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Isolation and purification of an anticoagulant from fermented red seaweed *Lomentaria catenata*

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Abstract

We attempted to isolate an anticoagulant polysaccharide from marine algae by fermentation of freeze-dried seaweed with nonselective microorganisms present in the seaweed itself. An anticoagulant compound was purified from red algae *Lomentaria catenata* and the molecular mass of the purified polysaccharide ranged from 100 to 500 kDa. The purified anticoagulant polysaccharide was mainly composed of galactose with small amounts of glucose and it is highly sulfated (21.76% as SO_4^{2-}). In addition, 9.42% of protein content proved the compound to be a proteoglycan. The anticoagulant activity of the purified compound was assayed by the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) assays and compared with the classical anticoagulant heparin. It demonstrated >1000 s of APTT activity at 40 μg/mL and the activity is greater than heparin (183 IU/mg, >1000 s of APTT activity at 62.5 μg/mL). The anticoagulant compound showed prolonged activity towards APTT and TT assays but low or no activity for PT. Therefore, the isolated compound may act on the intrinsic and/or common pathways of the blood coagulation system.

Keywords: *Lomentaria catenata*; Fermentation; Anticoagulant; Polysaccharide; Red algae

1. Introduction

The current leading causes of death are disorders of the cardiovascular system. Heparin has been identified and used for more than 50 years as a commercial anticoagulant. Recently, several side effects of heparin have been identified such as development of thrombocytopenia, hemorrhagic effect, ineffectiveness in congenital or acquired antithrombin deficiencies, incapacity to inhibit thrombin bound to fibrin, and more (Pereira, Melo, & Mourao, 2002). Furthermore, heparin is available in very low concentrations in pig intestine or bovine lungs from where it is primarily extracted (Pereira et al., 2002). Therefore, the necessity of discovering alternative sources of anticoagulants has been arisen with the increasing demand for safer anticoagulant therapy.

In recent years, plant originated macromolecules have been intensively investigated for their biological activities. Among those, polysaccharides have gained attention as biological macromolecules and polysaccharides with a sulfate group play a predominant role. These sulfated polysaccharides contain hemi-ester sulfate groups in their sugar residues (Shanmugam & Mody, 2000). Moreover, these compounds are commonly found in marine algae and higher animals but are absent in microbes and in higher plants (Shanmugam & Mody, 2000). Anticoagulant activity of sulfated polysaccharides has been widely studied. Two types of sulfated polysaccharides with anticoagulant properties are fucoidans, which are available in brown seaweeds (Chevolot et al., 1999; Colliec et al., 1991; Dobashi,

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Nishino, Fujihara, & Nagumo, 1989), and galactans, which are rich in red seaweeds (Carlucci et al., 1997; Kolender, Pujol, Damonte, Matulewicz, & Cerezo, 1997; Sen et al., 1994).

Sulfated galactans are primarily abundant in marine invertebrates (Mourão & Perlin, 1987; Pavão, Albano, Lawson, & Mourão, 1989), sea grasses (Aquino, Landeira-Fernandez, Valente, Andrade, & Mourão, 2005), and especially in seaweeds of the family Rhodophyta (Lahaye, 2001; Velde, Pereira, & Rollema, 2004). Sulfated galactans of algae are also known as carrageenan or agaran, whose structure is arranged by 4-linked β-galactopyranose and 3-linked α-galactopyranose in an alternating pattern (Lahaye, 2001). Since sulfate esters, methyl groups, or pyruvic acid substitute the various hydroxyl groups, the structure of the sulfated galactans of marine algae becomes more complex (Usov, 1998).

The blood coagulation pathway is divided into an intrinsic pathway and extrinsic pathway. Anticoagulation occurs mainly due to the inhibition of key coagulation serine proteases, thrombin, and factor Xa by the activity of serine protease inhibitor antithrombin III (Shanmugam & Mody, 2000). The anticoagulant activity of sulfated galactans is exhibited via the thrombin inhibition in the intrinsic pathway of blood coagulation (Shanmugam & Mody, 2000).

