



Anticoagulant Activity of Rhamnan Sulfate Isolated from Commercially Cultured *Monostroma nitidum*

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Abstract: The green seaweed, *Monostroma nitidum*, is widespread in Japan. In Okinawa Prefecture, the production of seaweed is performed using culture-nets that are seeded artificially. Algae contain a soluble polysaccharide, rhamnan sulfate. To estimate its applicability, the anticoagulant activity of rhamnan sulfate was investigated. Rhamnan sulfate was fractionated by ion-exchange chromatography on a DEAE-sepharose column, and two fractions (A and B) were obtained. Partially hydrolyzed rhamnan sulfates with different molecular mass (C1, C2 and C3) were also prepared. The activated partial thromboplastin time (APTT) test, prothrombin time (PT) and thrombin time (TT) were applied using human plasma and compared with standard heparin (174 units/mg). The native rhamnan sulfate (molecular mass, 630 kDa; sulfuric acid content, 22.7%), fraction A (12.4%) and fraction B (27.8%) showed approximately 73% APTT activity in comparison with that of standard heparin, but fraction C2 (molecular mass, 450 kDa) had a higher activity than that of the standard (107%). On the other hand, in the PT assay, all fractions except fraction C2 and C3 (370 kDa) showed higher activity approximately 120-155% greater than that of standard heparin. The TT activity of rhamnan sulfate depended on the sulfate content, and that of fraction B, which has high sulfuric acid content (27.8%), was 135-173% greater than that of heparin. The sulfate groups of L-rhamnosyl residues and carboxyl group of D-glucuronosyl residue on the trisaccharide side chains of the rhamnan sulfate might interact strongly with the active site of thrombin molecules. The results and discussion suggested that rhamnan sulfate from commercially cultured *Monostroma nitidum* could be a potential anticoagulant polysaccharide.

Keywords: *Monostroma nitidum*, Commercially Cultured, Seaweed Polysaccharide, Rhamnan Sulfate, Anticoagulant Activity

1. Introduction

We isolated agar [1], methylated agar (agarose) [2], fucoidan [3-7], alginate [5, 8, 9], κ -carrageenan [10], ι -carrageenan [11], rhamnan sulfate [12], ulvan [13] and pyruvated glucogalactan sulfate [14] from some seaweeds grown in Okinawa Islands, Japan. Specifically, the novel acetyl fucoidan from commercially cultured *Cladosiphon okamuranus* was isolated and characterized [5, 15, 16]. An

acetyl fucoidan exhibits some biological activities, such as antitumor [15] and immune-enhancing abilities [16]. An over-sulfated acetyl fucoidan, the sulfate content of which was 32.8%, showed a significant antitumor activity *in vitro* [15]. The results suggested that the over-sulfated acetyl fucoidan was applicable as an anticancer drug. Acetyl fucoidan is now used as a supplement in the health food, food and cosmetic industries worldwide.

On the other hand, one of the authors, Tako, discussed the

structure-function relationship from the viewpoint of the rheological characteristics of algal polysaccharides and the proposed gelation mechanism of κ -carrageenan [17-19], ι -carrageenan [20, 21], agarose [22], and alginate [23, 24]. Consequently, there are some basic thermodynamic and theoretical rules in gel-formation processes of polysaccharides including water molecules in principle [25-27].

The green seaweed, *Monostroma nitidum*, is widespread in nature between Korea, China and Japan. In Okinawa Prefecture, Japan, production of seaweed is performed using culture-nets that are seeded artificially. The annual production of algae in Okinawa was approximately 100 t in 2016. Recently, because *Monostroma nitidum* is used in health foods, salads, soups and other items, its utilization in the food industry has increased.

