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Anticoagulant and Anticancer Activities of Sulfated Polysaccharides Purified from *Ecklonia cava* and *Grateloupia filicina*

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GRADUATE SCHOOL

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CONTENTS

요 약 문

제주 연안에 대량으로 자생하는 감태(*Ecklonia cava*)와 지누아리(*Grateloupia filicina*)로부터 해조 기능성 다당류를 분리하고 정제하여 항혈액응고활성과 항암활성을 검토하였다. 해조류 다당류 추출은 시판되고 있는 식품용 당분해 효소로 해조류 조체의 세포벽을 분해하여 원심분리 후 상층액으로 알코올 침전법을 이용하였다. 이들 해조 다당류를 음이온교환 크로마토그래피(DEAE-cellulose)와 겔여과 크로마토그래피(sepharose-4B)로 항혈액응고 활성 물질을 순수 분리 하였다.

감태에서 분리한 황산화 다당류는 fucose 함량이 약 82%를 차지하고 있으며, 갈락토오스는 약 14%, 그리고 총당에 대한 황 함량비는 0.95 인 후코이단인 것으로 밝혀졌다. 감태에서 순수 분리한 황산화 다당류로 혈액의 응고 지연 시간을 측정하여 항혈액응고 활성을 검토한 결과는, 감태 유래 항혈액응고 물질 (*Ecklonia cava* anticoagulant; ECA) 농도가 2 배에서 시판 항혈액응고제인 헤파린과 아주 유사한 결과를 나타내었다. ECA 는 activated coagulation factor assay 로 검토한 결과, serine proteases II, X 및 VII 의 생리학적인 저해 활성(serine protease 에 의해 응고활성이 활성화가 되는데 감태 다당류가 serine proteases 와 결합함으로서 혈액 응고를 방해한다)으로 응고 단계반응을 강하게 억제시켜 항혈액응고 활성을 나타낸다는 사실이 밝혀졌다. 표면 플라즈몬 공명(SPR: surface Plasmon resonance)으로 ECA/ATIII (antithrombin-III)와 혈액응고 인자인 VIIa (FVIIa), factor Xa (FXa), thrombin (FIIa) 결합친화력과 kinetic 을 분석한 결과는, KD 값이 FVIIa 에서 15.1, FXa 에서 45, FIIa 에서는 65 nM 로 나타났다(ECA 와 ATIII 가 먼저 결합을 하여 결합친화력을 증가시켜 응고를 활성화 시키는 serine protease 와의

결합을 유도하여 응고 활성을 억제시킴). 이러한 결과를 종합해 볼 때, 감태에서 순수 분리한 다당류는 외래 요인과 공통 응고 경로 모두에서 ATIII 를 매개하여 혈액응고를 저해하는 활성을 증가시킨다는 것을 확인하였다. ECA 는 in vitro 상에서 높은 항혈액응고 활성을 나타낼 뿐만 아니라 내피세포(ECV-304)에서 독성을 나타내지 않아, *in vivo* 상에서 항혈액응고 활성을 알아 보기 위하여 rat 에 경구 투여한 결과, 시판 항혈액응고제인 헤파린과 동일한 농도에서 항혈액응고 활성을 나타냈으며, 대조구보다 아주 우수한 효과를 나타내었다. 따라서 ECA 는 높은 항혈액 응고활성을 가지고 있다는 사실이 명백하게 밝혀져 향후 천연 항혈액 응고제로서 개발이 가능할 것으로 판단된다.

지누아리에서 순수 분리한 항혈액응고 다당류(*Grateloupia filicina* anticoagulant: GFA)는 galactose 함량이 98%이고 총당에대한 황산기의 함량비가 0.42 인 황산화 갈락탄인 것으로 밝혀졌으며, 분자량은 약 1,357 kDa 을 가지고 있었다. 지누아리에서 순수 분리한 황산화 갈락탄의 항혈액응고 활성을 검토한 결과는, activated partial thrombin time (APTT)와 thrombin time (TT)에서 긍정적인 항혈액응고 활성을 나타내었다. GFA 는 단백질과 당의 상호 작용에 의한 응고 반응 단계에서 factor X 가 92%, factor II 가 82% 및 factor VII 이 68% 항혈액응고 활성을 나타내었으며, 여기서도 감태 유래 ECA 와 마찬가지로 ATIII 에 의해서 항혈액응고 활성이 증가하였다. GFA 는 serine proteins 과 혈전 응집과정에서 지속적으로 결합하여 응고를 감소시켰고, SPR 결과는 FXa (58.9 nM), FIIa (74.6 nM), 그리고 FVII (109.3 nM)로 각각 나타났다.

독성이 거의 없는 해양 유래 천연 다당류의 장점은 제약 공정에서 부작용을 줄일 수 있어 유리하게 작용할 수 있지만, 아직까지 해조류 유래

다당류를 이용한 항혈액응고 기전에 대한 명확한 연구가 진행되지 않아, 현재로서 당장 제약시장에서 상용화 될 수는 없지만 향후 충분한 가능성이 있을 것으로 판단된다.

높은 항혈액응고 활성을 갖는 감태에서 분리한 황산화 다당류인 ECSP 가 항암효과를 가지는지 알아보려고 대표적인 시판 황산화 다당류인 fucoidan 과 비교하여 여러 종류의 암세포에 대한 항암효과를 비교하였다. ECA 는 대체적으로 암세포 증식을 억제 하였고, U-937 과 HL-60 에서 뛰어난 암세포 증식 억제 활성을 나타내었다. 특히 U-937 세포에서는 36.2 μg/ml 로 fucoidan (43.9 μg/ml) 보다 낮은 농도에서 암세포 증식 억제 활성이 나타낸 것을 확인 할 수 있었다. apoptosis 유도 활성에서는 ECA 를 15 μg/ml 와 30 μg/ml 로 세포에 각각 처리하였을 때에 sub G1 함량이 매우 높아 암세포에 대한 사멸효과는 apoptosis 유도에 기인하는 것으로 판단된다. 또한 혈액암세포인 U-937 에 ECA 를 24 시간 동안 반응 시켰을 때 ECA 의 농도가 증가할수록 DNA fragmentation 이 증가하여 암세포 억제 활성을 보였다.

항암활성에 의한 다당류의 메커니즘을 확인하려고 caspase-7, caspase -8, Bax, Bcl-xL 및 PARP 등의 항체를 이용하여 Western blot 을 수행하였다. 그 결과는 caspase -7 와, caspase -8 및 PARP 경로에 의해 절단된 단백질을 확인하였고, 이러한 절단 단백질에 의하여 apoptosis 가 유도되었다는 것을 예측할 수 있었다. 이와 함께 Bax 와 Bcl-xL 과 같은 세포 수송분자를 조절한 것을 확인 할 수 있었다. 모든 결과를 종합해 볼 때 황산화 다당류는 세포증식억제 효과를 통하여 apoptosis 를 유도한 것으로 예측된다. 따라서 신호전달 경로와 연관된 다당류에 의한 apoptosis 에 대한 연구는 더 필요하다.

SUMMARY

Seaweeds collected from the sea shores of Jeju Island of Korea were enzymatically digested to prepare the extracts and investigated for their anticoagulant activity in several blood coagulation assays namely, activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT). Of the investigated algal species, the enzymatic extracts of *Ecklonia cava* (brown alga) and *Grateloupia filicina* (red alga) showed a promising anticoagulant activity. The selected enzymatic extract of each algal species was applied on DEAE-cellulose (anion exchange chromatography) and Sepharose-4B (gel filtration chromatography) to purify the active compound.

The highly sulfated (0.95 sulfate/total sugar) anticoagulant polysaccharide (ECA), purified from *E. cava* was mainly composed of fucose (82%), galactose (14%) and small amount of mannose and xylose with 0.01% yield. The purified compound strongly interferes with coagulation cascade by inhibiting biological activity of serine proteases II, X and VII. The binding affinity between ECA/ATIII (antithrombin-III) and blood coagulation factors was in the order of VIIa (FVIIa) > factor Xa (FXa) > thrombin (FIIa) and the kinetics analysis elucidated that the K_D values of ECA for FVIIa, FXa, and FIIa were 15.1, 45.0 and 65.0 nM, respectively. Therefore, the sulfated polysaccharide purified from the enzymatic extract of *E. cava* strongly enhance antithrombin III (ATIII) mediated coagulation factor inhibition selectively (FVII, FX, and II) in both of the extrinsic and common coagulation pathways. This may contribute for its high anticoagulant activity *in vitro*, and low cytotoxicity of ECA on venous endothelial cell line (ECV-304) expands its value in the future studies *in vivo*. The *in vivo* anticoagulant activity of ECA was investigated in Wistar rats and compared the results with that of heparin. Even though, the result of ECA was relatively lower than that of the commercial compound like heparin at the same concentration, but was quite

stronger than that of saline alone treated sample. Therefore, as a natural anticoagulant compound ECA will be a promising candidate for future drug discovery in pharmaceutical industry.

Sulfated galactan isolated from *Grateloupia filicina* (0.42 sulfate/total sugar) possessed approximately 1,357 kDa molecular weight and composed mainly of galactose 98% and 1-2 % of glucose. The purified *G. filicina* anticoagulant (GFA) showed a potential anticoagulant activity on anticoagulant assays such as APTT and TT. GFA inhibited the coagulation factor X (92%), factor II (82%) and factor VII (68%) of the coagulation cascade, the molecular interaction (protein-polysaccharide) was highly enhanced with the presence of ATIII. The dissociation constant of polysaccharide towards serine proteins decreased in the order of FXa (58.9 nM)> FIIa (74.6 nM)> and FVII (109.3 nM) respectively. The low/less cytotoxicity of the polysaccharide benefits its use in pharmaceutical industry, however further studies which would help us to elucidate the mechanism of its activity are needed to address.

A sulfated polysaccharide purified from a brown alga, *E. cava* (ECSP) having high anticoagulant activity was investigated for its antiproliferative effect on several cancer cell lines and compared the effect with that of the commercial fucoidan. The sample had remarkable inhibitory effects against some selective tumor cell growth, especially its effect on human leukemic monocyte lymphoma cell line (U-937) and human promyelocytic leukemia cell (HL-60) was promising. The IC_{50} value of ECSP on U-937 was 43.9 μg/ml. The presence of the sample in the U-937 cell culture stimulated the induction of apoptosis as it was revealed by nuclear staining with Hoechst 33342. Moreover, the apoptosis induction was confirmed by the cell cycle analysis, clearly a pronounced sub G1 phase arrest was observed when the cells were treated at 15 μg/ml and 30 μg/ml. To rule out action mechanism of the polysaccharide for its anticancer

activity, a western blot analysis was conducted with antibodies like caspase -7, caspase -8, Bax, and Bcl-xL. Taken together, it can be assumed that cleaved caspase-7 and 8 promoted PARP cleavage and they are directly responsible for the apoptosis induced by ECSP on U-937 cells. Moreover, it was revealed that ECSP sample regulated the Bax and Bcl-xL signals appropriately and contributed to control the normal U-937 cell growth.

Above results clearly demonstrate the possibility of algal polysaccharide for their potential application in pharmaceutical industry. However, further structural and functional studies on the sulfated polysaccharides isolated from the two seaweeds tested in this study are needed to utilize them successfully.

LIST OF FIGURES

Fig. 1-14. Schematic illustration of surface plasmon resonance. The sensor surface is gold with antibodies attached to it. As the complementory antigen (analyte) binds to the antibodies, the refractive index shifts and the SPR-dip moves to larger angles. The movement of the SPR-dip is the actual monitored signal, and the movement over time forms the sensorgram. Quoted from Shankaran et al., Sensors and Actuators B 121 (2007) 158–177.

Fig. 1-15. Binding affinity assay of ECA to blood coagulation factors using surface plasmon resonance (SPR) spectrometer. Black columns express resonance unit (RU) values of real-time sensorgram in the absent of antithrombin III (ATIII). Gray columns express resonance unit (RU) values in the present of ATIII.

Fig. 1-16. Real-time surface plasmon resonance (SPR) sensorgram during amine-coupling immobilization of FXa on the dextran surface of CM5 at the optimum pH of 4.5. Phase 1 Activation of the dextran surface of the CM5 sensor chip with 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M N-ethyl-N′-(3-diethylaminopropyl) carbodiimide (EDC); Phase 2 immobilization of ligand protein FXa (5,963.9±8.7 RU), Phase 3 deactivation of residual esters by addition of 1.0 M ethanolamine hydrochloride, pH 8.5, Phase 4 removal of non-covalently bond materials.

Fig. 1-17. Surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factor FXa in dose-dependent manners (0 – 7140 nM of ECA). Phase 1; association sensorgram and phase 2;

dissociation sensorgram.

Fig. 1-18. Surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factor FVIIa in dose-dependent manners (0 – 7140 nM of ECA). Phase 1, association sensorgram and phase 2, dissociation sensorgram.

Fig. 1-19. Surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factor FIIa in dose-dependent manners (0 – 7140 nM of ECA). Phase 1, association sensorgram and phase 2, dissociation sensorgram.

Fig. 1-20. Anticoagulation mechanism of ECA in human blood coagulation pathway. The dotted line means weak-negative effects of ECA on target factor (FXa).

Fig. 1-21. Some proposed structures for average fucoidans. (Patankar et al. 1993)

Fig. 1-22. Field Emission scanning electron microscopy of sulfated polysaccharide isolated from *E. cava*. (A) 1 x 10, 000; (B) 1 x 18, 000; (C) 1 x 80, 000

Fig. 2-1. Purification of the potential anticoagulant polysaccharide from the red alga *G. filicina* by DEAE cellulose. A crude polysaccharide (500 mg) was applied to a DEAE-cellulose column (17 x 2.5 cm) equilibrated in 50 mM sodium acetate (pH 5.0) and washed with 500 ml of the same buffer containing 0.2 M NaCl. Thereafter, the column was eluted by a gradient prepared by mixing 50 mM sodium acetate containing from 0.2 M NaCl to 2 M NaCl in the same buffer. Fractions of 4 ml were collected and checked for carbohydrate content by phenol-H_2SO_4.

Fig. 2-2. Partially purified anticoagulant polysaccharide of *G. filicina* (100 mg) were re-chromatagraphed on a new DEAE cellulose column (10 x 1.7 cm). Partially

purified polysaccharide was applied to a new DEAE-cellulose column equilibrated in 50 mM sodium acetate (pH 5.0) and washed with 500 ml of the same buffer containing 0.2 M NaCl. Thereafter, the column was eluted by a gradient prepared by mixing 50 mM sodium acetate containing from 0.2 M NaCl to 2 M NaCl in the same buffer. Fractions of 4 ml were collected and checked for carbohydrate content by phenol-H_2SO_4.

Fig. 2-3. Purification of the anticoagulant polysaccharide from the red alga *G. filicina* by Sepharose 4B. Solution fraction (1 mg/ml in water) was applied to a Sepharose 4B column (72 x 2 cm) equilibrated and eluted with water at a room temperature at a flow rate 1 ml/min. Fractions (2 ml) were collected and assayed for metachromatic property at 525nm and for total carbohydrate contents. Carbohydrate content (◊); Metachromatic property (■); Blue dextran (V_o) and Cresol red (V_1).

Fig. 2-4. Agarose gel electrophoresis of the anticoagulant polysaccharide of *G. filicina*. The purified polysaccharide (~10 μg) was applied to a 0.5% agarose gel and run for 1h at 110 V in 0.05 M 1, 3-diaminopropane/acetate (pH 9.0). The sulfated polysaccharides were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution.

Fig. 2-5. The HPLC chromatograms for the monosugar composition of the purified polysaccharide of *G. filicina*. Chromatogram for the sugar standards (A); Chromatogram for the *G. filicina* polysaccharide sample (B).

Fig. 2-6. Calibration curve of dextran standards for the determination of the average molecular weight of the *G. filicina* sample. The retention time is plotted against the molecular weight of the dextrans.