We identified anticoagulant property from several marine algae species. This study was conducted to purify the anticoagulant compound from the red seaweed *Lomentaria catenata*. The anticoagulant property of *L. catenata* belonging to the family Champiaceae (Rhodymeniales) has not been studied. As a cost effective method of digestion, we performed natural fermentation using nonspecific microorganisms. Although the enzymatic digestion is a quick method, its substrate specificity, pH adjustments, and residual effects increase the production cost. Natural fermentation is a cheap and effective method for polysaccharide hydrolysis. Here, we present the evidence of anticoagulant activity of purified sulfated polysaccharide from fermented *L. catenata*.

2. Materials and methods

Lomentaria catenata was kindly donated by Prof Y. J. Jeon, Bio-resource Lab, Cheju National University. After removing salt, sand, and epiphytes by washing with water, samples were subjected to freeze drying and stored at −20 °C for further testing. Activated partial thromboplastin time (APTT) reagent, $CaCl_2$ solution, prothrombin time (PT) reagent, and thrombin time (TT) reagent were purchased from the Fisher Scientific Company (VA, USA). DEAE-cellulose and Sepharose-4B resins were purchased from Sigma–Aldrich, USA. Sodium barbital was purchased from Wako Pure Chemical Industries, Japan. All other chemicals used were of analytical grade.

2.1. Fermentation of seaweeds

Freeze-dried red seaweed samples (1.5 g) and 45 g of sugar were mixed with 300 mL water in glass jars. The jars containing the algal mixture and control samples were incubated at 25 °C for 10 weeks to carry out the fermentation process. From each sample, 5 mL were drawn from the supernatant after shaking, before the fermentation process started, and during the fermentation at 2-weeks interval.

Yield estimation was done by oven drying the sample (1 mL) at 105 °C for 24 h with and without sugar in preweighed aluminium plates. After reducing the sugar content, the weight difference was expressed as yield in mg/mL.

2.2. Anticoagulation assay

Anticoagulant activity was determined by activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) assays according to the manufacturer's guidelines (Fisher Scientific Company, USA) using a dual-channel clot-2, (SEAC, Italy) and the activity was compared with heparin, the commercial anticoagulant. Human plasma was prepared with slight modifications of Shida et al. (2001). Briefly, blood was collected and pooled from 10 healthy individual donors into conical tubes with 2.5% sodium citrate solution (9:1 v/v). The plasma was separated from blood cells by centrifuging at 3000 rpm at 4 °C for 20 min. The plasma was stored at −60 °C for further testing.

2.3. Extraction of crude polysaccharides

The aqueous fraction of the fermented algal mixture (150 mL) was mixed with 300 mL of absolute ethanol (99.9%). After 24 h, the soluble polysaccharide content was precipitated by centrifugation at 10,000 rpm at 4 °C for 20 min (Iacomini et al., 2005). The recovered polysaccharide was dissolved in distilled water and freeze dried.

2.4. Anion exchange chromatography

The crude polysaccharide sample (200 mg) was purified by anion exchange chromatography using DEAE-cellulose column as described by Pereira, Mulloy, and Mourao (1999). The sample was applied to a DEAE-cellulose column (18 × 1 cm) linked to FPLC system from ProTeam LC™ (Lincoln, USA), which was pre-equilibrated with 50 mM sodium acetate (pH 5.0) and washed with 200 mL of the same buffer containing 0.2 M NaCl. The column was eluted by a linear gradient prepared by mixing 150 mL of 50 mM sodium acetate (pH 5.0) containing 0.2 M NaCl with 150 mL of 2 M NaCl in the same buffer with the flow rate of 60 mL/h. Then, 5-mL fractions were collected and total polysaccharide content and metachromatic properties were monitored by phenol–H_2SO_4 assay

and 1,9-dimethylmethylene blue assay (Chaplin, 1994). Anticoagulant activity was determined by APTT assay and fractions with high activity were pooled, dialyzed against distilled water, and concentrated.