We previously reported the chemical structure of rhamnan sulfate (octa-saccharide repeating units) that isolated from commercially cultured *Monostroma nitidum* [12]. It was the first report proposed that the entire chemical structure of the polysaccharide. Rhamnan sulfate consists of 1,3-linked α -L-rhamnopyranosyl residues on the main chain, a part of which contains β -D-glucopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranosyl trisaccharide side-chains at the C-2 position on the main chain. The sulfate groups are substituted at the C-4 position of the L-rhamnopyranosyl residues on the main chain as well as at the C-3 position on the side chains, as shown in Figure 1.

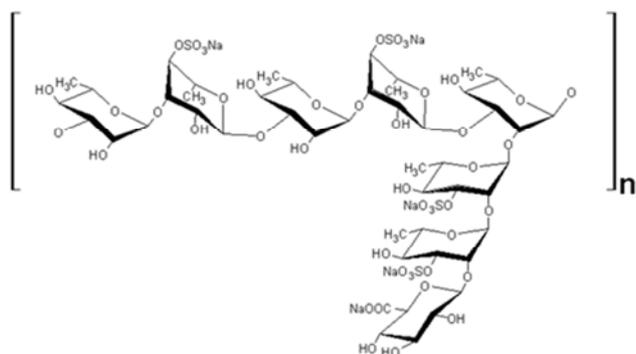


Figure 1. Chemical structure of rhamnan sulfate.

Many sulfated polysaccharides are known to have anticoagulant activities [28-34]. However, a few report on rhamnan sulfate isolated from *Monostroma nitidum* have been published [34]. Here, we report on the anticoagulant activities of rhamnan sulfate isolated from commercially cultured *M. nitidum* in Okinawa, Japan.

2. Material and Methods

2.1. Materials

Monostroma nitidum, which was commercially cultured on nets (1.5 \times 20 m) at sea off of the central part of Okinawa Island (Kitanakagusuku Village), was used in this study. Heparin (sulfate content, 27.8%; molecular mass, 17-19 kDa.; 174 units/mg) from porcine intestinal mucosa, normal

human plasma and human thrombin were purchased by Cosmo Bio Co. Ltd, Tokyo, Japan. APTT reagents including phospholipid and PT reagent, which included rabbit brain thromboplastin were obtained from Eidia Co. Ltd., Tokyo, Japan.

2.2. Preparation of Rhamnan Sulfate

Algae were washed with tap water and air dried at 40 $^{\circ}$ C for 48 h. An air-dried seaweed sample (20 g) was suspended in distilled water adjusted to a pH 3.0 with 0.05 M hydrochloric acid and stirred at 60 $^{\circ}$ C for 3 h to extract the polysaccharide. The extract was neutralized with a 0.05 M NaOH solution, and then centrifuged at 13,000 g for 20 min. The supernatant was filtered through Celite 545 (Nakarai, Japan). The filtrate was precipitated by adding 2 volumes of ethanol, and the resulting solid was dried *in vacuo* [12-14].

The crude polysaccharide was dissolved in distilled water and the solution was passed through Celite 545. The filtrate was deionized by passage through a cation exchange column composed of Amberlite 120A H⁺ (Organo, Japan). After neutralization, the solution was subsequently lyophilized.

2.3. Determination of Total Carbohydrate, Uronic Acid and Sulfuric Acid of Rhamnan Sulfate

The composition of the total carbohydrate and uronic acid was determined by the phenol-sulfuric acid method [35] and the carbazole-sulfuric acid method [36] using L-rhamnose and D-glucuronic acid as standards, respectively. Sulfuric acid was determined by the turbid-metric method described by Dodgson and Price [37].

2.4. Fractionation of Rhamnan Sulfate

Fractionation of rhamnan sulfate was performed by passage via anion exchange chromatography (DEAE Sepharose: ϕ 25 \times 380 mm). After applying sample (100 mg/mL), distilled water (100 mL) was eluted, and a gradient of sodium chloride solutions was from 0 to 3.5 M.

