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Letter to the Editors-in-Chief

# Alterations of fibrin networks mediated by sulfated polysaccharides from green seaweeds

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## 1. Introduction

Sulfated polysaccharides (SP) extracted from marine green algae are becoming a focus of interest due to their anticoagulant activities [1]. It has been reported that sulfation degree and, consequently electronegative-charge densities, are important structural determinants for the anticoagulant activity, however, other structural features could also influence their anticoagulant effect. Therefore, in order to contribute to the potential development of new antithrombotic agents, the knowledge of specific structural characteristics of these products and their interaction with the different proteins involved in the hemostatic system must be considered. We focused the present work on two different polysaccharides extracted from cell walls of green seaweeds. Fernández et al. [2] reported the isolation and characterization of a highly sulfated linear (1 → 3)-β-L-arabinan, obtained from *Codium vermilara* (Bryopsidales), Ab1 (Suppl. Fig. 1A), as well as the analysis of its anticoagulant behavior. On the other hand, we have previously reported that a polysaccharide extracted from *Penicillus capitatus* (Bryopsidales), PF1, a sulfated pyruvylated (1 → 3) (1 → 6)-β-D-galactan (Suppl. Fig. 1B) [3], also exerted anticoagulant activity. It is well known that formation and lysis of the blood clot play an essential role in hemostasis, and changes in these physiological processes can modulate the resulting fibrin network features, rendering a clot more or less thrombogenic [4]. The aim of our study was to evaluate the in vitro effects on formation, structure, and lysis of plasma fibrin networks, given by Ab1 and PF1.

## 2. Materials and methods

PF1 and Ab1 were prepared as described before [2,3]. Aliquots of normal pooled citrated plasma (platelet-depleted) were mixed with each of the polysaccharides samples in a ratio 9:1 (final concentration in test solution 0.125 to 10 μg/mL) and incubated for 1 min at 37 °C. Saline solution was used as control. All the experiments were carried out at pH 7.4, in the absence of calcium.

Fibrin formation kinetic assays were performed by adding thrombin to the preincubated plasmas. In order to quantify the fibrin formed, plasma clots were weighted. Observations of plasma fibrin networks were carried out in a scanning electron microscope (SEM). Lysis were performed in one-stage assays, by adding recombinant tissue plasminogen activator (rt-PA) and thrombin to each preincubated plasma and the results were expressed as *half-lysis-time* ($t_{L1/2}$). (Details of experimental procedures are given as Supplementary material).

Results were expressed as mean ± standard deviation (SD) or median and range, and compared with Student's *t*-test or Mann-Witney-Wilcoxon, using the statistical program Statistix 8.0 (Analytical Software, Tallahassee, FL, USA).

## 3. Results

Important alterations on plasma fibrin formation kinetic assays were shown by both SP fractions, in a concentration dependent manner (Table 1). PF1 and Ab1 caused increased lag phase and decreased fibrin formation rate and $OD_{Max}$ regards to control. Significant changes in the kinetic parameters were observed with concentrations equal or higher than 2.5 μg/mL for PF1 and 0.25 μg/mL for Ab1. No plasma coagulation was detected when PF1 was added to reach a 10 μg/mL concentration, neither with 1.25 μg/mL of Ab1; hence, these concentrations were considered as positive controls of the anticoagulant effect. Moreover, effects of PF1 on a purified system were evaluated. When fibrinogen was employed instead of plasma, statistically significant changes in the kinetics parameters respect to control were also observed. In particular, at 25 μg/mL, increase in the *lag phase* [(16.2 ± 0.8) vs. (0.0 ± 0.0)] min, decrease in the slope [(9.0 ± 0.0) vs. (14.0 ± 0.0)] $min^{-1}$, and decrease in the $OD_{Max}$ [(0.226 ± 0.015) vs. (0.338 ± 0.008)] were detected.

*Abbreviations:* rt-PA, recombinant tissue plasminogen activator; SP, sulfated polysaccharides

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**Table 1**
Effects of PF1 and Ab1 on fibrin formation parameters, weight of fibrin and fibrinolysis.