2.5. Gel filtration chromatography

The partially purified polysaccharide was applied to gel filtration chromatography on Sepharose-4B column, equilibrated with distilled water. The column was eluted with distilled water with the flow rate of 15 mL/h. Then, 5-mL fractions were collected and total polysaccharide content and the metachromatic properties were monitored by the phenol–H_2SO_4 assay and 1,9-dimethylmethylene blue assay (Chaplin, 1994), respectively. The anticoagulant activity was monitored by APTT assay and highly active fractions were pooled and concentrated.

2.6. Agarose gel electrophoresis

The purity of the algal polysaccharide was analyzed by agarose gel electrophoresis according to the method described by Pereira et al. (1999). Purified compound (~10 μg) was applied to a 0.5% agarose gel, run for 1 h at 100 V in 0.05 M 1,3-diaminopropane/acetate (pH 9.0), and fixed with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v).

2.7. Polyacrylamide gel electrophoresis

The molecular weight of the purified compound was estimated by polyacrylamide gel electrophoresis (PAGE) (Pereira et al., 1999). Standards and the sample (~10 μg) were applied to a 6% 1-mm-thick polyacrylamide gel in 0.02 M sodium barbital (pH 8.6). After electrophoresis (100 V for 30 min), the gel was stained with 0.1% toluidine blue in 1% acetic acid and then washed for about 4 h in 1% acetic acid.

2.8. Neutral sugar content analysis

The hexose content of the purified sample was measured by the phenol–H_2SO_4 assay (Chaplin, 1994). The neutral sugars (fucose, galactose, glucose, and mannose) and amino sugars (glucosamine and galactosamine) were separated and quantitated on a CarboPac PA10 column (4.5 × 250 mm) with a Bio-LC DX-600 (Dionex Co., Sunnyvale, CA, USA) using the Carbopac PA1 cartridge (4.5 × 50 mm).

2.9. Protein content analysis

The purified sample (30 μg) was hydrolyzed in 0.5 mL of 20% trifluoroacetic acid in screw capped polypropylene tubes. Protein present in soluble polysaccharide was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

2.10. Sulfate content analysis

Sulfate content was determined by rhodizonate method described by Silverstri, Hurst, Simpson, and Settine (1982) with a sulfate standard. Briefly, 100 μL of hydrolyzed sulfated polysaccharide were mixed with 600 μL Barium buffer (2.5 mL of 2 M acetic acid, 0.5 mL of 0.01 M $BaCl_2$, and 2 mL of 0.02 M $NaHCO_3$, which were diluted to 75 mL with ethanol) and 300 μL rhodizonate reagent (5 mg rhodizonate and 100 mg ascorbate in 20 mL deionized water then dilute to 100 mL with ethanol). The absorbance was measured at 520 nm after 10 min of incubation at room temperature.

3. Results and discussion

3.1. Seaweed fermentation

Carrageenans of red seaweeds need to be chemically modified or depolymerized to before being used as an anticoagulant (Shanmugam & Mody, 2000). Several methods such as acid base hydrolysis and enzymatic hydrolysis can depolymerize the polysaccharide of seaweeds. In this study, we introduced natural fermentation as a cheap and effective method of hydrolyzing polysaccharides. Four red seaweeds (*Lomentaria catenata*, *Gracilaria verrucosa*, *Gloiopeltis furcata*, and *Gracilaria textorii*) available in Cheju Island were fermented and their anticoagulant activity was assayed (Table 1). *L. catenata* showed higher anticoagulant activity (>1000 s of APTT activity) compared to other seaweeds during their peak anticoagulant activity (Table 1). Sulfated galactans are water soluble polysaccharide found in red seaweeds (Takano, Shiomoto, Kamei, Hara, & Hirase, 2003). With the fermentation, sulfated galactans content in the supernatant of the algal mixture increases. Therefore, the anticoagulant activity of the seaweed mixture increased with the fermentation of *L. catenata* and peaked at the 4th week of fermentation (Table 2).