2.5. Partial Hydrolysis of the Rhamnan Sulfate

Rhamnan sulfate (100 mg) was dissolved in formic acid solutions adjusted to pH 3.5, 2.5 and 1.5. Each solution was heated at 70 $^{\circ}$ C for 2 h, and then neutralized by the addition of 0.05M sodium hydroxide. The hydrolyzed solutions were dialyzed using a cellulose membrane with a molecular weight of 3,500 (Membrane Filtration Products, Inc., USA) in distilled water for 24 h. Samples prepared from pH 3.5, 2.5 and 1.5 formic acid were referred to as C1, C2 and C3, respectively.

2.6. Molecular Mass Determination of Rhamnan Sulfate

The molecular mass of rhamnan sulfate (1 mg/mL) was determined by high-performance liquid chromatography (HPLC), using an LC-6A chromatograph (Shimadzu, Kyoto Japan) on a TSK-gel GMPW column (7.8 \times 200 mm, Tosoh Corporation, Tokyo, Japan). HPLC analysis was performed at

a flow rate of 0.3 mL/min with refractive index detection (RID-6A, Shimadzu) at room temperature. The column was conditioned with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2), and elution was conducted with the same buffer. Standard pullulans (Showa Denko Co. Ltd., Tokyo, Japan) including P-800 (molecular mass, 8.1×10^5 Da), P-400 (4.0×10^5), P-200 (2.2×10^5) and P-100 (1.1×10^5) were used as molecular mass markers [12-14].

2.7. Assay for Anticoagulant Activity

The anticoagulant activities of various types of rhamnan sulfates were measured by three different *in vitro* assays, including the activated partial thromboplastin test (APTT), prothrombin time (PT) and thrombin time (TT). The values were the means of three separate measurements by visual inspection of generated white turbidity after gently pendular movement and expressed as a % in relation to that of heparin (174 units/mg: 100%), which was used as a standard.

2.7.1. Activated Partial Thromboplastin Time (APTT)

The APTT clotting assay was performed using normal human plasma according to the method of Anderson et al. [38]. Normal plasma (100 μ L) was incubated for 1 min at 37°C with 10 μ L of a solution of rhamnan sulfate or heparin (75 or 150 μ g in 1 mL of physiological saline), and then, APTT reagent was added and allowed to incubate for 2 min at 37°C. Prewarmed 20 mM CaCl₂ (100 μ L) was added and measured during clot formation.

2.7.2. Prothrombin Time (PT)

The PT clotting assay was performed by the method of Quick [39]. One hundred microliters of plasma and 10 μ L of samples (75 and 150 μ g/mL of physiological saline) were mixed and warmed for 1 min at 37°C. Then, 100 μ L of PT rabbit brain thromboplastin was added to the mixture. After 2 min of incubation at 37°C, 100 μ L of 20 mM CaCl₂ was added to the mixture and the clotting time was measured.

2.7.3. Thrombin Time (TT)

The TT clotting assay was performed by the method of Denson and Bonnar [40]. Plasma from humans (100 μ L) was incubated with 10 μ L of sample (75 and 150 μ g/mL of physiological saline). After 3 min of incubation at 37°C, 100 μ L of human thrombin was added to the mixture and the clotting time was measured.

3. Results

3.1. Chemical Characteristics and Molecular Mass of Rhamnan Sulfate

The yield of rhamnan sulfate from commercially cultured *Monostroma nitidum* was 23.0% and 4.3% (W/W) on the basis of dried and wet algae, respectively. The total carbohydrate, D-glucuronic acid and sulfate contents of rhamnan sulfate were estimated to be 63.1%, 12.5% and 22.7%, respectively. The molecular mass of the polysaccharide (native) was 630 kDa.

3.2. Fractionation and Sulfate Content of Rhamnan Sulfate

As shown in Figure 2, two fractions of rhamnan sulfate were obtained and referred to Fraction A and B. The sulfate contents of Fraction A and Fraction B were 12.4% and 27.8%, respectively.

