Overview of Anticoagulant Activity of Sulfated Polysaccharides from Seaweeds in Relation to their Structures, Focusing on those of Green Seaweeds

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Abstract: The anticoagulant behavior of sulfated polysaccharides from seaweeds is reviewed based on their chemical structures. Analysis of the literature suggested that the driving force for the formation of the sulfated polysaccharide/protein complex is the non-specific polar interaction between the negatively and positively charged groups in the polysaccharide and protein, respectively and that the complex is further stabilized 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 sulfated monosaccharide units with the highest potential for anticoagulant activity should have two sulfate 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-disulfated units, 4-linked 2,3-disulfated units and 2-linked 3,4-disulfated residues. These types of units have the possibility, through the equilibrium of the chair conformations, to place their sulfate groups in adequate spacial positions to interact with basic groups of the protein. The anticoagulant activity is mainly attributed to thrombin inhibition mediated by antithrombin and/or heparin cofactor II, with different effectivenesses depending of the compound. Other mechanisms are 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.

Keywords: Sulfated polysaccharides, anticoagulant activity, chemical structure, structure-activity relationship, green seaweed, disulfated structural units.

INTRODUCTION

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, [1], bleeding complications [2, 3], and so on. Furthermore, the incidence of prion-related diseases in mammals and the increasing requirements of anticoagulant therapy indicate that we may need to look for alternative sources of anticoagulant and antithrombotic compounds.

Most seaweeds synthesize sulfated polysaccharides, those from brown seaweeds (fucans and fucoidans) [4] and from red seaweeds (carrageenans, agarans and DL-hybrid galactans) [5] have been thoroughly studied. All of them show, in different degrees, biological activities [6]. Their antiviral and anticoagulant effects have been investigated in some detail [7-12], while others, as the antiangiogenic [13, 14] and immunomodulating effects [15, 16], are less known.

Marine macrophytes belonging to Chlorophyta (green seaweeds) are widely distributed in the coastal waters. Some of them are extremely invasive and proliferate in great amounts in eutrophicated lagoons [17, 18]. Utilization of this biomass could be based on specific properties of their sulfated polysaccharides, between others, their anticoagulant properties. There are, however, fewer reports of anticoagulants from green algae than from brown and red seaweeds and in most of the cases the structure of those products were not investigated [11, 12].

Identification of specific structural features of seaweed polysaccharides, necessary to evaluate their anticoagulant activities are limited by the fact that these products show, due to their biosynthetic pathways, the phenomenon of structural dispersion. This fact means that the molecules do not have a unique regular structural sequence, but that the samples are mixtures of similar, but not identical molecules and that the structures determined are only average ones.

In this review, attempts will be made, in spite of the scarce data, to identify the type of polysaccharides synthesized by green seaweeds, their gross structures and anticoagulant properties and the structural factors which determine them. Structural factors and mechanistic models obtained from nearby fields (mainly glycosaminoglycans and sulfated polysaccharides from brown and red seaweeds) will be used for a better understanding of the data produced with sulfated polysaccharides from green seaweeds.

Control of the Anticoagulant Activity

Even if a new, cell-based, model of hemostasis has been proposed in the last years [19], the coagulation "cascade" is still in use, as it models very well the screening coagulation

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laboratory tests, which have been used in the control of the anticoagulant properties of the sulfated seaweed polysaccharides.

Anticoagulant activity of the sulfated polysaccharides was usually tested by the general coagulation tests: prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and by assays of amydolytic activity of thrombin or factor Xa using chromogenic substrates [20]. Since these tests record interactions with different stages of the coagulation process, they provide information about the mode of action of the anticoagulants. The PT and APTT evaluate the extrinsic and the intrinsic coagulation pathway, respectively. The TT is the test for the last coagulation step, the thrombin-mediated fibrin formation. Amydolytic techniques are used to investigate direct or indirect inhibition of thrombin or factor Xa by anticoagulant compounds. Comparison of the results of tests obtained between different authors or with different products is sometimes difficult, as different ways to express the results are used. Sulfated polysaccharides exhibit anticoagulant effects in all of those assays, but display the highest activity in APTT and TT. Since PT is a technique that involves a very quick kinetics reaction, only very important anticoagulant effects can be detected by this method.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are long unbranched anionic polysaccharides consisting of repeating disaccharide units, formed by an uronic acid and a hexosamine which are differently *O*- and *N*-sulfated. GAGs bind to a wide variety of proteins and signaling molecules in the cellular environment and modulate their activity, thus impinging on fundamental biological processes. In particular, the anticoagulant activities of GAGs with important physiological role, such as heparin and dermatan sulfate will be discussed.

Heparin is a naturally occurring glycosaminoglycan that accelerates the inactivation of coagulation proteases. Heparin produces little anticoagulant effect directly. Rather, its action is mediated by several plasma proteins known as serpins (serine protease inhibitors). These serpins include antithrombin, usually known as antithrombin III, heparin cofactor II (HCII), and tissue factor pathway inhibitor. Antithrombin is considered to be the primary inhibitor of blood clotting proteinases and when antithrombin is bound to soluble heparin or to heparan sulfate in the vessel wall, it rapidly inhibits thrombin and the activated coagulation factors Xa and IXa, among others [21]. This accelerating activity results from a sequence-specific pentasaccharide region, present in about one-third of heparin polysaccharide chains, which binds antithrombin with high affinity and induces an activating conformational change in the inhibitor. This conformational change appears to be primarily responsible for heparin acceleration when factor Xa is the target proteinase [22, 23]. However, this change would not be critical for heparin acceleration when thrombin is the target proteinase, since the pentasaccharide has negligible activity to accelerate the inactivation of this enzyme by antithrombin. Instead, heparin rate enhancement of thrombin inhibition appears to be mostly due to a zone of the polysaccharide, greater than 18 saccharide units, bridging antithrombin and thrombin in a ternary complex, in which both the inhibitor and proteinase are bound nonspecifically to the same polysaccharide chain [24, 25]. This complex is assembled by electrostatic interactions between thrombin and 5-6 anionic residues contained in a hexasaccharide binding site of heparin [26].

The three-dimensional structure of heparin is complicated by the fact that iduronic acid units may be present in either of two low-energy conformations ($^2S_0$ and $^1C_4$), being this conformational equilibrium influenced by the sulfation state of the iduronate residue and that of the adjacent glucosamine [27]. Nevertheless, the solution structure of a dodecasaccharide composed solely of six [GlcNS(6S)→IdoA(2S)] repeat units has been determined [28]. Two models were constructed, one on which all IdoA (2S) were in the $^2S_0$ conformation and one in which they were in the $^1C_4$ conformation. Both models show extended helix conformations capable to pack by nesting; their rotation places cluster the sulfate groups at regular intervals of about 17 Å on either side of the helical axis.

Dermatan sulfate is a GAG that increases the rate of inhibition of thrombin by heparin cofactor II ~1,000 fold. Unlike antithrombin, which inhibits several serine proteases of the coagulation system, the known protease specificity of HCII is rectricted mainly to thrombin. Both dermatan sulfate and heparin enhance HCII anticoagulant activity, but, in comparison with antithrombin, HCII binds to heparin with a lower affinity. By contrast, dermatan sulfate highly increases the rate of inhibition of thrombin by HCII, but has no appreciable effect on the rate of inhibition of thrombin by antithrombin [29]. Although HCII interacts non-specifically with heparin oligosaccharides (20 monosaccharide units are required for optimal inhibition), it binds only to a small percentage of dermatan sulfate oligosaccharides $\geq$ 6 monosaccharide units in length, containing 4 or 6 sulfate groups, which are composed by three IdoA (2-sulfate)→GalNAc (4-sulfate) [30]. Dermatan sulfate obtained from pig skin contains an amount, not negligible, of IdoA→GalNAc (4,6-sulfate) which contribute to the activity expressed by the above major sequence [31]. It has been reported that dermatan sulfate activation of HCII would be an allosteric mechanism. Thus, binding of dermatan sulfate to HCII could induce a conformational change, with the displacement of the N-terminal acidic domain allowing it to interact with thrombin molecule [32]. Nevertheless, other researchers have proposed that thrombin – HCII reaction is also stimulated by a bridging mechanism, in which both proteins bind to a single chain of GAG, either heparin or dermatan sulfate [33].

GENERAL STRUCTURAL FACTORS DETERMINING ANTICOAGULANT ACTIVITY OF POLYSACCHARIDES

Several biological activities of GAGs and, in general, of sulfated polysaccharides are mediated by interactions with proteins. In particular, the anticoagulant properties of the majority of sulfated polysaccharides are dependent of non-specific binding zones which can be constituted by different sequences of monosaccharides and distribution of anionic groups. Those properties appear from a combination of different structural factors depending on: 1) The carbohydrate backbone, and 2) The anionic groups and their counter-ions.

The Carbohydrate Backbone

The change of conformation (random coil → ordered shape) of the binding zones makes the polysaccharide/protein complexes possible. It is influenced by the flexibility/rigidity balance and substitution of the binding zones of the polysaccharides. Molecular weight of the polysaccharide determines the probability of finding binding zones as a function of the length and composition of the polysaccharide chain, and those of binding zone. Polysaccharide/protein complexes can be stabilized through short-range interactions, as hydrophobic, hydrogen-bonding and Van der Waals forces.

Glycosidic Linkage

In solution, the carbohydrate chain is fluctuating continuously between different local and overall conformations, generating what is known as a random coil. For a chain with a regular sequence of structural units, the interaction energies between consecutive residues determine the ordering of the chain. However, the characteristics that determine the shape (conformation) of an ordered chain are the geometrical relationships (type of cycle and linkages) within each structural unit. The isolated ordered chain is usually unstable and in equilibrium with the random coil, and it is stabilized building up tertiary or quaternary structures (i.e double helices in the first case or complexes with proteins in the second one) [34-36].

Thus, β-(1*e*→4*e*)-linked xylans/glucans with sugar units in a $^4C_1$ conformation give rise, when ordered, to a ribbon-like conformation, while β-(1*e*→3*e*)-linked galactans/glucans or α-(1*a*→4*e*)-linked glucans in the same conditions produce helices. In both cases, the ordered molecules are extended and aligned and can easily get in contact with other molecules. This is not obtained easily with molecules of the "crumpled" family (i.e. β-(1→2)-glucans), or those with (1→6)-linked backbones, in which the extra freedom of rotation of the (1→6)-linkage makes ordered conformations more difficult [36]. Nevertheless, heavily sulfated, flexible, dextran molecules (α-(1→6)-linked glucans, DS 2.3), in low ionic strength solutions, would be fully extended due to the repulsion of the charges of the negative sulfate groups [37]; this repulsion of the negative charges in the same sugar unit would invert the pyranose cycle to a $^1C_4$ conformation placing the sulfate groups in trans axial positions. Both conditions would be adequate to produce binding zones able to contact easily other molecules as in the examples given above.

Length and Structure of Binding Zones

15 oversulfated oligosaccharides with different carbohydrate backbones were assessed for anticoagulant activity (APTT, Table **1**) [38]. The role of the oligosaccharide chain length is apparently determinant, as shown by the finding that chain elongation by a single monosaccharide residue from oversulfated maltotetraose to maltopentaose, resulted in a 19-fold increment in the anticoagulant activity; a further increase in chain length did not produce a significant change in the anticoagulant effect. This result provides evidence of a threshold effect, where a minimum chain length is required for the activity, rather than a consecutive increase in the activity occurring with chain elongation.

On the other hand, the importance of monosaccharide composition is highlighted by the fact that sulfated chitosan hexamer, composed by β-(1→4)-linked glucosamine residues, exhibits negligible activity compared with the β-(1→4)-linked cellohexaose sulfate [38].

Table 1. Anticoagulant Activity of Different Sulfated Oligosaccharides as Measured by the APTT test[a]

Compound	Monosaccharide constituents	Linkage	Degree of sulfation[b]	APTT[e]
Unfractionated heparin	GlcA, IdoA, GlcN	β(1→4), α(1→4)	2.1-2.4/5[c]	29.69
LMWH	GlcA, IdoA, GlcN	β(1→4), α(1→4)	2.4-2.6/5[d]	11.20
Maltose sulfate	Glc	α(1→4)	6/8	0.31
Maltotriose sulfate	Glc	α(1→4)	9/10	0.25
Maltotetraose sulfate	Glc	α(1→4)	11/14	0.28
Maltopentaose sulfate	Glc	α(1→4)	9/17	5.25
Maltohexaose sulfate	Glc	α(1→4)	16.6/20	5.85
Maltoheptaose sulfate	Glc	α(1→4)	n.d.	5.9
Isomaltopentaose sulfate	Glc	α(1→6)	14/17	1.85
Isomaltohexaose sulfate	Glc	α(1→6)	12.8/20	6.56
Cellotetraose sulfate	Glc	β(1→4)	n.d.	4.2
Cellopentaose sulfate	Glc	β(1→4)	n.d.	3.5
Cellohexaose sulfate	Glc	β(1→4)	n.d.	4.8
Laminaritetraose sulfate	Glc	β(1→3)	n.d.	2.9
Laminarihexaose sulfate	Glc	β(1→3)	n.d.	0.9
Laminariheptaose sulfate	Glc	β(1→3)	n.d.	0.5
Chitosan hexamer sulfate	GlcNAc	β(1→4)	n.d.	0.4

[a]From Wall *et al*. 2001 [38]. [b]This is the actual number of sulfate groups attached/theoretical maximum number of sulfate groups that can be coupled to each molecule. In cases where degree of sulfation was not determined (n.d.), oligosaccharides were sulfated under conditions that resulted in maximum sulfation. [c]Volpi 1993 [39]. [d]Guerrini *et al*. 2007 [40]. [e]Data were expressed as increase in APTT (sec.) resulting from a 1mg/ml increase in concentration of the compound.

The series of different sulfated gluco-oligosaccharides emphasizes the major influence of the monosaccharide glycosidic linkage on anticoagulant activity. Thus, the sulfated laminaran series, constituted by β-(1→3)-linked glucose units exhibits much lower activity compared to the sulfated α-(1→4), α-(1→6), and β-(1→4)-linked glucose-containing oligosaccharides. Sulfated alkyl laminaran oligosaccharides have also exhibited little or no antocoagulant activity [41].

Hydrophobic Zones

Data on the influence of hydrophobic pockets on the anticoagulant properties of sulfated polysaccharides is very scarce. It was reported that the anticoagulant ability is higher for derivatives of dextran sulfate bearing benzamide groups [42]. Also, that the activity of chitosan sulfate was improved by *N*-propionyl and *N*-hexanoyl substituents [43]. Nevertheless, the interaction of heparin with different peptides, suggested that, while some hydrophobic bonds are formed upon heparin binding, the majority of the binding energy is not due to hydrophobic interactions [44].