Table 1
The maximum APTT, PT, and TT clotting times of selected fermented red seaweeds

Seaweed	Anticoagulant activity (s)		
	APTT	PT	TT
Lomentaria catenata	>1000	20.9	>1000
Gracilaria verrucosa	38.6	25.3	13.2
Gloiopeltis furcata	51.7	31.6	13.6
Gracilaria textorii	49.1	24.7	13.1
Control	36.8	11.7	12.3

The activity was expressed by the time (s) taken to clot the human plasma. Water was used as the control. All the values were the mean of triplicates.

Table 2
The increase in anticoagulant activity of *L. catenata* during the fermentation

Sample	Fermentation period (weeks)	APTT (s)
Lomentaria catenata	0	137.2
	2	781.5
	4	>1000
	6	>1000
	8	>1000
	10	>1000
Heparin[a] (62.5 μg/mL)		>1000
Water[b] (control)		36.8

APTT activity is expressed by time (s). All the values were the mean of triplicates.
[a] Heparin (183 IU/mg) is used as the positive control.
[b] Water was the control.

The soluble polysaccharide content was extracted at 4th week of fermentation had reached its maximum anticoagulant activity. The recovered soluble crude polysaccharide content accounted for 17% of the seaweed's dried weight. The papain digestion of red seaweeds *Gracilaria cornea* (Melo, Feitosa, Freitas, & de Paula, 2002) and *Botryocladia occidentalis* (Farias, Valente, Pereira, & Mourao, 2000) yielded 11% and 4% of soluble sulfated polysaccharides, respectively. Therefore, fermentation can be suggested as an efficient method to extract soluble polysaccharides.

3.2. Purification of anticoagulant compound

The crude polysaccharide was purified by anion exchange chromatography into a single peak at the NaCl gradient 0.35–0.4 M (Fig. 1). The fractions showing strong anticoagulant activity were pooled and further purified by Sepharose-4B gel filtration column (Fig. 2). The purity of the algal polysaccharide was determined by studying the migration pattern on an agarose gel. The anticoagulant of *L. catenata* showed a single clear spot on the agarose gel (Fig. 3A) and it revealed the high purity purified compound.

3.3. Molecular mass of the purified anticoagulant

The Sepharose-4B column eluted the polysaccharide into a single peak at the initial stage of the fractionation (Fig. 2). It suggested the molecular weight of the purified compound to range from 100 to 500 kDa. This estimate was corroborated by PAGE (Fig. 3B). The band of the algal polysaccharide lies between chondroitin 6 sulfate sodium salt (60 kDa) and dextran sulfate sodium salt (500 kDa) on the polyacrylamide gel. The anticoagulant sulfated galactan identified from green alga *Codium pugniformis* also reported as 100–500 kDa in molecular mass (Matsubara, Matsuura, Hori, & Miyazawa, 2000). The purified polysaccharide results a broad band on PAGE (Fig. 3B). It suggested that the purified anticoagulant polysaccharide of *L. catenata* behaves as a heterogeneous system, similar to the polysaccharides identified from red

