was measured by fluorescence spectroscopy. CD spectra of the Ab1-complex suggested that ligand binding induced a small conformational change on HT. Ab1-thrombin interactions were studied by molecular dynamic simulations using the persulphated octasaccharide as model compound. Most carbohydrate-protein contacts were postulated to occur by interaction of sulphate groups with basic amino acid residues in the surface of the enzyme, being more than 60% of them performed by the exosite 2-composing residues. In these interactions, the sulphate groups on C-2 showed to interact more intensely with thrombin structure. In contrast, the disulphated oligosaccharide does not promote major conformational modifications at the catalytic site when complexed to exosite 1. This pyranosic sulphated arabinan exerts its anticoagulant activity by a mechanism different to that of heparin, which interacts with exosite 2 of thrombin, but it only inhibits the enzyme by an AT-dependent mechanism, meanwhile dermatan sulphate potentiates HCII without binding to thrombin. The unusual mechanism described for Ab1, direct thrombin inhibition by interaction with exosite 2 that alters active site without mediation of AT, could be of interest in the

context of development of antithrombotic strategies targeting thrombin (Fernández et al., 2013).

9.4.2 Antiangiogenic Activity

Angiogenesis, the formation of new blood vessels, plays an important role in physiological processes. In addition, angiogenesis is involved in several pathological conditions, including tumour growth and metastasis, atherosclerosis and diabetic retinopathy (Folkman, 1995). Inhibition of angiogenesis suppresses the tumour growth and metastasis.

It has been shown that fucoidans (Guerra Dore et al., 2013; Liu et al., 2012; Matou, Helley, Chabut, Bros, & Fischer, 2002; Pomin, 2012) and sulphated galactans from red seaweeds (De Souza et al., 2012) have antiangiogenic activity. However, to the best of our knowledge, only the anticoagulant sulphated galactan isolated from *Codium cylindricum* was investigated in this sense (Matsubara, Mori, Matsumoto, Hori, & Miyazawa, 2003). This galactan suppressed microvessel formation in an *ex vivo* serum-free matrix culture model using rat aortic ring. It also inhibited human umbilical vein endothelial cells (HUVEC) tube formation on reconstituted basement membrane gel. These effects were already evident at a concentration of 5 μg/ml of the polysaccharide. These results show the interest of further studies in this area of research.

9.4.3 Antiviral Activity

Several sulphated seaweed polysaccharides show antiviral activity against enveloped viruses, including important human patogens as Herpex simplex virus (HSV), human immunodeficiency virus (HIV), dengue virus and others (Damonte, Matulewicz, & Cerezo, 2004). Their antiviral activity appears to be based on the ability to interfere with the initial attachment of the virus to the target cell, blocking the viral entry. This activity was mainly reported for polysaccharides from red seaweeds (carrageenans, agarans and xylomannans) and brown seaweeds (fucoidans). In 1999, Lee, Hayashi, Maeda, & Hayashi, 2004 described the anti-HSV-1, anti-HCMV and anti–HIV-1 activity of a rhamnnan from the green seaweed *Monostroma latissimum*. Later, the same group assayed for anti-HSV-1 activity 11 polysaccharides from green algae, including two arabinans from *C. latum* and *C. fragile* and an arabinoxylogalactan from *C. adhaerens*. Although no detailed structural information about these polysaccharides was given, differences in antiviral potency among arabinans appear to be related with sulphate content. Arabinan from *C. latum*, as well as other compounds evaluated, showed the highest activity when added

to the medium at the time of viral infection, but it maintained its activity at high levels even when added to the medium 8 h post-infection.

Finally, a highly branched galactan from *C. fragile,* FG, inhibited the replication of HSV-2, possibly by interfering in the early steps such as virus adsorption and penetration into host cells. FG suppressed virus production most efficiently when added at the same time as virus infection and throughout the incubation thereafter. Pretreatment of host cells with FG showed no inhibitory effects, and less anti-HSV-2 effect was observed when added only during viral infection. FG maintained antiviral activity at higher levels when added to the medium even after 6 h of virus infection. In addition, FG was found to make the virion lose its infectivity. In contrast, FG did not show anti-influenza A virus effect (Ohta et al. 2009).

9.4.4 Immunostimulating Activity

Different cell types are involved in innate and adaptive immune responses. Among them, macrophages have a central role, as they act removing pathogens, presenting antigens to lymphocytes and releasing biologically active molecules that regulate other cell's activity (Leiro, Castro, Arranz, & Lamas, 2007). They also participate in inflammatory processes, promoting them, as well as releasing anti-inflammatory cytokines. Some sulphated polysaccharides are capable of mimicking the protein-binding characteristics of mammalian glycocongujates (Nika, Mulloy, Carpenter, & Gibbs, 2003), thus modifying the activity of macrophages and exerting immunomodulatory effects. Sulphated polysaccharides can also interact with several cytokines and exert direct or indirect modulating effects (Nika et al., 2003). In some cases, antitumour activity reported for seaweed polysaccharides was found to be associated with macrophages, by means of the release of certain cytokines such as tumour necrosis factor-α (TNF- α) (Leiro et al., 2007).

The same branched galactan from *C. fragile*, described above as having antiviral activity (Ohta et al., 2009), was found to stimulate the production of nitric oxide (NO) by activation of inducible nitric oxide syntase (iNOS), at the mRNA and protein levels (Lee, Ohta, Hayashi, & Hayashi, 2010). Nitric oxide may inhibit an early stage in viral replication, and thus prevent viral spreads. In addition, this galactan also enhances the production of both pro-inflammatory (IL-1, IL-6, IL-12 and TNF-α) and anti-inflammatory (IL-10) cytokines.

Crude cell walls, as well as galactose and mannose-rich fractions from *C. fragile*, were reported to improve macrophage cells proliferation (Tabarsa et al., 2013). Besides, they also stimulate cytokines (COX-2, IL-1β, IL-6,

IL-10 and TNF-α) and NO release by these cells. Increased levels of NO were related with enhanced mRNA and protein expression of iNOS in the presence of the algal polysaccharides. This stimulation effect on macrophages could be due to the activation of NF-κB and MAPK pathways.

Nika et al. (2003) assayed the binding of a sulphated arabinogalactan from *C. fragile* to IL-2, IL-7 and gamma interferon (INF- γ). This polysaccharide appears to bind cytokines to a similar extent as λ-, κ- and ι-carrageenans. Sulphated polysaccharides could be useful for cytokine-based therapies, as they help to concentrate cytokine molecules close to their site of secretion and also protect them against proteolytic degradation (Nika et al., 2003).

9.5 CONCLUSION

In summary, *Codium* species biosynthesise sulphated polysaccharides with very distinct structural features, which have been elucidated in recent years. Some of them have different biological activities with a great potential in pharmaceutical applications. However, a lot of work is still pending for the development of drugs suitable for use in human health

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