Carbohydrate Backbone as a "Carrier" and "Positioner" of Sulfate Groups and Hydrophobic Sites

Ionic interactions between the negative charges of sulfate groups of a polysaccharide and the positive charges of basic amino acids in the target proteins, as well as any other type of short range interactions (van der Waals forces, hydrogen bonds, hydrophobic forces, etc.) imply the close approach of zones of the polysaccharide to the binding sites in the target protein and the positioning of their complementary functional groups for the intermolecular interactions to be built up. These interactions between complementary groups are increased if binding zones of the sulfated polysaccharide not only approach to the target protein surface, but adapt to it and produce in it also a complementary conformational adaptation. The adaption was described in the interaction between fibroblast growth factor and heparan sulfate when, even if the overall helical structure of the heparan sulfate is mantained, changes in the backbone torsion angles of the oligosaccharide binding site are induced upon protein binding. These changes result in a local deviation in the helical axis that provide optimal ionic and Van der Waals contact with the protein [45]. In this sense, it has been suggested [46] that the conformational flexibility of the ordered binding region plays an important role in the biological properties of sulfated polysaccharides. Examination of the structures of active compounds suggests that all of them are random coils at room temperature, but can adopt ordered flexible forms (ribbon, helix) under compelling circumstances (*i.e.* formation of the polysaccharide/protein complexes). The complexation of fucoidan fractions by Ca^{2+} [47] and the interactions between mu/nu- and lambda-carrageenans [48] are other examples of complexation of sulfated polysaccharides.

The Anionic Groups and their Counter-Ions

Carboxyl and/or sulfate are the anionic groups usually present. Besides, divalent cations can play a role in competitive complexation.

Most of the polysaccharides with anticoagulant properties carry sulfate as anionic groups. The presence of these groups is an essential requirement for the anticoagulant activity, since desulfated products loose their activity. Usually, increasing the degree of sulfation is beneficial for the activity [49]. Thus, a fucan, consisting in a backbone of (1→3)-linked α-L-fucose with sulfate groups on C-4, showed potent anticoagulant activity. Solvolytic desulfation indicated that only fucans with a ratio sulfate/total sugar residues greater than one displayed significant anticoagulant activity [50]. On the other hand, oversulfation of the same fucan increased the APTT by 10-20% and the TT by 8-40% [51]. The anticoagulant activity of a pullulan sulfate, measured as TT, dramatically increased up to a level similar to that of heparin when the DS changed from 0.47 to 1.80 [49]. However, this is not the only requirement, as for highly sulfated dermatan sulfates with different sulfation patterns, very different activities were found [52].

Although, it is usually accepted that high anticoagulant activities are associated to high sulfate content, examination of a few examples shows that this straightforward correlation is not always maintained. Thus, κ/ι- and μ/ν-carrageenans with 32 % and 33 % sulfate (as SO_3 Na), respectively showed TT values that doubled the control only at 200 μg/ml concentration [53], while the "proteoglycan" isolated from *Codium fragile* ssp. *atlanticum* (18 % sulfate) showed TT values 10 times higher than the control, at 15.65 μg/ml. However, another sulfated polysaccharide from the same seaweed with 10.2% sulfate needed a concentration of 125 μg/ml to produce the same result and a third sulfated polysaccharide with even less sulfate content (7.5 % sulfate) showed at 1,000 μg/ml concentration a TT only 5.55 times of the control [54], showing clearly the influence of the sulfate content for similar backbone structures. On the other hand, fractions of a sulfated arabinan and a sulfated arabinogalactan from other seaweed of the same genus with 41.45% and 31.85% sulfate, showed TT values 10 times higher than the control at 5 μg/ml and 10 μg/ml, respectively [55].

A minimum charge density is essential for any anticoagulant effect, but it should be taken into account that, for similar charge density, another essential factor is the chain length. Thus, linear short chain β-(1→3)-glucan sulfates require DS higher than 0.7 to give any activity. In contrast with this result, the same kind of products, but with high molecular weight were active with DS as low as 0.5 [56]. The fact that there are many factors that affect the anticoagulant behaviour of these compounds and, in most of the studies, only a few are analized, makes the comparison of data from different sources difficult.

The other anionic group present in GAGs is the carboxyl of the uronic acid residues, but it would not have an important role in the anticoagulant activity. Nevertheless, it has been claimed that carboxyl and sulfate groups have synergistic action [57]. Thus, inhibition of thrombin activity by sulfated chitin was increased considerably by the introduction of carboxyl groups in the molecule. The highest degree of inhibition was given by a sulfated, carboxylated chitin in which the levels of both anionic substituents were equal [58]. Nevertheless, in the case of fucoidans and fucans, where both sulfate groups and uronic acids are present, to the best of our knowledge, there are no data suggesting that the carboxyl groups from the uronic acids have any role in the production of anticoagulant compounds.

In crude fucoidan fractions, the main counter-ions are usually Ca^{2+} or Mg^{2+}, either due to the tendency of the seaweeds to concentrate divalent cations from sea water, or to purification procedures involving treatment with calcium chloride solutions in an effort to eliminate possible alginic acid contaminations. These divalent cations could produce polysaccharide complexes by linking anionic groups (carboxylate and sulfate) and hydroxyl groups from different molecules [47], altering some properties as apparent molecular weight, three dimensional shapes, etc. Formation of these complexes explains why in these cases Ca^{2+} could not be exchanged by Na^{+}, even in the hardest conditions and that the molecular weight of the complexes was much higher than those determined by chemical methods [47] masking the actual anticoagulant properties of these compounds.

These results show that the anticoagulant activity of sulfated polysaccharides is not merely a consequence of their charge density and/or sulfate content, but that the structural requirements for interaction of the polysaccharide with coagulation cofactors and their target proteases involves some degree of specificity.

Effect of Oversulfation on the Conformation of the Sugar Units in the Polysaccharide

Oversulfation of neutral polysaccharides with no biological activities transforms them into anticoagulants [34, 56] or, in the cases of naturally sulfated polysaccharides, like dermatan sulfate [59] and chondroitin sulfate [60], change their anticoagulant properties. The appearance or change of anticoagulant activity with the increase of sulfate content was explained by the possibility of higher sulfation of the polysaccharide to produce new sulfate distribution patterns [59]. It has been suggested that biospecificity can be achieved by random substitution with the sulfate groups [35]. Such oversulfated polymers would contain arrangements of groups which mimic natural biospecific sites and the probability of occurrence of such arrangements would depend of the average degree of substitution (% of sulfate groups) of the polymer [35].

An additional explanation for this effect, as shown for different oversulfated polysaccharides [34, 61], is that the overcrowding of sulfate groups on the monosaccharide structural unit, especially if the sulfates are adjacent equatorial groups, changes the conformation of the pyranose ring ($^4C_1 \rightarrow {}^1C_4$) in an attempt to set the negative charges as apart as possible [61] (Fig. (**1A**)). This conformational change produces a transformation in the geometrical disposition of the glycosidic linkage and, as a consequence, in the conformation of the polysaccharide (Fig. (**1B**)). The change from equatorial to axial of the charged groups, that produces a change in distance and position, would influence their interaction with the amino groups in the basic amino acids of the target protein and, consequently, the formation of the polysaccharide-protein complex.

Full chemical sulfation of neutral polysaccharides [β-(1→4)-xylan, β-(1→4)-glucan (cellulose), α-(1→4)-glucan (amylose), β-(1→3)-glucan (curdlan)] gives rise to anticoagulants. A sulfated β-(1→3)-galactan was also oversulfated

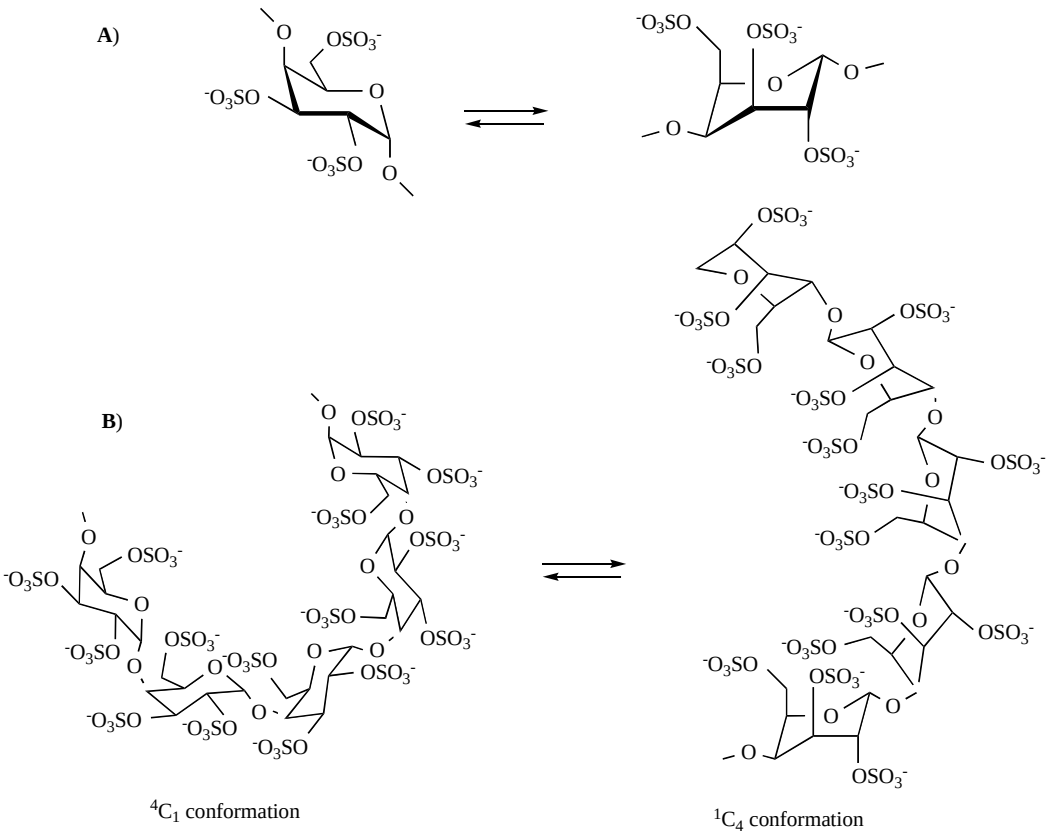

Fig. (1). A) Equilibrium between $^4C_1$ and $^1C_4$ chair conformations of an oversulfated (1→4)-α-D-galactose unit in a galactan backbone. **B**) Change in the galactan backbone shape with the inversion of the configuration of the pyranose cycle of the sugar unit.

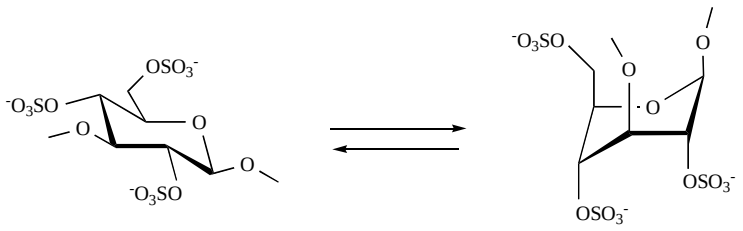

Fig. (2). Equilibrium between $^4C_1$ and $^1C_4$ chair conformations in an oversulfated curdlan: β-D-glucopyranose 2,4,6-trisulfate units.

producing a derivative with little anticoagulant activity, which was attributed to its low molecular weight (8.3 kDa against 16.0-39.0 kDa of the other polysaccharides) [34]. In both (1→4)- and (1→3)-linked polysaccharides, the unit conformation changed from $^4C_1$ to $^1C_4$ with full sulfation.

6-Desulfation of the (1→4)-linked polysaccharides did not change significantly the activity, while in the case of curdlan, a (1→3)-glucan, ~ 41-57% of the anti-thrombin activity was significantly lowered. The loss of anticoagulant activity with the C-6 desulfation in the above (1→3)-linked polysaccharides could be determined by the return to the $^4C_1$ conformation of the structural units after elimination the interactions between the sulfate groups on C-4 and C-6 (Fig. (**2**)).

Based on these results, it is emerging that a couple of neighboring sulfate groups, such as those at C-2/C-3 or C-4/C-6 might be required for the anticoagulant activity.

In conclusion, the presence of adjacent sulfates at C-2 and C-3 would be a requirement to inhibit thrombin in (1→4)-linked glycans, while the contribution of the sulfate group on C-6 might be important to increase the activity of the (1→3)-linked polysaccharides disulfated on C-2 and C-4. The conformational flipping of core sugar residues ($^4C_1 \rightarrow {}^1C_4$) caused by the negative charges of the sulfate groups, which change from equatorial to axial positions, was observed for different persulfated polysaccharides, including fully sulfated GAGs. These conformational changes produce a substantial increase in anti-thrombin activity.

However, it is important to note that the repulsion between neighbour sulfate groups in the same unit is not the only factor determining this activity, as shown by the fact that a curdlan sulfated only in positions 2 and 4, with interactions between the equatorial sulfate groups on C-2 of two consecutive units or between C-2 and C-4 of two consecutive units, which restrict the flexibility of the glycosidic likage, showed an important anticoagulant activity [34].

SEAWEEDS SULFATED POLYSACCHARIDES AS ANTICOAGULANTS

A better comprehension of the anticoagulant activity/structure relationship of sulfated polysaccharides from brown and red seaweeds could be of great help to understand this relationship in the less studied polysaccharides of green seaweeds. For these reasons, selected examples of the former were chosen without any pretension of being exhaustive. Another reason to use this material as background to study the anticoagulant activity of green seaweeds is that all of them are seaweed polysaccharides and, as a consequence, according to their biosynthetic pathway, they all show the phenomenon of compositional and structural dispersion, which is not found in polysaccharides from animal tissue [62]. The actual structures of the polysaccharides from seaweeds are usually more complex than those depicted here and simplification of some fine structural details was carried out in order to focus on those factors that possibly determine the anticoagulant properties.

Sulfated polysaccharides from other sources, like marine invertebrates, have been studied from the structural and biological points of views and the results have been the motive of excellent reviews [63,64]. Thus, these results together with those obtained from synthetic sulfated polysaccharides, will be mentioned only when they reinforce those that are the subject of study in this review.

Red Seaweeds: Sulfated Galactans

These polysaccharides have structures based on linear chains of alternating 3-linked β-D-galactopyranosyl residues (A-units) and 4-linked α-galactopyranosyl residues (B-units). The B-units may include residues of the D- and the L-series, many times occurring as 3,6-anhydrogalactopyranosyl moieties. A first classification is made according to whether the configuration of the B-unit is D or L, in the first case they are called carrageenans and in the second, agarans. Carrageenans and agarans will be treated separately for matters of clarity and use, but comparisons between them can be made, as data available about biological activity of these compounds [7] suggest that the configuration of the B-units does not influence the anticoagulant activities. The existence of a third group in which B-units with the D- and L-configurations are interspersed on the same molecule (DL-galactan hybrids) is under discussion [5], but considering the above statement, they would not differ in anticoagulant properties from the other sulfated galactans with similar substitution patterns.

When the B-units in the backbone of these "mixed linkage" galactans are cyclized as 3,6-anhydro-derivatives ($^4C_1 \rightarrow {}^1C_4$), the ordered conformation of the galactans (agarose, κ- and ι-carrageenans) is a helix, but if these units are non-cyclized galactoses ($^4C_1$) (agarans, λ-carrageenans), the conformation is a flat ribbon [66] (Table **2**). The freedom of rotation between adjacent sugar residues in the polysaccharide chains increases, together with the difficulty to acquire an ordered conformation, in the sequence λ-, ι- and κ-carrageenans [66] concomitant with the decrease of the anticoagulant activity.