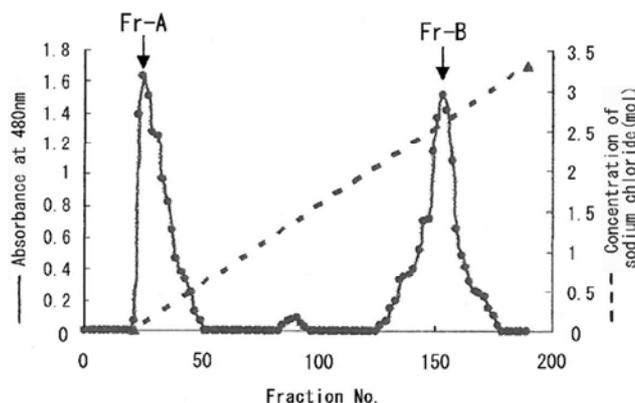


Figure 2. Fractionation of the rhamnan sulfate isolated from *M. nitidum* by DEAE-Sepharose exchange column.

3.3. Partially Hydrolysis and Molecular Mass of Rhamnan Sulfate

Rhamnan sulfate was hydrolyzed into smaller fragments with different molecular weights by a formic acid solution adjusted to pH 3.5, 2.5 and 1.5; the products were referred to as C1, C2 and C3. The molecular masses of the C1, C2 and C3 fractions of rhamnan sulfate were 630, 550, 450 and 370 kDa, respectively, as determined by high performance gel chromatography.

3.4. Anti-coagulant Activity

Heparin is a linear acidic polysaccharide that has been used in anticoagulant therapy for a long times. The polysaccharide consists of 1,4-linked α -D-glucosamine and uronic acids (β -D-glucuronic acid and β -L-idulonic acid). Sulfate groups were attached to the C-2 and C-6 position of sugar residues. When blood clotting is inhibited in the intrinsic and/or the extrinsic systems, each clotting factor (proenzyme) is activated in a stepwise sequence until thrombin is formed and subsequently fibrinogen is converted to fibrin. It has been reported that the action of heparin as a plasma anticoagulant is the potentiation of the rates of inhibition of the activated clotting factors, such as factor II (prothrombin), X and XII.

In this investigation, the anticoagulant activities of rhamnan sulfates with different sulfate contents or different molecular weights were measured by *in vitro* assays including the activated partial thromboplastin time test (APTT), prothrombin time (PT) and thrombin time (TT), and compared to heparin (174 units/mg), which was used as a standard. The concentrations of the sample and standard heparin, 75 and 150 μ g/mL, were determined from a published paper [31].

3.4.1. Activated Partial Thromboplastin Time (APTT)

The anticoagulant activities of native rhamnan sulfate, and

those with different sulfate contents (fraction A and B) and different molecular weights (C1, C2, and C3) were assayed with respect to APTT, which is related to the intrinsic coagulant and/or common pathway using normal human plasma. The APTT test reflects the activities of coagulation factors XII, XI and IX in the intrinsic coagulant pathway, as well as coagulation factors in the common pro-coagulant pathway, which include factors II (prothrombin), X and V. Figure 3 shows the effect of native rhamnan sulfate (sulfate content: 22.7%), fraction A (12.4%) and fraction B (27.8%) as well as heparin at concentrations of 75 and 150 $\mu\text{g/mL}$ on the APTT. The generated white turbidity was expressed as a relative percentage of heparin. The anticoagulant activities of rhamnan sulfate and its fragments were weaker than those of heparin approximately 60-78% at a concentration of 75 $\mu\text{g/mL}$ and 30-35% at 150 $\mu\text{g/mL}$, respectively. As shown in Figure 4, partially hydrolyzed rhamnan sulfates C1 (550 kDa), C2 (450 kDa) and C3 (370 kDa) also exhibited weaker activity than standard heparin except C2 (107%) at 75 $\mu\text{g/mL}$. The results suggested that the interaction between the rhamnan sulfate and intrinsic factors was not as strong as standard heparin.