Fig. 2-7. Infrared analysis of the purified sulfated polysaccharides isolated from *G.*

determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

Fig. 3-3. The effect of the sulfated polysaccharides on the growth of the human promyelocytic leukemia (HL-60) cancer cell line. Cells were seeded onto 96-well plate at 1×10^3 cells /well and were treated with sulfated polysaccharides at different concentrations, and percentage of cell viability was determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

Fig. 3-4. The effect of the *E. cava* sulfated polysaccharide on the growth of the mouse melanoma (B-16) cancer cell line. Cells were seeded onto 96-well plate at 1×10^3 cells/well and were treated with sulfated polysaccharides at different concentrations, and percentage of cell viability was determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

Fig. 3-5. Effect of *E. cava* sulfated polysaccharide on morphological changes in U-937 cell line. Cells were treated in the absence of (**A**) or in the presence of 75 μg/ml of sulfated polysaccharide for 24 hrs, stained with Hoechst 33342, and observed by fluorescence microscopy. Arrows on photo **B** indicate a typical apoptotic cell with apoptotic body. Apoptotic nuclei appear brighter and more fragmented than healthy nuclei, which are less bright and intact.

LIST OF TABLES

BACKGROUND

Seaweeds as well-balanced, harmless, natural sources with a high degree of bioavailability of trace elements are strongly advised for fast grown children and pregnant women (Booth, 1964; Choi et al., 1986). In contrast to their use as a source of food, marine algae are widely used in the life science as the source of compounds with diverse structural forms and biological activities. Over the years marine algal species offer the biological diversity for sampling in discovery-phase of new drug development (Munro et al., 1987; Chung et al., 1997). Therefore, it is clearly documented that, pre-clinical pharmacological research with new marine compounds continued to be extremely active in resent history (Mayer et al., 2003). Recently, several biologically important seaweed species at Jeju Island of Korea have been reported (Athukorala et al., 2003, 2005; Siriwardhana et al., 2003; Heo et al., 2005; Karawita et al., 2005).

Jeju Island is located in the southwest sea of the Korean peninsula and is highlighted for its uniqueness. Especially, in the coastal area of this Island the seawater level fluctuates rapidly. Therefore, the algal species present along the shoes of Jeju Island may require high endogenous biological protection as an adaptative response to this milieu. As it has been reported, 432 species of seaweeds are available in Jeju Island, among them 62 species belong into Chlorophyta, 116 into Phaeophyta and 254 species belong into Rhodophyta (Lee and Kang 2001). However, yet there are few or less systematically studied reports regarding the potential biological activities of Jeju Island's seaweeds.

In 1913, scientists investigated blood anticoagulant properties of marine brown algae (Killing, 1913). Even if it is difficult to elucidate the exact structure of the anticoagulant polysaccharides isolated from algae, the research interest to isolate anticoagulant compounds from marine seaweeds is continuously increased in the field

of pharmaceutical industry (Koo et al, 1995). Heparin is the drug of the choice in prevention of thromboembolic disorders. But recently alternative drugs for heparin are in high demand due to its bad and long-term side effects. Therefore, as an alternative source, seaweed polysaccharides gain much attention in the pharmaceutical industry to develop better and safe drugs with low or less side effects. Recently, there was a case study on the changes of the haemorrhage, plasma cholesterol and albumin and clinical effects in 36 children with refractory nephrosis after treatment with fucans. The results of the study suggest that fucan might be used in the anticoagulant treatment of refractory nephrosis (Shanmugam and Mody, 2000). Therefore, algal anticoagulants in future may add a new dimension in vascular disorders.

Popular anticoagulants like heparin, low molecular weight heparin and dermatan sulfate, chondroitin sulfate and dextran sulfate mediate their high anticoagulant activity through antithrombin III (Desai, 2004). As a major blood clotting regulator, antithrombin inhibits almost all proteinases of the coagulation cascade including thrombin and FXa. Interestingly, under normal physiological condition, antithrombin does not show substantial coagulant factor inhibition (Olson et al., 1993). However, the presence of the heparin and other anticoagulant polysaccharides with antithrombin dramatically enhance coagulation factor inhibition. Especially, heparin together with antithrombin mediate 2,000 fold activity enhancement on thrombin inhibition compared to that of antithrombin alone (Jordan et al., 1980). Natural anticoagulants like sulfated polysaccharides share structure similarities with heparin, therefore most of them have shown similar mode of action to that of heparin on blood coagulation (Jung et al., 2006). As a promising natural anticoagulant, sulfated polysaccharides isolated from algae have been studied for years to utilize as a safe drug (Yang et al., 2006; Shanmugam and Mody, 2000; Matsubara et al., 2001).

Epidemiological studies have indicated that the consumption of seaweeds is a protective factor against some types of cancer (Cho et al., 1990; Park et al., 2006). The potential beneficial association between consumption of seaweeds and breast cancer can be traced back to the ancient Egyptian 'Ebers Papyrus' dating from approx. 1534 B.C., which mentioned that seaweeds were administrated as a treatment to breast cancer patients (Teas, 1981). Compounds that blocks mitosis which occur during cell proliferation have become some of the most important anticancer agents. By the means of immuno-activation, algal polysaccharides have been showed their potentiality as anti-tumor agents (Yim et al., 2005; Bae 2004). A marine red alga, *Marginisporum crassissimum* is rich with both immuno-stimulating and suppressive effects against tumor cells. Also, fucoidan, a sulfated polysaccharide isolated from brown algae, has been shown potential antitumor activity through the inhibition of tumor inducing angiogenesis. The ability of algal polysaccharides to induce tumor cell proliferation has been well documented. Moreover, polysaccharides isolated from Japanese seaweeds like *Laminaria japonica*, *Undaria pinnatifida*, *Eisenia bicyclis* and *Hijikia fusiforme* were identified as potential anti-genotoxic substances (Okai et al., 1993; Okai and Higashi-Okai, 1994).

Fucoidan isolated from *Cladosiphon novae-caledonias Kylin, Fucus vesiculosus* and *Undaria pinnatifida Sporophylls* have been shown high anticancer activity by inhibiting IgE production, **ε**GT, and the nuclear translocation of NF_kB p52 in B cells induced by IL-4 and anti-CD40 antibodies in vitro. Fucoidans optimize the natural killer cell (NK) function, first line of immune defense. Fucoidan promotes a healthy immune response by increasing macrophage production, and enhancing its phagocytic activity which help the body get rid of viruses, bacteria, fungus, and parasites. Fucoidan helps to maintain healthy upper respiratory mucous membranes which when weakened may increase the

incidence of bronchitis, asthma, chronic obstructive pulmonary disease (COPD) and other pulmonary diseases. Fucoidan strengthens the mucosal lining of the digestive and urinary tracts. Fucoidan is a powerful free-radical neutralizer. Fucoidan is important for normal blood sugar level, and to support liver function. Fucoidan is important for healthy hair, nails and skin tone (Davis TA, 2004).

Therefore, it is postulated that as a highly abundant renewable natural source, polysaccharide isolated of marine algae are important for further studies in pharmaceutical industry (Kwon and Nam 2007). But due to some of limitations, it's hard to purify exactly biologically active compounds. Some of the biggest hurdles include the low concentration, instability and difficulty in separation and detection of these bioactive compounds. As an alternative technique, here we used in- expensive carbohydrate digestive enzyme to digest algal bio-mass to extract marine plant photochemical materials. As previously mentioned, this enzymatic extraction possesses much more advantages than conventional techniques. According to our lab previous experiments, several brown algal species were enzymatically digested with several carbohydaces and proteases to investigate their potential bioactivities. The degree of enzymatic hydrolysis was different with the treated enzymes, especially, in that study AMG extract of *E. cava* showed the highest extraction yield (41.52%) among the tested enzymatic hydrolysates (Heo et al., 2003). Therefore the digestion of alga by enzymes increase the extractable polysaccharide and meantime release polysaccharide attached polyphenolic compounds to the medium. From this point of view, the present study was designed to isolate sulfated polysaccharide derived from enzymatically hydrolyzed *Ecklonia cava* and *Grateloupia filicina* to characterize a purified compound with respect to its anticoagulant and anticancer activity.

Part I

Anticoagulant Activity of Sulfated Polysaccharide Purified from *E. cava*: an *in vitro* and *in vivo* study

ABSTRACT

In this study, seven brown algal species (*Ecklonia cava, Ishige okamurae, Sargassum fullvelum, Sargassum honeri, Sargassum coreanum, Sargassum thunbergii and Scytosiphon lomentaria*) were enzymatically hydrolyzed using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and screened for their potential anticoagulant activity. E. cava was the most potent anticoagulant inhibitor of the seven species. The active polysaccharide fraction of the AMG enzymatic extract of E. cava was purified using anion exchange chromatography on DEAE-cellulose and gel filtration chromatography on Sepharose-4B. The highly sulfated (0.92 sulfate/total sugar) active sample, purified from E. cava was mainly composed of fucose and small amount of galactose with 0.01% yield. The anticoagulant compound studied was detected by prolongation of activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT). As it was revealed by APTT assay, the pure sulfated polysaccharide from E. cava at 0.7 μg/ml showed almost similar anticoagulant activity to that of heparin. According to the results of activated coagulation factor assay, the purified compound strongly interferes with coagulation cascade by inhibiting biological activity of serine proteases II, X and VII. According to the SPR study data, binding affinity between ECA/ATIII (antithrombin-III) and blood coagulation factors was in the order of VIIa (FVIIa) > factor Xa (FXa) > thrombin (FIIa) and the kinetics analysis elucidated that the K_D values of ECA for FVIIa, FXa, and FIIa were 15.1, 45.0 and 65.0 nM, respectively. Therefore, sulfated polysaccharide purified from the enzymatic hydrolysate of E. cava strongly enhance antithrombin III (ATIII) mediated coagulation factor inhibition selectively (FVII, FX, and II) in both of the extrinsic and common coagulation pathways. This may contribute for its high anticoagulant activity in-vitro, also low cytotoxicity of ECA on venous endothelial cell line (ECV-304)

expands its value in future studies in-vivo. To rule out the iv-vivo anticoagulant activity of the ECA, the samples was administrated into male Wistar rats and compared the results with that of heparin. Taken together, the results were relatively low than that of commercial compound like heparin at the same concentration, but was quite stronger than that of saline alone treated sample. Therefore, as a natural anticoagulant compound ECA will be a promising candidate for future drug discovery in pharmaceutical industry.

INTRODUCTION

Heparin is widely used for the prevention of venous thromboembolic disorders, moreover heparin is employed during extracorporeal circulation, such as in major vascular surgery and in hemodialysis (Mourano and Pereira, 1999). Heparin is a 1, 4 linked linear copolymer of glucosamine (2-amino-2-dexyglucopyranose) and uronic acid (pyranosyluronic acid) residues that are variously sulfated. The β-D-glucosamine may be N-sulfated or acetylated and may also contain sulfates at 2-3 and 6 positions whereas the uronic acid residues may be either β-D-glucouronic acid or α-L-iduronic acid and mainly be sulfated at 2-.position (Dasai, 2004). The anticoagulant activity of this heparin is due to its special polyanionic character (Huntington, Read, & Carrell, 2000). This structure, called glycosaminoglycan (GAG), is obtained by chemical processing of proteoglycan heparin present in porcine or bovine intestinal mucosa and lung. Interestingly, it is the most negatively charged compound in human body (Dasi, 2004). The potential anticoagulant action of heparin is achieved mainly by potentiation of antithrombin and heparin cofactor II (Pereira at al., 1999).

As it has been previously reported, heparin chains interacts with platelet 4 (PF4), a plasma protein, to form a tetrameric complex that initiates an immune response. This interaction is the basis for heparin induced thrombocytopenia (Boneu, 2000; Dasi, 2004). Heparin also has a propensity to bind non-specifically to other plasma proteins. Because plasma levels of these heparin-binding proteins vary from patient to patient, the anticoagulant response to heparin is unpredictable and careful laboratory monitoring is necessary to ensure that an adequate anticoagulant effect is achieved (Theroux et al., 1992). In addition to these problems with heparin therapy, it has been found that heparin can't inactivate clot bound thrombin. Furthermore, heparin is mostly extracted from pig intestine and bovine lung where it occurs in low concentration.

These disadvantages, associated with heparin have opened up a new area of antithrombotic research for discovering novel anticoagulant agents. Therefore, new alternative anticogulant agents, which can exhibit rapid and predictable onset and offset kinetics are in high demand.

There is a grater incidence of anticoagulant activity in the extracts of marine brown algae than red and green algae (Patanker et al., 1993; Chevolvet et al., 1999). As it has been previously reported, it is clear that polysaccharides from seaweeds are good alternative sources for anticoagulative drug production (Matsubara, 2004; Nishino et al., 2000; Church et al., 1989). Therefore, algal polysaccharides have attracted the attention of biomedical scientists. Interestingly enzymatic digestion of algae gains 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 also this method do not adapt any toxic chemicals. Therefore, cheep and food grade enzymes may useful in future to extract pharmaceutically important compounds from algal bio-mass (Heo et al., 2003). Accordingly, in this study, algal samples were digested using food grade enzymes to investigate their potential anticoagulant activity.

In our preliminary experiments, a marked anticoagulant activity was observed in enzymatic extracts of *E. cava*. However, few/no reports had dealt with producing enzymatic extracts of seaweeds and investigating their anticoagulative activities. From this point of view, the present study was designed to isolate anticoagulative polysaccharide derived from enzymatically hydrolyzed *Ecklonia cava* and to characterize a purified compound with respect to its anticoagulant activity. *E. cava* anticoagulant showed a similar anticoagulant activity to that of heparin, and strongly interfered with coagulation cascade by inhibiting biological activity of the activated

blood coagulation FII, FX and FVII as serine proteases. To understand molecular interaction of the highly sulfated polysaccharide of *E. cava*, binding assay and kinetics study using surface plasmon resonance (SPR) spectormeter are focused in the present study.

MATERIALS AND METHODS

Algae samples and reagents

Marine brown algae used in this study were collected close to the shores of Jeju Island in Korea during March and October 2006 (Fig. 1-1). Salt, sand and epiphytes were removed using tap water. Finally, seaweed samples were rinsed carefully with fresh water and freeze-dried at -20^{o}C for further experiments. APTT (ellagic + bovine phospholipids) and $CaCl_2$ solution were obtained from International Reagents Corporation (Japan), PT (rabbit thromboplastion) and TT reagents were purchased from Fisher Scientific Company (USA). Carbohydrates such as Viscozyme L (a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase), Celluclast 1.5 L FG (catalyzing the breakdown of cellulose in to glucose, cellobiose and higher glucose polymers), AMG 300 L (an exo1, 4-alpha-d-glucosidase), Termamyl 120 L (a heat stable alpha – amylases), Ultraflo L (a heat stable multi-active beta-glucanase) were obtained from Navo Co. (Novozyme Nordisk, Bagsvaed, Denmark). Heparin, low molecular weight dextran sulfate (*Dex* 8) (8 kDa), chondroitin 6-sulfate from shark cartilage (*C-6-S*) (60 kDa), high molecular weight dextran sulfate (500 kDa), sepharose 4B (MW range, 30, 000 -5, 000,000), 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 grater purity.

Digestion of algae samples

The preparation of enzymatic extracts was followed as previously reported (Heo et al., 2003). Dried alga sample was ground (MFC SI mill, Janke and Kunkel Ika-Wreck,

Staufen, Germany) and sieved through a 50 standard testing sieve. A 100 g of alga sample was homogenized with water (2 L), and then 1 g or 1 ml enzyme was mixed. The enzymatic hydrolytic reactions were performed for 12 h to achieve optimum degree of the hydrolysis. Before the digestion pH of the homogenate was adjusted to its optimal pH value, also after digestion the digests were boiled for 10 min at 100^{o}C to inactive the enzyme. Each sample was clarified by centrifugation (3000 rpm, for 20 min at 4^{o}C) to remove the residue. All samples were kept in -20^{o}C for further experiments.

