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# Evaluation of Biomolecular Interactions of Sulfated Polysaccharide Isolated from *Grateloupia filicina* on Blood Coagulation Factors

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**An edible marine red alga, *Grateloupia filicina*, collected from Jeju Island of Korea was hydrolyzed by cheap food-grade carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) to investigate its anticoagulant activity. Among the tested enzymatic extracts of *G. filicina*, a Termamyl extract showed the highest anticoagulant activity. Anion-exchange chromatography on DEAE-cellulose and gel-permeation chromatography on Sepharose-4B were used to purify the active polysaccharide from the crude polysaccharide fraction of *G. filicina*. The purified sulfated polysaccharide (0.42 sulfate/total sugar) showed ~1,357 kDa molecular mass and was comprised mainly of galactose (98%) and 1–2% of glucose. The sample showed potential anticoagulant activity on activated partial thromboplastin time (APTT) and thrombin time (TT) assays. The purified *G. filicina* anticoagulant (GFA) inhibited the coagulation factor X (92%), factor II (82%), and factor VII (68%) of the coagulation cascade, and the molecular interaction (protein-polysaccharide) was highly enhanced in the presence of ATIII (antithrombin III). The dissociation constant of polysaccharide towards serine proteins decreased in the order of FXa (58.9 nM) >FIIa (74.6 nM) >FVII (109.3 nM). The low/less cytotoxicity of the polysaccharide benefits its use in the pharmaceutical industry; however, further studies that would help us to elucidate the mechanism of its activity are needed.**

**Keywords:** *Grateloupia filicina*, enzymatic hydrolysis, anticoagulant activity, ATIII, surface plasmon resonance

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In the developing world, cardiovascular diseases still cause severe health problems. In 1995, heart diseases and stroke caused approximately 44% of the world's death toll [19]. As a treatment, heparin has been utilized to treat thromboembolic disorders for more than 50 years. However, limitations associated with heparin therapy have created a big demand for alternative drugs [3, 9, 19]. According to previous reports, algal polysaccharides are good natural compounds to replace this long-needed product. Therefore, scientists have focused their attention on algal polysaccharides to utilize/modify them as anticoagulative drugs [6, 16].

Among other algal groups, Rhodophyta is well documented for its potential anticoagulant activity. Carrageenans and D-galactan have been identified as the key anticoagulative compounds of this seaweed group. Sulfated galactans/carrageenans are located in the cell wall of the seaweed [23]. When algal samples are evaluated for their potential anticoagulant activity, the algal samples are normally extracted using water as a solvent, and therefore, only the water-soluble active compounds may come into the water extract. Utilization of cell wall breakdown enzymes (carbohydrases) on algal biomass is expected to be a useful technique to extract all possible bioactive compounds from the algal sample. According to our previous experiments, an enzymatic digestion of algal biomass gains better advantages than conventional extraction techniques [12]. However, so far, few reports related to the enzymatic digestion of algal samples are available for the evaluation of their potential bioactive compounds. As we have observed before, the digestion of marine algal materials from the carbohydrases increases the extraction yield, and

thereby enhances bioactive availability. Furthermore, common problems associated with organic solvent extraction can be overcome by this technique [1]. Therefore, the investigation of bioactive compounds after an enzymatic digestion will be fruitful for the in pharmaceutical industry.

In this study, our aim was to utilize several food-grade carbohydrate enzymes (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) on *G. filicina* to extract plant material for its anticoagulant activity. The anticoagulant compound of the *G. filicina* enzymatic extract was purified and characterized, and its activity compared with that of heparin. The compound exhibited good anticoagulant activity, inhibiting coagulation factors of the coagulation cascade. Overall, the sample exhibited ATIII-mediated anticoagulant activity without showing toxic or side effects on normal cell growth.

## Materials and Methods

### Algae Samples and Reagents

Marine red algae used in this study were collected close to the shores of Jeju Island in Korea during March and October 2005. Salt, sand, and epiphytes were removed using tap water. Finally, seaweed samples were rinsed carefully with fresh water and freeze-dried at -20°C for further experiments. APTT (ellagic+bovine phospholipid) and $CaCl_2$ solution were obtained from International Reagents Corporation (Japan), and PT (rabbit thromboplastin) and TT reagents were purchased from Fisher Scientific Company (USA). Carbohydrate-degrading enzymes such as Viscozyme L (a multienzyme complex containing a wide range of carbohydrate-degrading enzymes, including arabinase, cellulase, beta-glucanase, hemicellulase, and xylanase), Celluclast 1.5 l FG (catalyzing the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers), AMG 300 l (an exo1,4-alpha-D-glucosidase), Termamyl 120 l (a heat-stable alpha-amylase), and Ultraflo L (a heat-stable multi-active beta-glucanase) were obtained from Navo Co. (Novozyme Nordisk, Bagsvaed, Denmark). Heparin, sepharose 4B, *N*-cetyl-*N,N,N*-trimethylammonium bromide, toluidine blue, agarose, and DEAE-cellulose were purchased from Sigma. All other chemicals used in this study had 90% or greater purity.