Carrageenans

Different carrageenans originate on their sulfation pattern and/or the appearance of B- units as 3,6-anhydrogalactose

Table 2. Chemical Analysis and Anticoagulant Activity of Carrageenans Extracted from *Gigartina skottsbergii,* their Cyclized Derivatives and Carrageenans from other Sources

Compound	Carrageenan type	B[b]	Sulfate as SO_3Na	Molecular Weight	TT[c]				
					Concentration (μg/ml)				
			%	KDa	0.5	5	50	100	200
$1C_1$[a]	κ/ι	0.100	33.1	75	1.0	1.0	1.5	1.5	2.0
$1C_2$[a]	κ/ι	0.100	31.4	124	1.0	1.0	1.5	1.7	2.1
$1C_3$[a]	Partially cyclized μ/ν		33.6	198	1.0	1.0	1.7	2.0	2.4
$1C_1T$[a]	κ/ι	0.100	34.5	73	1.0	1.0	1.3	1.3	1.2
$1C_2T$[a]	κ/ι	0.100	35.3	18	1.0	1.0	1.0	1.0	1.0
$1C_3T$[a]			31.4	112	1.0	1.0	1.6	1.0	1.0
1T[a]	λ	0.053	38.5	n.d.[d]	1.3	1.4	3.6	>11.5	>11.5
$1T_1$[a]	λ	0.053	40.0	83	1.3	2.2	>11.5	>11.5	n.d.
$1T_2$[a]	λ	0.053	39.2	121	1.4	2.1	>11.5	>11.5	n.d.
λ-carrageenan[e]	λ	0.053			1.7	2.4	>11.5	n.d.	n.d.
F_2[f]	λ-type		48.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
F_3[f]	λ-type		56.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$1T_1T_1$[a]	cyclized λ	0.083	35.9	66	1.1	1.1	1.2	2.0	2.2
$1T_2T_1$[a]	cyclized λ	0.083	35.8	17	1.0	1.0	1.9	1.3	1.5
DS8000	-			8	1.2	1.5	1.9	3.0	>11.5
DS500000	-			500	2.3	>11.5	>11.5	n.d.	n.d.
heparin	-		36		>11.5	>11.5	n.d.	n.d.	n.d.

[a]From *Gigartina skottsbergii* [53]. [b]B=flexibility parameter [66]. [c]TT ratio relative to control sample without compound. [d]n.d.= not determined. [e]Data extracted from a plot [67]. [f]Fractions obtained from *Botryocladia oxidentalis*, APTT ratio ~ 4.55 for a concentration of 0.1 μg/ml [69].

derivatives [5], that give important differences in their biological properties.

Taking into account the sulfation pattern of the A-units, they are grouped into families, each of them comprising different idealized structures:

The κ-family comprises 4-sulfated β-D-galactose units in polysaccharides built up from the structural unit [→3)-β-D-Gal*p* 4-sulfate-(1→4)-α-D-Gal*p*-(1→]. Four "idealized" carrageenans belong to this family: κ-, ι-, μ- and ν-carrageenans (Fig. (**3**)).

The λ-family includes the polysaccharides with 2-sulfated β-D-galactose units, built up from the structural unit [→3)-β-D-Gal*p* 2-sulfate-(1→4)-α-D-Gal*p* 2,6-disulfate-(1→] and its B-cyclized derivative (Fig. (**4**)).

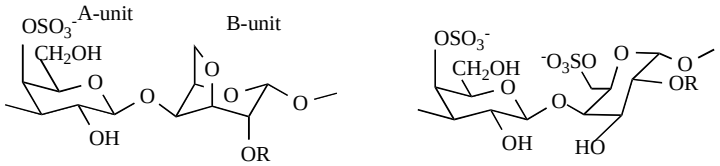

Fig. (3). Idealized structures of carrageenans of the κ-family.

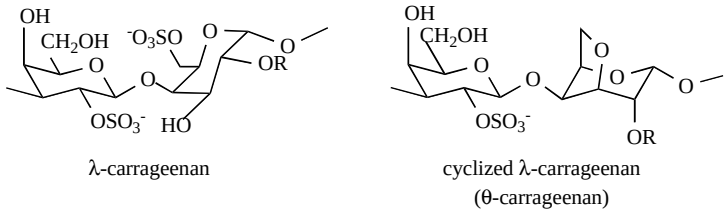

Fig. (4). Idealized structures of carrageenans of the λ-family.

Only few members of the β- and ω-families, which comprise carrageenans with nonsulfated or 6-sulfated A-units, respectively have been studied and, to the best of our knowledge, no determinations of anticoagulant properties have been carried out. They will not be treated any further.

Carrageenan molecules, as those of the other galactans, in solutions at low concentrations, may be considered as isolated, without intermolecular interactions, and in a conformational equilibrium determined by a balance of the entropy (favoring random coil conformations) and energy (favoring ordered conformations) terms of their free energy:

$$\text{Random coil} \rightleftharpoons \text{ordered chain conformation} \quad (1)$$

These molecules have a strong tendency of the random coil to stretch out due to the repulsion of the negative charges of the sulfate groups, and forming helices or ribbon shapes [36, 66] when the ordered conformation is stabilized through intra and intermolecular interactions.

Actually, in most "natural" carrageenans sequences with the above "ideal" repeating structures are separated by regions having different types of regularity or no regularity at all (see later). "Natural" carrageenans are, then, hybrids containing in the same molecule, regions formed by sequences of different "ideal" structures. The so called κ/ι-hibrid carrageenans are the most usual ones [5].

Besides, in the "natural" κ- or ι-carrageenan blocks, the alternating A-B sequence is interrupted from time to time by disaccharide units containing α-D-galactopyranosyl 6-sulfate or α-D-galactopyranosyl 2,6-disulfate residues replacing 3,6-anhydro-α-D-galactopyranosyl- or 3,6-anhydro-α-D-galactopyranosyl 2-sulfate units, respectively. This has profound implications for the overall shape of the molecule, as it produces the replacement of a unit in the $^1C_4$ conformation linked by two equatorial bonds (3,6-AnGal) by one which is in the $^4C_1$ conformation and linked by two axial bonds (α-D-Gal). This replacement introduces a kink into the regular helical sequence and the molecule should be seen as having regions of regular sequences separated by other units or sequences acting as kinks.

In λ-carrageenan molecules the equilibrium (1) is more displaced to an ordered flat ribbon conformation because of the stabilization of the linkage conformation by intramolecular interactions (hydrogen bond between sulfate on C-2 of the A-unit and hydroxyl group on C-3 of the B-unit [65] and restriction of the glycosidic linkage flexibility by the steric interaction of the sulfate equatorial groups on C-2 of adjacent units [36]. Cyclization of the α-D-galactose 2,6-disulfate units produces θ-carrageenans (Fig. (**4**)) with all the conformational characteristics discussed for ι-carrageenan (Fig. (**3**)), but with a less flexible chain (Table **2**).

Members of the κ-family have minor anticoagulant properties, in spite of their high content of sulfate groups (~33 % as SO_3Na) (Table **2**) [53, 67, 68]. Only λ-carrageenans (38.5-40.0 % sulfate) showed significant activity (Table **2**), which cannot be explained only by the increase of sulfate content, almost 20 % more than in κ-carrageenans, and similar to that of dextran sulfate (MW $5x10^5$) and heparin (Table **2**). This activity decreases after cyclization of the B-unit forming θ-carrageenans, which is in the order of those of the κ/ι-carrageenans and of their completely cyclized derivatives (Table **2**) indicating that the loss of the sulfate group on C-6 of the B-unit or the conversion of a rather rigid, ribbon conformation to a more flexible helix was deleterious for the anticoagulant behavior. The knowledge that the anticoagulant activity is not affected by the presence or absence of the 6-sulfate in (1→4)-linked polysaccharides [34] favors the latter hypothesis.

A linear polysaccharide isolated from a sea urchin, composed of 3-linked α-L-galactose 2-sulfate units, similar to those of a λ-carrageenan, showed an anticoagulant activity of 10 units/mg, while under the same conditions (APTT test), a λ-carrageenan has an activity corresponding to 77 units/mg [69] suggesting that a λ-disaccharide structure is necessary as "minimal binding structure". The pattern of sulfation [sulfate on C-2 of the A-unit → sulfate on C-2 (and C-6) of the B-unit] on a backbone with restrained A→B flexibility that facilitates the ordered conformation, would be the structural basis for anticoagulant activity of λ-carrageenans.

Carrageenans of the λ-family isolated from the carrageenophyte *Botryocladia occidentalis,* showed very high anticoagulant properties [69]. Sulfation of the B-units was studied, showing that 1/3 were 2,3-disulfated, while another 1/3 of them were 2-sulfated and the rest of the units were not sulfated. Reexamination of the ^{13}C NMR spectra suggested high sulfate substitution at C-2 of the A-units. If this is true, this carrageenan would belong to the λ-family with C-3 sulfation instead of C-6 sulfation on the B-units. Sulfation on C-2 and C-3 of the B-units would produce an inversion of the chair conformation of the pyranose cycle ($^4C_1 \rightarrow {}^1C_4$) (Fig. (**5**)) in an attempt to place the negative charges of the sulfate groups as far as possible, while the 2-sulfated and the non-sulfate B-units would remain in the more stable $^4C_1$ form. Moreover, the $^4C_1$ conformation would be destabilized by the interactions between substituents on $C\text{-}2_e$ and $C\text{-}3'_e$ and $C\text{-}2_e$ and $C\text{-}2'_e$ of neighboring units, while with the change of $^4C_1 \rightarrow {}^1C_4$ conformation, these interactions would be relieved.

Two extreme conformations can be envisaged for these molecules, namely: a) a block "copolymer" with blocks of disaccharidic units having disulfated B-units with helicoidal shape, together with blocks of disaccharidic units with mono- or non-sulfated B-units having a ribbon form and b) a random coil-like conformation constituted by disaccharidic units having disulfated B-units interspersed by one less sulfated disaccharide. Somewhat in between these extremes,

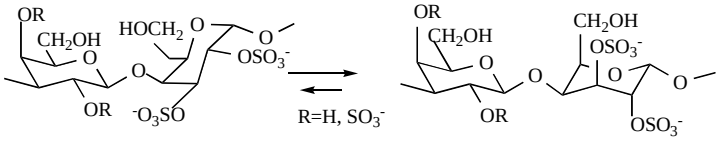

Fig. (5). Sulfated galactan from *Botryocladia occidentalis*. Conformational equilibrium of the 4-linked 2,3-disulfated α-D-galactose units.

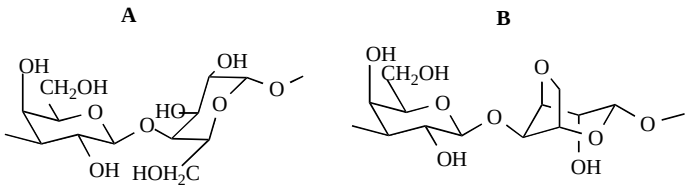

Fig. (6). Structure of **A**) the basic backbone of agarans and **B**) agarose.

helical blocks with the axial sulfates protruding from the helix and flat ribbon zones with equatorial sulfates, would justify the strong anticoagulant activity of these polysaccharides

Agarans

Due to the complexity and diversity of substitution patterns, it has not been possible to classify agarans into "ideal structures" as neatly as carrageenans. Actually, only the term agarose has strict chemical sense, whereas many other polysaccharides of the group are usually termed according to the algal species from which they were isolated. All the agarans can be formally obtained by substitution with groups as sulfate, pyruvic acid ketal, methyl and single monosaccharide side chains, of two extreme structures: agarose and the "basic backbone", being the latter precursor of the former through C-6 sulfation of the B-unit and further cyclization of this unit by a specific enzyme (sulfate eliminase) (Fig. (**6**)).

A fraction (F_2) of the sulfated galactan isolated from the agarophyte *Gelidium crinale* with high APTT at very low concentrations (0.1-0.4 μg/ml) (Table **3**), studied by ^{1}H and ^{13}C NMR spectroscopy of the product and of its desulfated derivative, showed signals corresponding to an alternate structure [→3)-β-D-Gal*p*-(1→4)-α-L-Gal*p*-(1→] corresponding to an agaran [70]. The α-L-galactose moieties are non-sulfated, 2-sulfated or 2,3-disulfated in a molar ratio 0.40:0-45:0.15 (^{1}H NMR spectrum), while a reinterpretation of the NMR spectra suggests that major amounts of the A-units have no substitutions and only minor percentages are 2-sulfated [70]. The agaran does not contain 3,6-anhydro-α-L-galactose ($^{1}C_4$ B-units) and its ordered backbone when stabilized, would be a ribbon conformation. The only possible interruption in the regularity of the chain is that the α-L-galactose 2,3-disulfate residues change their $^{4}C_1$ chair conformation with equatorial sulfate groups into a $^{1}C_4$ (trans diaxial sulfates) (Fig. (**7**)) in an attempt to avoid the repulsion of the 2,3-equatorial sulfate groups.

Extracts from *Acanthophora spicifera* have low anticoagulant activity [71]. Structural studies [72] indicated that the extract contained a sulfated agaran (12.8-26.6% sulfate) made up of A-units highly substituted with sulfate groups on C-2 (28-30%), some of them containing also 4,6-*O*-(1′- carboxyethylidene) groups (9-15%) and unsubstituted residues. B-units were formed mainly by 3,6-anhydro-L-galactose (15-16%) and its precursor α-L-galactose 6-sulfate (10-17%), together with minor amounts of other units. Agarose has, as κ- and ι-carrageenans, a helical ordered conformation but with inverted chain sense. When the 3,6-anhydro-α-L-galactopyranose unit ($^{1}C_4$ conformation) is replaced by the α-L-Gal*p* residue ($^{4}C_1$ conformation), the ordered conformation of the backbone changes to a ribbon one. Like carrageenans, the conformations of sulfated agaroses, in low concentrations, would be in equilibrium:

$$\text{Random coil} \rightleftharpoons \text{ordered chain conformation} \quad (1)$$

in which the random coil would be stretched by the repulsion of the sulfate groups.

The disaccharide structural units of sulfated agaran contain both types of B-units: the 3,6-anhydro-α-L-Gal*p* ($^{1}C_4$) and the α-L-Gal*p* ($^{4}C_1$), together with different A-units sulfated on C-2. All the evidence suggests that the different disaccharides formed by these B-units are interspersed in the backbone forming regions with different types of regularity or no regularity at all. The consequence would be that the conformation of the chain would tend to stretch random coils with difficulties to produce an ordered form, and the small amount of single stubs of β-D-xylose would also contribute to disorganize these chains. This is consistent with their minor anticoagulant activities as measured by the TT and APTT tests (Table **3**).