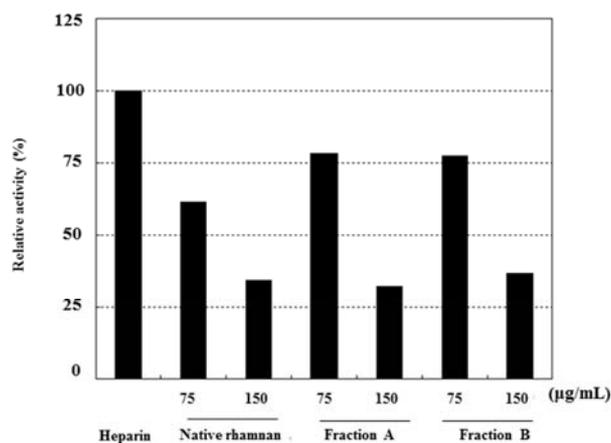


Figure 3. APTT assay of the fractionated rhamnan sulfate isolated from *M. nitidum*. Expressed as % in relation to that of heparin (174 units/mg: 100%) as a standard. Values are means the average of the measurements in three times.

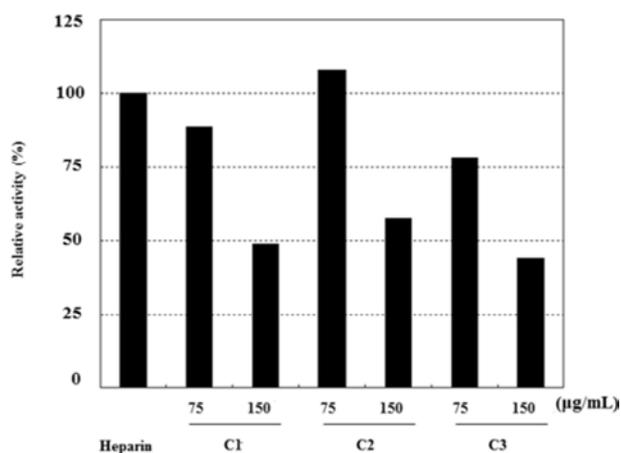


Figure 4. APTT assay of the partially hydrolyzed rhamnan sulfate isolated from *M. nitidum*. Expressed as % in relation to that of heparin (174 units/mg: 100%) as a standard.

3.4.2. Prothrombin Time (PT)

The PT test reflects the activities of the extrinsic and/or common coagulant pathway, which includes factors VII for former pathway, and factors X, V and II for latter. The anticoagulant activity of native and fractionated (A and B) and partially hydrolyzed (C1, C2 and C3) rhamnan sulfates were assayed with respect to PT and are presented in Figure 5 and 6. The native and fractionated (A and B) rhamnan sulfates showed stronger inhibition than standard heparin approximately 120-123% at a concentration of 75 $\mu\text{g/mL}$. However, weaker inhibition was observed at a concentration of 150 $\mu\text{g/mL}$, approximately 60-70% (Figure 5). These results suggest that inhibition of the rhamnan sulfate depends on the polysaccharide concentration. Figure 6 shows partially hydrolyzed rhamnan sulfate (C1, C2 and C3). Only C1 (Molecular mass: 550 kDa) exhibited stronger inhibition approximately 120-155%, but C2 (450 kDa) and C3 (370 kDa) exhibited weak inhibition (15-78%). The results suggest that the PT prolongation depends on the molecular mass of rhamnan sulfate. Rhamnan sulfate with long molecular mass > 550 kDa interacted with thromboplastin and/or prothrombin molecules, resulting in the prolongation of the PT time.