### Enzymatic Extraction of *G. filicina*

The preparation of enzymatic extracts was followed as previously reported [12]. The freeze-dried *G. filicina* (100 g) was homogenized with water (2 l), and then 1 g or 1 ml of enzyme was added. The enzymatic hydrolytic reactions were performed for 12 h to achieve the optimum degree of hydrolysis. Before the digestion, the pH of the homogenate was adjusted to its optimal pH value. After the digestion, the digests were boiled for 10 min at 100°C to inactive the enzyme. Each sample was clarified by centrifugation (3,000 rpm, for 20 min at 4°C) to remove the residue. The supernatant was kept at -20°C for further experiments [24, 27].

### Crude Polysaccharide Separation

Each algal enzymatic extract (240 ml) was mixed well with 480 ml of 99.5% ethanol [10]. Then, the mixture was allowed to stand for 30 min at room temperature, and crude polysaccharides were collected by centrifugation at 10,000 ×*g* for 20 min at 4°C [17]. The precipitated crude polysaccharide was freeze-dried and kept at -20°C for further experiments

### Anion-Exchange Chromatography

The crude polysaccharide of the Termamyl hydrolysate of *G. filicina* (500 mg), obtained using the procedures described above, was applied to a DEAE-cellulose column (17× 2.5 cm) equilibrated in 50 mM sodium acetate buffer (pH 5.0) and washed with the same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate of 15 ml/h with a linear gradient of 0.2–1.2 M NaCl containing 50 mM sodium acetate (pH 5.0). Fractions of 5 ml were collected and measured for polysaccharide by the phenol-$H_2SO_4$ and carbazole reactions and by its metachromatic property [5]. Fractions showing strong anticoagulant activity were collected, dialyzed against distilled water, and concentrated to 5 ml by rotary evaporation under reduced pressure below 40°C. The partially purified concentrated polysaccharide fraction was rechromatographed on new DEAE-cellulose column, under the same experimental condition. The active fractions were pooled, dialyzed, and freeze-dried for gel filtration chromatography.

### Gel Filtration Chromatography

Purified sample (10 mg/ml in water) was applied to a Sepharose 4B column (72×2 cm) equilibrated and eluted with water at room temperature, at a flow rate of 1 ml/min. Fractions (2 ml) were collected and assayed for metachromatic property at 525 nm and for total polysaccharide contents.

### Agarose Gel Electrophoresis

The purity of the purified polysaccharide sample was examined using agarose gel electrophoresis. About 5 μg of the purified polysaccharides was applied to a 0.5% agarose gel in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0) for 1 h at 110 V. The polysaccharide in the gel was fixed with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide in water. Polysaccharides were stained after 12 h using 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v).

### Neutral Sugar Analysis

The purified polysaccharide was hydrolyzed in a sealed glass tube with 2 M trifluoroacetic acid for 4 h at 100°C to analyze neutral sugars. In order to analyze the amino-sugars, the sample was digested using 6 N HCl for 4 h. Then, 0.055 μg and 2.75 μg of sample were separately applied to CarboPac PA1 (4.5×250 mm; Dionex, Sunnyvale, CA, U.S.A.) with a CarboPac PA1 cartridge (4.5×50 mm) column to analyze neutral and amino sugars, respectively. The column was eluted using 16 mM NaOH at 1.0 ml/min flow rate. Each sugar of the sample was detected by using an ED50 Dionex electrochemical detector and data were analyzed by Peak Net online software.

### Sulfate Content Analysis

After acid hydrolysis of the purified polysaccharide, the sulfate content was measured by the $BaCl_2$/gelation method [22].

### Determination of the Molecular Mass of the Sample

In order to determine the molecular mass of the sample, the freeze-dried sample was introduced into a PL-Aquaz OH 40 column and eluted with deionized water at 0.8 ml/min flow rate (23°C). Dextran

standards (48.6, 148, 273, 410, 830, and 2,000 kDa) were also introduced into the column under the same experimental condition for comparison purposes. The retention time was plotted against average molecular mass of the dextrans, and thereby the molecular mass of the sample was calculated.

**Blood Coagulation Assays**

Normal pooled plasma was made from 10 individual healthy donors, without history of bleeding or thrombosis. Nine parts of blood collected by venipuncture were drawn into one part of 3.8% sodium citrate. Blood was centrifuged for 20 min at 2.400 ×*g*, and the plasma was stored at −60°C until use. All coagulation assays were performed with four individual replicates using Dual-channel clot-2 (SEAC, Italy) and mean values were taken. For activated partial thromboplastin time (APTT) assay, citrated normal human plasma (90 μl) was mixed with a solution of algal extract (10 μl) and incubated for 1 min at 37°C, and then APTT reagent (100 μl) was added to the mixture and incubated for 5 min at 37°C. Thereafter, clotting was induced by adding 0.025 mol/l $CaCl_2$ (100 μl) and the clotting time was recorded. In prothrombin time (PT) assay, citrated normal human plasma (90 μl) was mixed with a solution of algal extract (10 μl) and incubated for 10 min. Then, prothrombin time reagent (200 μl), pre-incubated for 10 min at 37°C, was added and the clotting time was recorded. For thrombin time (TT) measurement, citrated normal human plasma (190 μl) was mixed with a solution of algal extract (10 μl) and incubated for 2 min. Then, pre-incubated TT reagent (10 min, at 37°C) was added (100 μl) into the mixture and the clotting time was recorded. All algal extracts including heparin were dissolved in water.