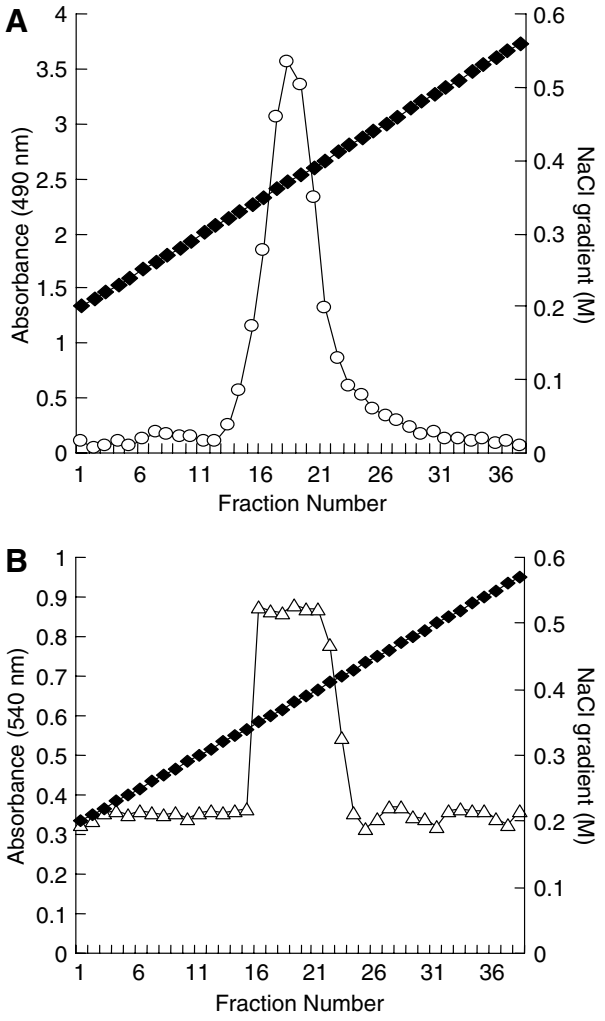

Fig. 1. Purification of anticoagulant from red seaweed *L. catenata* by DEAE-cellulose column under the NaCl gradient (♦). Fractions of 5 mL were collected and assayed by phenol–H_2SO_4 assay (○), (A); 1,9-dimethylmethylene blue assay (Δ), (B).

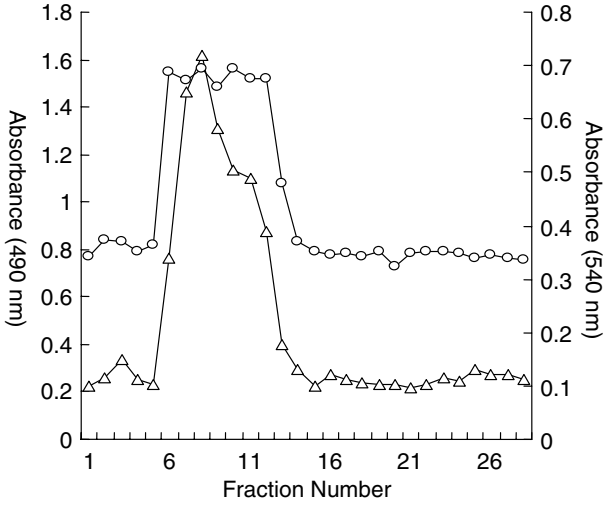

Fig. 2. Purification of anticoagulant from red seaweed *L. catenata* by Sepharose-4B column. Fractions of 5 mL were collected and assayed by phenol–H_2SO_4 (△) and 1,9-dimethyl methylene blue assay (○).

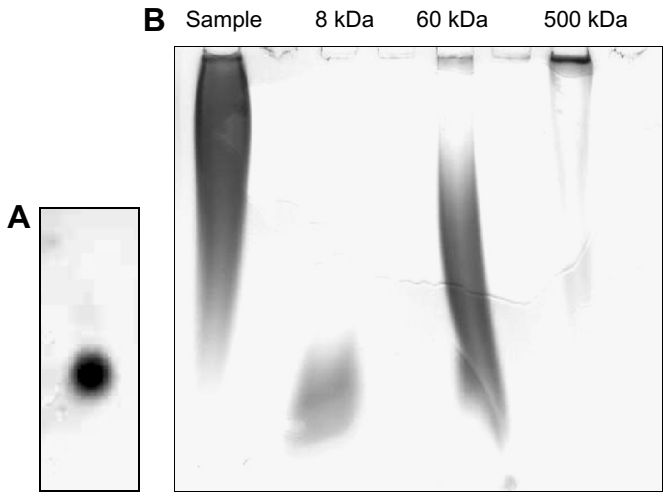

Fig. 3. Agarose gel electrophoresis (A) and polyacrylamide gel electrophoresis (PAGE) (B) of the purified polysaccharide from *L. catenata*. Dextran sulfate sodium salt from *Leuconostoc* spp. (8 kDa), chondroitin 6 sulfate sodium salt from shark cartilage (60 kDa), and dextran sulfate sodium salt (500 kDa) were used as standards for PAGE.

alga *B. occidentalis* (Farias et al., 2000), *Gelidiuim crinale* (Pereira et al., 2005), and *G. cornea* (Melo et al., 2002). The similar behavior of the sulfated galactans was also reported in marine invertebrates (Pavão et al., 1989; Santos, Mulloy, & Mourão, 1992). Higher molecular weight carragenans with high sulfur content showed higher anticoagulant activity than those with low molecular weight with low sulfur content (Shanmugam & Mody, 2000). Low molecular weight carragenans have low anticoagulant activity and potent anti-HIV activity (Yamada, Ogamo, Saito, Uchiyama, & Nakagawa, 2000).