Comparison of the different disaccharide structural units of the galactans from red seaweeds (Fig. (**8**)) suggests that the structural factors responsible for strong anticoagulant properties are: a) accumulation of equatorial sulfate groups on vicinal carbon atoms (C-2 and C-3) in the $^{4}C_1$ conformation of the B-units, which produces inversion of the chair conformation to place the sulfate groups in axial positions, b) restrinction of the A→B flexibility by the interaction between equatorial groups on C-2 of two neighbor units and between C-2 and C-3 of two neighbor units, and c) the concomitant change of an ordered backbone shape from ribbon to extended helix.

Binding Zone of Galactans

If the backbone of galactans from *B. occidentalis* and *G. crinale* are built up by the statistical distribution of the struc-

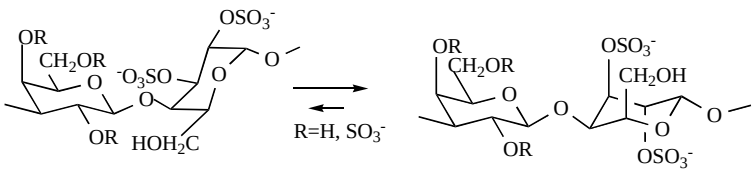

Fig. (7). Sulfated galactan from *Gelidium crinale*. Conformational equilibrium of the 4-linked 2,3-disulfated α-L-galactose units.

Table 3. Chemical Analysis and Anticoagulant Activity of Agaran Fractions from *Acanthophora spicifera*[a,b]

Fraction	Carbohydrate %	Sulfate as $NaSO_3$ %	Pyruvic acid %	Monosaccharide composition (mol %)					APTT/TT[c] Concentration (μg/ml)		
				Gal	AnGal	6-Gal	2-Gal	Xyl	250	500	1000
H-2	44.5	18.2	2.6	65.9	21.9	3.6	3.9	4.8	1.2/1.2	1.5/1.3	2.0/1.5
H-3	56.0	24.9	2.4	75.2	17.2	1.4	1.4	4.8	2.0/1.3	2.1/1.4	> 3.8/1.5
H-4	56.0	26.4	0.6	80.5	10.8	1.7	1.8	5.2	2.0/1.5	2.7/1.7	> 3.8/1.8
H-5	38.3	19.2	nd	64.5	9.5	4.4	5.1	5.9	2.4/1.5	3.2/1.8	> 3.8/2.1
H-6	30.0	17.2	1.0	73.3	11.9	2.6	2.1	5.8	1.6/1.4	2.0/1.4	2.6/1.5
H-7	37.0	4.7	nd	50.4	11.6	7.7	5.6	10.2	1.2/1.2	1.2/1.3	1.4/1.3

[a]APTT for heparin (5 μg/ml): >120 s. TT for heparin (5 μg/ml) : > 100 s [72]. [b]APTT ratios for a galactan from *Gelidium crinale*: ~ 2.5, 4.2, 6.0 and 7.5 for 0.1, 0.2, 0.3, and 0.4 μg/ml, respectively [70]. [c]APTT and TT ratios relative to control sample without agaran.

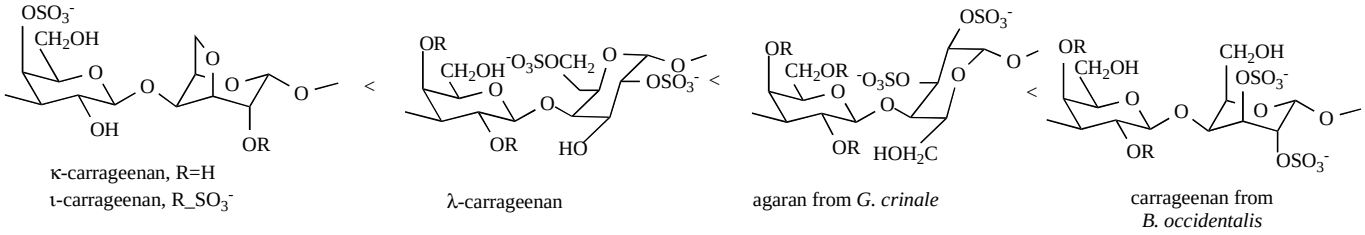

Fig. (8). Comparison of the galactan structures with increasing anticoagulant activity.

tural units, the disaccharide containing disulfated B-unit would appear one time each three and seven disaccharide residues, respectively. The probability of finding, in the first case, one disaccharide with a B-disufated unit is about 33%, of two clustered disaccharides with B-disulfated units is 11% and of three of them is 4%. In the case of the galactan from *G. crinale*, the probabilities of the di-, tetra- and hexasaccharide of the above type are 15%, 2% and 0.3%, respectively. Nevertheless, these sulfated galactans have a considerable anticoagulant activity, measured as APTT, aproximately 2/3 and 1/3, respectively of that of heparin.

These results indicate that: a) the interacting zone in the polysaccharide with the target protein should be small, being possible to define "minimal binding structures" as those hypothysized for the antiviral properties of these sulfated polysaccharides [7] and in the order of those produced statistically or b) some of the polysaccharide chains are biosynthesized containing blocks of disaccharides with disulfated B-units in spite of its low probability in a statistical distribution, as was suggested for the dermatan sulfate [30].

Both before mentioned possibilities are compatible, having the molecules of the galactans a small binding size (~ 3 disaccharides) effective for the interaction with the protein in spite of its low statistical occurrence in the molecule.

Mechanisms Involved in the Anticoagulant Activities of Galactans

Both carrageenans and agarans exhibit anticoagulant action through different mechanisms. Earlier studies showed that λ-carrageenan had the highest anticoagulant activity between these compounds, although it was lower than that of heparin [73,74]. It was found that this activity was not reduced in antithrombin deficient plasma, so antithrombin appeared to play little or no role in that action [67]. It could be thought that anticoagulant properties of these galactans are related to a direct inhibition of thrombin. Nevertheless, other mechanisms have been described; in particular, those involved in the action of sulfated galactans from red algae *Botryocladia occidentalis*. These galactans proved to increase thrombin and factor Xa inhibition by antithrombin and/or HCII [75]. Furthermore, recently the molecular mechanism involved in the potentiation of thrombin inactivation by antithrombin was postulated in this case [76]. It was found that these sulfated galactan and heparin increase protease inactivation by antithrombin at similar molar concentrations, although they differ markedly in the molecular size required for their activities; the galactan catalyzes the formation of a covalent complex between antithrombin and the protease, having a higher affinity for thrombin than antithrombin. It was proposed that, firstly the polysaccharide binds to the protease through a high-affinity interaction; antithrombin is then added to the complex and the protease is inactivated by covalent interactions. Finally, the antithrombin-thrombin complex dissociates from the polysaccharide chain.

Anticoagulant mechanism of sulfated galactans from *Gelidium crinale* has also been studied [70, 77]. These polysaccharides showed lower anticoagulant activity regards to those from *B. occidentalis*. Although both of them similarly inhibit thrombin by a mechanism mediated by antithrombin, a significantly higher concentration of the sulfated galactan from *G. crinale* than of polysaccharide from *B. occidentalis*,

is required to inhibit thrombin by HCII. On the contrary, when factor Xa instead of thrombin is used as the target protease, the galactan from *G. crinale* is a more potent anticoagulant. Therefore, slight differences in the proportions and/or distribution of sulfated residues along the galactan chain may be crucial for the distinct interaction of the polysaccharide with plasmatic proteases and inhibitors, giving different anticoagulant activity.

Brown Seaweeds: Fucans and Fucoidans

Brown seaweeds biosynthesize, among other polysaccharides, a complex system of fucans and fucoidans, in which the quantitative and qualitative variation of the components and distribution patterns, as well as the difference in structural details, are not only due to heterogeneity of the samples, but also to extreme compositional and structural dispersion [4]. Fucans have been defined as the homopolymers of fucose, while fucoidans are those in which sulfated "fucan" chains containing other sugars, mainly glucuronic acid, as side chains, are linked to backbones of blocks of β-D-galactose and/or β-D-mannose units [4]. Several "fucans" have been investigated, however they usually represent a small part of the system of polymers biosynthesized by the seaweed, that is, they are samples selected due to their high content of fucose, sulfate and/or biological activities.

Algae of the orders Chordariales and Laminariales (Phaeosporophyceae) synthesize linear backbones built up of 3-linked α-L-fucopyranose residues, sulfated at C-4 and, sometimes, at C-2 and C-4. Thus, a fucan from *Ecklonia kurome*, with relevant anticoagulant activity, has a (1→3)-α-L-fucopiranosyl backbone sulfated mainly at C-4 and also at C-2, with (1→2)-α-L-fucosyl branches [50, 78]. Fucans having similar backbones were also isolated from *Cladosiphon okamurans*, *Laminaria saccharina*, and *Chorda filum*, all of them mainly sulfated on C-4; the first one also substituted at C-2 by α-D-glucuronic acid [79], the second and the third one partially sulfated at C-2 of some of the fucose units, with some branching at C-2 of other units [80, 81].

In contrast, fucans from *Ascophyllum nodosum* [82-84], *Fucus vesiculosus* [85] and *Fucus evanescens* [86], all of the order Fucales (Cyclosporophyceae), have a backbone built up of alternating 3-linked α-L-fucopyranose residues partially substituted at C-4 and 4-linked α-L-fucopyranose units sulfated at C-2 and C-3 or only at C-2. This backbone is highly branched with short chains formed by major amounts of 4-linked fucose and 4-linked glucuronic acid**.**

A study of the anticoagulant activities showed considerable differences in APTT values among fucoidans obtained from different seaweeds (Table **4**) [87]. The fucoidans tested can be divided into three groups according to their activities. The most active anticoagulants were fucoidans from *Laminaria saccharina*, *L. digitata*, *Fucus distichus* and *F. serratus*, whose activities exceeded 19 heparin U/mg. Fucoidans of the second group, which includes *F. evanescens*, *F. spiralis*, *A. nodosum* and *F. vesiculosus*, exhibited aproximately half activity: 9-15 U/mg. It is remarkable that the fucoidan from *C. okamuranus* had virtualy no anticoagulant effect. The absence of activity could be explained by the fact that this preparation contains low percentages of sulfate or by the presence of vicinal 2,3-branching points formed by 2-*O*-α-D-glucuronyl substituents [79, 88].

Brown seaweed *Sargassum stenophyllum* (Fucales, Sargassaceae) [4] biosynthesizes two different sets of fucoidans. One of them (Table **5**, fractions F1-F4) is characterized by higher percentages of glucuronic acid and fewer sulfate groups, which are situated on different sugar units. (1→3)- and/or (1→4)-α-L-fucose units are the major monosaccharide components, but other sugars, like (1→6)-β-D-galactose, (1→2)-β-D-mannose, α-D-glucuronic acid, α-D-glucose and terminal β-D-xylose are also present in substantial amounts; this set shows very low anticoagulant activities, as other fucoidans of the order Fucales with similar chemical characteristics [85]. Fucoidans from the second set (Table **5**, fractions F5 and F6) have only fucose and galactose as major components, they contain high percentages of sulfate, which are concentrated on the fucose residues, and only small amounts of α-D-glucuronic acid; these fractions show higher anticoagulant activity (Table **5**). The small core is made mainly of (1→6)-linked galactose units, with large "fucan" chains of (1→3)-linked fucose units and minor amounts of 4- and 2-linked fucose residues. Most of the sulfate groups in the "fucan" domain are linked to C-2 and C-4 or only at C-4 of the 3-linked fucose units, and at C-2 of the 4-linked fu-

Table 4. Chemical Composition and Anticoagulant Activity of Fucoidans from Different Brown Seaweeds[a]

Seaweed source	Fuc	Xyl	Man	Glc	Gal	Uronic acid	Sulfate as SO_3Na	APTT U/mg[b]
Laminaria saccarina	36.7	1.2	1.0	2.2	4.6	4.8	29.6	33.0 ± 2.0
Laminaria digitata	30.1	1.9	1.7	1.4	6.3	7.0	27.5	24.2 ± 1.2
Cladosiphon okamuranus	30.9	0.7	-	2.2	-	23.4	15.1	0.5 ± 0.1
Fucus evanescens	58.7	1.6	-	-	1.6	< 1	36.3	15.1 ± 0.9
Fucus vesiculosus	26.1	2.4	3.1	2.2	5.0	10.3	23.6	9.4 ± 1.2
Fucus serratus	24.8	2.4	2.1	2.0	4.8	6.2	29.2	19.1 ± 1.6
Fucus distichus	40.8	0.8	-	-	0.8	< 1	34.8	26.9 ± 1.7
Fucus spiralis	33.0	2.8	1.4	1.2	3.0	8.2	25.9	13.6 ± 1.4
Ascophyllus nodosum	26.6	4.4	2.6	1.1	4.7	9.4	24.4	13.4 ± 1.1

[a]From Cumashi *et al*. 2007 [87]. [b]Anticoagulant activity determined as APTT relative to the heparin standard with an activity of 140 U/mg.

Table 5. Chemical Analysis and Anticoagulant Activity of Fucoidans from *Sargassum stenophylum*[a]

Fraction	Carbohydrate %	Uronic acid %	Sulfate as $NaSO_3$ %	Protein %	Monosaccharide composition (mol %)					APTT/TT[cb] Concentration (μg/ml)			
					Fuc	Xyl	Man	Gal	Glc	25	50	100	200
F1	66.3	17.3	17.7	9.6	35.5	28.0	5.0	20.7	10.8	1.1/1.0	1.2/1.0	1.3/1.1	1.4/1.2
F2	78.3	11.0	18.8	11.1	60.0	9.0	9.9	21.0	tr.	1.2/1.2	1.2/1.3	1.6/1.6	2.3/2.2
F3	68.0	10.2	20.7	11.8	52.4	6.6	16.6	23.4	tr.	1.3/1.2	1.7/1.8	2.3/2.4	3.2/2.7
F4	59.6	10.1	22.4	12.5	46.4	13.6	6.2	29.6	4.3	1.5/1.7	2.0/2.0	2.9/3.3	3.9/3.4
F5	73.8	2.5	28.5	7.5	59.6	4.8	1.9	31.5	2.1	2.2/3.5	2.6/>5.0	>4.0/>5.0	>4.0/>5.0
F6	54.0	1.7	28.3	6.7	65.0	3.0	2.0	28.6	tr.	2.7/3.6	>4.0/>5.0	>4.0/>5.0	>4.0/>5.0

[a]From Duarte *et al*. 2001 [4]. [b] APTT/TT ratios relative to control sample without fucoidan.

cose residues. Disulfated units in the galactose backbone contain sulfate groups at C-2 and C-3 or C-3 and C-4 of the 6-linked residues.

A sample of commercial fucoidan from *F. vesiculosus* was fractionated by gel filtration and ion-exchange chromatography (Table **6**). All the fractions contain fucose as major monosaccharide component, in addition to galactose and xylose as minor ones. As in the case of the fucoidan from *S. sterophyllum* [4], those fractions that eluted at lower concentration of NaCl were richer in uronic acids and poorer in sulfate than the others and have little anticoagulant activity. It is worth of note that even in those fractions with the higher activity (Table **6**), this was about 1/10 of that of heparin [85].