Molecular weight fractionation of algal extract

Algal extracts were passed through micro-filtration membranes (5, 10 and 30 kDa) using Millipore's Lab scale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) to obtain different molecular weight fractions. Finally, all the fractions (>30, 30–10, 10–5 and <5 kDa) were separately processed to evaluate anticoagulant activity.

Crude polysaccharide separation

The enzymatic extract was (240 ml) mixed well with 480 ml of 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at a room temperature, crude polysaccharides were collected by centrifugation at 10,000 g for 20 min at 4^{o}C (Kuda et al., 2002; Matsubara et al., 2000).

Anion-exchange chromatography

The crude polysaccharide from *E. cava* (500 mg) obtained using the procedures described above was applied to a DEAE-cellulose column (17 x 2.5 cm) equilibrated in

50 mM sodium acetate (pH 5.0) and washed with the same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate 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, carbazole reactions and by metachromatic property (Chaplin and Kennedy, 1994). Fractions showing strong anticoagulant activity were collected, dialyzed against distilled water, and concentrated to 5 ml by rotary evaporation under reduced pressure bellow 40°C. The partially purified concentrated polysaccharide fraction was re-chromatographed on new DEAE-cellulose column (10 · 1.7 cm), under 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 x 2 cm) equilibrated and eluted with water at a room temperature at a flow rate 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 sulfated polysaccharide sample was examined using agarose gel electrophoresis. About 5 μg of sulfated polysaccharides was applied to a 0.5% agarose gel in 0.05 M in 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 hr using 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v/v).

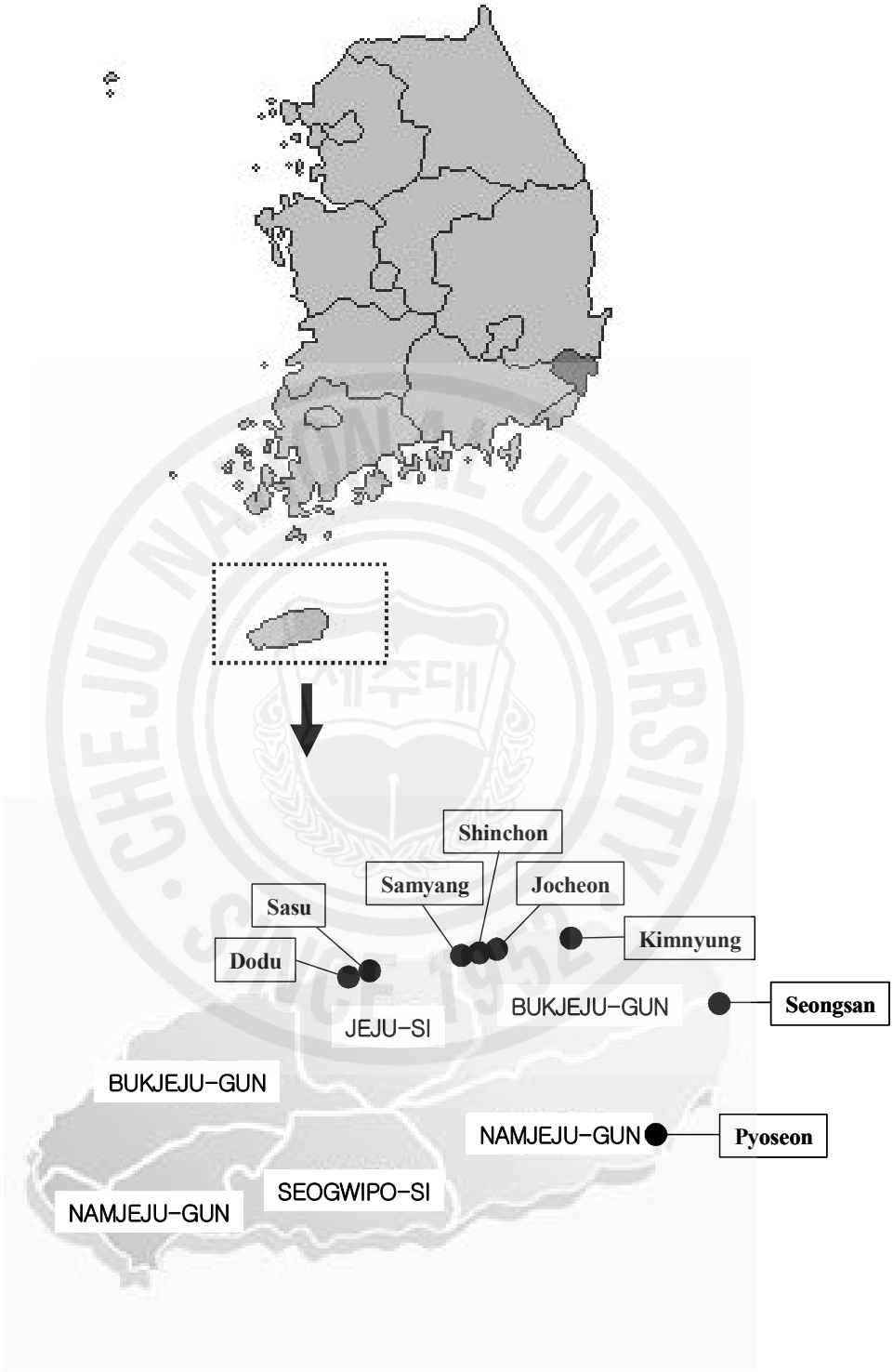

Fig. 1-1. Geological location of Jeju Island in Korea. The black circles (●) indicate seaweed sample collected locations in Jeju Island.

Table 1-1. Characterization and optimum hydrolysis conditions of particular enzymes.

Enzyme	Enzyme characterization	Optimum conditions	
		pH	Temp (^{o}C)
Viscozyme	Arabanase, celluiase, β-glucanase, hemi-cellulase and Xyianase	4.5	50
Celluclast	Catalyzing the breakdown of cellulose into glucose cellobiose and higher glucose polymer	4.5	50
AMG	An exo-1,4-α-D-glucosidase	4.5	60
Termamyl	A heat stable α-amylase	6.0	60
Ultraflo	A heat stable multi-active β-glucanase	7.0	60

Polyacrylamide gel electrophoresis

Purified polysaccharide sample was applied on polyacrylamide gel electrophoresis in order to evaluate molecular mass. Approximately 5 μg of sample was introduced to 6% polyacrylamide gel slab (1 mm thickness) in 0.02 M sodium barbital (pH 8.6) and run for 30 min at 100 V. Thereafter, the gel was stained with 0.1% toluidine blue in 1% acetic acid and then washed about 4 h in 1% acetic acid.

Determination of the molecular weight of the sample

In order to determine the exact molecular weight of the sample, the freeze-dried sample was introduced into a PL-Aquaz OH 40 column and eluted with de-ionized water at 0.8 ml/min flow rate (23^{o}C). Dextran standards (48.6, 148, 273, 410, 830, and 2000 kDa) were also introduced into the column under same experimental condition for comparison purposes. The retention time was plotted against average molecular weight of the dextrans and thereby molecular weight of the sample was calculated.

IR-spectrum study

Freeze dried algal polysaccharide sample (0.1 g) approximately was mixed with potassium bromide powder (100-200 g) then pressed into thin disc under hydraulic power and used as a sample for FT-IR measurement. The spectrum was arranged at mildest infrared region.

Field emission electron microscopic study

The purified polysaccharide was freeze dried and posited on a carbon tape coated with aluminum stubs, vacuum dried sputted with gold and the structure characters were investigated under field emission electron microscope operated at 5 kV.

Neutral sugar analysis

The purified polysaccharide was hydrolyzed in a sealed glass tube with 2 M of 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 of HCl for 4 h. Then, 0.055 and 2.75 μg of sample were separately applied to CarboPacc PA1 (4.5 x 250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartridge (4.5 x 50 mm) column to analyze neutral and amino sugar, respectively. The column was eluted using 16 mM of NaOH at 1.0 ml/min flow rate. Each sugar of the sample was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

Sulfate content analysis

After acid hydrolysis of the purified polysaccharide, the sulfate content was measured by the $BaCl_2$/gelation method (Saito et al, 1968).

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 2400 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, 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 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 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 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) and was slightly changed according to previously known methods of Jung et al, 2002; Rajapaksha et al, 2005.

Surface plasmon resonance (SPR) spectroscopy

To identify binding affinity and kinetics of ECA for blood coagulation factors, surface plasmon resonance (SPR) sensorgrams were determined by a BIAcore 2000® system (Pharmacia Biosensor). This method detects binding interactions in real time by measuring changes in the refractive index at a specific surface of biomolecules and enables association and dissociation rate constants to be calculated. Target factors (FIIa, FVIIa, FXa) interfered by ECA in the previous study (Athukorala et al, 2006) were directly immobilized on a CM5 sensor chip by coupling through 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 the method of Shobe et al (1999) after pH scouting. Sensor chip surface was coated with ligand

proteins in 10mM sodium acetate buffer at optimal pH, and un-reacted groups were blocked with 1 M ethanolamine, 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 ECA) was injected onto the surface of 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 (*ka*, *kd*, and *K*D), ECA solution at various concentrations (0 – 7,500 nM) with or without adding 1,000 nM of antithrombin III (ATIII) was injected to coagulation factors, and real-time sensorgrams were evaluated using BIA evaluation software (version 3.2). Association rate constant (*ka*) was calculated from multiple sensorgrams, representing at least five different concentrations of analyte for each experiment. Dissociation rate constant (*kd*) was calculated from the initial dissociation phase of the binding curves, and equilibrium dissociation constant (*K*D) equaled the ratio of *kd*/*ka*.

Cytotoxicity assay

Venous endothelial cell line (ECV-304) were maintained in RPMI 1640 or DMEM medium 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 highly sulfated polysaccharide ECA on normal endothelial cell (ECV-304) was measured by using MTT assay (Mossman, 1983; Carmichael et al, 1987). The cells were seeded in a 96-well plate at the concentration of 2×10^4 cells/ml using RPMI medium. After 16 h (at 37°C, in a humidified atmosphere of 5% CO_2),

ECA was treated to the wells at a concentration range 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., USA). For treated cells, viability was expressed as a percentage of control cells. All determinations were carried out in triplicate.

***Ex vivo* determination of anticoagulant activity on Wistler rats**

The experiment was conducted on male Wistar rats (~300 g), before start the experiment the rat was anesthetized by injecting a mixture of Ketamine and Xylasine (intraperitoneally) with a dose of 100 and 16 mg/ Kg respectively. The sample was injected as it was previously published (Herrero et al., 2005). The carotid artery was dissected and carefully separated from the surrounding tissues, heparin, sample and saline were administrated. During the each time interval (5, 30 and 60 min) the blood samples were taken out into sodium citrated tube and immediately separated the plasma by centrifugation at 2500 g at room temperature. Then, the APTT, TT and PT activities were examined as it has been previously reported.

Tail transaction bleeding time

The samples were administrated through the carotid artery and the bleeding time was started to measure after cutting 3 mm from the tip of the tail. The tip of the tail was blotted on a tissue paper in a 30 sec intervals and the time take to stop the bleeding was recorded. Each component was administrated five min prior to tail dissection.

Statistics

Results are presented as mean ± SD of the mean (n = 3). Student's t-test was used to determine the level of significance unless otherwise stated.

RESULTS AND DISCUSSION

In this study, seven brown algal species (*E. cava*, *I. okamurae*, *S. fulvellum*, *S. horneri*, *S. coreanum*, *S. thunbergii* and *S. lomentaria*) were digested with carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo). The general information including optimum hydrolyzing conditions for the carbohydrases used in this study is summarized in Table 1-1. Interestingly enzymatic digestion gains 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 also this method do not adapt any toxic chemicals. Therefore, cheep and food grade enzymes may useful in future to extract pharmaceutically important compounds from algal bio-mass (Heo et al, 2003). Accordingly, in this study, algal samples were digested using food grade enzymes to investigate their potential anticoagulant activity.

All representative results are shown in Table 1-2. The enzymatic extracts of some species of brown algae (*E. cava, S. horneri* and *S. coreanum*) showed high anticoagulant activities while the other species showed low activities. All the tested enzymatic hydrolysates of *E. cava* showed >300 sec of APTT activity at 80 μg/ml. Viscozyme, Celluclast and Ultraflo extract of *S. honari* also showed good anticoagulant activities. Meanwhile, *S. coreanum* showed a mild anticoagulant activity in this study. Due to high anticoagulant activity, *E. cava* was subjected for further anticoagulative experiments. According to the results of Table 1-3, *E. cava* sample that was digested by AMG extract showed the highest APTT activity compared to its other counterparts.

Table 1-2. Anticoagulant activities of brown algae hydrolyzed with carbohydrases

Species	Activated partial thromboplastin time (sec), APTT				
	1[a]	**2**	**3**	**4**	**5**
E. cava	>300	>300	>300	300	>300
I. okamurae	35	32	38	31	35
S. fullvelum	32	35	32	32	33
S. horneri	>300	>300	120	165	>300
S. coreanum	125	152	114	131	115
S. thunbergii	30	35	32	31	36
S. lomentaria	45	35	61	45	52

Results are expressed as means of two determinations and the sample concentration is 80 μg/ml. [a] 1, Viscozyme; 2, Celluclast; 3, AMG; 4, Termamyl and 5, Ultraflo.

Table 1-3. Anticoagulant activities of enzymatic hydrolysates of *E. cava* measured by APTT, PT and TT assays (sec)

Sample	APTT	PT	TT
Viscozyme digest	320	11	108
Celluclast digest	348	12	26
AMG digest	1500	15	124
Termamyl digest	300	11	26
Ultraflo digest	350	11	120
Water	37	11	26
Heparin	1500	1202	>1500

Results are expressed as means of two determinations and the sample concentration is 80 μg/ml.

Table 1-4. Anticoagulant activities of molecular weight fractions from AMG extract of *E. cava*.

Sample (80 μg/ml)	APTT	PT	TT
Above 30 kDa fraction	>1500	20	>1500
30-10 kDa fraction	35	13	26
10-5 kDa fraction	37	30	26
Below 5 kDa fraction	37	11	26
Water	37	11	26
Heparin	>1500	1202	>1500

Results are expressed as means of two determinations.

In PT assay, almost all tested extracts did not show good activities, however AMG hydrolysate showed 15 sec PT value with that of 11 sec in control. However, in TT assay AMG hudrolysate showed 124 sec TT value, moreover Ultraflo and Viscozyme extracts also showed considerable activities (120 and 108 respectively). According to our lab previous experiments, several brown algal species were enzymatically digested with several carbohydaces and proteases to investigate their potential bioactivities. In that study AMG extract of *E. cava* showed the highest extraction yield (41.52%) among the tested carbohydraces (Heo et al, 2003). AMG is able to digest 1, 4 and 1,6-α linkages of the plant cell wall materials. The rate of hydrolysis depends on the type of linkage and on chain length. Especially, AMG hydrolyze 1, 4- α linkages more easily than 1, 6- α linkages. This especial chain breaking ability of AMG may inversely relate with its high anticoagulant activity. Therefore, AMG is the enzyme of the choice for further experiments to digest *E. cava*. In order to recognized molecular weight distribution of the active fraction, the AMG digest of *E. cava* was passed through ultrafiltration membranes (5, 10 and 30 kDa) and the relevant molecular weight cut-off fractions were separated and evaluated for anticoagulant activity (Table 1-4). It is interesting to mention that >30 kDa fraction of AMG recorded the highest anticoagulant activity. Especially it was very effective in prolonging the APTT and TT compared to its other counterparts. Therefore, >30 kDa fraction of AMG extract was applied to further experiments. According to previous records the anticoagulant activity of most brown algal species are due to sulfated polysaccharides (Shanmugam & Mody, 2000). Hence the crude polysaccharide fraction of the AMG extract was separated by ethanol precipitation technique.