| | Lag phase (min) | $V_{Max}$ ($min^{-1}$) | $OD_{Max}$ | Fibrin weight (mg) | $t_{L1/2}$ (min) |
|---|---|---|---|---|---|
| | 0,0 ± 0,0 | 64,5 ± 9,9 | 0,373 ± 0,010 | 2.149 ± 0.287 | 24.2 ± 3.1 |
| Control | | | | | |
| PF1 (μg/mL) | | | | | |
| 1.25 | 0,1 ± 0,1 | 62,0 ± 9,9 | 0,368 ± 0,002 | 1.967 ± 0.198 | |
| 2.5 | 0,7 ± 0,1* | 29,0 ± 4,2* | 0,329 ± 0,008* | 1.401 ± 0.085* | 22.9 ± 5.8 |
| 5 | 4,1 ± 0,9* | 7,3 ± 1,0* | 0,278 ± 0,006* | 0.934 ± 0.065* | 15.7 ± 1.3* |
| 10 | No coagulation was detected | | 0,210 ± 0,007* | No coagulation | |
| Ab1 (μg/mL) | | | | | |
| 0.125 | 0,4 ± 0,2 | 51,5 ± 9,1 | 0,368 ± 0,029 | 1.880 ± 0.108 | |
| 0.25 | 1,5 ± 0,0* | 41,5 ± 0,7* | 0,350 ± 0,001* | 1.286 ± 0.089* | 17.3 ± 0.3* |
| 0.5 | 1,5 ± 0,35* | 4,0 ± 0,0* | 0,243 ± 0,001* | 0.820 ± 0.062* | 15.3 ± 2.5* |
| 1.25 | No coagulation was detected | | 0,202 ± 0,001* | No coagulation | |

Curves of $OD_{405}$ vs. time were characterized by: *lag phase* (time required for initial protofibril formation), *slope* which corresponds to the maximum velocity achieved ($V_{Max}$), and the *final network OD* ($OD_{Max}$), which is influenced by the number of protofibrils per fiber. Quantification of fibrin was performed by gravimetric method. Fibrinolysis activated by rt-PA (25 IU/mL). Results are expressed as mean ± SD (n = 4).

* Statistically significant differences ($p < 0.05$). Student's *t*-test was used to compare the curves' parameters, the weights of fibrin clots and half-lysis-time values between SP incubated samples and control.

In order to quantify the fibrin obtained in the presence of increasing concentrations of the SP, the weight of the plasma clots was measured. The amount of fibrin formed diminished with increasing concentrations of the PF1 and Ab1 concentrations (Table 1); in particular, significant differences were obtained with concentrations equal or higher than 2.5 μg/mL and 0.25 μg/mL, respectively.

Plasma fibrin networks obtained in the presence of the SP (concentrations tested were 0.125–10 μg/mL) and the control were evaluated by SEM. Images of the networks corresponding to the minimum concentration of each polysaccharide that showed significant differences were chosen as an