A similar commercial fucoidan from *F. vesiculosus* was fractionated by precipitation from a 0.25M NaCl solution with acetone, and the anticoagulant activity was determined for the original product and for three fractions by APTT and PT tests. The APTT test showed high activity at 5 μg/ml for the original product and for two fractions. PT test showed high activity only at 50μg/ml for one of the fractions. Another fraction, with low molecular weight (15.2 kDa) and 26.1% of sulfate, had little effect by these two *in vitro* tests [89].

Anticoagulant properties of fucoidans are mainly determined by the sulfated fucose chains, [90-92] specially by the disulfated fucosyl units; namely, 3-linked α-L-fucose 2,4-disulfate and 4-linked α-L-fucose 2,3 disulfate, [92] and not by the blocks of lightly sulfated D-galactose or D-mannose residues or by the carboxyl groups of the uronic acids.

Both, the (1*a*→3*e*)- and (1*a*→3*e*) (1*a*→4*a*)-linked α-L-fucose chains are in the equilibrium (1), which would be displaced to the ordered conformation with the formation of the sulfated polysaccharide/protein complex. Sulfation on C-2 and C-4 of a 3-linked α-L-fucose unit maintains the $^1C_4$ conformation with an equatorial sulfate on C-2 and an axial sulfate on C-4 in equilibrium with the $^4C_1$ conformation, in which the position of the sulfate groups is reversed (Fig. (**9**)). This could be a mechanism that would place any of the sulfate groups in the best position for the interactions with the protein lysines. Sulfation on C-2 and C-3 of 4-linked α-L-fucose units would invert the cycle to a $^4C_1$ form due to the repulsion of the equatorial sulfate groups; in this case, there would not be a change in the ordered helix conformation of the backbone, but both sulfate groups would be in axial positions (Fig. (**9**)). It is noteworthy that the antithrombin-binding region of heparin is also disulfated at C-2 and C-3 of

Table 6. Chemical Analysis and Anticoagulant Activity of a Commercial Fucoidan from *Fucus vesiculosus*, and of Fractions Obtained by Gel Permeationchromatography and Further Anion Exchange Chromatography[a]

Compound	M_{app} x 10^4	Sulfate %	Monosaccharide composition (molar ratios)						Anticoagulant activity[b]	
			Fuc	Gal	Man	Xyl	Uronic Acid	Sulfate	APTT U/mg	TT U/mg
Fucoidan	>68	23	1.00	0.03	0.02	0.04	0.20	1.20	9	12
$I_{1.8}$	56	32.5	1.00	0.06	0.02	0.03	0.05	1.13	13	10
$II_{0.8}$	51	14.8	1.00	0.01	0.10	0.25	0.43	0.57	6	1
$II_{1.15}$	14	23.8	1.00	0.03	0.01	0.04	0.06	0.78	8	12
$II_{1.35}$	16.5	33.2	1.00	0.05	-	0.01	-	0.91	11	19
II_2	17.5	35.9	1.00	0.05	Tr	0.01	-	1.23	6	16
III-$1_{0.9}$	7.9	18.6	1.00	0.03	0.05	0.13	0.20	0.71	6	6
III-$1_{1.5}$	6.2	33.8	1.00	0.05	Tr	0.02	-	0.96	12	18
III-1_2	14	29.1	1.00	0.05	-	tr	-	0.98	9	15

[a]Nishino *et al*. 1994 [85]. [b]Relative to the heparin standard with an activity of 167 units/mg.

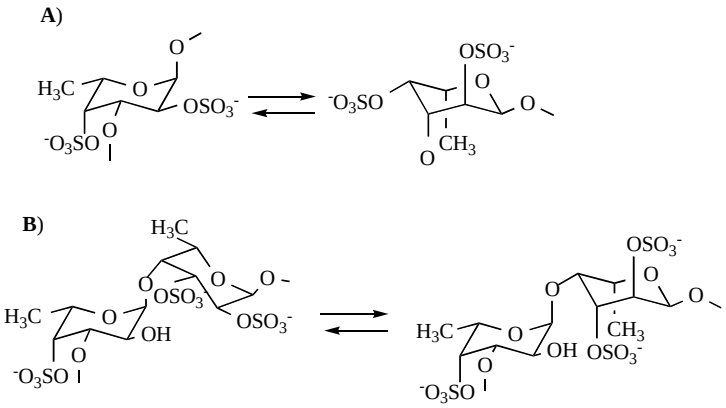

Fig. (9). Conformation of disulfated units in fucans and fucoidans: **A**) 3-linked α-L-fucose 2,4-disulfate units, **B**) 4-linked α-L-fucose 2,3-disulfated residues.

one of the units, and this sulfation pattern is known to be essential for activity [93].

Mechanisms Involved in the Anticoagulant Activities of Fucans and Fucoidans

Sulfated fucans isolated from brown algae are the most widely studied algal anticoagulants. Since these polysaccharides have complex and randomly heterogeneous structure, diverse anticoagulant mechanisms may be involved. Thus, Church *et al.* reported that fucoidan enhances the HCII-thrombin reaction more than 3,500-fold by formation of a ternary complex, which implies binding of both proteinase inhibitor and proteinase to fucoidan [94]. A purified fucoidan from *Ecklonia kurome* also showed anti-thrombin activity mediated by potentiation of HCII, depending of both sufate content and molecular weight of the polysaccharide. Moreover, this polysaccharide also binds to fibrinogen, blocking thrombin action [95, 96]. Fucoidans from *Ascophyllum nodosum* were described to inhibit thrombin mainly by potentiation of HCII, but also *via* antithrombin and their mechanistic model would be dependent on the polysaccharide concentration [97]. Later, it was described that this fucoidan also shows an important inhibitory effect on the generation of thrombin by preventing the factor Xa and the prothrombinase complex formation [98]. Studies performed with fucans isolated from *Fucus vesiculosus* and *Laminaria brasiliensis*, indicated that these polysaccharides directly inhibited thrombin [99]. More recent studies are in agreement with those that described that the main anticoagulant action of fucans is through potentiation of serpins. Thus, polysaccharides extracted from *Laminaria cichorioides* increased thrombin inhibition by HCII in concentrations similar to heparin; although, they also inhibited thrombin and factor Xa by potentiation of antithrombin, but in a lower degree [100].

GREEN SEAWEEDS: SULFATED RHAMNANS AND SULFATED ARABINOGALACTANS

Green Seaweeds other than those of the Genus *Codium*

Only a few sulfated polysaccharides from green seaweeds have been thoroughly studied from the structural point of view. Additionally, there is limited information about their biological behavior, particularly, about their anticoagulant activity. Some of them, like ulvans, sulfated polysaccharides extracted from *Ulva* and *Enteromorpha*, have their fine structural details determined, as well as their conformational and rhelogical characterization [101]. Simplifying, these polysaccharides are composed by chains of alternating 4-linked α-L-rhamnose 3-sulfate and β-D-glucuronic acid or α-L-iduronic acid and sequences of alternating 4-linked α-L-rhamnose 3-sulfate and β-D-xylose, partially sulfated on C-2; single stubs of β-D-glucuronic acid are linked to some rhamnose units. Unfortunately, to the best of our knowledge, no information about the anticoagulant behavior of the characterized compounds has been published. However, sulfated polysaccharides from *Ulva conglabata*, which contained major amounts of rhamnose and uronic acids, showed anticoagulant activity [102]. Some other extracts from seaweeds of the Ulvales also showed certain activity [103]. The structure of the active compounds was not determined in any of these papers.

On the other hand, some work has been carried out on the anticoagulant properties of aqueous extracts from green seaweeds. The extracts from 23 green seaweeds were found to have anticoagulant activity, being that of *Monostroma nitidum* very active (Table **7**). After purification, a sulfated rhamnan was isolated from this seaweed, which had an anticoagulant activity six-fold higher than that of heparin [104]. Also, sulfated galactans isolated from *Caulerpa okamurai* and *C. brachypus* had a specific HCII-dependent thrombin inhibition, while sulfated rhamnans isolated from *Monostroma nitidum* and *M. latissimum* exhibited an 8-fold difference in the IC_{50} for thrombin inhibition also in the presence of HCII [105].

Both, structures and anticoagulant properties, have been determined for sulfated rhamnans isolated from two seaweeds of the genus *Monostroma, M. nitidum* [106] and *M. latissinum* [107] and for several Bryopsidales of the genus *Codium* (see later), and in those cases a correlation between structure and anticoagulant activity is attempted.

Sulfated Heterorhamnans from Green Seaweeds of the Genus Monostroma

A purified sulfated polysaccharide obtained from *M. latissimum*, mainly composed by rhamnose, with 11 % uronic acids, 21 % sulfate and molecular weight of 725.4

Table 7. Chemical Analysis of Crude Sulfated Polysaccharides with Anticoagulant Properties Obtained from Hot Water Extracts of Chlorophyta[a]

Species	Yield %	Major sugars	Sulfate %	ATA[b]
Ulotricales				
Monostroma nitidum	19.6	Rha (Xyl)	25.0	3.3
M. zostericola	8.2	Gal (Xyl)	23.8	2.1
M. angicava	11.3	Rha,Xyl, Gal	19.3	1.7
M. lattissimum	9.5	Rha,Xyl, (Glc, Gal)	16.5	1.3
M. pulchrum	12.7	Gal, Xyl	6.2	0.5
M. groculandicum	6.8	Xyl (Rha)	5.5	0.2
M. fusem	14.5	Glc, Man	16.7	2.4
M. grevillei	7.2	Glc, Man, Gal	17.1	2.2
Spongomorpha duriuscula	4.2	Xyl, (Gal)	7.8	0.8
Ulvales				
Enteromorpha compressa	5.6	Man, Xyl	15.8	0.7
E. intestinalis	7.8	Gal, Xyl	3.6	0.1
Blidingia minima	6.4	Gal, Xyl	6.6	0.0
Ulva arasaki	13.6	Rha, Man	16.2	1.5
Cladophorales				
Cladophora densa	3.2	Gal, Glc	18.2	2.2
C. rugulosa	7.6	Gal, Glc, Man, (Xyl)	20.8	2.3
Chaetomorpha spiralis	6.4	Man (Gal, Xyl)	7.2	0.5
C. crassa	5.2	Man, Glc, Gal	19.2	2.2
C. media	3.8	Xyl, Man, (Gal)	4.2	0.3
Bryopsidales				
Caulerpa okamurai	8.7	Xyl, Glc	7.2	0.7
Bryopsis maxima	10.3	Glc, Xyl	0.0	0.1

[a]From Maeda *et al*. 1991 [104]. Seaweed classification is given according to the present criteria. *Codium* sps., informed in the original table, were not included in this table (see later). [b]ATA: anti-thrombin activity relative to heparin.

KDa had high anticoagulant activity. Sequential reduction of the molecular weight to 10.6 KDa showed that the activity was maintained until 61.9 KDa, after that, further decrease in the molecular size of the molecule drastically reduced the activity [108].

Two fractions (WF1 and WF3) were obtained from the crude extract of *M. nitidum* by fractionation through gel permeation chromatography. Data shown in Table **8** indicated that the polysaccharides were mainly composed of L-rhamnose. Methylation analysis indicated major amounts (about 86% in WF1 and 76% in WF3) of 2-linked 3,4-disulfated L-rhamnose units, together with 2-linked and 4-linked L-rhamnose residues in WF1 (7-8% and 7%, respectively), while WF3 shows 2-, 3- and 4-linked rhamnose residues (8%, 7% and 6%, respectively). The anticoagulant activities of both fractions were significant, but weaker than that of heparin. Thus, in the APTT test, solutions of 5μg/ml showed clotting times of about 45s, 100s and 200s for WF1, WF3 and heparin, respectively, with similar relationships in the TT test [106].

The sulfated rhamnan from *M. latissimun* was extracted and purified in similar conditions as those of the rhamnan from *M. nitidum* (Table **8**). Methylation analysis indicated the presence of 2- and 3-linked L-rhamnose units in 19% and 11%, respectively, with major amounts of 2-linked 4-sulfate (34%), 2-linked 3-sulfate (10%) and 2-linked 3,4-disulfate (27%) L-rhamnose units. APTT and TT were effectively prolonged by this rhamnan, but less than heparin, and its signals for clotting times became saturated at 20 μg/ml concentration (Table **8**). No clotting inhibition was observed in PT assay [107].

The "2-linked α-L-rhamnans" from *Monostroma* species, as well as that from *Gayralia oxysperma,* a related species [106-109] contain also significant amounts of 3- and 4-linked α-L-rhamnose units, together with small amounts of uronic acid. This sugar and linkage heterogeneity, together with the fact that an important quantity of the 2-linked units are disulfated and would be in a $^1C_4$ conformation, resulting from a balance between the repulsion of the equatorial sulfate groups (in a $^4C_1$ conformation) and the 1,3-diaxial (3-

Table 8. Chemical Analysis and Anticoagulant Activity of Fractions WF1 and WF3 from *Monostroma nitidum*[a] and La from *M. latissimum*[b]

Comp.	Sulfate %	Protein %	Uronic acids %	Mw kDa	$[\alpha]_D$ [c] °	Monosaccharide composition mol %					APTT/TT[d] Concentration (μg/ml)				
						Rha	Xyl	Glc	Gal	Man	2	5	10	20	50
WF1	28.2	1.0	7.9	870	- 37.2	79.4	5.2	10.1	5.3	-	1.2/1.2	1.4/1.4	1.8/1.5	2.5/2.0	3.8/3.2
WF3	34.4	0.7	6.8	70	- 64.8	78.2	14.7	3.7	-	3.4	1.8/1.7	3.0/2.6	4.3/3.7	>5.6/5.1	>5.6/>6.6
La	23.6	2.3	3.2	55	nd	86.8	6.3	6.9	-	-	1.5/1.4	2.1/2.7	3.1/>6.6	>5.6/>6.6	>5.6/>6.6

[a]Mao *et al*. 2008 [106]. [b]Mao *et al*. 2009 [104]. [c]The rhamnan from *G. oxysperma* [106] has similar rotations. [d]For WF1 and WF3, values were obtained from a plot. APTT/TT ratios relative to control sample without the compound.

sulfate, 5-methyl) steric hindrance in a $^1C_4$ conformation, indicate that the molecule would be in a ramdon coil conformation in aqueous solution. This dispersion of structural factors should not be an obstacle for the formation of an ordered binding zone in the polysaccharide, considering the high percentage of 2-linked disulfated units and the small size of the binding zone.

Mechanisms Involved in the Anticoagulant Activities of Ulvans and Rhamnans

It has been reported that the anticoagulant activity of ulvan from *U. conglobata* is due to the direct inhibition of thrombin and the potentiation of HCII [102]. As indicated above, sulfated rhamnans isolated from *M. nitidum* rendered a sixfold higher action than that of heparin [106]. It was reported that distinct anticoagulant activities from fractions of those algae were related to different molecular size, charge density, sulfate positions and linkage pattern of rhamnose residues [106]. Those fractions had a powerful anti-thrombin activity mediated by HCII in a stronger manner than that of heparin, meanwhile a weak inhibition of factor Xa was through antithrombin. Sulfated polysaccharides isolated from *M. latissimum* also had a significant inhibitory effect on thrombin activity mediated by HCII and this ability was similar to that of heparin. A lower inhibition of thrombin and factor Xa by potentiating antithrombin was described [107].