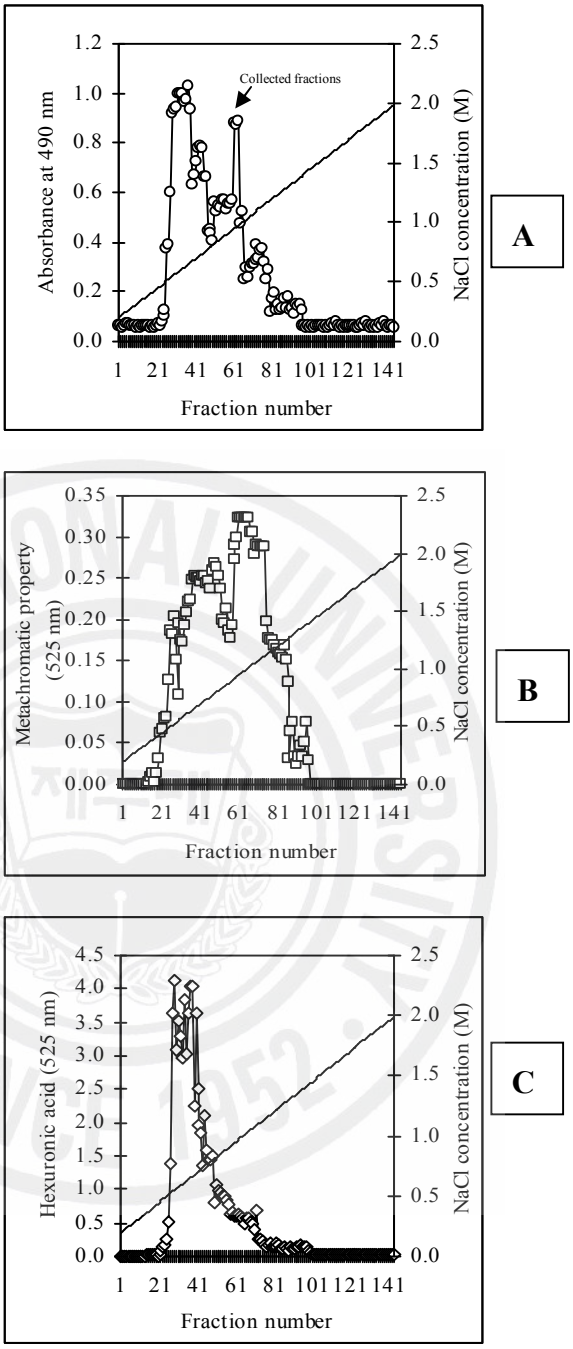

Fig. 1-2. Purification of anticoagulant polysaccharide from the brown alga, *E. cava* on DEAE cellulose anion chromatography. Fractions of 4 ml were collected and sugar content was checked by phenol-H_2SO_4 (A) metachromacia using 1, 9-dimethylmethylene blue (B) and carbasole reaction (C).

Thereafter, the crude polysaccharide fraction of *E. cava* was purified by a combination of ion exchange chromatography on DEAE-cellulose and Sepharose-4B. The profile of DEAE-cellulose chromatography is shown in Fig. 1-2. Anion exchange chromatography on a DEAE- cellulose column separated the major anticoagulant peak from large polysaccharide peaks. The main peak eluted at the beginning of the salt gradient, containing high hexuronic acid content (Fig. 1-2, A and C), had low/less anticoagulant activity. According to the results of the first DEAE-cellulose chromatography, the anticoagulant was a minor polysaccharide content in the alga. However, those fractions containing higher anticoagulant activity had high metachromatic property (Fig. 1-2, B). The metachromatic activity depends largely upon the charge density of the whole molecule. A large number of sulfate groups or anionic groups relatively close to each other display high metachromatic activity, whereas molecules with fewer sulfate groups exhibit low activities. Therefore metachromatic property indicates the complex binding properties of the isolated polysaccharide (Baumann and Rys, 1999). Normally, anticoagulant compounds isolated from brown algae have relatively high metalachromatc property (Pereira et al., 1999) and similar results were observed in this study. The active polysaccharide fractions were pooled, dialyzed and freeze dried. Then more purified anticoagulant polysaccharide was isolated by re-chromatography on another new DEAE-cellulose column (Fig. 1-3). In re-chromatography experiment the active compound appeared as a very clear and distinct one peak with strong metalachromatic property (Fig 1-3, A and B). Moreover, the final active compound composed with low hexuronic acid content as it was revealed by the carbazole reaction results.

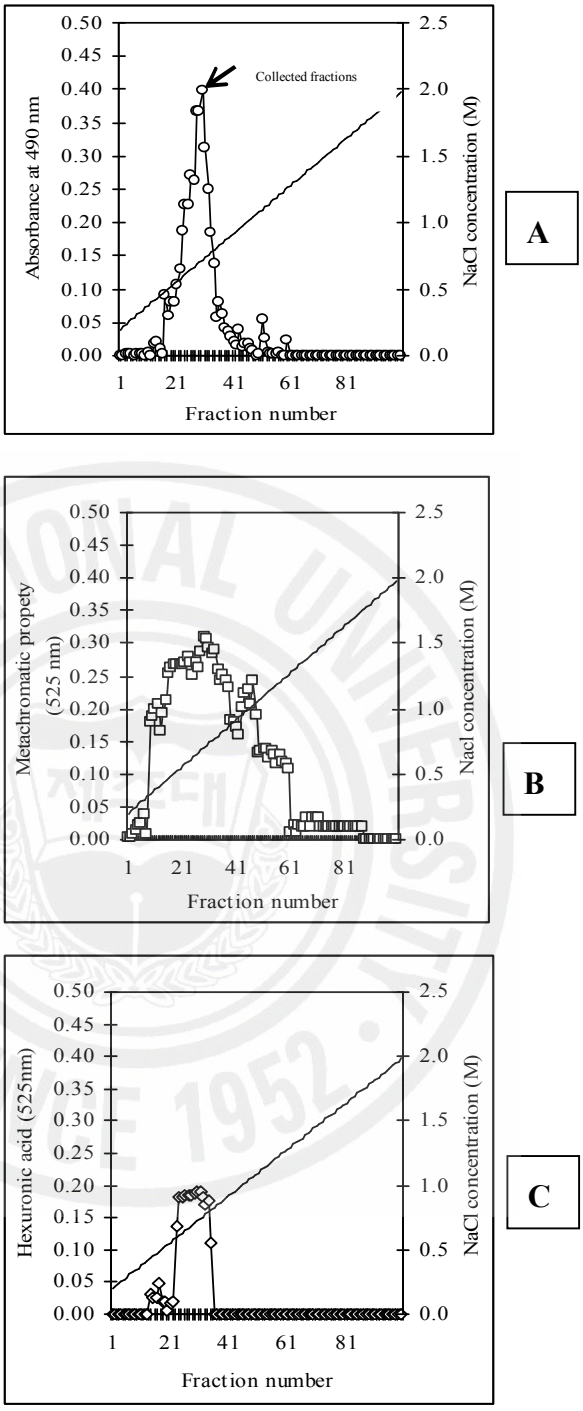

Fig. 1-3. Re-chromatography of anticoagulant polysaccharides from the brown alga, *E. cava* on new DEAE cellulose. Fractions of 4 ml were collected and sugar content was checked by phenol-H_2SO_4 (A); metachromacia using 1, 9-dimethylmethylene blue (B); and carbasole reaction (C).

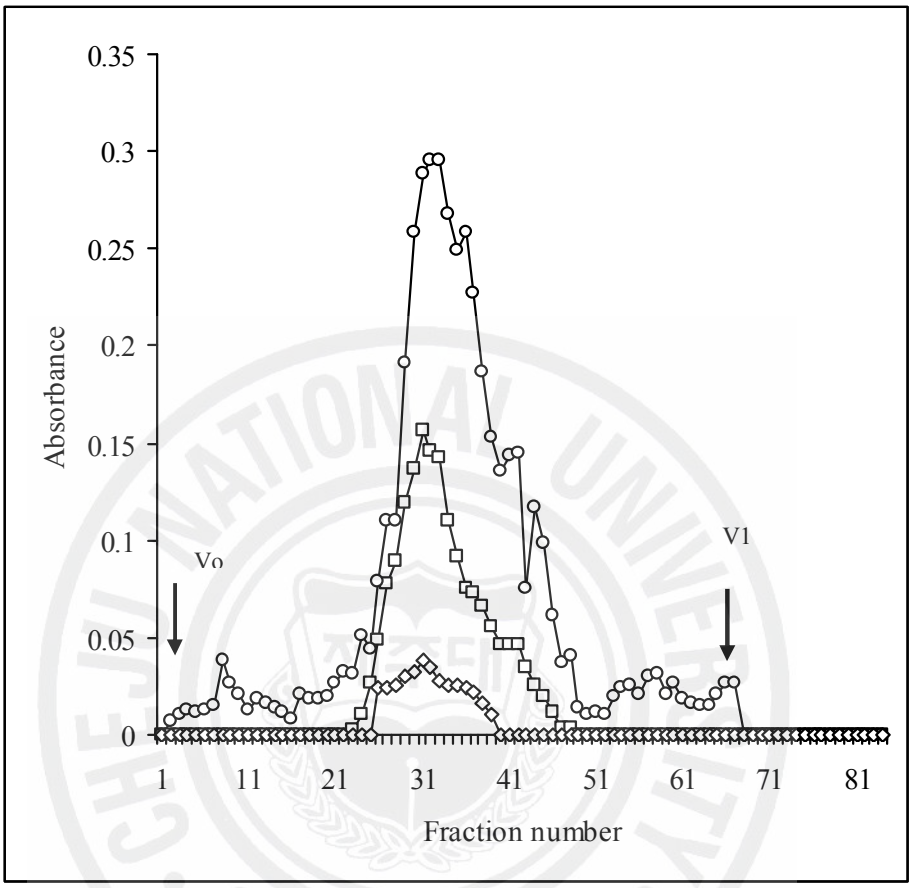

Fig. 1-4. Purification of the sulfated polysaccharides from the brown alga *Ecklonia cava* on Sepharose 4B. Fractions (2 ml) were collected and assayed for metachromatic property at 525 nm, for total polysaccharide contents and for hexuronic acid contents. Carbohydrate content (⬭) Metachromatic property (□) Hexuronic acid content (◇), Blue dextran (V_0); Cresol red (V_1).

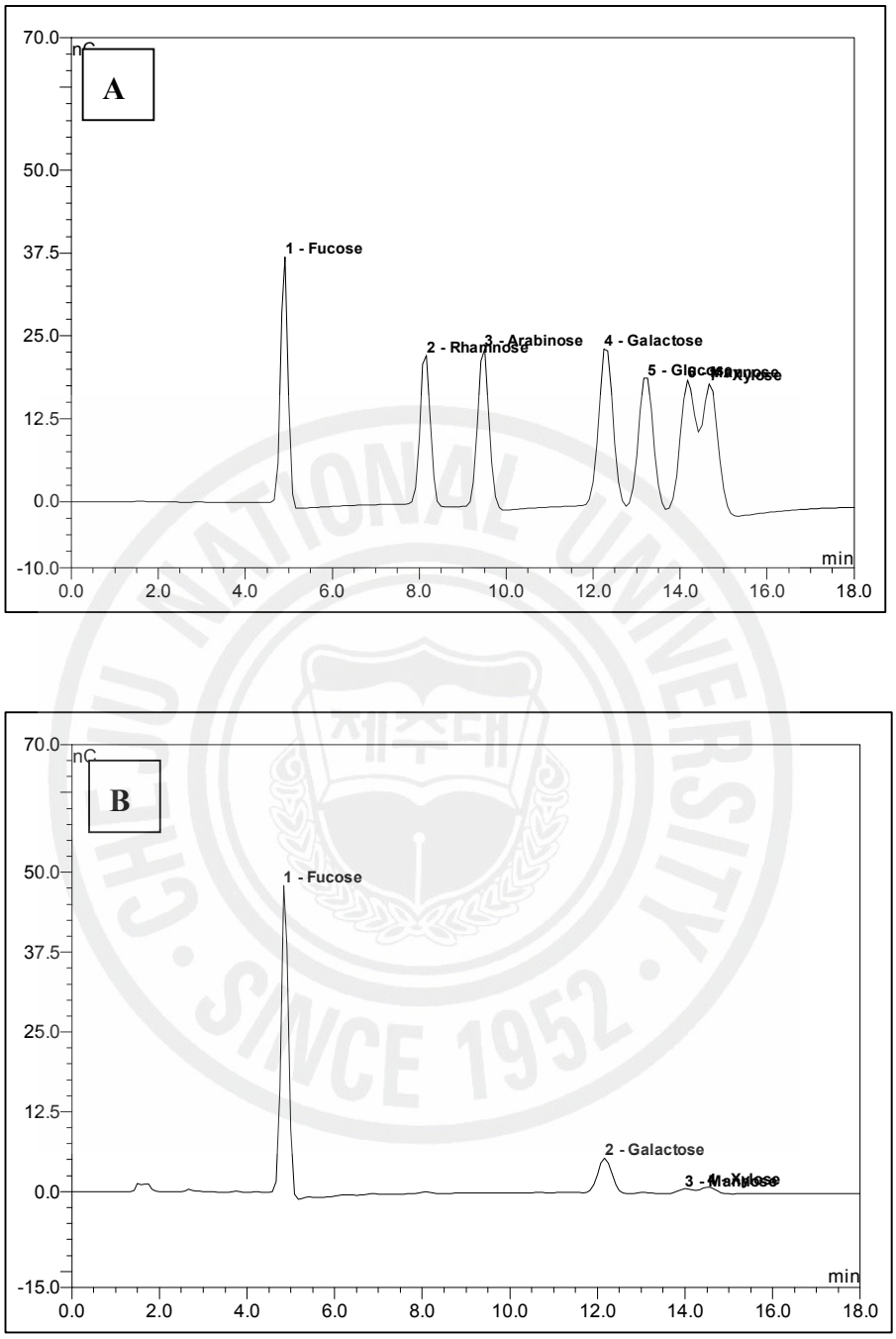

Fig. 1-5. The HPLC chromatograms for the monosugar composition of the purified polysaccharide of *E. cava*. The chromatogram for the sugar standards (A); The chromatogram for the *G. filicina* polysaccharide sample (B).

Table 1-5. Monosugar constituents and degree of sulfation of polysaccharide isolated from *E. cava.*

Sugar	(%)
Fucose	82.1
Galactose	14.2
Mannose	2.31
Xylose	3.19
Sulfate/total sugar[b]	0.95

[b] The mean degree of substitution of sulfate ester per anhydro sugar residue

Table 1-6. Comparison of anticoagulant activity of *E. cava* with that of heparin

	Clotting time (sec)						
		E. cava anticoagulant (μg/ml)			Heparin (μg/ml)		
	Control	0.7	1.4	2.8	0.7	1.4	2.8
APTT	37	60	110	>300	78	>300	>300
PT	11	15	30	68	58	82	>300
TT	26	55	98	>300	80	>300	>300

Results are means of three determinations.

After being evaporated under vacuum, the dialyzed sample was applied on Sepharose 4B column to purify active compound in terms of molecular mass (Fig. 1-4). The active polysaccharide fraction on this chromatograph was observed as a one-polysaccharide peak when determined by the phenol-H_2SO_4 reaction with high metachromatic property and low hexuronic acid. Then, the active fraction was pooled, dialyzed and freeze dried to give dry polysaccharide powder. Fig. 1-5 shows the electrophoretic mobility of the purified polysaccharide on agarose gel. The clear one spot on agarose gel after stained with toluidin blue confirmed the homogeneity of the active compound. In this study, the molecular mass of the purified compound was determined by polyacrylamide gel electrophoresis (Fig. 1-6). The figure of this experiment indicates that the sample have high molecular weight. The marker dextran sulfate (500 kDa) and the purified polysaccharide stayed at the same line after electrophoresis. Therefore, the purified compound is high molecular weight compound with good anticoagulant activity. To investigate the exact molecular weight, the sample was applied on HPLC gel filtration chromatography and the retention time of the sample was plotted with known dextran standards. The molecular weight of the samples was 1381 kDa (Fig 1-7). As it has been reported previously, anticoagulant polysaccharide purified from brown algae like *Laminaria brasiliensis* and *Ascophyllum nodosum* also have similar results under similar experimental conditions (Pereira et al, 1999). According to previous reports, the anticoagulant activity of the fucoidan depends on its molecular weight. Normally, fucoidans with 50-100,000 Da MW are considered to be potential anticoagulants, whereas fractions with >850,000 Da MW are usually demonstrated low anticoagulant activities (Shanmugam and Mody, 2000).