3.4. Anticoagulant activity of the purified polysaccharide and its chemical composition

The blood coagulation system consists of intrinsic and extrinsic pathways, where a series of factors affect the mechanism. Anticoagulants inactivate or restrict the activity of factors that affect either one or both of these pathways. APTT is a measure of the intrinsic pathway depending-clotting time and PT is the extrinsic pathway depending-clotting time.. The TT revealed the thrombin inhibition–dependent clotting time, which is common to intrinsic and extrinsic pathways. The anticoagulant activity of the purified *L. catenata* proteoglycan was assayed by APTT, PT, and TT (Table 3). APTT and TT were prolonged at 40 μg/mL of polysaccharide concentration; however, no clotting inhibition was observed in PT assay. The anticoagulant activity of the *L. catenata* polysaccharide was greater than that of heparin (183 IU/mg) and it effectively acts on the intrinsic and the common pathways of the blood coagulation system. The lack of effect on PT suggests that it does not inhibit the extrinsic pathway of coagulation.

The correlation between the structure and anticoagulant activity of sulfated galactans has been studied by Pereira et al. (2002). Anticoagulant activity of sulfated galactans depends on the nature of the sugar residue and the sulfation position of the structure (Melo, Pereira, Foguel, & Mourão, 2004). Pereira et al. (2002) demonstrated 2-*O*-sulfated 3-linked α-L-galactan has potent anticoagulant activity whereas 3-*O*-sulfated 4-linked α-L-galactopyranose does not have. Furthermore, the *O*-sulfated 3-linked α-galactans enhanced the inhibition of thrombin and factor Xa by antithrombin and/or heparin cofactor II (Pereira et al., 2002) in the intrinsic pathway of blood coagulation. Structural studies on the sulfated polysaccharides isolated from *L. catenata* will play an indispensable role in the understanding of the mechanism of anticoagulant activity. However, the detailed mechanism of anticoagulant action of the sulfated polysaccharide from *L. catenata* is still being investigating.

Table 3
Anticoagulant activity of purified polysaccharide from fermented *L. catenata* and comparison with heparin (183 IU/mg)

Sample	Concentration (μg/mL)	Clotting time (s)		
		APTT	PT	TT
L. catenata	12	348	14.3	670
	25	576	18.2	841
	40	>1000	19.8	>1000
Heparin[a]	31.25	409	197	574
	62.5	>1000	779	>1000
Water[b]		36.8	11.4	12.1

All the values were the mean of triplicates.
[a] Heparin (183 IU/mg) is used as the positive control.
[b] Water was the control.

From analysis of neutral sugars and amino sugars, the predominant sugar of the polysaccharide was galactose, followed by glucose and glucosamine. The approximate molar ratio of galactose, glucose, and glucosamine were 0.94, 0.40, and 0.01, respectively. It has been reported that an anticoagulant polysaccharide isolated from enzymatic hydrolysate of brown seaweed, *Ecklonia cava* contained fucose, glucosamine, galactose, and mannose in approximate molar ratio of 0.81, 0.01, 0.16, and 0.01, respectively (Athukorala, Jung, Vasanthan, & Jeon, 2006). Moreover, the anticoagulant polysaccharide isolated from *L. catenata* was highly sulfated containing 298 μg of sulfate as SO_4^{2-} (21.76%) per mg polysaccharide. It also contained 114 μg of protein (9.42%) per mg polysaccharide. Therefore, the anticoagulant of *L. catenata* was a sulfated proteoglycan (Matsubara et al., 2000). The sulfate content and protein content of the isolated anticoagulant is higher than that of the anticoagulant polysaccharide of red seaweeds *G. cornea* (Melo et al., 2002) and green algae *C. cylindricum* (Matsubara et al., 2001).