Green Seaweeds of the Genus *Codium*: Sulfated Arabinogalactans

The cell walls of the green seaweeds of the genus *Codium* contain different types of polysaccharides, namely: β-(1→4)-linked mannans and sulfated galactans and arabinans (or arabinogalactans), together with small amounts of arabinogalactan proteins [111,112]. Part of these products is extracted with water at room temperature or with hot water (or aqueous solutions) and usually they are contaminated with amylose-type glucans. Only the sulfated polysaccharides have anticoagulant properties [11] and so, discussions will be restricted to them.

Table **9** gives a list of the seaweeds, whose water extracts showed anticoagulant activity [113-115, 104]. No identification of the products responsible for these activities was carried out, so these products will not be treated any further.

Table **10** concerns to sulfated polysaccharides obtained from water extracts and submitted to fractionation procedures, as ion-exchange and/or gel permeation chromatography. The only proof of homogeneity and/or dispersion is that they were eluted as a single peak in gel permeation chromatography or gave a single spot on electrophoresis, and usually the choice criterion was the anticoagulant activity. They contain L-arabinose and/or D-galactose, sometimes together

Table 9. Extracts Obtained from the Green Seaweeds of the Genus *Codium* Only Tested as Anticoagulants

Codium species	Extraction-Fractionation Procedure	Chemical characteristics	Anticoagulant characteristics
C. fragile ssp. *tormentosoides*[a]	Extraction at rt. with aqueous buffer at pH=7.2	No determination was carried out	TT in plasma and fibrinogen, PT, Caolin Cephalin clotting time.
C. istmocladum[b]	Extraction at rt. with aqueous buffer at pH=7.3	No determination was carried out	Haemostatic activity compared with that of heparin, ratio 0.79
C. fragile[c]	Extraction with water at 100ºC	Glc, Gal (Man, Xyl)[e], 10.2 % sulfate	Anti-thrombin activity, relative to heparin: 0.9
C. divaricatum[c]	Extraction with water at 100ºC	Man, Gal (Ara), 11.7 % sulfate	Anti-thrombin activity, relative to heparin: 0.8
C. latum[c]	Extraction with water at 100ºC	Glc, Gal, Ara (Man), 13.6 % sulfate	Anti-thrombin activity, relative to heparin: 1.2
C. fragile[d]	Extraction at 70º	80 % carbohydr. and 19 % proteins. MW>30 KDa	APTT,TT

[a]From Deacon-Smith *et al.* 1985 [113]. [b]From Blunden *et al.* 1991 [114]. [c]From Maeda *et al*. 1991 [104]. [d]From Athucorala *et al.* 2007 [103]. [e] In parenthesis, minor quantities to traces.

Table 10. Extracts Obtained from the Green Seaweeds of the Genus *Codium* Fractionated and Fractions Submitted to Chemical and Biological Characterizations

Codium species	Extraction-Fractionation Procedure	Chemical characteristics	Anticoagulant characteristics
C. frgile ssp. *atlanticum*[a]	Extraction at rt. Purification by GPC and, for the polysaccharides, byIEC	Ara and Gal by tlc, proteoglycan with 18.4 % sulfate and 8.6 % protein, 2 polysaccharides with 7.5 % and 10.2 % sulfate	Thrombin inhibition by potentiation of HCII
C. latum[b]	Extraction at rt and IEC and GPC or by precipitation with 0.2 M KCl	(1→5)-α-L-arabinan sulfate, furanosic, 20.7 % sulfate, active to 8% sulfate, minor quantities of Gal. NMR spectrum incorrectly assigned	TT
C. dwarkense[c]	Extraction with water at rt and purif. by IEC and GPC	Sulfated arabinan furanosic (41.45 % sulfate), sulfated arabinogalactan (Ara 61.25 % Gal 35.75 %, 31,85 % sulfate). Structural determination based in that of *C. latum*	APTT, TT
C. pugniformis[d]	Extraction with water at rt and purify. by IEC and GPC	Glc (72.7%), Ara (17.3 %) and Gal (10.0 %), 32.6 % sulfate (SO4) and 5.2 % protein	Direct inhibition of thrombin activity
C. divaricatum[e]	Extraction at rt and 100º. Purif. By IEC (x2) and GPC	Ara (Glc, Xyl) DS 0.6	Activation of HCII. Different HCII-binding site to that of heparin and dermatan sulfate
C. adhaerence[e]	Extraction at rt and 100º. Purif. by IEC (x2) and GPC	Ara (Glc, Xyl) DS 0.8	Activation of HCII. Different HCII-binding site to that of heparin and dermatan sulfate
C. latum[e]	Extraction at rt. Purif. by IEC and GPC	Ara DS 0.8	Activation of HCII. Different HCII-binding site to that of heparin and dermatan sulfate
C. fragile[e]	Extraction at rt and 100º. Purif. by IEC (x2) and GPC	Ara (Xyl) DS 0.5	Activation of HCII. Different HCII-binding site to that of heparin and dermatan sulfate
C. cylindricum[f]	Extraction with water at rt and purif. by IEC and GPC	Gal (89 %), Glc (11 %), 7.8 % protein	Inhibition of fibrin polymerization, did not inhibit coagulation enzymes, factor Xa or thrombin

[a]From Rogers *et al*. 1990 [115] and Jurd *et al*. 1995 [54]. [b]From Uehara *et al*. 1992 [116]. [c]From Siddhanta *et al*. 1999 [55]. [d]From Matsubara *et al*. 2000 [117]. [e]From Hayakawa *et al*. 2000 [105]. [f]From Matsubara *et al*. 2001 [118].

with D-glucose and/or D-xylose, they were obtained in very small yields and do not represent the whole family of sulfated polysaccharides synthesized by the seaweed. They also contain medium-to-high percentages of sulfate, small amounts of protein and show anticoagulant activity weaker than that of heparin [54,55,116-119,105].

Generally, the small amount of glucose (~ 10%) was attributed to a contamination with the amylose-like glucans mentioned above, but in one case, a product (CP) was obtained from *C. pugniformis*, after ion exchange and gel permeation chromatography, which contained 72.7% glucose, 17.3% arabinose and 10.0% galactose with 32.6 % sulfate (as SO_4^{2+}) and 5.2 % protein [118]. No yields were given, but the product was named as a "minor polysaccharide". In this case, glucose was considered part of the sulfated polysaccharide, which was supposed to be a "new anticoagulant" proteoglycan in the *Codium* genus. This product is formally similar to those obtained from *C. fragile* and *C. vermilara* (M1, M2 and W1, W2, repectively) by hot-water extraction of the residue of the room-temperature water extraction of these seaweeds, as they also contain important percentages of glucose. [111]. Analysis and monosaccharide composition of these products are given in Table **11**, together with those of the products from *C. pugniformis* (CP) [118] and *C. cylindricum* (CC) [119]. M1-M2 and W1-W2 contained, apart from arabinose and galactose, major percentages, not only of glucose, but also of mannose. Methylation analyses of M1-M2 and W1-W2 indicated that both glucose and mannose constituted 4-linked chains, while the ^{13}C NMR spectra showed that mannose was part of the β-(1→4)-linked mannan, coexisting in the cell wall with the "arabinogalactans" and glucose was derived from the amylose-type reserve glucans [111]. The molar ratio monosaccharide/sulfate was then calculated on the basis that all the sulfate groups were in the "arabinogalactan" moiety (Table **11**). M1 and M2 showed molar ratios Ara+Gal/sulfate of 1/0.7-0.8, while W1 and W2, of 1/1.2-1.6. When similar calculations were carried out on CP a monosaccharide/sulfate ratio of 1/1.9 was obtained. These ratios suggest the presence of an arabinogalactan with high anticoagulant activity, which shows up, in spite of the contaminating neutral polysaccharides. M1-M2 and W1-W2 were supposed to be aggregates or complexes of the cell wall and reserve products formed during the extraction, this is consistent with the high percentages of divalent cations (Ca^{2+} and Mg^{2+}) present in the extracts [111].

CC was obtained from *C. cylindricum* by an extraction and purification procedure in 0.4 % yield (Table **11**) [119]. It was composed by galatose as major monosaccharide, but also glucose (11 %) and sulfate (13.1 %). On the basis that glucose would derive from a contaminating glucan, the product would be a galactan with a galactose/sulfate molar ratio 1/0.3.

Table 11. Chemical Characterization of Fractions Obtained from *Codium* Species, Rich in Glucose, with Anticoagulant Properties

Sample	Carbohydrate	Sulfate as SO_3Na	Protein	Monosaccharide composition (mol %)				APTT/TT					
								Concentration (μg/ml)					
	%	%	%	Gal	Ara	Man	Glc	500	250	100	50	25	2.5
CP[a]	71.3	25.0	3.7	10.0	17.3	-	72.7	-	-	-	-	>10/>10	3.6/2.3
M1[b]	74.0	7.2	14.4	10.5	9.2	50.1	28.9	>10/>10	3.1/>10	1.4/4.7	1.4/2.2	1.3/2.0	1.0/1.0
M2[b,c]	72.0	10.8	6.2	18.2	10.3	37.0	34.5	>10/>10	3.3/>10	1.1/1.2	1.0/1.2	1.0/1.2	1.0/1.0
$W1_d$[b,]	58.4	22.0	15.9	27.3	20.7	31.7	21.2	7.5/>10	6.0/>10	4.9/>10	1.8/5.3	1.4/3.7	1.0/1.0
W2[b]	62.5	21.9	15.5	17.6	13.5	60.7	8.2	3.1/7.2	2.5/7.0	2.2/6.7	1.3/6.2	1.2/2.4	1.0/1.1
CC[e]	n.i.[f]	13.1	7.8	89	-	-	11	-	-	-	-	>10/>10	2.4/3.7

[a]Glucan from *C. pugniformis*. Inferred from data obtained for concentrations of 15 μg/ml and 3 μg/ml, respectively. APTT/TT for 1.5 μg/ml, were 2.1/1.3 [117]. [b]Fractions from *C. fragile* and *C. vermilara* [111]. [c]Small amounts of Fuc were present. [d]Small percentages of Rham and Xyl were detected. [e]Galactan from *C. cylindricum*. Inferred from data obtained for concentrations of 15 μg/ml and 3 μg/ml, respectively. APTT/TT for 1.5 μg/ml, were 1.6/1.6 [118]. [f]n.i.=not informed (≤79.1 %).

Room-temperature extraction of *C. latum*, DEAE-cellulose, gel-permeation chromatographies and KCl precipitation produced an arabinan with anticoagulant activity [116]. Based on IR and ^{1}H NMR spectroscopy (anomeric proton δ 5.240 (D_2O)), this product was considered a sulfated furanosic α-L-arabinan. It contained 20.7 % sulfate (molar ratio Ara/sulfate 1/0.3-0.4), but the partially desulfated derivative retained anticoagulant activity until > 8% sulfate ($[\alpha]_D$ = -21.6°, molar ratio Ara/sulfate 1/0.13). The ^{13}C NMR spectrum of the desulfated derivative showed carbon values [106.15 ppm (C-1), 82.0 (C-4), 81.6 (C-2), 77.5 (C-3) and 66.2 (C-5)] that were supposed to be in aggreement with an (1→5) linked α-L-arabinan [120,121].

After extensive fractionation of the cold-water extract (CWE) of *C. dwarkense*, a sulfated arabinan (A2a) with 41.45% sulfate (molar ratio Ara/sulfate 1/1) and a sulfated arabinogalactan (A2b) with 31.85% sulfate were obtained in ~ 0.3 % and ~ 0.4 % yield, respectively [117] (Table **12**). After repeated DEAE-cellulose and gel permeation chromatography, and further fractionation by precipitation with KCl of the same extract, the product (Jia) with the highest anticoagulant activity was a sulfated arabinan (molar ratio Ara/sulfate 1/1), that by IR and ^{1}H NMR spectrometry was assigned as a furanosic α-L-arabinan without any specification of the position of the sulfate groups (only the anomeric proton in the original product δ 5.21 (D_2O) and desufated derivative δ 4.93 (DMSO) were informed [55]. On the bases that both fractions (A2a and Jia) were sulfated arabinans, they were considered the same product, a sulfated polymer constituted by α-L-arabinofuranose units.

In a screening of inhibition of thrombin by sulfated polysaccharides isolated from green algae, arabinans were extracted from *C. divaricatum* (DS 0.6), *C. adharence* (DS 0.8), *C. latum* (DS 0.8), *C. fragile* (DS 0.5). No structural details were determined [105]. Table **13** gives the analysis of the samples and the concentration to obtain 50 % inhibition *via* HCII or antithrombin.

Table **14** gives a list of the sulfated polysaccharides from *Codium* that have been studied from a structural point of view [122-127], only a few were tested to determine their anticoagulant activity. Some of them can be considered as homopolysaccharides of galactose or arabinose, taking into account certain considerations, as described later.

Table 12. Anticoagulant Activity of the Arabinan (A2a) and Arabinogalactan (A2b) from *C. dwarkense* Compared with that of Heparin[a]

Concentration μg/ml	PT[b]			APTT[b]			TT[b]		
	A2a[c]	A2b[d]	heparin	A2a	A2b	heparin	A2a	A2b	heparin
1	1.00	1.00	1.00	1.00	1.00	1.00	1.44	1.43	1.23
5	1.00	1.00	1.00	1.20	1.20	2.54	>10	9.61	>10
10	1.00	1.00	1.00	1.54	1.52	>10	>10	>10	>10
30	1.00	1.00	1.00	8.57	7.84	>10	>10	>10	>10
100	2.60	1.13	1.60	>10	>10	>10	>10	>10	>10
500	>10	1.73	6.60	>10	>10	>10	>10	>10	>10
1000	>10	4.06	>10	>10	>10	>10	>10	>10	>10

[a]Siddhanta *et al*. 1999 [55]. [b]Expressed as ratios relative to control sample without the compound. [c]Ara was the only monosaccharide detected; sulfate content 41.45 %. [d]Ara, 61.25 %, Gal 38.75 %; sulfate content, 31.85 %.

Table 13. Chemical Characterization of Sulfated Polysaccharides from *Codium* Species and Thrombin Inhibition Under Different Experimental Conditions[a]

Sulfated polysaccharide	Species	Major sugar constituents	Degree of sulfation[b]	Apparent molecular mass	IC_{50}[c]	
					HC II	AT III
AS-1	*Codium divaricatum*	Ara (Glc, Xyl)	0.6	n.d.	0.02	0.05
AS-2	*Codium adhaerence*	Ara (Glc, Xyl)	0.8	n.d.	0.02	0.10
AS-3	*Codium latum*	Ara	0.8	2.75×10^5	0.02	0.02
AS-4	*Codium fragile*	Ara (Xyl)	0.5	n.d.	0.02	0.16

[a]Hayakawa *et al.* 2000 [105]. [b]Per anhydrosugar. [c]Concentration (μg/ml) of the polysaccharide to achieve 50 % inhibition of thrombin with antithrombin III and HC II.