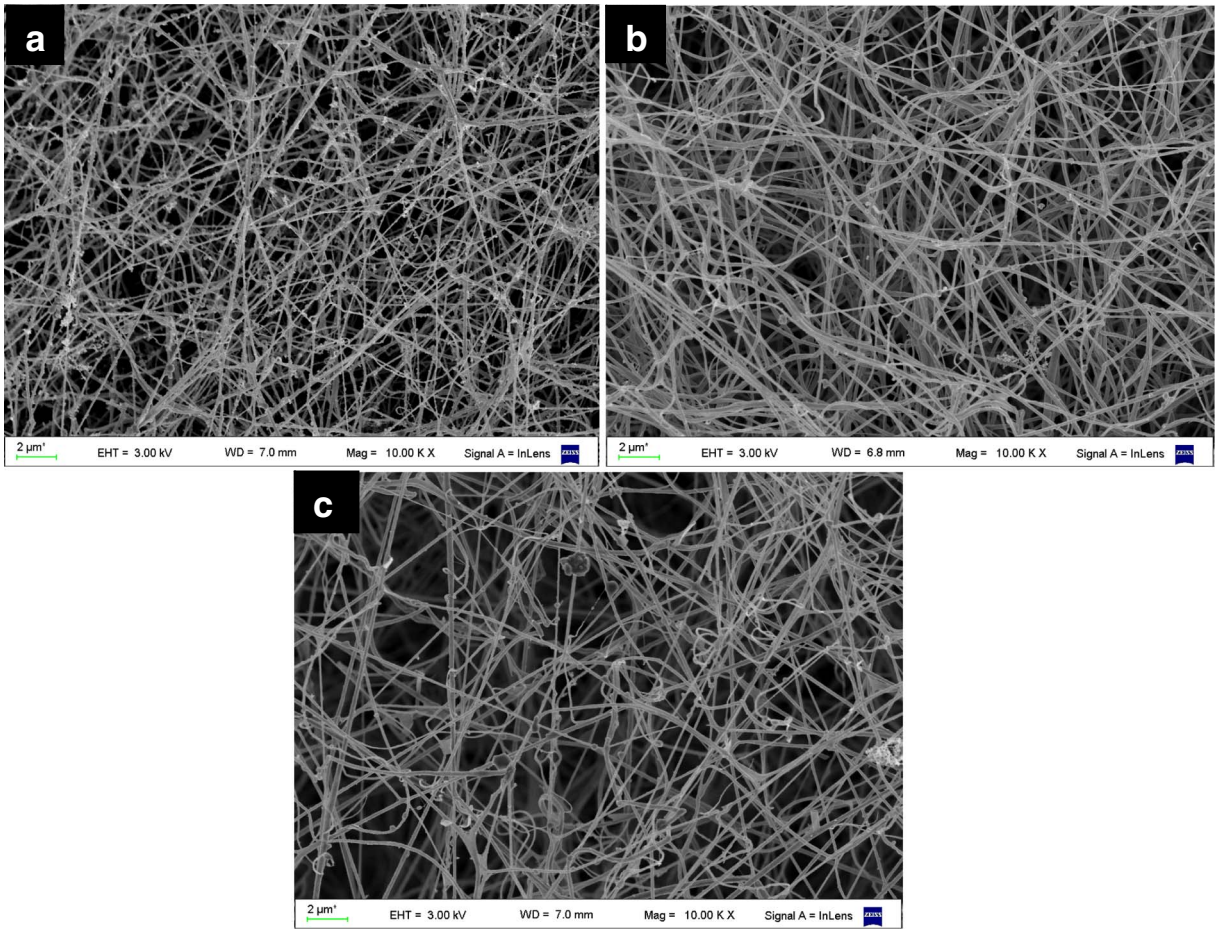


**Fig. 1.** Fibrin structure by scanning electron microscopy (SEM).
Images of plasma fibrin networks obtained in the presence of a) Saline solution as control, b) PF1 5 μg/mL, c) Ab1 0.25 μg/mL by adding thrombin (0.5 IU/mL). Bars indicate the size reference (μm). Stabilized gels were fixed in glutaraldehyde 2% overnight, dehydrated in graded ethanol-water solutions (50–100%), dried by critical point procedure and gold-palladium coated. Observations at 5 kV were photographed at 10,000 ×. Quantitative analysis was performed using ImageJ 1.48v software. Networks were characterized by the number of fibers per field, network percentage (ratio between total surface of the fibers and total field area × 100), width and length of the fibers.

example (5 μg/mL for PF1 and 0.25 μg/mL for Ab1). Fig. 1 shows representative pictures of the tested fibrin networks, denoting a very clear difference between plasma incubated with each of the SP and control. Quantitative analysis of the photographs (Supplementary Table 1) showed that the network percentage and the number of fibers per field were statistically significantly lower in fibrin networks in the presence of PF1 and Ab1, than in the control, rendering a more opened structure. Moreover, fibrin clots in the presence of both SP were composed by significantly thicker and longer fibers than control.

Plasma fibrin networks produced in the presence of different concentrations of PF1 and Ab1 were lysed by adding rt-PA, and the dissolution of the clots by the generated plasmin was studied by the changes of turbidity. *Half-lysis-time* ($t_{L1/2}$) of plasma fibrin network obtained in the presence of each sample resulted shorter than control (Table 1). Moreover, lysis rate was higher when the concentration of the SP was increased.

## 4. Discussion

Fibrin clot formation, final step in blood coagulation, plays a crucial role in the hemostatic system. Under certain conditions, alterations in formation, structure and lysis of fibrin networks can induce pathological consequences, such as thrombotic episodes. It has been widely demonstrated that fibrin architecture is involved in the lysis rate and a recent work has shown that, particularly the intrafibrillar structure of fibrin, that is, the way protofibrils are arranged inside the fibrin fibers, regulates fibrin function, conditioning the subsequent thrombogenic properties of the clot [5].

Previously, we demonstrated that PF1 exerted anticoagulant effects, and a possible direct thrombin inhibition was postulated [3]. In another study, Ab1 proved to have important anticoagulant activity by a mechanism, among others, involving direct thrombin inhibition and that effect was mostly associated to the presence of a sulfate group on C-2 of the β-L-arabinopyranose units [2]. The fact that galactan structures from *Codium* species [6], that are not sulfated on C-2, do not have important anticoagulant action, and that galactans from *P. capitatus*, with similar carbohydrate backbone, but sulfated on C-2 show anticoagulant effect, suggests that the active structure in the galactans studied here could be related to sulfation on C-2 of the main chain.

Kinetics of fibrin formation was affected by both SP, showing delayed polymerization curves, in a concentration dependent manner. Increased times required for initial protofibrils formation (*lag phase*) and decreased maximum velocities achieved (*slope*) observed in our assays are related to an anticoagulant behavior. On the other hand, in the presence of increasing concentrations of each of the SP, decreases in the amount of fibrin generated were detected, denoting a reduced quantity of clotted fibrinogen, consequence of the anticoagulant action of the SP. This behavior of PF1 and Ab1 would be due to the thrombin inhibition mediated by the SP, as was mentioned above.