**Determination of Specific Factor Assay**

The specific activity of activated coagulation factors was determined by modified clotting assays of APTT using IL test factor assay kits (Instrumental Laboratory Co., Lexington, MA, U.S.A.) and was slightly changed according to previously described methods [15, 21].

**SPR Binding Studies**

Surface plasmon resonance (SPR) experiments were performed at 25°C, using a BIAcore 2000 system (Pharmacia Biosensor). Target factors (FIIa, FVIIa, FXa) interfered by GSG in the previous study were directly immobilized on a CM5 sensor chip by coupling through the free amino group to a carboxylated dextran matrix, activated with a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(3-diethylaminopropyl) carbodiimide (EDS) according to a previously described method [25], after pH scouting. The sensor chip surface was coated with ligand proteins in 10 mM sodium acetate buffer at optimal pH, and unreacted groups were blocked with 1 M ethanol amine, pH 8.5. Among four flow cells in the sensor chip, each first flow cell, similarly activated and blocked without immobilization of protein, served as a control surface. After immobilization of coagulation factors on the sensor chip, an analyte (100 μg/ml of GSG) was injected onto the surface of the sensor chip in HBS buffer (Hepes-buffered saline containing 1 mM CHAPS, 0.005% surfactant P20, 5 mM $CaCl_2$, pH 7.4) at 25°C, at a flow rate of 30 μl/min for 3 min, followed by 2 min of dissociation. Resonance was monitored as a function of time and shown as resonance units in real time. To determine kinetic binding constants ($k_a$, $k_d$, and $K_D$), GSG solution at various concentrations (0–7,500 nM) with or without adding 1,000 nM antithrombin III (ATIII) was injected to the coagulation factors, and real-time sensorgrams were evaluated using BIAevaluation software (version 3.2). The association rate constant ($k_a$) was calculated from multiple sensorgrams, representing at least five different concentrations of analyte for each experiment. The dissociation rate constant ($k_d$) was calculated from the initial dissociation phase of the binding curves, and the equilibrium dissociation constant ($K_D$) equaled the ratio of $k_d/k_a$.

**Cytotoxicity Assay**

The Chinese hamster fibroblast cell line/normal cell line (V79-4) was maintained in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C under 5% $CO_2$ in the air. Toxic effect of GFA on V79-4 was measured by using the MTT assay [4, 18]. The cells were seeded in a 96-well plate at the concentration of $2\times10^4$ cells/ml DMEM. After 16 h (at 37°C, in a humidified atmosphere of 5% $CO_2$), GFA was added to the wells at a concentration ranging from 5 to 500 μg/ml. The cells were then further incubated for an additional 72 h at 37°C. MTT stock solution (50 μl of 2 mg/ml in PBS) was then added to each well for a total reaction volume of 250 μl. After incubating for 4 h in a humidified atmosphere of 5% $CO_2$ at 37°C, the plate was centrifuged at 800 ×*g* for 5 min and the supernatants were aspirated to remove untransformed MTT. The formazan crystals in each well were dissolved in 150 μl of DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Ultramark Microplate Imaging System 110/230 V; Bio-Lab Co., U.S.A.). For treated cells, the viability was expressed as a percentage of control cells. All determinations were carried out in triplicate.

## RESULTS

### Purification of Anticoagulant Compound of *Grateloupia filicina*

A popular edible alga, *G. filicina*, was enzymatically extracted by several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) to examine its anticoagulative efficacy. All the enzymatic extracts of *G. filicina* showed good anticoagulant potential compared with that of the control sample (Table 1). Of the tested extracts, the Termamyl and AMG extracts of *G. filicina* showed an outstanding anticoagulant activity on APTT assay. However, the former sample showed pronounced APTT activity and was therefore

**Table 1.** Anticoagulant activity of enzymatic extracts from *G. filicina*.

| Sample (80 μg/ml) | APTT | TT | PT |
|---|---|---|---|
| Viscozyme | 312 | 50 | 11 |
| Celluclast | 205 | 53 | 11 |
| AMG | 520 | 68 | 11 |
| Termamyl | >600 | 101 | 11 |
| Ultraflo | 350 | 68 | 11 |
| Control | 32 | 26 | 11 |

*Results are expressed as the means of two determinations.