Table 14. Extracts Obtained from the Green Seaweeds of the Genus *Codium* Fractionated and Fractions Submitted to Structural Determinations

Codium species	Extraction-Fractionation Procedure	Chemical characteristics	Anticoagulant characteristics
C. fragile[a]	Extraction at rt and 90º. Purif. by treatment with α-amylase and IEC. Partial acid hydrolysis	sulfated arabinogalactan, sulfate on C-4 and/or C-6 of 3-linked galactopyrasnose units and C-2 or C-3 of arabinopyranose units- uronic acids, protein→proteoglycan	-
C. fragile[b]	Extraction with cold water. By IEC a HMW compound containing carbohydrates, sulfate and protein and a LMW compound containing only carbohr. (mainly Gal and small amount of Ara) and sulfate	The LMW compound was constituted by β-Galp partially sulfated on C-6 and α-Arap, pyruvic acid was also detected.	-
C. yezoense[c]	Extraction with water at rt. Purification by IEC	pyruvylated β-D-galactan sulfate (14.4 % sulfate and 5.3 % protein) with 1→3, 1→6 linkages, sulfate mainly on C-4 and also on C-6 and pyruvate forming a 5-membered ring with O-3 and O-4 of β-D-galactose terminal units was eluted at 0.8 M NaCl..	-
C. fragile var. *novae-zelandiae*[d]	Extraction with water at rt and 90º. Purification by IEC	Extracts with ratio Gal:Ara 3:1-1:1. Pyruvilated arabinogalactan sulfates β-D-galactose units with 1→3, 1→6 linkages, sulfate mainly on C-4 and also on C-6 and pyruvate forming a 5-membered ring with O-3 and O-4 of β-D-galactose terminal units. 3-linked Arap units sulfated on C-2, C.4, and on C-2 and C-4, variable quantieties of protein. Sulfated arabinans, galactans and/or arabinogalactans?	APTT, TT, dual effect anticoagulant, but pro-aggregant.
C. vermilara[d]	Extraction with water at rt and 90º.	Similar structural units to those of *C. fragile* var. *novae-zelandiae*, but higher amounts of Ara and sulfate.	APTT, TT, more active than *C. fragile* var. *novae-zelandiae*, anticoagulant, but pro-aggregant.
C. istmocladum[e]	Extraction at pH=8 and 60º, proteolitic digestion, precipitation with increasing volumes of acetone and IEC of the fraction obtained with 1:0.9 water:acetone (Gal:Ara 0.42:0.50	Pyruvylated galactan sulfate, predominantly linear, with 3-linked 4-sulfated β-D-galactose units. Minor quantities of 3-Gal 4,6S, 6-Gal 4S and terminal 3,4-Pyr Gal units were also detected.	-
C. fragile[f]	Extraction with water at 100º. Purification by IEC and GPC.	Pyruvylated galactan sulfate, with 3-linked β-D-galactose units partially sulfated at C-4 and partially branched at C-6 with major amounts of single stubs of 3,4-Pyr Gal units.	-

[a]From Love & Percival 1964 [121]. [b]From Bilan *et al*. 2006, 2007 [123,124]. [c] Nika *et al.* 2003 [122]. [d]From Ciancia *et al*. 2007 [111]. [e]From Farias *et al*. 2008 [125]. [f]From Ohta *et al*. 2009 [126].

Galactans

A pyruvylated galactan sulfate (G-II) was isolated from *C. yezoense* in ~ 1.5 % yield after room-temperature water extraction and ion-exchange chromatography (Tables **15** and **16**). The backbone was constituted by β-(1→3)-D-galactose units with some β-(1→6)-galactose residues, branching points were found mainly at C-6 of the backbone units and could be constituted by single stubs or short oligosaccharide chains, sulfates were attached mainly to C-4 and to lesser extend to C-6. In addition, it contained a five-membered cyclic pyruvate ketal linked to C-3 and C-4 of non-reducing terminal galactose units and minor quantities of 3-linked 4,6-*O*-carboxyethylidene-β-D-galactose units [124,125].

Sulfated galactans (SG1 and SG2) were obtained from *C. isthmocladum* after aqueous extraction at 60˚C and pH 8.0, fractional precipitation with acetone and further ion-exchange

Table 15. Chemical Analysis and Anticoagulant Activity of the Products Obtained from *Codium* Species[a]

Sample	Sulfate as SO_3Na	Protein	Monosaccharide composition Mol %					MW	APTT/TT[c] Concentration (μg/ml)				
	%	%	Gal	Ara	Man	Glc	Xyl	KDa	500	250	100	50	25
A1	20.3	11.3	62.5	23.4	3.9	4.9	3.2	11	>10/>10	>10/>10	2.4/2.4	1.5/1.7	1.1/1.5
A2	15.7	17.3	32.9	29.0	27.1	6.1	3.6	12	>10/>10	>10/>10	2.1/2.0	1.3/1.4	1.1/1.3
F1	12.2	10.5	65.2	22.4	2.1	7.3	1.4	16	4.0/>10	3.3/4.7	1.8/1.4	1.1/1.5	1.0/1.0
F6	11.0	tr.[d]	84.8	2.5	1.2	3.2	2.5	n.d.[e]	1.1/1.3	1.0/1.2	1.0/1.2	1.0/1.3	1.0/1.0
F7	19.4	tr.	87.1	12.9	tr.	tr.	tr.	10	8.0/6.7	3.9/>10	1.9/1.7	1.0/1.3	1.0/1.0
F8	24.2	tr.	44.1	51.5	-	1.5	1.9	12	>10/>10	>10/>10	3.4/3.7	1.2/1.3	1.0/1.0
F9	25.5	tr.	60.7	37.3	tr.	2.0	tr.	13	>10/>10	>10/>10	3.1/3.3	1.2/2.2	1.0/1.0
V1	30.4	16.2	49.8	44.7	3.6	1.9	-	66	>10/>10	>10/>10	>10/>10	7.0/>10	3.1/>10
V2	30.2	14.4	32.1	36.2	27.7	4.0	-	13	>10/>10	>10/>10	>10/>10	3.1/10	1.7/5.7
G-II	14.4	5.3	90.2	4.6	0.9	1.3	3.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SG-1	47[f]	n.d.	98	<1	<1	-	-	14	n.d.	n.d.	n.d.	n.d.	n.d.
SG-2	64[f]	n.d.	98	<1	<1	-	-	20	n.d.	n.d.	n.d.	n.d.	n.d.
FG	11	tr.	98	2	-	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.

[a]From Ciancia *et al*. 2007 [111], Bilan *et al*. 2006 [123], Farias *et al*. 2008 [125], Ohta *et al*. 2009 [126], Estevez *et al*. 2009 [112]. [b]Small amounts of Rham, Fuc, 2-*O*-methyl-Gal were also found. [c]APTT/TT ratios relative to control sample without the sample. [d]tr.= traces. [e]n.d.=not determined. [f]Calculated from molar ratios, considering Gal and sulfate as the only two constituents of the sample.

Table 16. Methylation Analysis of Extracts and Fractions Obtained from *C. fragile, C. vermilara, C. yezoense* and *C. isthmocladum*[a]

Permethylated monosaccharide[b,c]	Sample[d]																		
	A1	Ds A1	F1	Ds F1	F6	Ds F6	F7[e]	F8[e]	F9	V1	Ds V1	G-II	Ds G-II	SG1	Ds SG1	SG2	Ds SG2	FG	Ds FG
2,3,5-Ara	tr.	-	tr.	2	tr.	tr.	-	5	12	-	-	-	-	-	-	-	-	-	-
2,3-Ara							-	7	11	-	-	-	-	-	-	-	-	-	-
2,4-Ara	-	29	5	19	tr.	3	9	26	10	-	54	-	-	-	-	-	-	-	-
2-Ara	10	1	5	tr.	tr.	-	2	18	4	10	3	-	-	-	-	-	-	-	-
4-Ara	-	-	5	4	3	1	1	2	4	-	-	-	-	-	-	-	-	-	-
Ara	24	9	8	-	tr.	-	2	4	2	45	tr.	-	-	-	-	-	-	-	-
2,3,4,6-Gal	tr.	tr	tr.	tr.	1	-	-	-	11	tr.	tr.	-	-	-	-	-	-	-	3.5
2-AG	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,4,6-Gal	9	20	19	21	23	26	22	10	10	8	8	5	35	10	34	7	36	16.2	34.1
2,3,6-Gal	1	-	1	-	tr.	-	-	-	1	tr.	-	4	2	-	-	-	-	-	-
2,6-Gal	21	22	23	29	37	32	24	9	9	10	11	11	22	40	41	32	38	40.2	33.1
2,3-Gal	3	-	tr.	tr.	3	3	1	tr.	6	tr.	tr.	-	1	-	-	-	-	-	-
2,4-Gal	6	14	18	19	11	18	20	6	8	3	14	2	22	tr.	25	tr.	22	6.0	27.1
6-Gal	tr.	tr.	tr.	1	tr.	2	-	-	-	2	5	-	-	-	-	-	-	-	-
2-Gal	23	2	15	6	19	10	10	4	7	19	3	8	-	50	tr.	61	4	37.6	2.4
3-(4-) Gal	3	3	tr.	tr.	tr.	4	4	2	3	7	3	70	14	-	-	-	-	-	-

[a]From Ciancia *et al*. 2007 [111], Estevez *et al*. 2009 [112], Bilan *et al*. 2007 [124], Farias *et al*. 2008 [125], Ohta *et al*. 2009 [126]. [b]Numbers indicate position of methyl groups. [c]Small percentages to trace amounts of Glc and/or Man derivatives were detected in most of the samples, but they were considered as deriving from contaminant polysaccharides and therefore, they were not included. [d]A1 and FG were obtained from *C. fragile*, V1, from *C. vermilara*, F1, F6-F9 are fraction obtained from A1 by ion exchange chromatography, G-II was obtained from *C. yezoense* and SG1 and SG2, from *C. isthmocladum*. Ds=desulfated derivatives. [e]Unpublished results.

Table 17. Partially Methylated Galactose Units Found by Methylation Analysis of Extracts and Fractions Obtained from *C. fragile* and *C. vermilara* Considered as Pure Galactans[a]

Permethylated monosaccharide[b]	Deduced structural units	Sample[c]							
		A1	DsA1	F1	DsF1	F6	DsF6	V1	DsV1
2,4,6-Gal	3Gal	15	34	25	28	26	30	20	22
2,6-Gal	3,4-PyrGalT (major), 3,4Gal, 3Gal4S	36	38	31	39	41	37	25	31
2,4-Gal	3,6Gal, 3Gal6S, 3,6Gal4S	10	24	24	25	12	21	8	39
2-Gal	3,6Gal4S, 3,4Gal6S, 3Gal4,6S, 3Gal4,6-Pyr	39	3	20	8	21	12	48	8

[a]From Ciancia *et al*. 2007 and Estevez *et al*. 2009 [111,112]. [b]Numbers indicate position of methyl groups. [c]A1 and V1 are extracts obtained from *C. fragile* and *C. vermilara*, F1 and F6 are fractions obtained from A1 by ion exchange chromatography. Ds=desulfated derivatives.

chromatography, no yields were given [126]. Their analyses (Table **15**) and structures (Table **16**) were essentially similar to that of G-II from *C. yezoense* with quantitative variations (Tables **15** and **16**), as it was mainly constituted by 3-linked β-D-galactose 4-sulfate units, with minor quantities of 3-linked β-D-galactose 4,6-disulfate, β-D-galactose 4-sulfate substituted also at C-6 and non-reducing galactose units containing 3,4-*O*-(1´carboxy)ethylidene substituents. The main differences with G-II were that this product did not have 3,6-linked galatose units and no six-membered cyclic ketal groups attached to C-4, C-6 positions. Recently, a sulfated galactan with similar structural characteristics was isolated from *C. fragile*; this product, which contained 12 % pyruvic acid and 11 % sulfate, showed an important antiviral activitic against HSV-2 in mice [128]. Unfortunately, no anticoagulant activities were determined for these galactans.

There are two cases [122,111] in which, even if the galactans were not isolated in "pure" form, experimental data permits some considerations on their structures. An "arabinogalactan" from *C. fragile* produced after partial hydrolysis only disaccharides composed by galactose or arabinose. The first one was identified as 3-*O*-β-D-galactopyranosyl-D-galactose. It was also determined that the sulfate groups, on the galactose units, were on C-4 and C-6 [124] in agreement with the above structures. Other extracts and fractions obtained from *C. fragile* and C. *vermilara* also gave "arabinogalactans" [111] (Table **15** and **16**).

The pattern of partially methylated monosaccharides in the methylation analysis of these products and some of their desulfated derivatives is shown on Table **16**. On the other hand, spectroscopic analysis suggested that there were no galactose or/and arabinose structural units interpersed in the polysaccharide chains and that the scheme of partially methylated sugars could be easily interpreted as derived from mixtures of galactans and arabinans or from block copolymers of these structures. No mater which of those is the real arrangement, it is possible to consider the structure of the galactans and arabinans separately. Thus, this can be carried out working out separately the galactose and arabinose methylated derivatives (Tables **15-17**).

According to these data, green seaweeds of the genus *Codium* synthesize a family of sulfated β-(1→3)-D-galactans with additional structural variations, being the most important the presence of β-(1→6)-linkages in the backbone and/or side chains, sulfate groups on C-4 and/or C-6, although usually not in the same unit, and β-D-galactose side chains, composed by single stubs or short oligosaccharide chains, comprising ketal groups of pyruvic acid attached to C-3 and C-4 of the non-reducing end chain. Some possible structural galactan sequences are shown in Fig. (**10**).

A sulfated β-(1→3)-D-galactan would conform, in compelling circumstances, an extended helix which could pack to another extended backbone. Several structural factors would condition the ordered conformation, namely: presence of β-(1→6) linkages, sulfate groups in the backbone and steric restriction of the flexibility of the glycosidic linkages. The first factor would enhance the flexibility of the sugar chain tending to a random coil, while the repulsion of the sulfates and the steric interactions of substituents in adjacent sugar units would straight it. Also, the steric effect of side chains

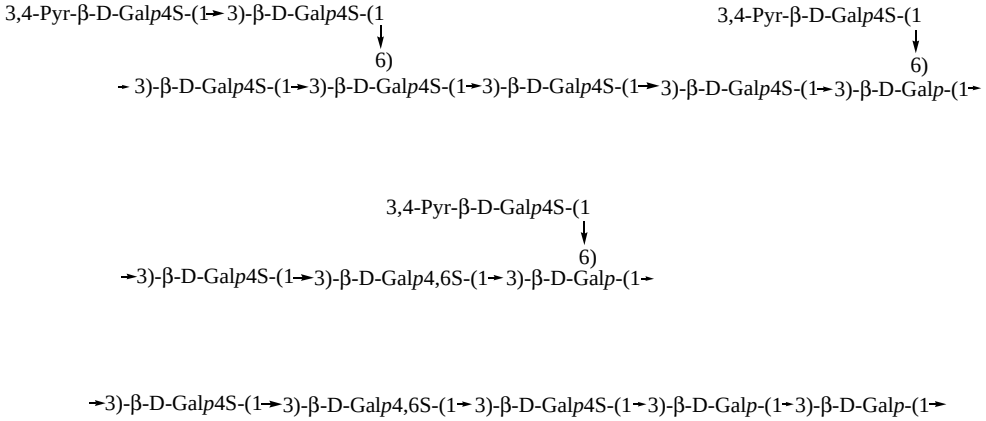

Fig. (10). Some possible structural unit sequences found in galactans biosynthesized by *Codium* seaweeds.