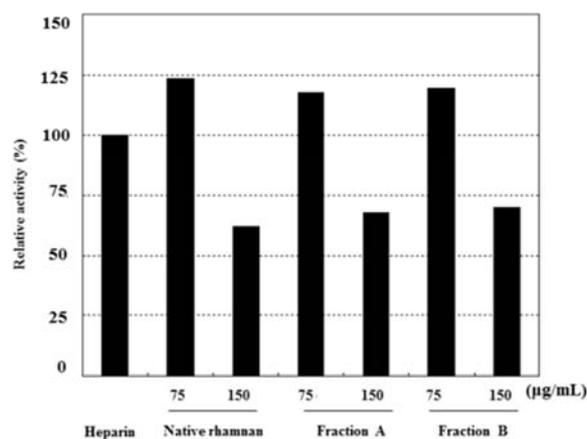


Figure 5. PT assay of the fractionated rhamnan sulfate isolated from *M. nitidum*. Expressed as % in relation to that of heparin (174 units/mg: 100%) as a standard.

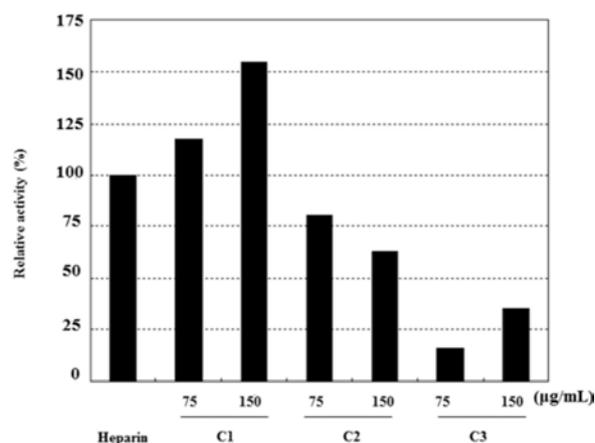


Figure 6. PT assay of the partially hydrolyzed rhamnan sulfate isolated from *M. nitidum*. Expressed as % in relation to that of heparin (174 units/mg: 100%) as a standard.

3.4.3. Thrombin Time (TT)

TT is an important test because it checks to see if different blood clotting factors II (prothrombin), VII and X are present. The TT assay is related to thrombin activity or fibrin polymerization resulting in the extrinsic coagulant pathway which inactivates several enzymes of the coagulation system, such as factor II (prothrombin), VII and X in plasma. The results of the TT assay were presented in Figure 7 and 8. Native rhamnan sulfate showed a high potency of inhibition at a concentration of 75 $\mu\text{g/mL}$ (167%), but remained at a low value, 70%, at a high concentration of 150 $\mu\text{g/mL}$. As for the high sulfate fraction (B), the most potent inhibition was observed (138-170%), while the low sulfate fraction (A) showed very weak inhibition (30-75%), as shown in Figure 7. The results indicate that TT prolongation depends on the sulfate content. The activity increased as the sulfate content increased. The results suggest that highly sulfated rhamnan sulfate (Fraction B) inhibited thrombin activity. The effect of the molecular mass of the rhamnan sulfate on TT inhibition is shown in Figure 8. Fractions C2 and C3 retained almost the same degree of inhibition (100-105%) as standard heparin at concentrations of 150 $\mu\text{g/mL}$ for the former and at 75 $\mu\text{g/mL}$ for the latter. Harada and Maeda reported that a rhamnan sulfate isolated from *Monostroma nitidum* collected in Shizuoka Prefecture, Japan, had strong anti-thrombin activity about 5 times higher than that of standard heparin [34]. Thus, the results suggest that highly sulfated rhamnan sulfate interacted strongly with thrombin molecules resulting in the prolongation of the TT time.