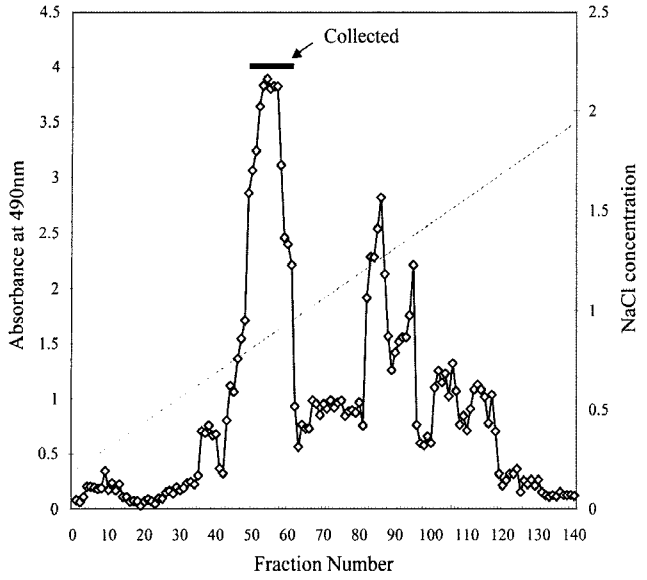


**Fig. 1.** Purification of the potential anticoagulant polysaccharide from the red alga *G. filicina* by DEAE-cellulose chromatography.

selected for further purification purposes. All the enzymatic extracts showed slight activity on TT assay; however, the *G. filicina* extracts were devoid of PT activity.

Because of high activity of the Termamyl extract, a crude polysaccharide fraction of the sample was separated by the ethanol precipitation technique. The freeze-dried crude polysaccharide sample was introduced to a DEAE-cellulose column with NaCl gradient to separate the anticoagulant fraction. According to the first anion-exchange chromatograph (Fig. 1), the main prominent peak that eluted at around the 50th fraction showed high anticoagulant activity (APTT); however, the rest of the peaks, eluted after the main peak, were devoid of anticoagulant activity. Therefore, we assumed that the peak was a main polysaccharide, as the anticoagulant compound present in the *G. filicina* is a main polysaccharide. The active fractions were collected, concentrated, dialyzed, and freeze-dried. Then, ~100 mg of the partially purified sample was rechromatographed on new DEAE-cellulose column to enhance the purity of the sample. The clear distinct peak of the second anion chromatograph confirms the purity of the sample (Fig. 2). After being evaporated under a rotary evaporator, the dialyzed sample was further purified by gel-filtration chromatography on Sepharose 4B (Fig. 3). The application of the sample on Sepharose 4B yielded a single polysaccharide peak with high metachromatic property. Metachromatic property is an indicator of the polyanionic power of the active compound. Samples with a high metachromatic power have strong binding property with other compounds. However, the samples were devoid of hexauronic acid like compounds (data not shown). As a method to confirm the homogeneity of the purified compound, we subjected the purified sample to 0.5% agarose gel electrophoresis. After staining with toluidine blue, the sample appeared as a clear single spot on the agarose gel (Fig. 4). The sugar composition of the active compound was investigated by HPLC and compared with the absorption spectra of standard sugars (Fig. 5A). The purified compound was composed of a high amount of galactose (~98%) and less amount of fucose (1–2%)

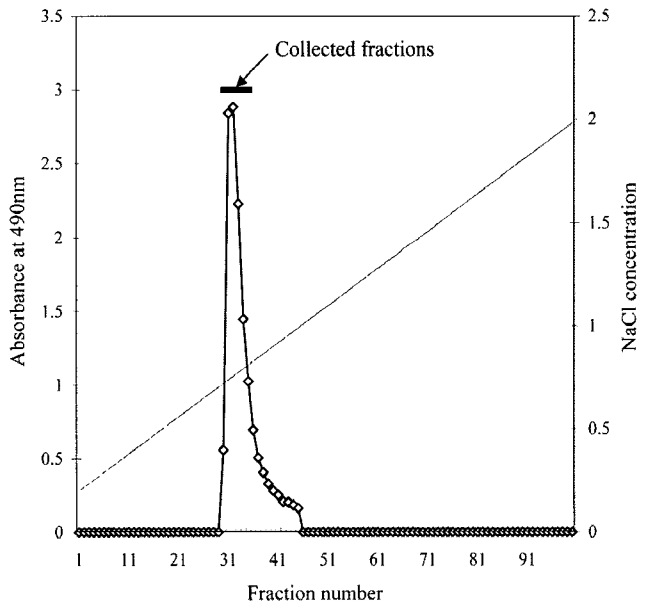


**Fig. 2.** Purification of the potential anticoagulant polysaccharide from the red alga *G. filicina* on a new DEAE-cellulose column (10×1.7 cm).