These results are in agreement with our previous data obtained by global coagulation assays for PF1 and Ab1, which showed anticoagulant effects [2,3]. The impaired plasma fibrin polymerization could be attributed to modifications of fibrinogen and/or other proteins of the hemostatic system. When the action of PF1 on the polymerization process with pure fibrinogen instead of plasma was evaluated, a similar effect was observed. In the assay with purified proteins, the only coagulation factors present were fibrinogen and thrombin. Hence, the significant changes detected in the kinetic parameters indicate that PF1 could impair thrombin activity and/or alter fibrinogen molecule. These results are in accordance to those previously obtained by TT-like assays (Thrombin Time using fibrinogen instead of plasma) [3]. Besides that direct inhibition, an inhibition of thrombin mediated by antithrombin and/or heparin cofactor II should not be discarded. Regarding the possible effects of PF1 on fibrinogen it could be proposed that the presence of the SP, highly negatively charged molecules, interferes with the noncovalent interactions involved in the polymerization step. On the other hand, we previously reported that the mechanisms involved in the anticoagulant effect of Ab1 were direct thrombin inhibition by interaction with exosite 2, and indirect action through potentiation of its physiological inhibitors (antithrombin and heparin cofactor II) [2]. When equal concentrations of each polysaccharide were employed in fibrin formation assays, very different results were observed. In particular, at a concentration of 5 μg/mL an important anticoagulant activity was demonstrated for PF1, but this effect was so important in the case of Ab1, that no coagulation was detected (Suppl. Fig. 2).

In addition, in fibrin formation curves lower final optical densities respect to control were registered in the presence of the SP. It is well known that the final turbidity measurement is related to the weight-averaged mass per unit length (mass/length ratio) of fibrin fibers composing the clot, allowing to infer the structure of the network [7]. It should be noted that the optical density value achieved is also influenced by the branching within the fibrin matrix. Analysis of the clot structure by SEM showed a less branched fibrin than control, composed by wider and longer fibers, being the degree of length increase much higher than that of the width. Thus, the mass/length ratio proved to be lower, and consequently, gave a lower optical density value. Moreover, the delayed kinetics observed in our fibrin polymerization assays is associated to prolonged lateral aggregation times, yielding clots with few branch points and thicker fibers [8].

In the presence of PF1 and Ab1 accelerated fibrinolysis rates were observed, mostly attributed to the reduced amount of fibrin formed. This fact is the result of diminished thrombin activity, mediated, direct or indirectly, by these SP, which induces clotting of a lesser amount of fibrinogen and consequently generates less fibrin. It must be noted that when similar $t_{L1/2}$ values (15 min approximately) were achieved, ten-fold more concentrated solutions of PF1 than Ab1 were employed.

On the other hand, SEM showed important differences between networks. The final fibrin structure obtained in the presence of PF1 (5 μg/mL) and also in the presence of Ab1, but in this case, at a lower concentration (0.25 μg/mL), was less compact than control, composed by thicker and longer fibers. These changes in fibrin architecture could also contribute to the improved fibrinolysis observed when plasminogen was activated by rt-PA. Thus, in these conditions, important decreases of the $t_{L1/2}$ regards to control were obtained. It has been previously reported that fibrin meshwork configuration has a strong effect on the fibrinolysis rate [9]. Several studies have shown that opened fibrin networks are characterized by an increased lysis rate, likely because of a favored transport of fibrinolytic agents, such as plasminogen and t-PA, within the clot. In addition, fibrin fibers diameter is also involved in that process. Thus, thick fibrin fibers provide greater surface for adsorption of more plasminogen and t-PA, favoring the assembly of the fibrinolytic complex and therefore, rendering faster fibrinolytic rates [10]. Moreover, effects of the SP on proteins of the fibrinolytic system should not be discarded.

To our knowledge, the relationship between chemical structure, fibrin formation and lysis was only described by our group for xylogalactoarabinans from *Cladophora falklandica* [11]. More recently, Li et al. demonstrated that a rhamnan-type sulfated polysaccharide from the green seaweed *Monostroma angicava* exhibited high fibrinolytic effect and thrombolytic activity [12].

In summary, the structural and functional features of the clots generated in the presence of PF1 and Ab1 are consequences of the anticoagulant action of these SP which, direct or indirectly, can reduce the thrombin activity. In particular, lower concentrations (expressed as mass/vol) of Ab1 than of PF1 were needed to produce similar anticoagulant effects. Our results show different characteristics of the fibrin, resulting from the action of

SP, contributing to the understanding of their anticoagulant activity, and to the development of new compounds with antithrombotic therapeutic properties.

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## Disclosure of conflict of interests

The authors state that they have no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2017.09.014.

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