Fig. 1-6. Agarose gel electrophoresis of the sulfated polysaccharides from *E. cava*. The purified polysaccharide (~10 μg) was applied to a 0.5% agarose gel and run for 1h at 110 V in 0.05 M 1, 3-diaminopropane/acetate (pH 9.0). The sulfated polysaccharides were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution.

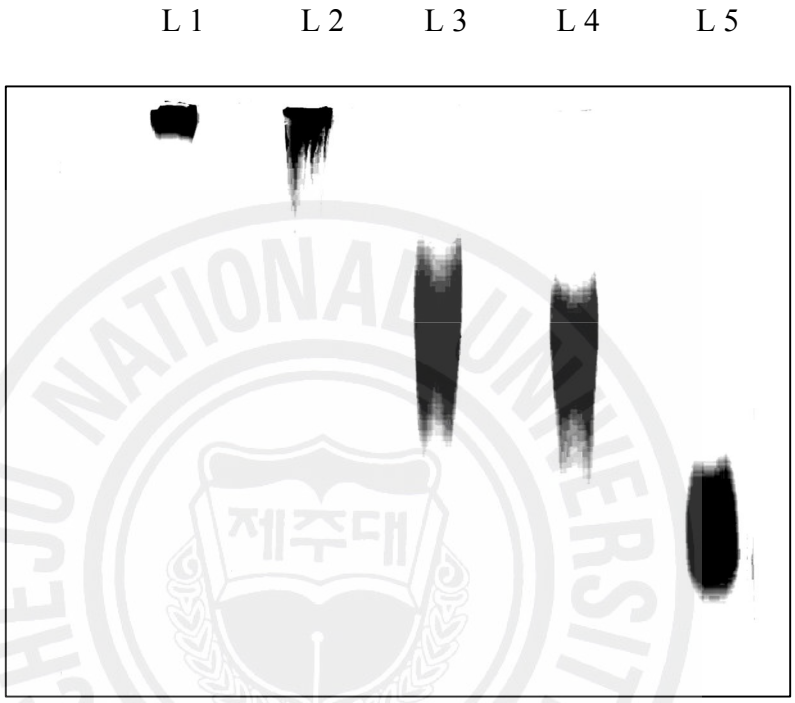

Fig. 1-7. Polyacrylamide gel electrophoresis of the sulfated polysaccharides from *E. cava*. Purified sample (L1); dextran sulfate, 500 kDa (L2); chondroitin 6-sulfate, 60 kDa (L3); chondroitin sulfate, 50 kDa (L4) and deaxtran sulfate, 8 kDa (L5).

However, fucoidan isolated from *Fucus vesiculosus,* with molecular weight of 7.4 x 10^4 Da showed 60-80% of the activity of heparin in the recalcification tests and 15-18% heparin activity in the whole human blood. In this study the isolated active polysaccharide fraction was composed of >80 % fucose and small amount of galactose and glucose (Table 1-5 and Fig. 1-8). The total sulfate content of the tested sample was 0.95 (sulfate/ total sugar). Fourier transform infrared spectroscopy (FT-IR) is a new technique to analyze the polysaccharides from various origins. The spectra obtained from the wave number 400-4000 cm^{-1} give some inside information of the tested compound therefore; the method is popular in identifying vibrational structure of materials. In this study, crude polysaccharide fraction and the purified polysaccharide compound of *E. cava* was compared with that of commercial fucoidan. The crude polysaccharide fraction showed completely different pattern to that of other polysaccharides (Fig. 1-9). Interestingly, the purified polysaccharide of *E. cava* showed almost similar infrared absorption properties to that of commercial fucoidan (Fig. 1-10 and 1-11). As it has been reported, intense absorption band at 1240-1272 cm^{-1} common to all sulfate esters (S=O), meanwhile band at 828 cm^{-2} indicate the presence of C-O-S (secondary axial) sulfate. Therefore, it is assumed that the main sulfate groups occupy positions C-2 /or C-3 and lesser part of sulfate in located at C-4 of fucopyranose residue. The other major absorption band at 3500 and the sharp absorption peak at 1030-1200 cm^{-1} are belongs to O-H and COO- stretching respectively. Taken together these results clearly illustrate the similarity of *E. cava* polysaccharides with that of commercial fucoidan. When we compare the results of the purified sample and the commercial compound, very small differences were observed, this may be due to species differences and technique of the isolation procedure. Moreover this compound contains a slight amount of hexouronic acid.

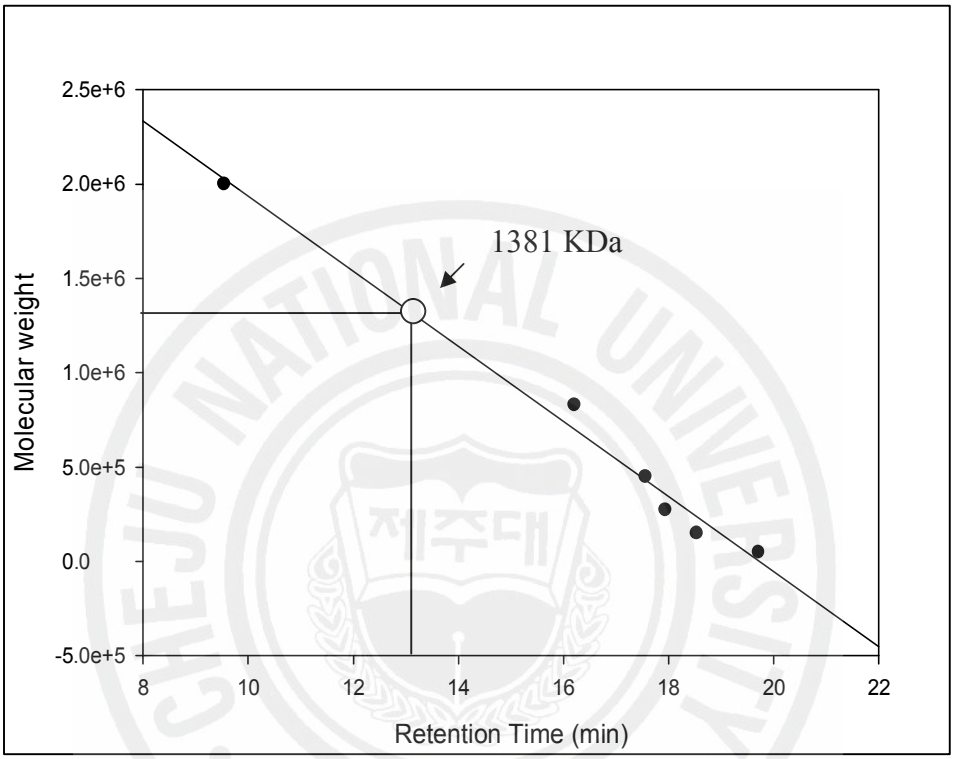

Fig. 1-8. Calibration curve of dextran standards for the determination of the average molecular weight of the *E. cava* sample. The retention time is plotted against the molecular weight of the dextrans.

This may explain the high anticoagulant activity of the isolated sulfated polysaccharide, according to the previous reports, fucan sulfate with high sulfates and low uronic acid content are well documented for high anticoagulant activity than those with high uronic acid and low sulfate content (Shanmugam and Mody, 2000). However the anticoagulant action of polysaccharides is mainly depending on the monosugar composition, sulfate position and molecular weight of the compound. Anticoagulant potency of the purified sample was evaluated and compared with that of heparin, a known commercially available anticoagulant (Table 1-6). According to the activity of purify compound, the sample activity of APTT is almost similar or slightly less than that of commercial counterpart at same concentration (0.7 μg/ml) but with the increment of the sample concentration, heparin APTT activity increase rapidly than that of the isolated compound. Moreover, the tested sample shows considerable activity on TT assay whereas a low clotting inhibition was observed in PT assay. Therefore, in this study isolated anticoagulant polysaccharide effectively prolonged APTT and TT. Normally high APTT activity of anticoagulants is due to the inhibition of the intrinsic and / or common pathway, whereas prolongation of TT indicates inhibition of thrombin activity or fibrin polymerization. The extrinsic pathway is another pathway for the activation of the clotting cascade of the human body. It provides a very quick response to tissue injury. The extended PT time correlates with the degree of inhibition of the extrinsic pathway factors. The PT is sensitive to deficiency of extrinsic pathway factors X, VII, V, II, and fibrinogen (Roberts and Escobar, 2002). Therefore, main pathways (intrinsic, common and extrinsic pathways) of the coagulations cascade are effectively affected by the isolated polysaccharide, hence the tested compound has high potential as a anticoagulant agent.

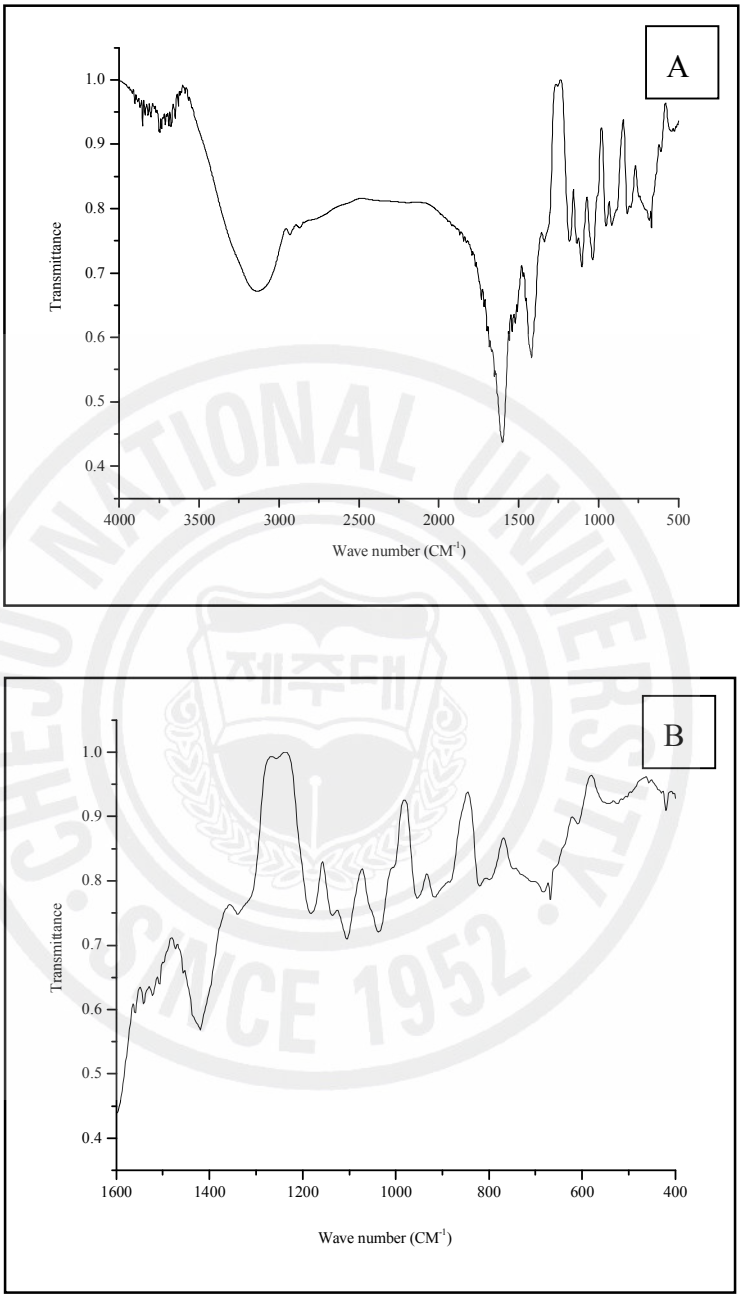

Fig. 1-9. Infrared analysis of the crude polysaccharide separated from the *E. cava*. The sample was scanned between 4000 and 400 cm^{-1} (A); The sample scanned between 1600 and 400 cm^{-1} (B).

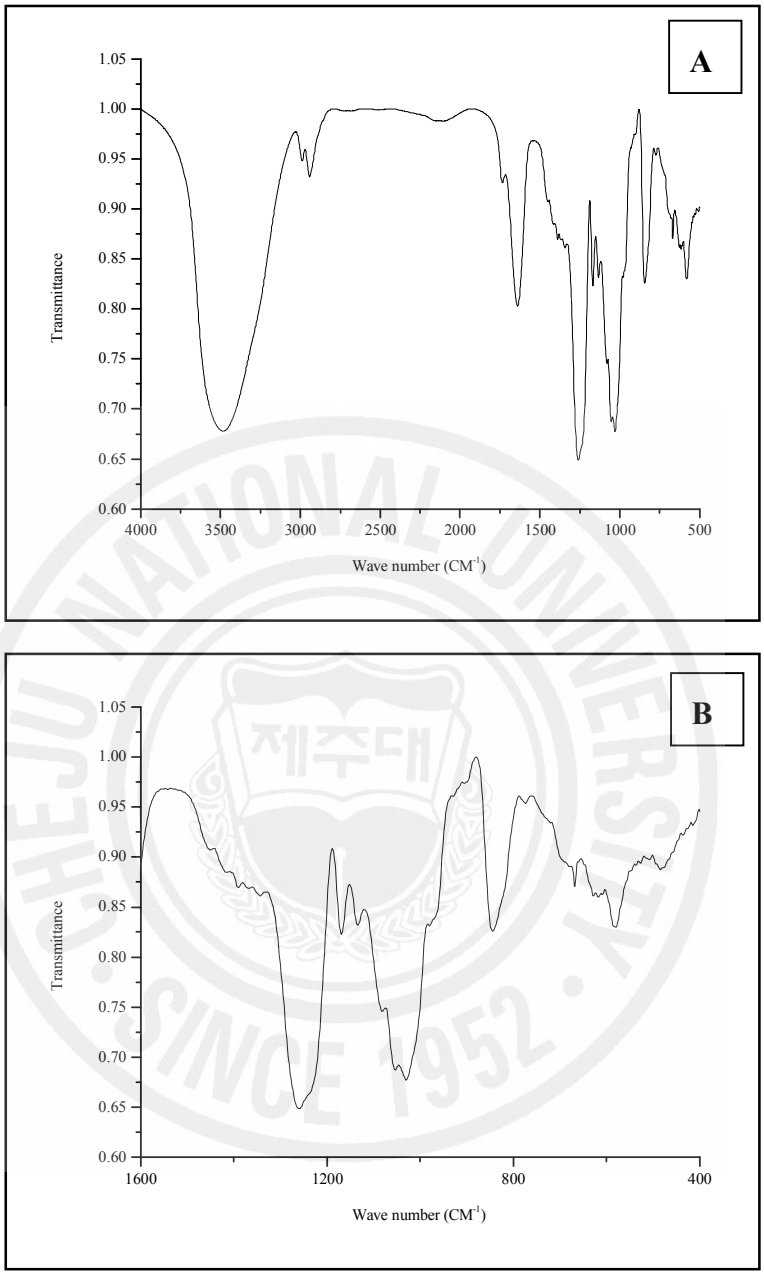

Fig. 1-10. Infrared analysis of the commercial fucoidan purified from the *Fucas vessiculosis*. The sample was scanned between 4000 and 400 cm^{-1}(A); The sample scanned between 1600 and 400 cm^{-1}(B).