We purified the sulfated polysaccharide from *L. catenata*, which showed anticoagulant activity. The compound acts on the intrinsic or common pathways of coagulation system and the mechanism may be through the inhibition of thrombin or factor Xa. The structure of the purified compound is being studied to elucidate the mechanism of anticoagulation by which the purified compound works.

The therapeutic applications of high molecular weight sulfated polysaccharides have been limited. Therefore, possible methods to produce of low molecular weight polysaccharides from the purified *L. catenata* sulfated polysaccharide and its anticoagulant activity will be studied.

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References

Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohydrate Polymer, 66*, 184–191.

Aquino, R. S., Landeira-Fernandez, A. M., Valente, A. P., Andrade, L. R., & Mourão, P. A. S. (2005). Occurrence of sulfated galactans in marine angiosperms: Evolutionary implications. *Glycobiology, 15*, 11–20.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Carlucci, M. J., Pujol, C. A., Ciancia, M., Noseda, M. D., Matulewicz, M. C., Damonte, E. B., & Cerezo, S. A. (1997). Antiherpetic and anticoagulant properties of carrageenans from the red seaweed *Gigartina skottsbergii* and their cyclized derivatives: Correlation between structure and biological activity. *International Journal of Biological Macromolecules, 20*, 97–105.

Chaplin, M. F. (1994). Monosachcharides. In M. F. Chaplin & J. F. Kennedy (Eds.), *Carbohydrate analysis: A practical approach* (pp. 2–176). New York: Oxford University Press Inc..

Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A. M., & Boisson-Vidal, C. (1999). Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity. *Carbohydrate Research, 319*, 154–165.

Colliec, S., Fischer, A. M., Tapon-Bretaudiere, J., Boisson, C., Durand, P., & Jozefonvicz, J. (1991). Anticoagulant properties of a fucoidan fraction. *Thrombosis Research, 64*(2), 143–154.

Dobashi, K., Nishino, T., Fujihara, M., & Nagumo, T. (1989). Isolation and preliminary characterization of fucose-containing sulfated polysaccharides with blood-anticoagulant activity from the brown seaweed *Hizikia fusiforme*. *Carbohydrate Research, 194*, 315–320.

Farias, W. R. L., Valente, A. P., Pereira, M. S., & Mourao, P. A. S. (2000). Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from the red algae *Botryocladia occidentalis* and comparison of its anticoagulant action with that of a sulfated galactans from invertebrates. *Journal of Biological Chemistry, 275*, 29299–29307.

Iacomini, M., Serrato, R. V., Sassaki, G. L., Lopes, L., Buchi, D. F., & Gorin, P. A. J. (2005). Isolation and partial characterization of a pectic polysaccharide from the fruit pulp of *Spondias cytherea* and its effect on peritoneal macrophage activation. *Fitoterapia, 76*, 676–683.

Kolender, A. A., Pujol, C. A., Damonte, E. B., Matulewicz, M. C., & Cerezo, A. S. (1997). The system of sulfated alpha-(1→3)-linked D-mannans from the red seaweed *Nothogenia fastigiata*: Structures, antiherpetic and anticoagulant properties. *Carbohydrate Research, 304*, 53–60.

Lahaye, M. (2001). Developments on gelling algal galactans, their structure and physico-chemistry. *Journal of Applied Phycology, 13*, 173–184.

Matsubara, K., Matsuura, Y., Hori, K., & Miyazawa, K. (2000). An anticoagulant proteoglycan from the marine green alga, *Codium pugniformis*. *Journal of Applied Phycology, 12*, 9–14.

Matsubara, K., Matsuura, Y., Bacic, A., Liao, M. L., Hori, K., & Miyazawa, K. (2001). Anticoagulant properties of a sulfated galactan preparation from a marine green alga, *Codium cylindricum*. *International Journal of Biological Macromolecules, 28*, 395–399.