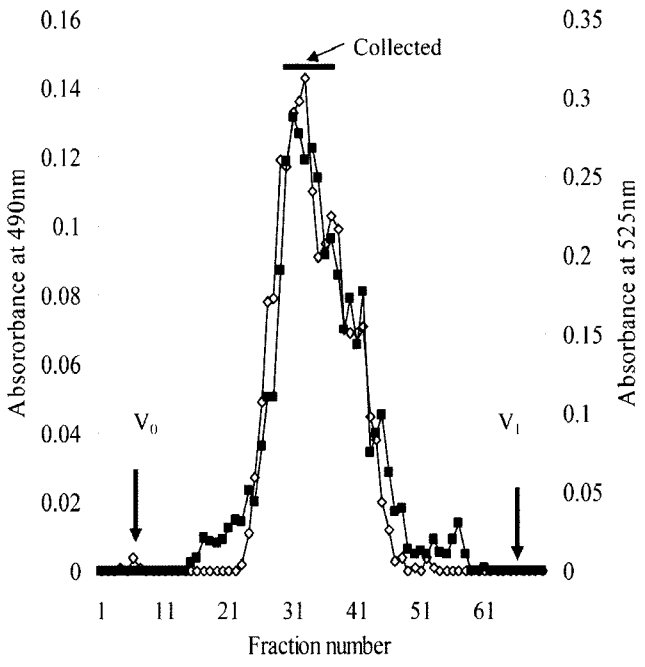


**Fig. 3.** Purification of the anticoagulant polysaccharide from the red alga *G. filicina* by Sepharose 4B.
Carbohydrate content (◇); Metachromatic property (■); Blue dextran ($V_0$); Cresol red ($V_t$).

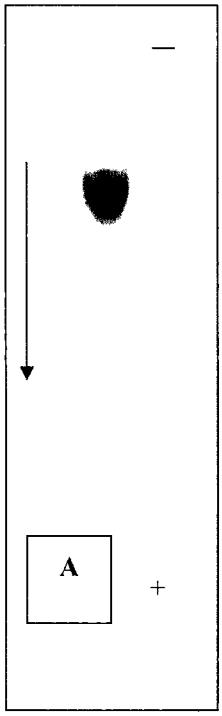


**Fig. 4.** Agarose gel electrophoresis of the sulfated polysaccharides from *G. filicina.*

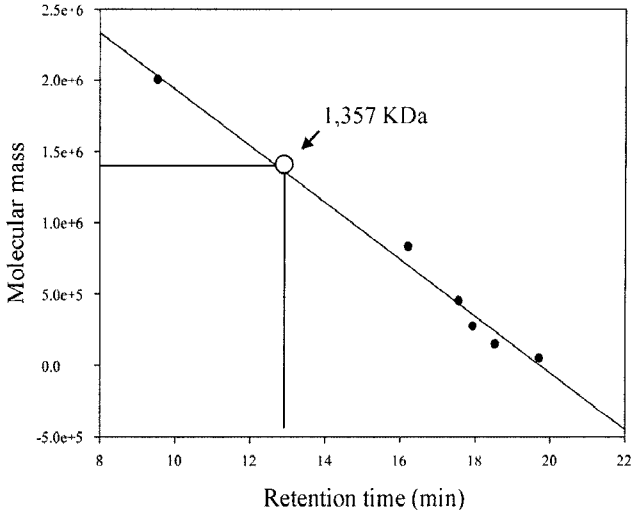


**Fig. 6.** Calibration curve of dextran standards for the determination of the average molecular mass of the *G. filicina* sample.
The retention time is plotted against the molecular mass of the dextrans.

(Fig. 5). The average molecular mass of the polysaccharide sample was calculated as 1,357 kDa according to the calibration curve with standard dextrans (Fig. 6). Furthermore, according to the $BaCl_2$ technique, the purified polysaccharide constituted a considerable amount of sulfate (0.42 sulfate/total sugar).

### Biomolecular Interaction of GFA on Blood Coagulant Factors

The sample showed good dose-dependant anticoagulant activity on APTT assay; however, the APTT activity of the compound was slightly less than that of heparin at the same concentration (Table 2). Furthermore, the purified compound showed considerable activity on TT assay, but had much less activity on PT assay. In specific coagulation factor assay, the purified sample showed strong interference with factor X (Stuart factor), factor II (prothrombin), and factor VII (proconvertin) (Fig. 7). Hence, presumably, the sample selectively inhibits coagulation cascade enzymes and thereby potentiates its anticoagulant activity. However, the sample had less/slight inhibition potential on other tested coagulation factors (factors V, IX, and XI). In order to dissect the mechanism of the GFA for its anticoagulant activity, a SPR study was carried out and the binding affinities on the human blood coagulant factors were analyzed for real-time sensorgraphic information (BIAcore 2000 system). The blood coagulations factors and ATIII