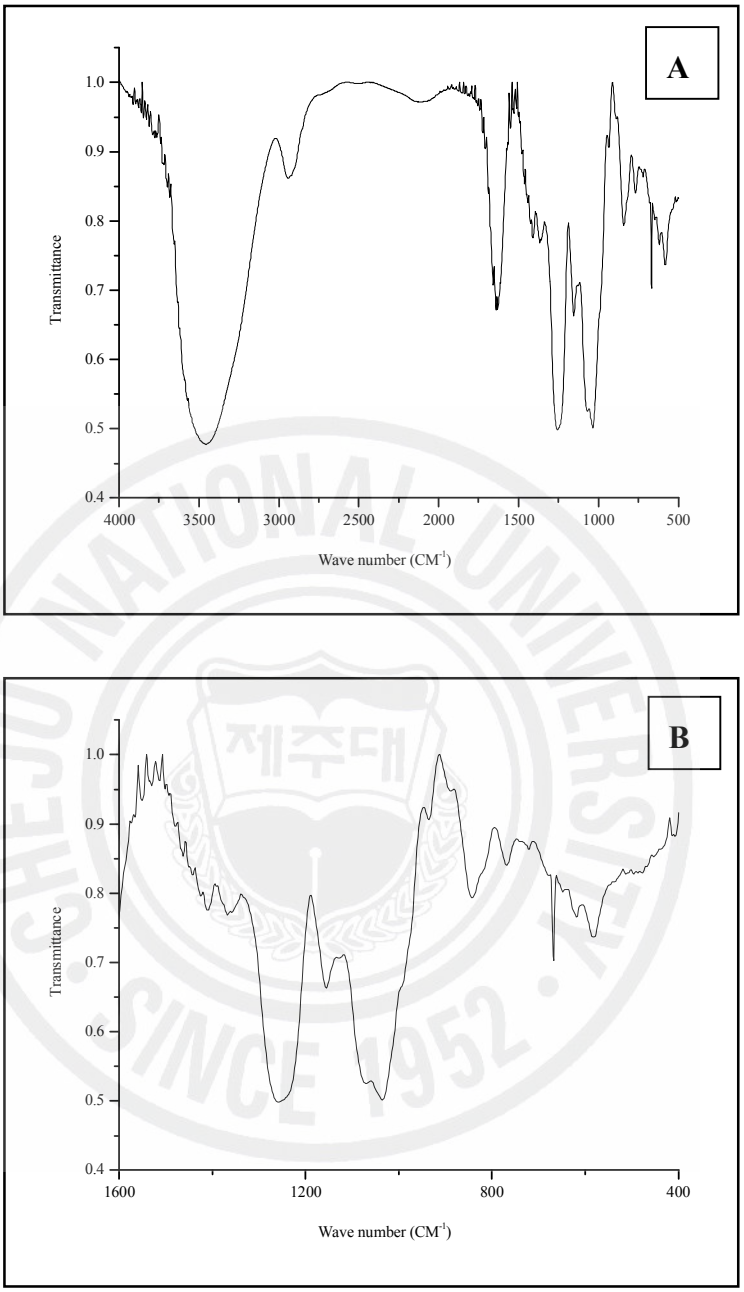

Fig. 1-11. Infrared analysis of the anticoagulant polysaccharide isolated from *E. cava*. The sample was scanned between 4000 and 400 cm^{-1} (A); The sample scanned between 1600 and 400 cm^{-1} (B).

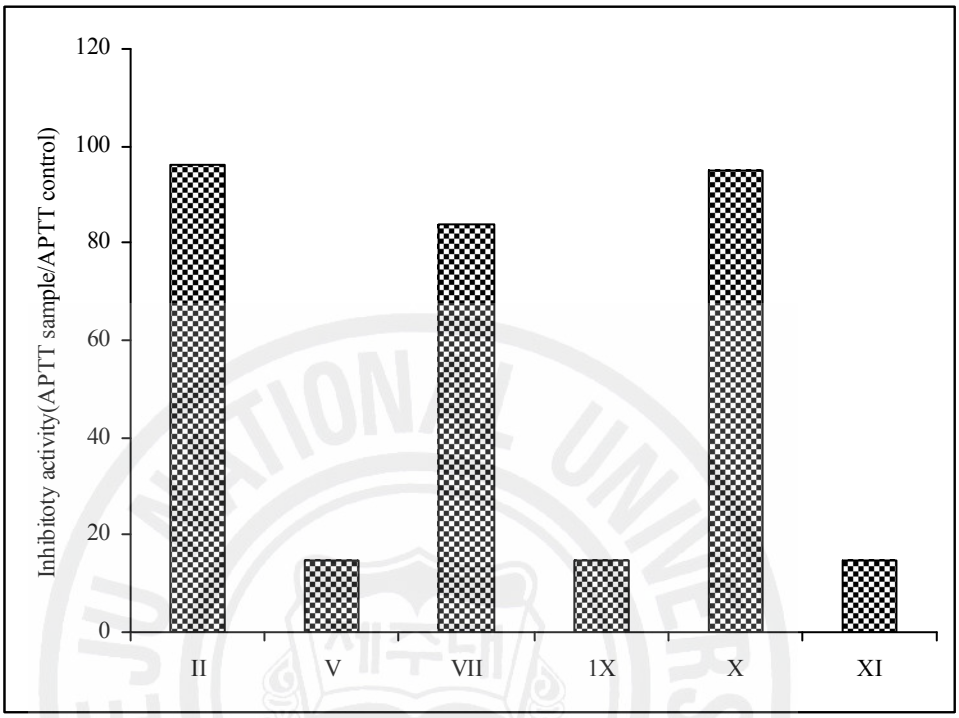

Fig. 1-12. Specific factor inhibitory pattern of the E. cava anticoagulant (ECA) on blood coagulant factors.

Blood coagulation pathway involves a series of zymogen (precursor protein) activation reactions Table 1-7. The factors associated with coagulations pathway interact with calcium and phospholipids surface to make platelet plug and stops bleeding until tissue repair can occur. Among blood coagulant factors, especially, II, VII, VIII, IX and X with

γ-carboxyglutamic acid domain play a key role in calcium mediated formation of phospholipids-factor complex like intrinsic factor tenase, extrinsic factor tenase and prothrombinase complex (Dave at al, 1979). Some algal polysaccharides can interfere with blood coagulation because they inhibit the biological activity of some plasmatic serine proteases either by direct or plasmatic factor interaction, especially polysaccharides isolated from brown algae have been investigated for years to utilize them in the field of pharmacology (Table 1-8). To understand the inhibitory mechanism, ECA treated plasma was examined on specific clotting factors. The results of this study are shown in Fig. 1-12. According to the results, factor II (prothrombin), X (stuart factor) and VII (proconvertin) were strongly inhibited by the tested purified polysaccharide respectively and no considerable influences of isolated compound were observed on factors IX, V and XI. Therefore, the addition of the purified compound to the media selectively prevent or control the generation of factor II, X and VII in the coagulation pathway and thereby exert high blood coagulation activity. Anticoagulants show different factor inhibitory abilities. Some critical structural motifs with the anticoagulant compound are responsible for this kind of special factor activities. More commonly, the ability of glycosaminoglycan to inactivate various serine proteases is linked to polysaccharide structure, molecular weight, the ratio of iduronic/glucuronic acid, the presence of particular specific sequences and degree of sulfation (Barbucci et al, 1996).

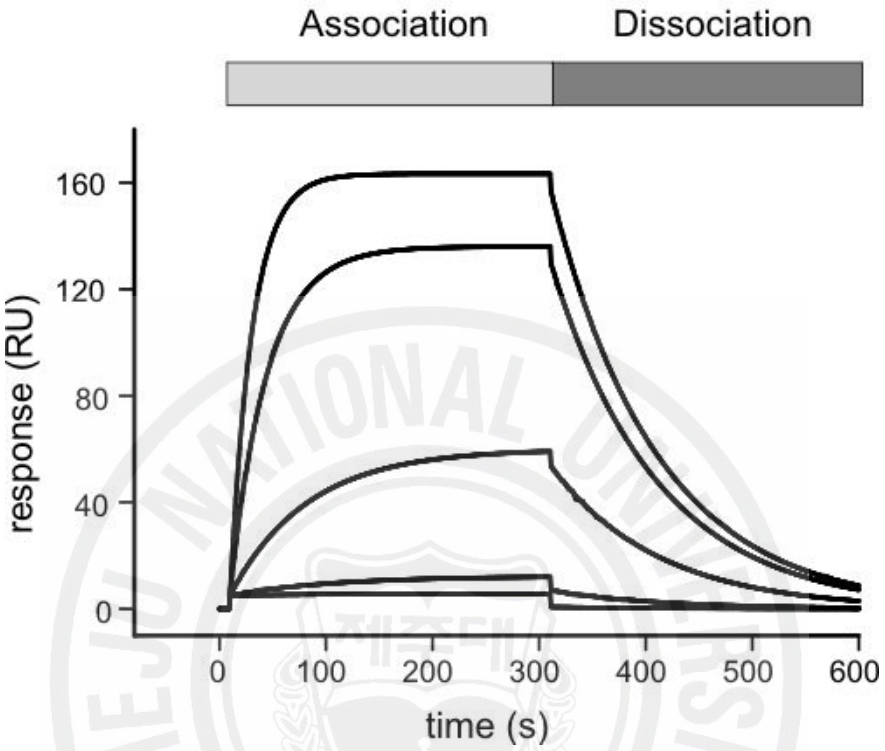

Fig. 1-13. Example of SPR Sensorgrams. Traces represent simulated SPR recordings showing an increase in resonance units (RU) when two binding partners associate and a decrease in resonance units when they dissociate. The concentration of the binding partner in the mobile phase (analyte) was varied from bottom to top as follows: 0.1 μM, 1 μM, 10 μM, and 100 μM. Dissociation constant K_D = 26.4 μM. Quoted from www.ifm.liu.se/applphys/sensor/spr.html

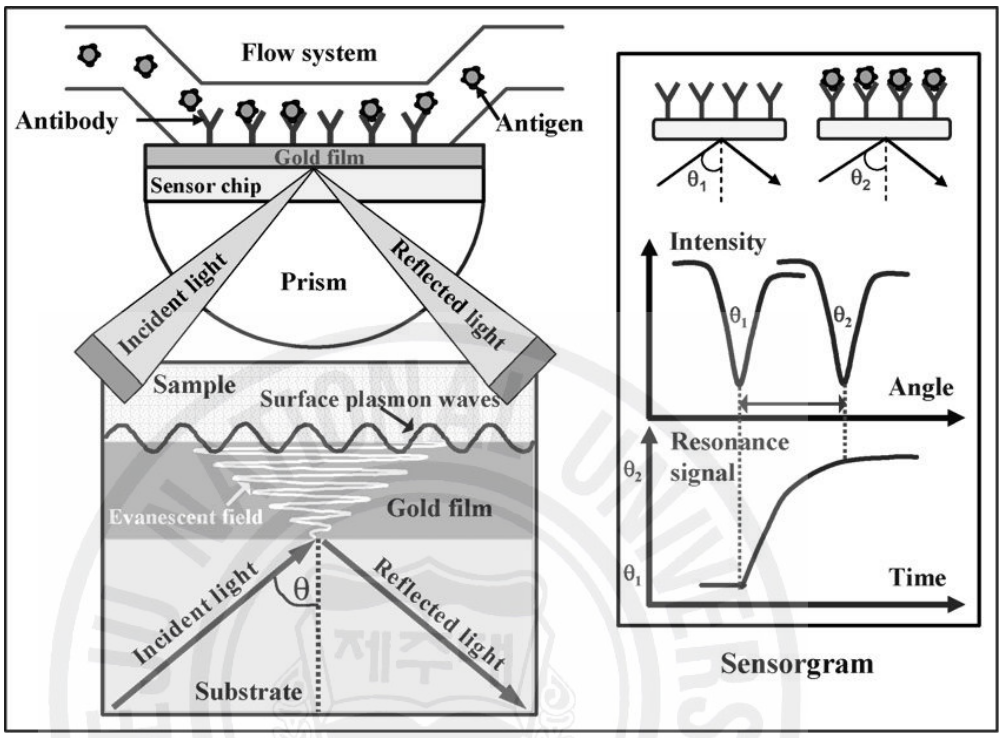

Fig. 1-14. Schematic illustration of surface plasmon resonance. The sensor surface is gold with antibodies attached to it. As the complementory antigen (analyte) binds to the antibodies, the refractive index shifts and the SPR-dip moves to larger angles. The movement of the SPR-dip is the actual monitored signal, and the movement over time forms the sensorgram. Quoted from Shankaran et al., Sensors and Actuators B 121 (2007) 158–177.

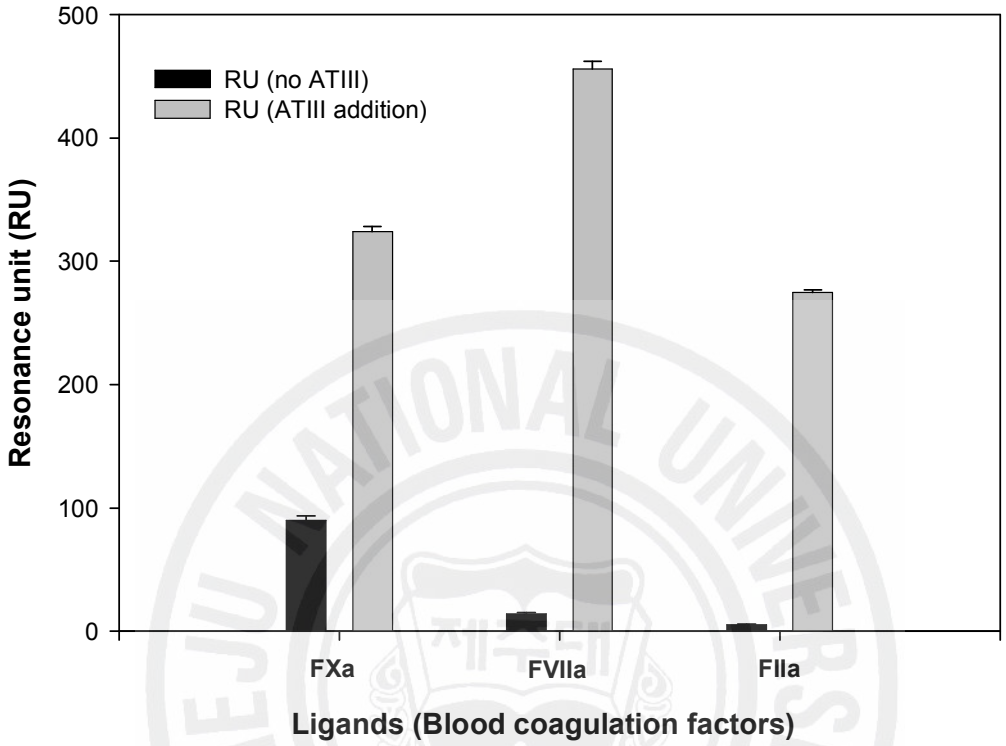

Fig. 1-15. Binding affinity assay of ECA to blood coagulation factors using surface plasmon resonance (SPR) spectrometer. Black columns express resonance unit (RU) values of real-time sensorgram in the absent of antithrombin III (ATIII). Gray columns express resonance unit (RU) values in the present of ATIII.