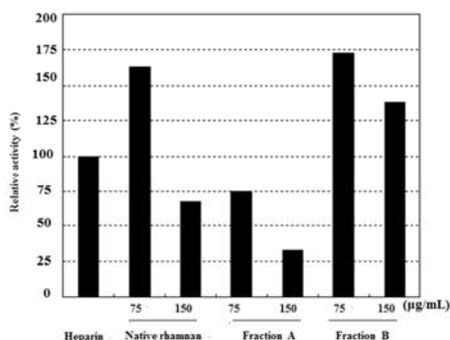


Figure 7. TT assay of the fractionated rhamnan sulfate isolated from *M. nitidum*. Expressed as % in relation to that of heparin (174 units/mg: 100%) as a standard.

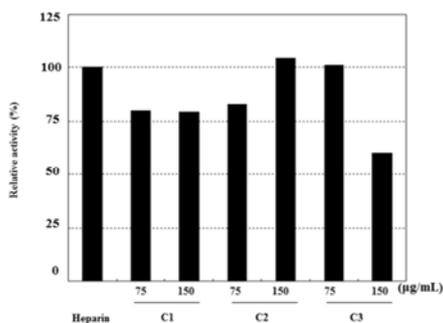


Figure 8. TT assay of the partially hydrolyzed rhamnan sulfate isolated from *M. nitidum*. Expressed as % in relation to that of heparin (174 units/mg: 100%) as a standard.

4. Discussion

The anticoagulant activity of a sulfated polysaccharide (rhamnan sulfate) mainly consists of L-rhamnopyransyl residues and sulfate (21.2%) isolated from *Monostroma latissimum*, as reported by Zhang et al. [31]. They prepared six rhamnan sulfates with different molecular masses from 725.4 (native) to 10.6 kDa. Those polysaccharides inhibited the intrinsic and/or common pathway of coagulation (APTT) and thrombin activity (TT), but not extrinsic pathway. They concluded that a greater molecular mass was necessary to achieve the coagulant inhibition of the sulfated polysaccharide. However, all polysaccharides exhibited lower activity than those of standard heparin.

In this investigation, we prepared three different sulfate contents (22.7, 12.4 and 27.8%) and four different molecular masses (630, 550, 450 and 370 kDa) of rhamnan sulfates from *Monostroma nitidum* and submitted them to APTT, PT and TT assays. Native rhamnan sulfate (molecular mass, 630 kDa; sulfuric acid content, 22.7%), fraction A (12.4%) and fraction B (27.8%) showed approximately 73% APTT activity in comparison with standard heparin, but the molecular mass decreased, (fraction C2, 450 kDa and C3, 370 kDa) inhibition showed higher activity than that of the standard (107%). On the other hand, PT depends on the molecular mass. The anticoagulant activity of the rhamnan sulfate with a greater molecular mass (native, fraction A, fraction B and C1 (550kDa) was higher than that of standard heparin (120-155%), but as the molecular mass decreased, (C2, 450kDa and C3, 370kDa) inhibition was reduced. The TT activity of rhamnan sulfate depended on the sulfuric acid content, and fraction B which has the highest sulfuric acid content (27.8%), showed TT activity that was 173% and 135% greater than that of heparin at concentrations of 75 and 150 $\mu\text{g/mL}$, respectively. The results indicated that rhamnan sulfate from commercially culture *Monostroma nitidum* showed moderate inhibition of intrinsic coagulant pathways and strong inhibition of the extrinsic and common path-ways relative to heparin. Consequently, rhamnan sulfate is applicable as an anti-coagulant. Further investigation is now in progress.

5. Conclusion

Rhamnan sulfate isolated from commercially cultured *Monostroma nitidum* prolonged the extrinsic and common pathways of coagulation by inhibiting thrombin activity relative to standard heparin. The results suggested that rhamnan sulfate with a molecular mass >550 kDa interacted with thromboplastin and prothrombin. In addition, the sulfate groups of L-rhamnosyl residues and carboxyl group of the D-glucuronosyl residue on the trisaccharide side chains of rhamnan sulfate might strongly interact with the active site of the thrombin molecules.

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