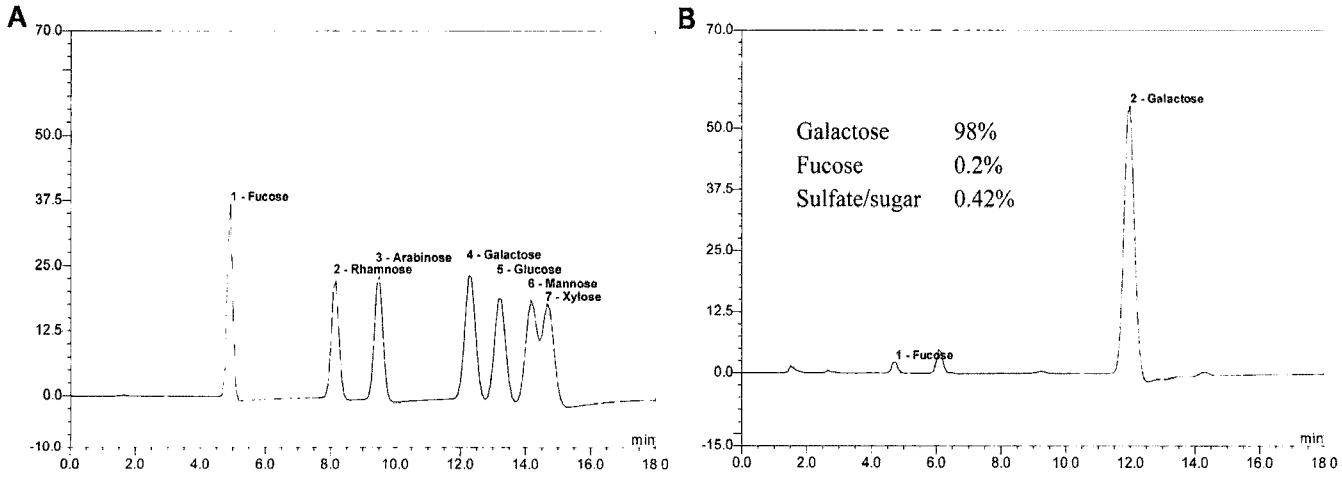


**Fig. 5.** HPLC chromatograms for the monosugar composition of the purified polysaccharide of *G. filicina.* **A.** Chromatogram of the sugar standards. **B.** Chromatogram of the *G. filicina* polysaccharide sample.

**Table 2.** Comparison of anticoagulant activity of the Termamyl extract from *G. filicina* with that of heparin.

| | Control | Clotting time (sec) | | | | | |
|---|---|---|---|---|---|---|---|
| | | *G. filicina* anticoagulant (μg/ml) | | | Heparin (μg/ml) | | |
| | | 0.7 | 1.4 | 2.8 | 0.7 | 1.4 | 2.8 |
| APTT | 37 | 52 | 98 | 289 | 70 | >300 | >300 |
| TT | 26 | 39 | 72 | 186 | 105 | >300 | >300 |
| PT | 11 | 11 | 16 | 36 | 55 | 93 | >300 |

*Results are the means of three determinations.

were immobilized onto the CM5 sensor chips using amine coupling after determining the optimal pH. Fig. 8 illustrates the difference of the binding affinities of the blood coagulant factors with the GFA alone and in the presence of ATIII. The coagulant factors interacted considerably with GFA in a varying degree, but in the presence of ATIII the biomolecular interaction was enhanced rapidly. Specifically, the addition of ATIII accelerated GFA-factor II interaction by two-fold. Presumably, the active compound has strong interaction with the ATIII-mediated anticoagulant pathway. The kinetic constants ($k_a$, $k_d$, and $K_D$) were evaluated by 1:1 Langmuirian analysis of the data from three separate experiments using BIAevaluation software (version 3.0) after analysis by the SPR program. The optimum pH values and RU units of the coagulant factors for the coupling reaction on sensor chip are listed in Table 3. Of the tested blood coagulant factors, FXa showed the lowest equilibrium dissociation constant with GFA than those of other plasma proteinases. The dissociation constant for the GFA extract was decreased in the order of FXa

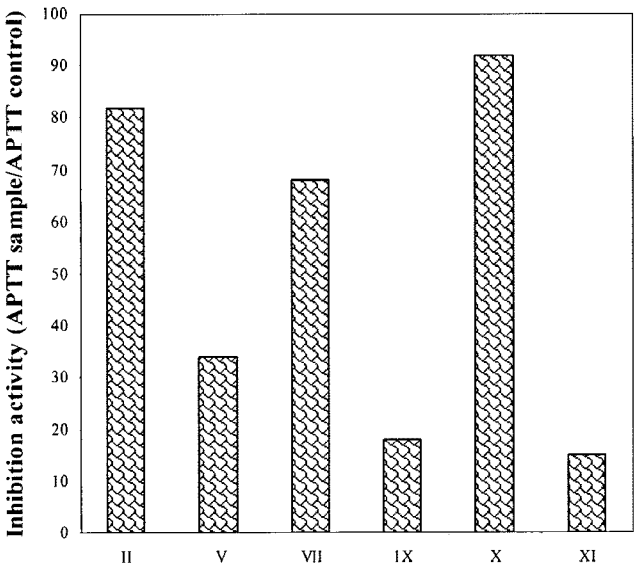


**Fig. 7.** Specific factor inhibitory pattern of the purified anticoagulant on blood coagulant factors.

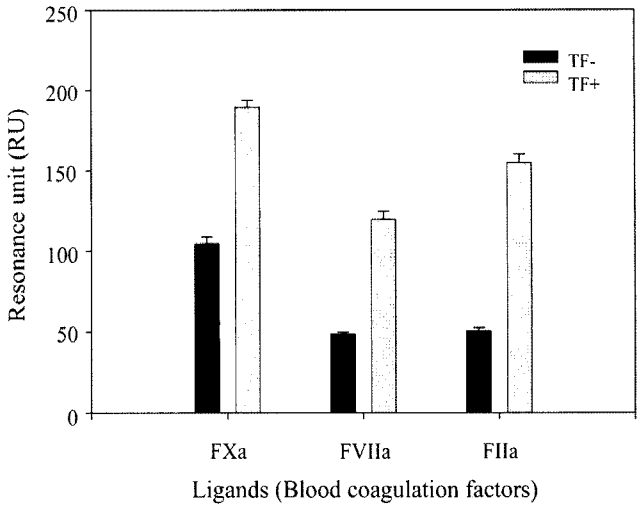


**Fig. 8.** Binding affinity assay of *G. filicina* anticoagulant (GFA) to blood coagulation factors using a surface plasmon resonance (SPR) spectrometer.
Black columns express resonance unit (RU) values of real-time sensorgram in the absent of tissue factor (TF). Gray columns express resonance unit (RU) values in the present of tissue factor (TF).