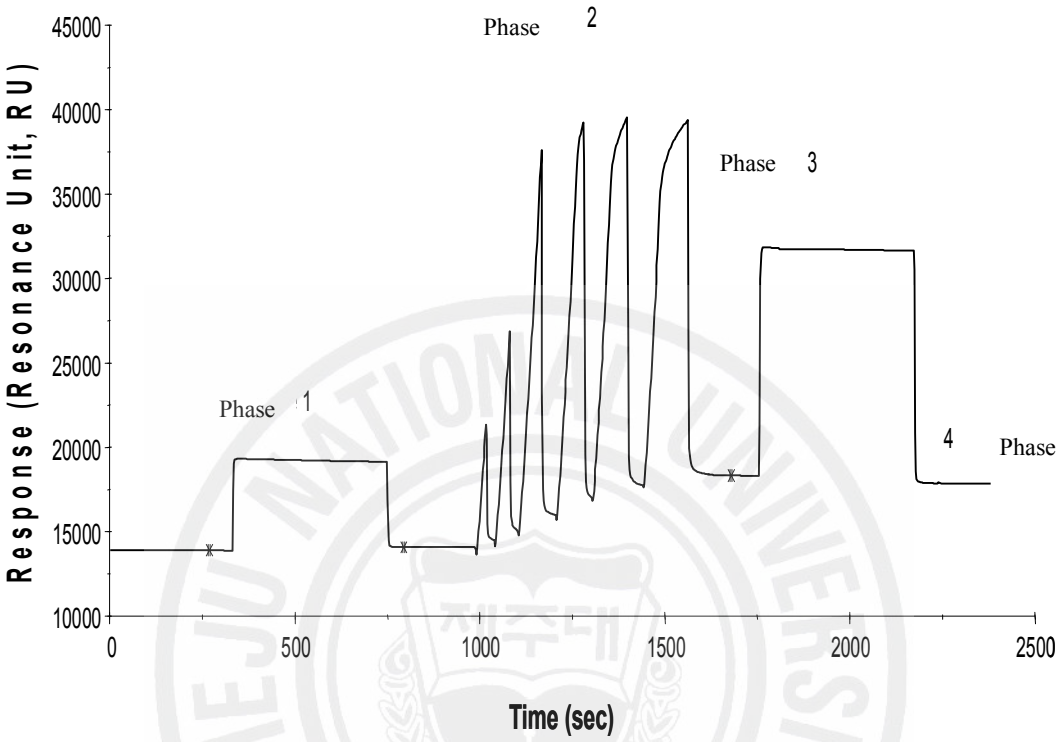

Fig. 1-16. The optimal pH scouting and immobilization of FXa on CM5 sensor chip. **Phase 1**, activation of the dextran surface of the CM5 sensor chip with 0.05 M NHS and 0.2 M EDC; **Phase 2**, Immobilization of ligand protein FXa (5,963.9 ± 8.7 RU) **Phase 3**; Deactivation of residual esters was performed by 1.0 M ethanolamine hydrochloride pH 8.5; **Phase 4**, Removal of non-covalently bond materials.

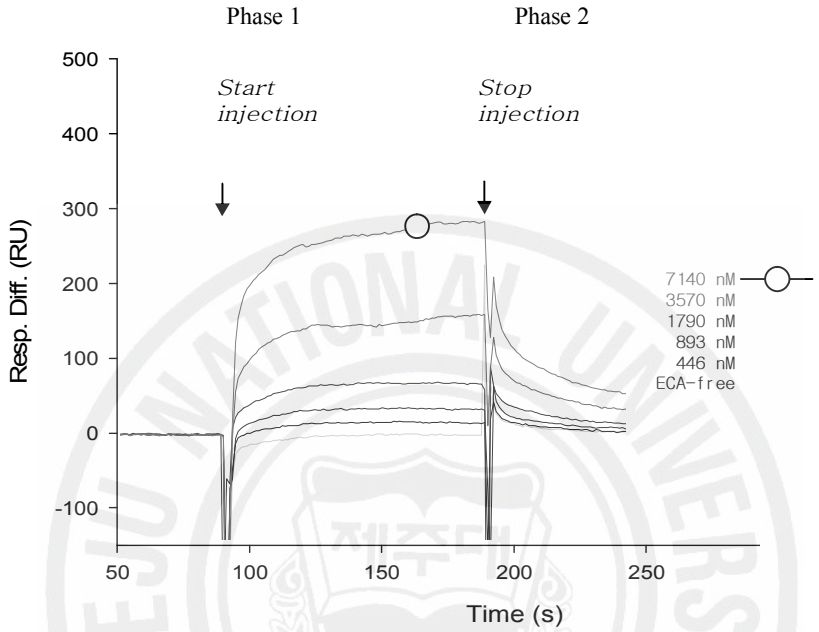

Fig. 1-17. Surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factor FXa in dose-dependent manners (0 – 7140 nM of ECA). **Phase 1**, association sensorgram and **phase 2**, dissociation sensorgram.

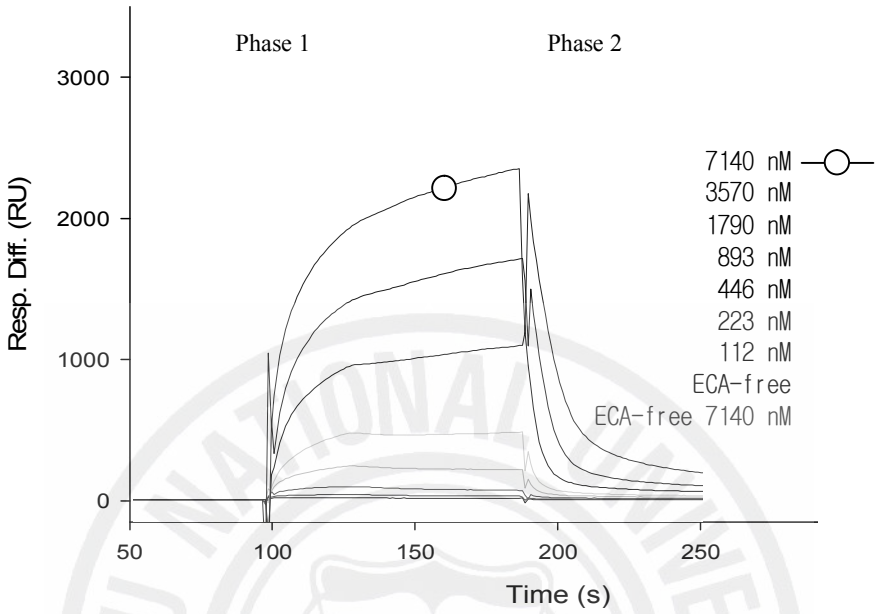

Fig. 1-18. Surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factor FVIIa in dose-dependent manners (0 – 7140 nM of ECA). **Phase 1**, association sensorgram and **phase 2**, dissociation sensorgram.

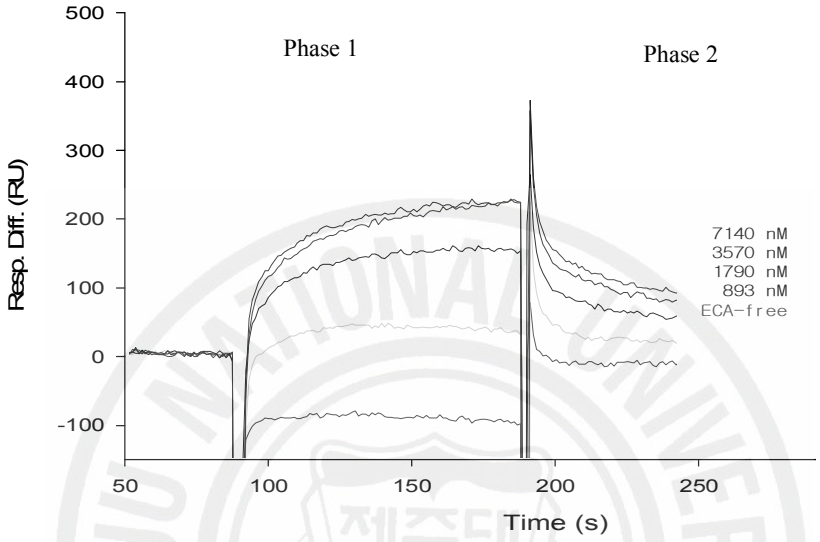

Fig. 1-19. Surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factor FIIa in dose-dependent manners (0 – 7140 nM of ECA). **Phase 1**, association sensorgram and **phase 2**, dissociation sensorgram.

Therefore, more intensive studies are needed to understand the anticoagulant behavior of the purified compound of *E. cava*. A surface plasmon resonance (SPR) study was performed to understand an intensive anticoagulant mechanism of ECA in the blood coagulation pathway. The basic operation technique, and the principle of the SPR technique is illustrated in Fig 1-13 and 14. Plane polarized light is directed through a glass prism to the gold/solution dielectric interface over a wide range of incident angles and the intensity of the resulting reflected light is measured-against the incident light angle with a detector. Using the SPR sensor graph produced by the devise the association and the dissociation constant of the tested ECA on serine proteases and be determined. To investigate binding affinities of ECA to blood coagulation factors using SPR spectrometer (BIAcore® 2000 system) for real-time sensorgraphic information without labeling of chromogenic or radioactive substance, ATIII and the activated blood coagulation factors (FIIa, FVIIa, FXa) as target proteins were immobilized on the dextran-coated gold surface of CM5 sensor chip using amine-coupling after scouting the optimal pH. For an example of pH scouting, FXa pre-incubated in various pH sodium acetate buffers (10 mM, pH 3.0-6.5) was coupled with the activated carboxyl group of dextran with EDC/NHS. The optimal pH for amine coupling between carboxyl group of dextran and amine group of ligand (FXa) was determined to be 4.5. Although the resonance unit or response unit (RU) of real-time sensorgram, corresponding to the rate of ligand association, showed the highest value at pH 4.0, the optimum was chosen at pH 4.5 to prevent protein denaturation by low pH.

Table 1-8. The optimal pHs for immobilization of blood coagulation factors and resonance unit (RU) values after coupling reaction on protein chip

Ligands	Molecular weight (kDa)	Optimum pHs for immobilization	Resonance units (RUs)
FIIa	58	5.0	6,123.5 ± 12.5
FVIIa	50	4.5	5,024.3 ± 09.1
FXa	46	4.5	5,963.9 ± 08.7

Table 1-9. Kinetic parameters for the blood coagulation factors with ECA using SPR sensorgraphy

Analyte	Ligand	Association rate constant (*ka*)	Dissociation rate constant (*kd*)	Equilibrium dissociation constant ($K_D = kdka^{-1}$)
		M^{-1} S^{-1}	S^{-1}	nM
ECA	FVIIa	2.19 x 10^3	3.30 x 10^{-5}	15.1
	FXa	0.58 x 10^3	2.61 x 10^{-5}	45.0
	FIIa	0.32 x 10^3	2.08 x 10^{-5}	65.0

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

As shown in Fig. 1-16, the ligand immobilization by amine-coupling on the dextran surface of sensor chip was performed with 0.05 M NHS and 0.2 M EDC (phase 1), then the ligand protein FXa (10 μg mL^{-1}) was injected to the activated chip at flow rate of 5 μL min^{-1}, pH 4.5 to reach the level of 5,963 RU (phase 2). Deactivation of residual esters was performed by 1.0 M ethanolamine, pH 8.5 (phase 3), and finally, non-covalently bond materials was removed by several injections of the running buffer (phase 4). The other factors were immobilized according to the same procedure, and the optimal pHs and RU values were summarized in Table 1-8. Direct binding affinity of the analyte (ECA) to ligands was also examined by SPR sensorgraphy.

As shown in Fig. 1-15, resonance unit values (RU values), corresponding to binding affinity between ECA and blood coagulation factors were highly elevated by addition of ATIII. The value of resonance unit was in the order FVIIa > FXa > FIIa. While the mechanism of action of most sulfated polymers of algae is not well defined, our samples strongly enhance antithrombin pathway in the present study. Fucoidan increased antithrombin activity concentration dependant manner in the presence and in the absence of the protease inhibitor (Nishino et al., 1991).

In the kinetics assay, the results of evaluation for association rate constant (*ka*), dissociation rate constant (*kd*), and equilibrium dissociation constant ($K_D = kd\ ka^{-1}$) were summarized in Table 1-9. The kinetic constants (*ka*, *kd*, 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 of SPR sensorgrams following the injection of each protein at different concentrations (0 – 7,140 nM) in the present of ATIII.

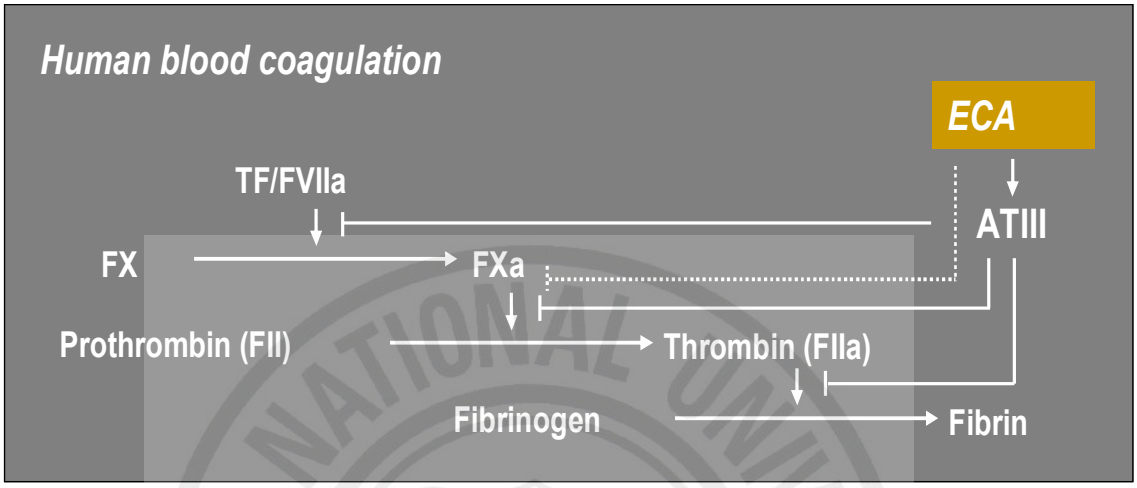

Fig. 1-20. Anticoagulation mechanism of ECA in human blood coagulation pathway. The dotted line means weak-negative effects of ECA on target factor (FXa).

The figures illustrate the surface plasmon resonance (SPR) real-time sensorgrams during interaction between ECA and human blood coagulation factors, (Fig.17) FXa, (Fig.18) FVIIa, and (Fig.19) FIIa in dose-dependent manners respectively. As shown in Table 1-9, the K_D value of ECA for FVIIa, FXa, and FIIa were 15.1, 45.0 and 65.0 nM, respectively. In these results, ECA interacted with human blood coagulation factors in the extrinsic pathway (FVIIa) and common pathway (FXa and FIIa) in ATIII-dependent manner. As reported by Lawson et al., (1993), unlike other serine proteases, factor VIIa alone is not readily inhibited by the ATIII/heparin complex. However, in the presence of ATIII/heparin exhibits significant inhibition of factor VIIa. ATIII is a single chain glycoprotein with a molecular weight of 58 kDa. It is a member of the serpin (serine protease inhibitor) super family and is considered to be the most important inhibitor in the coagulation cascade (Griffith, 1986; Rosenberg and Rosenberg, 1984). The rate of inhibition of serine proteases by ATIII is increased to varying degrees by heparin or heparin sulfated. In the case of FIIa or FXa, the interaction with ATIII is enhanced 3 orders of magnitude in the presence of heparin. The binding of ATIII to heparinoid structures on vascular endothelium has been demonstrated and shown to enhance the inhibition of FXa, and thrombin (FIIa). ATIII may also function in the complement cascade, and the binding of ATIII to fluid phase complement attack-complexes in sera has been demonstrated. Thrombin (FIIa) is a highly specific serine protease generated by proteolytic activation of the zymogen prothrombin (FII) (Lundblad et al., 1976). During coagulation, thrombin cleaves fibrinogen to form fibrin, leading to the ultimate step in coagulation, the formation of a fibrin clot. Thrombin is also responsible for feedback activation of the pro-cofactors FV and FVIII.