Melo, M. R. S., Feitosa, J. P. A., Freitas, A. L. P., & de Paula, R. C. M. (2002). Isolation and characterization of soluble sulfated polysaccharide from the red seaweed *Gracilaria cornea*. *Carbohydrate polymers, 49*, 491–498.

Melo, F. R., Pereira, M. S., Foguel, D., & Mourão, P. A. S. (2004). Antithrombin–mediated anticoagulant activity of sulfated polysaccharides. *The Journal of Biological Chemistry, 279*, 20824–20835.

Mourão, P. A. S., & Perlin, A. S. (1987). Structural features of sulfated glycans from the tunic of *Styela plicata* (Chordata-Tunicata). A unique occurrence of L-galactose in sulfated polysaccharides. *European Journal of Biochemistry, 166*, 431–436.

Pavão, M. S., Albano, R. M., Lawson, A. M., & Mourão, P. A. (1989). Structural heterogeneity among unique sulfated L-galactans from different species of ascidians (tunicates). *The Journal of Biological Chemistry, 264*, 9972–9979.

Pereira, M. S., Mulloy, B., & Mourao, P. A. S. (1999). Structure and anticoagulant activity of sulfated fucans. *Journal of Biological Chemistry, 274*, 7656–7667.

Pereira, M. S., Melo, F. R., & Mourao, P. A. S. (2002). Is there a correlation between structure and anticoagulant and sulfated fucans. *Glycobiology, 12*, 573–580.

Pereira, M. G., Benevides, N. M. B., Melo, M. R. S., Valente, A. P., Melo, F. R., & Mourão, P. A. S. (2005). Structure and anticoagulant activity of a sulfated galactan from the red alga, *Gelidium crinale*. Is there a specific structural requirement for the anticoagulant action? *Carbohydrate Research, 340*, 2015–2023.

Santos, J. A., Mulloy, B., & Mourão, P. A. S. (1992). Structural diversity among sulfated a-L-galactans from ascidians (tunicates). *European Journal of Biochemistry, 204*, 669–677.

Sen, A. K. S., Das, A. K., Banerji, N., Siddhanta, A. K., Mody, K. H., Ramavat, B. K., Chauhan, V. D., Vedasiromoni, J. R., & Ganguly, D. K. (1994). A new sulfated polysaccharide with potent blood anticoagulant activity from the red seaweed *Grateloupia indica*. *Journal of Biological Macromolecules, 16*, 279–280.

Shanmugam, M., & Mody, K. H. (2000). Heparinoid-active sulfated polysaccharides from marine algae as potential blood anticoagulant agents. *Current Science, 79*, 1672–1683.

Shida, K., Shiratori, I., Matsumoto, M., Fukumori, Y., Matsuhisa, A., Kikkawa, S., Tsuji, S., Okamura, H., Toyoshima, K., & Seya, T. (2001). An alternative form of IL-18 in human blood plasma: Complex formation with IgM defined by monoclonal antibodies. *The Journal of Immunology, 166*, 6671–6679.

Silverstri, L. J., Hurst, R. E., Simpson, L., & Settine, J. M. (1982). Analysis of sulfate in complex carbohydrates. *Analytical Biochemistry, 123*, 303–309.

Takano, R., Shiomoto, K., Kamei, K., Hara, S., & Hirase, S. (2003). Occurrence of carrageenan structure in an agar from the red seaweed *Digenea simplex* (Wulfen) C. Agardh (Rhodomelaceae, Ceramiales) with a short review of carrageenan–agarocolloid hybrid in the Florideophycidae. *Botanica Marina, 46*, 142–150.

Usov, A. I. (1998). Structural analysis of red seaweed galactans of agar and carrageenan groups. *Food Hydrocolloids, 12*, 301–308.

Velde, F., Pereira, L., & Rollema, H. S. (2004). The revised NMR chemical shift data of carrageenans. *Carbohydrate Research, 339*, 2309–2313.

Yamada, T., Ogamo, A., Saito, T., Uchiyama, H., & Nakagawa, Y. (2000). Preparation of *O*-acylated low-molecular-weight carrageenans with potent anti-HIV activity and low anticoagulant effect. *Carbohydrate Polymers, 41*, 115–120.