(58.9 nM)>FIIa (74.6 nM)> FVII (109.3 nM) (Table 4). In pharmaceutical research, the compounds with low equilibrium dissociation constant are in high demand, and in our previous research, a sulfated polysaccharide purified from *E. cava* showed a $K_D$ value of 15.mM towards FVIIa inhibition. Taken together, it is clear that GFA exerts high anticoagulant activity by inhibiting both the extrinsic and common pathways in an ATIII-mediated pathway. Antithrombin III inhibits all of the proteinases involved in the intrinsic coagulation pathway including Xa, IXa, XIa, XIIa, kallikren, and thrombin [28]. Therefore, the ability of a compound to accelerate the ATIII-mediated pathway is very important for further studies. Natural compounds are diverse with multiple bioactivities. Sometimes, the *in vivo* application of these compounds are limited owing to their side effects. Specivically, natural compounds should not induce cytotoxic effects on normal human cell lines while binding to their target compounds. To elucidate the potential cytotoxic activity of the GFA, in this study we introduced samples at different concentrations on normal cell lines and estimated its effects on cell growth by MTT assay (Fig. 9). Obviously, the sample had no any side

**Table 3.** The optimal pHs for immobilization of blood coagulation factors and resonance unit (RU) values after coupling reaction on protein chip.

| Ligand | Molecular mass (kDa) | Optimal pH for immobilization | Resonance unit (RU) |
|---|---|---|---|
| FIIa | 58 | 5.0 | 6,123.5±12.5 |
| FVIIa | 50 | 4.5 | 5,024.3±9.1 |
| FXa | 46 | 4.5 | 5,963.9±8.7 |

**Table 4.** Kinetic parameters for the blood coagulation factors with *G. filicina* anticoagulant (GFA) using SPR sensorgraphy.

| Analyte | Ligand | Association rate constant ($k_a$) | Dissociation rate constant ($k_d$) | Equilibrium dissociation constant ($K_D=k_d/k_a$) |
|---|---|---|---|---|
| | | $M^{-1}s^{-1}$ | $s^{-1}$ | nM |
| *G. filicina* anticoagulant | FVIIa | $5.33\times10^{3}$ | $5.83\times10^{-4}$ | 109.3 |
| | FXa | $1.97\times10^{3}$ | $1.16\times10^{-4}$ | 58.9 |
| | FIIa | $2.18\times10^{3}$ | $1.63\times10^{-4}$ | 74.6 |

*All data were expressed as mean values (n=3).

effects on normal cell growth, indicating its applicability in *in vivo* assays.

## DISCUSSION

Algal polysaccharides have been widely tested for their biological activities *in vitro* and *in vivo*. Because of their high biological activities, sulfated polysaccharides contained in algae are an alternative natural source for synthetic compounds in the pharmaceutical industry. Therefore, studies related to marine compounds have been continuously increased in recent history [13, 26, 29]. However, studies related to algal enzymatic extraction preparation for their biological activity are quite limited. Interestingly, enzymatic digestion gains a high bioactive yield and shows enhanced biological activity in comparison with water and organic extract counterparts. Enzymes convert water-insoluble materials into water soluble materials and this method also does not adopt any toxic chemicals. Therefore, enzymatic digestion can be utilized well for the biological activity evaluation of algae [2]. In this study, the anticoagulant activity of *G. filicina* collected from Jeju Island was investigated by utilizing the enzymatic hydrolysis technique.

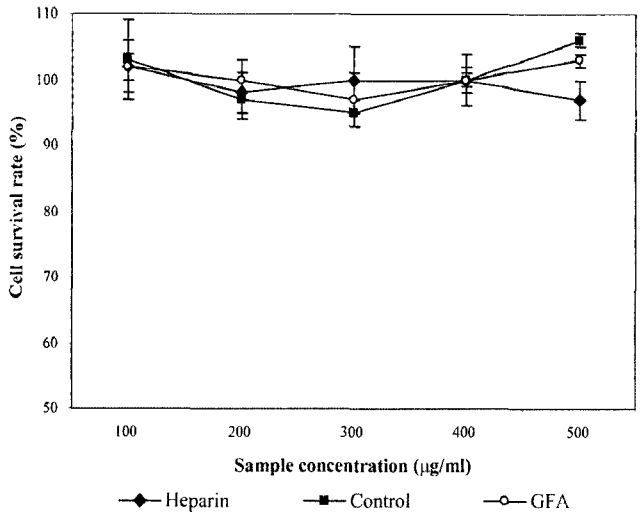


**Fig. 9.** Cytotoxicity of GFA on Chinese hamster fibroblast cell line/normal cell line (V79-4) in a dose-dependant manner.
*The control expresses cell viability (%) without any GFA.*