Table 18. Arabinan Structural Units Deduced from Methylation Analysis of Arabinogalactans from *Codium* Species[a]

Seaweed	Arabinan	Structural units			
		3Ara	3Ara 4S	3Ara 2S	3Ara 2,4S
C. fragile	A1	2	29	-	69
	M2	-	13	-	87
	F8[b]	52	37	4	7
	F9	53	18	21	8
C. vermilara	V1	-	13	-	87
	W2	65	11	-	24

[a]From Ciancia *et al*. 2007 and Estevez el al. 2009 [111,112] considering the arabinan and galactan moities as separated molecules. [b]Unpublished results.

would be against the interaction of the galactan with other molecules.

In no case anticoagulant activities were determined for pure galactans from *Codium* species of known structure, but the structures described above suggest at least in these cases, low activity.

Arabinans

Although arabinans have been detected in some species of *Codium*, to the best of our knowledge, no "pure" arabinans have been isolated and structurally studied in detail. The arabinans deduced from the methylation analysis (Table **16** and **18**) and ^{13}C NMR spectra of the extracts from *C. fragile* and *C. vermilara*, their desulfated derivatives and some of the fractions (F8 and F9) obtained from them showed a linear backbone of β-(1→3)-L-arabinopyranose units with major sulfate substitutions at C-2 and C-4 or only at C-4, and minor ones on C-2 (Table **16** and **18**) [111,112].

Non-sulfated or monosulfated arabinopyranose units, as those of arabinopyrans from *C. fragile* and *C. vermilara*, exist in a conformational equilibrium between two chairlike forms of unequal energy [127], being the $^4C_1$ form highly predominant. In the cases of the 3-linked 2,4-disulfated arabinose residues, both ($^4C_1$ and $^1C_4$) conformations, →3*e*)-β-L-Ara*p* 2S*e*, 4S*a* (1*a*→ ($^4C_1$) and →3*a*)-β-L-Ara*p* 2S*a*, 4S*e* (1*e*→ ($^1C_4$) have energies of the same order, so this structural units would be in equilibrium, as a way to place the adequate sulfate group in the most favorable spatial position for the formation of the polysaccharide/protein complex.

β-(1→3)-linked sulfated L-arabinan chain would adopt a helix-like conformation, which would be fully extended by repulsion of the sulfate groups. The flexibility of the glycosidic linkage is further restricted by the equatorial sulfates adjacent to the linkage and by the axial sulfates adjacent to the glycosidic oxygen (Fig. (**11**)).

Table **15** shows the anticoagulant activities of the water extracs from *C. fragile* and *C. vermilara*. No clotting inhibition was observed in the PT test of any of the samples and no effect was shown in the fibrinogen levels or in the Reptilase time by any of the extracts. All the fractions contained minor (A1 and V1) or major amounts of the non-sulfated β-(1→4)-mannans and/or α-(1→4)-glucans, which are devoid of anticoagulant properties. This is consistent with the higher activities of the fractions A1 and V1.

On the basis, previously suggested [26], that most of the anticoagulant activity of natural sulfated polysaccharides is based in the presence of disulfated structural units, it is hypothetized that the preponderant anticoagulant activity of the sulfated polysaccharides from green seaweeds of the genus *Codium* is determined by the sulfated arabinopyrans and/or the sulfated arabinopyran chains in a sulfated galactan/sulfated arabinopyran block copolymer.

Since APTT and TT values were prolonged, samples in Table **15** directly affect thrombin activity and/or increase the activity of physiological thrombin inhibitors.

Mechanisms Involved in the Anticoagulant Activities of Arabinogalactans from Codium

As it has been described above, green seaweeds of the genus *Codium* biosynthesize different and complex sulfated

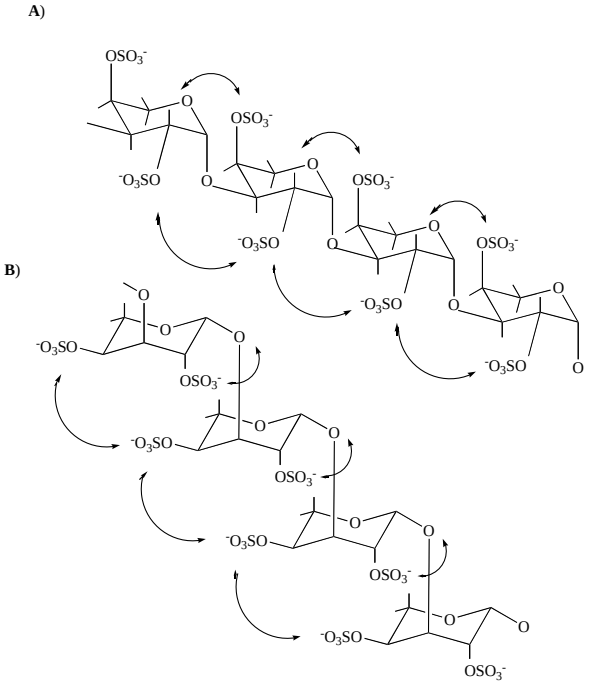

Fig. (11). General structure and conformations of β-(1→3)-L-arabinopyranose 2,4-disulfated units: **A**) With the pyranose cycle in a $^4C_1$ conformation, and **B**) With the pyranose cycle in a $^1C_4$ conformation. The restrictions caused by the sulfate groups are showed with arrows.

polysaccharides, mainly galactans and arabinans. This heterogeneity of structures has made their anticoagulant mechanism more difficult to rationalize. Two sulfated polysaccharides and a proteoglycan form *Codium fragile* ssp. *atlanticum* showed anticoagulant activity associated with higher sulfate content [54]. The proteoglycan fraction strongly proved to inhibit the thrombin activity in the presence of HCII, whereas the inhibition was weak in the presence of antithrombin. Moreover, no direct activity was observed against the active sites of either thrombin or factor Xa. On the contrary, the proteoglycan from *C. pugniformis* showed both direct and antithrombin mediated inhibition for thrombin activity, but it did not potentiate HCII [117]. Since sulfate and protein contents are similar in proteoglycans from those algal extracts, differences of anticoagulant mechanisms between them would be related mainly to their distinct polysaccharide structures, which were not investigated. Sulfated polysaccharides composed mainly by galactose were extracted from *C. cylindricum,* and they showed a particular anticoagulant action. These polysaccharides directly inhibited thrombin and no potentiation of HCII or antithrombin was observed [118]. It was proposed that one of the anticoagulant mechanisms for the sulfated polysaccharides from *C. cylindricum* could be the inhibition of fibrin polymerization. In another study, sulfated polysaccharides from other *Codium* species and related seaweeds exhibited thrombin inhibition and this effect was more potent for sulfated arabinans than for sulfated galactans [105]. Anticoagulant mechanism for all those polysaccharides proved to be by a HCII-dependent pathway with higher effectiveness than that of heparin or dermatan sulfate. It was suggested that binding of sulfated polysaccharides to HCII mainly induces conformational changes in the reactive site of this serpin, thereby optimizing its interaction with thrombin, its target protease. However, polysaccharides from *Codium* also showed antithrombin-dependent thrombin inhibition, although this effect was not as strong as that of heparin.

The differences found in the anticoagulant mechanisms of these polysaccharides are attributed to the important diversity of structures and to the fact that one compound may have more than one target protease.

CONCLUSIONS ABOUT SULFATED POLYSACCHARIDES/PROTEIN COMPLEXES

Based on the present knowledge about the behavior of GAGs as anticoagulants and of the results described here, conditions for high anticoagulant activity of sulfated polysaccharides from seaweeds are summarized as follows:

a) Non-specific electrostatic interactions [26] between clusters of negatively charged groups in the polysaccharide and clusters of cationic residues from the basic amino acids in the protein [128]. These functional groups interactions between parts of the macromolecules would determine the "binding zone", which would have to be constituted by conformationally adequate backbones (as helix or ribbon conformations in the polysaccharide and α-helix or β-strand in the protein) and interacting groups spacialy and sterically situated on them [128]. The structure of the binding zone in the polysaccharide would not necessarily be the same as the average structure determined by chemical and spectroscopical methods, due to the uneven distribution of substituents, as sulfate groups and others.

b) The backbone of the polysaccharide binding zone should have a random coil conformation in solution, with a symmetry determined by the steric interactions between sugar units and polar non-specific electrostatic interactions between sulfate groups.

c) As the interaction with the binding zone of the protein begins, the random coil conformation of the binding zone of the polysaccharide would turn into an ordered chain of the ribbon or helix type. At the same time, the binding zone of the protein would change to a helix or β-strand form.

d) The complex would aggregate further through the adaptation of the polysaccharide and protein binding zones into complementary fitting structures [128-131], to situate the interacting groups into the most favorable conditions [45] and it would be stabilized through short-range interactions: hydrogen bonding, van der Waals and hydrophobic interactions. While some hydrophobic interactions possibly occur upon complex formation, they would not play a major role in mediating binding between the sulfated polysaccharides and the antithrombin -based peptides [44].

e) The structural monosaccharide units with higher potential for biological activity in the polysaccarides studied have pyranose rings with their C-2, C-3 and C-4 in 2S,3R,4R or 2R,3S,4S configurations, respectively for arabinose, fucose and galactose and 2S, 3S, 4R for rhamnose. These carbon atoms carry a glycosidic linkage and two sulfate groups in three different distributions, namely: 3-linked 2,4-disulfated, 4-linked 2,3-disulfated and 2-linked 3,4-disulfate, as shown in Fig. (**12**).

f) The above structural units have the possibility to place their sulfate groups in axial and/or equatorial positions according to the spatial situation of the basic groups in the protein. The 3-linked units have C-2 equatorial and C-4 axial sulfate groups in a $^4C_1$ chair conformation of the cycle and C-2 axial/C-4 equatorial groups when the cycle is in $^1C_4$ form. Both forms have comparable energies and can be interchanged.

The 2-linked and 4-linked residues have both (3,4 and 2,3) substitutents in equatorial position, if neither of the sulfate groups is neutralized by a basic group, the repulsion of the negative charges would destabilize the $^4C_1$ chair conformation of the cycle to a $^1C_4$ form with both sulfates in axial positions.

g) As the binding zones would be composed by about 5-7 sugar residues and not all the sulfate groups would be involved in the formation of the complex, some of the units could be monosulfated, as in the case of the galactans.

It was shown that the solution conformation of heparin has clusters of sulfate groups down each side of the molecule, with a distance of about 17 Å between

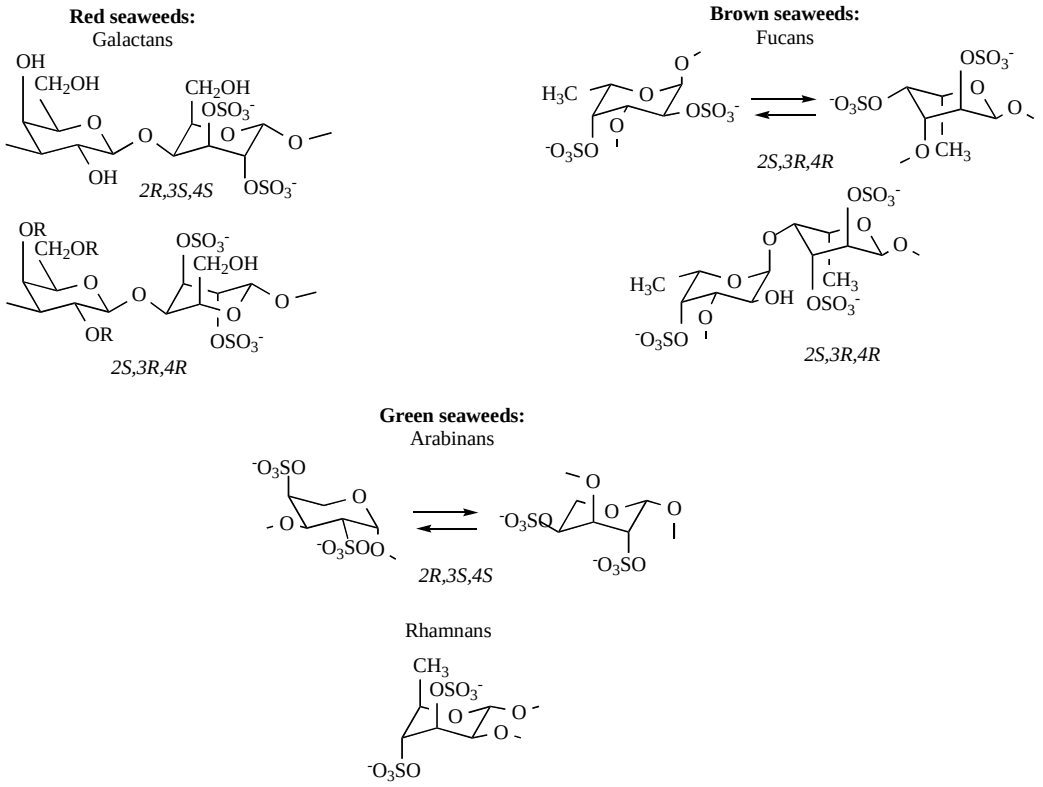

Fig. (12). Disulfated structural units present in sulfated seaweed polysaccharides that would be resposable for a high acticoagulant activity of the corresponding compound.

clusters. This spacing is suited to match the interval between basic side chains of residues presented by an α-helix, such as that formed when polylysine binds heparin. A specificity for heparin results from the presence on a protein surface of groups of basic residues 16-20 Å apart, whether the groups are presented by the same feature of secondary structure or whether they are distant in sequence, but located on adjacent features [132-134].

h) Sequences of sulfated polysaccharides can bind to different proteins, having specificity for each of them. This could result from the requirement of the proteins of different distributions of the interacting sulfate groups according to the distribution of their basic amino acids. The binding zone from each protein would require the non-interference of the sulfates required for the interaction with other proteins.

In summary, anticoagulant activity largely depends on the interaction affinity of protein-polysaccharide, which varies greatly according to the seaweed sulfated polysaccharide. That is because different algae biosynthetize polysaccharides differing on molecular size, monosaccharide composition, sulfate content and position. Thus, algal polysaccharides exert their anticoagulant activities through different mechanisms, they can inhibit thrombin directly, catalyse thrombin inhibition by antithrombin and/or HCII, inhibit generation of proteases and active complexes of the blood coagulation, interfere fibrin polymerization, and so on.

Knowledge of specific structural characteristics of seaweed polysaccharides and their relationship with the anticoagulant activity could contribute to the understanding of the regulation of the coagulation process and to the development of new antithrombotic therapeutic agents.

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ABREVIATIONS

a	=	axial position of A substituent
APTT	=	activated partial thromboplastin time
DS	=	degree of sulfation
e	=	equatorial position of A substituent
GAGs	=	glycosaminoglycans
HCII	=	heparin cofactor II
PT	=	prothrombin time
Serpin	=	serine protease inhibitor
TT	=	thrombin time

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