Several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo), as cheap food-grade enzymes, were used in this study to investigate the potential anticoagulant activity of *G. filicina*. As we expected, the enzymatic extracts of *G. filicina* showed different potentiality to extend blood coagulation time. Enzymes are compounds tailor-made to break down special linkages of the polysaccharides, and this may explain their different affinities to extend coagulation time. Taken together, in this study, all tested enzymatic extracts showed good anticoagulant activity, but the Termamyl extract showed the highest anticoagulant activity. Termamyl, a popular food-grade enzyme, is widely used in the food industry, and may break down plant cell wall materials such as polysaccharides or glycoproteins by hydrolyzing 1,4-alpha glycosidic linkages of the polysaccharides. This leads to a variety of biochemical compounds and some of them may be strong anticoagulants [2]. Sulfated galactans, available in marine red algae and in marine invertebrates, have been studied for their biological activities [23]. The structure and nature of galactans (carrageenans and agarans) present in the algal sample can be changed according to the species, collected area, and harvesting season. However, the structure of most galactans is composed of alternating 3-linked β-galactopyranose and 4-linked β-galactopyranose [20]. Sulfated galactans are known for its high anticoagulant activity. In this study, the galactose-rich sulfated polysaccharide showed considerable anticoagulant activity compared with that of heparin. Sulfated galactan (-4-α-D-Gal*p*-1→3-β-D-Gal*p*-1→) isolated from red alga (*Botryocladia occidentalis*) with variable sulfation pattern showed high anticoagulant activity, but was less potent than heparin [20]. Moreover, *Gelidium crinale*, a popular red alga composed of sulfated galactan (-4-α-Gal*p*-(1→3)-β-Gal*p*1), exhibited potent anticoagulant activity [11]. However, in most studies, red algal species show less anticoagulant potential than that of heparin. The anticoagulant activity of galactans is promoted by its proportion and distribution of sulfate groups. However, carrageenans, an important algal polysaccharide, isolated from red alga with high sulfate content and high molecular weight ($100-200\times10^{4}$), showed higher anticoagulant potential than those with low sulfur content and low molecular weight ($1.0\times10^{4}$) [23]. Interestingly, even if the molecular weight of heparin were low, the effect of heparin on thrombin

inhibition together with antithrombin is 2,000-fold that of antithrombin alone [14], and this is due to its special anionic power. Since heparin is the most highly anionic compound of our human body, it prolongs APTT, TT, and PT activities more effectively than those of synthetic and other related natural polysaccharides.

Sulfated polysaccharides enhance anticoagulant activity through direct or indirect interactions with blood coagulant factors, and the degree of plasmatic factor interactions depends on the structure of the polysaccharide [7]. The blood coagulation pathway of humans has several pathways; the potentiality of the anticoagulant on intrinsic/common pathway can be estimated by APTT assay, whereas the antithrombin activity and fibrin polymerization activity of the sample can be measured by TT assay. Moreover, the PT experiment is useful to estimate the efficacy of the sample for the inhibition of the extrinsic pathway [8]. The isolated potential anticoagulant compound from the enzymatic Termamyl extract of *G. filicina* was evaluated for its involvement in APTT, TT, and PT assays. The sample showed good anticoagulant activity on APTT assay, but showed less activity on TT assay. Moreover, the sample was devoid of PT activity. Therefore, the active compound selectively controls the blood serine proteases associated with the intrinsic/common pathway of the blood coagulation cascade. However, to study this further, the tested sample was subjected on specific factor assay to examine its effect on each clotting factor. The purified sulfated galactan of this study showed excellent activity on factor X (stuart factor), factor II (thrombin), and VII (proconvertin). Among the tested factors, the purified compound showed the most potent activity (92%) on factor X, which catalyzes the hydrolysis of prothrombin to thrombin. Hence, the purified compound has selective interactions with coagulation serine proteins of the clotting pathway. Antithrombin III inhibits all of the proteinases involved in the intrinsic coagulation pathway including Xa, IXa, XIa, XIIa, kallikren, and thrombin [28]. As was shown in the specific factor inhibition assay results, factors X (Stuart factor), II (prothrombin), and VII (proconvertin) were strongly inhibited by the tested purified polysaccharide, and showed low activity on factors IX, V, and XI. Therefore, several coagulation factors are associated with the observed activity of GFA. Hence, the interaction of GFA with serine proteases was investigated in the presence of ATIII. The presence of ATIII rapidly accelerated the molecular interaction of GFA with serine proteases in both extrinsic and common coagulation pathways.

GFA showed no side effect on normal cell growth, and therefore the compound is a promising agent for future drug development as a model compound in the pharmaceutical industry. However, the sample should be evaluated in other possible coagulation mechanism pathways to investigate its detailed interactions on the coagulation pathway.

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