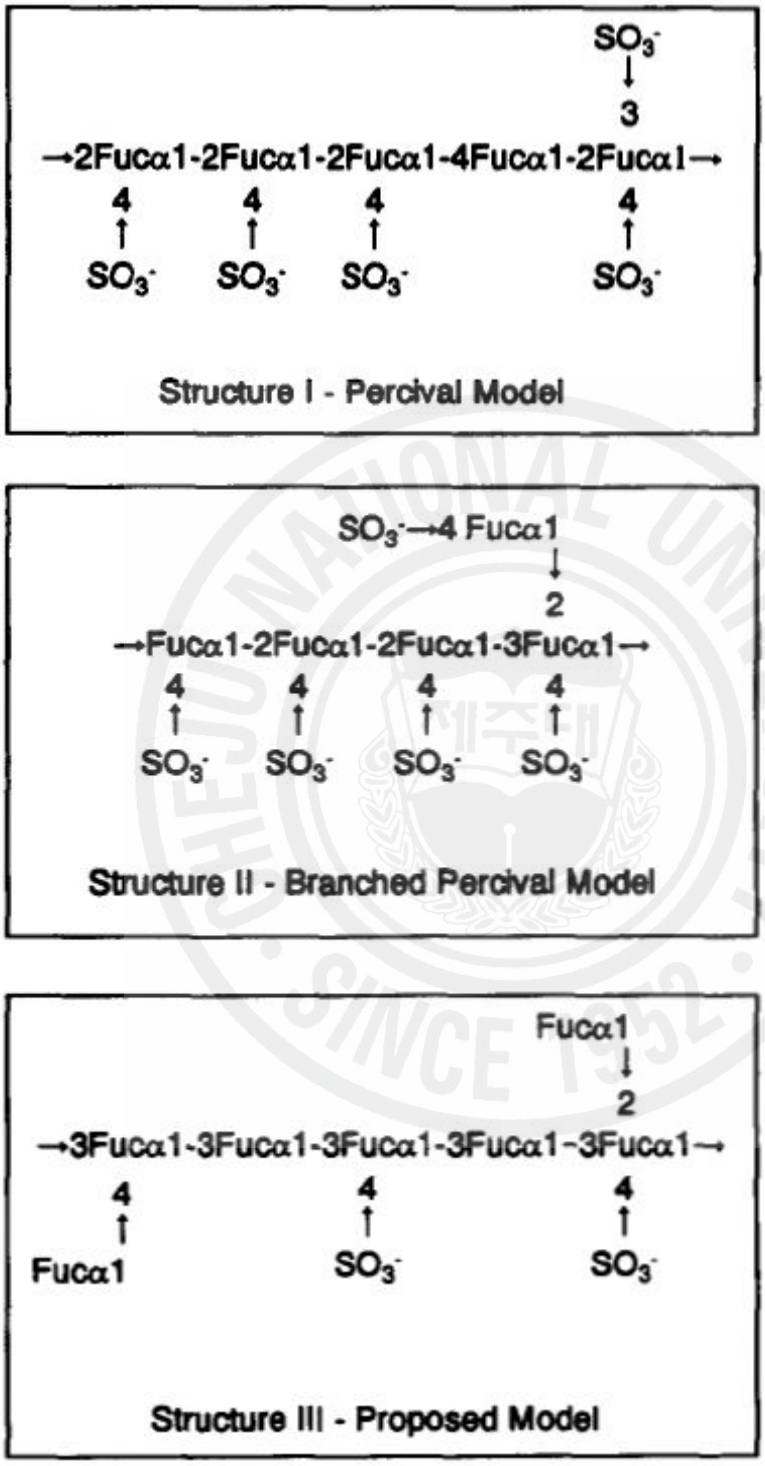

Fig. 1-21. Some proposed structures for average fucoidans. (Patankar et al., 1993)

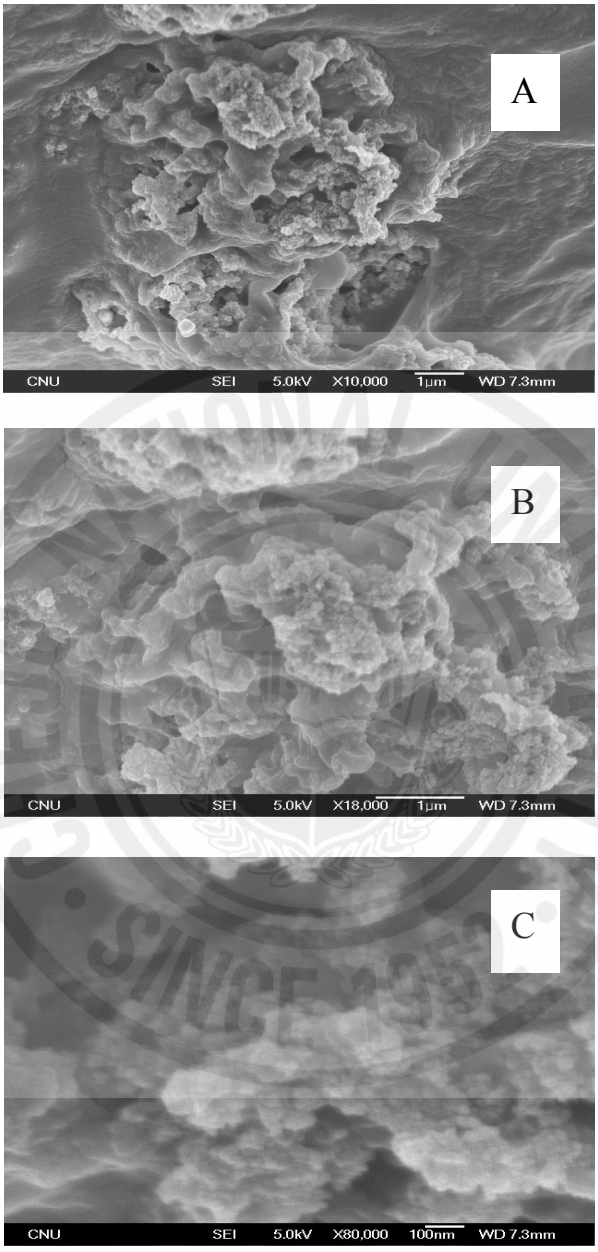

Fig. 1-22. Field Emission scanning electron microscopy of sulfated polysaccharide isolated from *E. cava*. (A) 1x 10, 000; (B) 1 x 18, 000; (C) 1 x 80, 000

Table 1-7. The Important characters of zymogens as coagulant factors in human blood coagulation system.

Factor	Traditional name	Molecular weight (Da)	Plasma Concentration (μg/ml)	Pathway	Organ
I	Fibrinogen	330,000	3000	Common	Liver
II	Prothrombin	72, 000	100	Common	Liver
III	Tissue factor			Extrinsic	Tissue
IV	Calcium			All	Plasma
V	Proaccelerin	300,000	10	Common	Liver
VI	Proconvertin	50,000	0, 5		Liver
VII	Antihemophlic	300,000	0, 1	Extrinsic	Res:
VIII	Thromboplastin	56, 000	5	Intrinsic	Liver
IX	F. Stuart	56,000	10	Intrinsic	Liver
X	Prethrombo:	160, 000	5	Common	Liver
XI	F. Hageman	76,000	30	Intrinsic	Liver
XII	Fibrin stabilizing	320,000	30	Intrinsic	Liver
XIII	Von Willebrand	040,000		Common	Endo:
PKLK	Prot C				Liver
HMWK	Prekallikrein	082,000	40	Intrinsic	
HMW	Kallikrein	108,000		Intrinsic	

* Compared to that of normal level (%)

Thrombin has also been reported to activate FXIII and platelets, and also functions as a vasoconstrictor protein. The procoagulant activity of thrombin is arrested in two ways: 1) inhibition by either heparin cofactor II or the ATIII/heparin complex; or 2) complex formation with thrombomodulin. In the present study, the results demonstrated that ECA interacts with FVIIa, FIIa, and FXa in the present of ATIII, and is able to directly inhibit FXa activity in the ATIII-independent manner Fig 1-20. Most of anticoagulants injected into venous vessel are faced with endothelial cells. The primary process of hemostatic mechanism (platelet aggregation) is set in motion, when the endothelial lining of blood vessels is denuded following vascular injury (Davie et al., 1991). To evaluate cytotoxicity of ECA containing high content of sulfur on normal cell line (venous endothelial cell line, ECV-304), ECA was treated to the normal cells at a concentration range from 5 to 500 μg mL^{-1}. As reported in the previous study (Athukorala, et al., 2006), enzymatic extracts of *E. cava* have potential antiproliferative activity against cancer cells and antioxidative activities against radical oxygen species. Moreover, treatment of *E. cava* on Chinese hamster fibroblast cell line/normal cell (V79-4) showed low cytotoxicity, hence the results confirm the ability of extracts to protect normal cells. Polysaccharides isolated from Japanese seaweeds like *Laminaria japonica*, *Undaria pinnatifida, Eisenia bicyclis* and *Hijikia fusiforme* were identified as potential anti-genotoxic substances (Okai et al., 1993; Okai and Higashi-Okai, 1994).

Table 1-10. The effect of *E. cava* anticoagulant (ECA) on the *ex-vivo* activated partial thrombin time (APTT) of Wistar rats.

		APTT *ex vivo*			
Compound	Dose	0 min	5 min	30 min	60 min
Sample	50 μg/Kg	20 ± 1.0	36 ± 1.2	38 ± 0.6	45 ± 0.1
	100 μg/Kg	20 ± 0.5	58 ± 1.2	67 ± 0.9	62 ± 0.9
	300 μg/Kg	18 ± 0.8	1 28 ± 1.1	156 ± 0.2	144 ± 0.7
Heparin	50 μg/Kg	19 ± 1.3	70 ± 0.8	211 ± 0.6	189 ± 1.2
	300 μg/Kg	23 ± 0.7	>300 ± 0.1	>300 ± 0.1	>300 ± 0.1
Saline		23 ± 0.3	25 ± 0.2	23 ± 0.1	22 ± 0.2

For each treatment group the mean APTT time ± SD was determined for n = 4/group.

E. cava showed potent anticoagulant activity as strongly interfering with coagulation cascade by inhibiting biological activity of serine proteases, the activated FII, FX and FVII. In the direct binding assay, binding affinity between ECA and blood coagulation factors (FVIIa > FXa > FIIa) was expressed potently in the present of ATIII, and the kinetics analysis elucidated that the K_D values of ECA/ATIII for FVIIa, FXa, and FIIa were 15.1, 45.0 and 65.0 nM, respectively. In the present study, it was indicated that natural *E. cava* anticoagulant (ECA) as a sulfated polysaccharide strongly inhibits activities of coagulation factors (FVII, FX, and II) by mediation of ATIII in the extrinsic and common coagulation pathway (Fig 1-20).

Sulfated polysaccharide isolated from marine alga has highly hetorogenious structure, therefore, structural elucidation of fucoidan is really difficult and. However some scientists already have proposed some possible structures according to NMR data. The proposed structures were not able to explain the diverse biological activities of fucoidan, the one research group (Patankar et al., 1993) proposed possible three structers for average fucoidan (Fig. 1-21). To take an idea of the ultrastructure of the purified polysaccharide, the sample was investigated through field emission electron microscopy and depicted in Fig. 1-22. When the magnification was increased, particles with polyhedrical morphologies were slightly observed. As it has been published previously, fucoidan investigated showed a polycrystalline nature with polyhedrical morphologies (Andrade et al., 2004), but we were not able to observe those structural information from our sample. This may be due to deviations of sample preparation technique.

To rule out the *in vivo* anticoagulant activity of the ECA, the samples was administrated into male Wistar rats (~300 g) and compared the results with that of heparin.

Table 1-11. The effect of *E. cava* anticoagulant (ECA) on the *ex vivo* thrombin time (TT) of Wistar rats.

Compound	Dose	TT *ex vivo*			
		0 min	5 min	30 min	60 min
Sample	50 μg/Kg	16 ± 0.3	17 ± 0.1	19 ± 0.23	19 ± 0.8
	100 μg/Kg	17 ± 0.4	21 ± 1.2	29 ± 1.0	20 ± 0.5
	300 μg/Kg	14 ± 0.9	85 ± 0.2	92 ± 0.4	89 ± 0 .6
Heparin	50 μg/Kg	16 ± 0.8	58 ± 1.2	70 ± 0.8	68 ± 0.4
	300 μg/Kg	18 ± 0.2	>300 ± 0.1	>300 ± 0.1	>300 ± 0.1
Saline		16 ± 0.2	14 ± 1.1	16 ± 0.1	16 ± 0.2

For each treatment group the mean TT time ± SD was determined for n = 4/group.

Table 1-12. The effect of *E. cava* anticoagulant (ECA) on the *ex vivo* prothrombin time (PT) of Wistar rats.

		PT ex vivo			
Compound	Dose	0 min	5 min	30 min	60 min
Sample	50 μg/Kg	10 ± 0.3	13 ± 0.5	12 ± 1.2	12 ± 1.1
	100 μg/Kg	12 ± 0.8	14 ± 0.3	13 ± 1.0	15 ± 1.0
	300 μg/Kg	11 ± 0.3	22 ± 0.8	28 ± 0.4	25 ± 0.9
Heparin	50 μg/Kg	10 ± 0.7	55 ± 0.9	69 ± 1.2	57 ± 0.2
	300 μg/Kg	13 ± 1.3	10 ± 0.2	120 ± 0.2	189 ± 0.4
Saline		12 ± 0.2	11 ± 1.0	10 ± 0.1	10 ± 1.2

For each treatment group the mean PT time ± SD was determined for n = 4/group.

The ECA sample at 300 µg/ml showed pronounced activity, the highest APTT activity was recorded after 30 min of application time (Table 1-10). Relatively, heparin showed much higher activity than that of ECA at same concentration level. TT value indicates the ability of a sample to prolong the blood coagulation by controlling the fibrinogen to fibrin conversion, the addition of TT reagent (human thrombin) to plasma stimulate that conversion but the presence of anticoagulants block the conversion by extending the coagulation time. As it has been reported previously, the sample had high affinity on factor II (prothrombin) therefore sample may have effect on fibrin polymerization which explain the extended TT activity on tested rat after injecting the sample. In the TT and PT *in vivo* experiment, ECA sowed slightly low activity than its APTT activity. Sample result for *in vivo* TT at 300 µg/ml was comparable with the results of Heparin at 50 µg/ml (Table 1-11). Taken together the ECA showed low activity on in-vivo PT assay in contrast TT and APTT assays (Table 1-12). However, heparin also at low concentration level showed low activity on PT assay, but that was even higher than that of ECA at 300 µl/ml. In PT assay, biomolecular interaction of the tested sample on II, V, VII, X and fibrinogen is estimated; these factors of the coagulation pathway are in conjunction with the intrinsic pathway. As it has been documented, abnormalaties of VII is much responsible for the extending the PT. Since the ECA has strong effect on VII, a considarable PT activity was observed in this study. Taken together, the results are relatively low than that of commercial compound like heparin, but was quite stronger than that of saline alone treated sample. Therefore, as a natural compound ECA will be a promising candidate for future drug discovery in pharmaceutical industry. Bleeding implications can be taken place with the treatment of anticoagulant drugs.

Fig. 1-13. *Ex vivo* anticoagulant activity of sulfated polysaccharide isolated from ECA on tail bleeding time of the Wistar rats.

Sample	Dose	Bleeding time (sec)
Saline		900 ± 20.1
Sample	50 μg/Kg	960 ± 10.1
	100 μg/Kg	990 ± 30.1
	300 μg/Kg	>1800 ± 05.1
Heparin	50 μg/Kg	1200 ± 15.2
	100 μg/Kg	>1800 ± 05.0

Tail bleeding time of sample treated rat was examined after the dissection of the tail extremely 2 mm from the tip. The tail was blotted with a tissue paper in every 30 sec and the time to stop bleeding was noted. For each treatment group the mean bleeding time ± SD was determined for n = 4/group.

Animal experiments are not suitable way of evaluating bleeding problems, however tail bleeding is still the choice of many scientists to investigate the antihemostatic effect of anticoagulant compounds. After 5 min of the sample administration, exactly 3 mm from the tip of the tail of mice was dissected and the time to cessation of bleeding was noted. As it has shown in the Table 1-13, the injection of the sample and heparin extended the tail transection bleeding time dose dependently.

ECA showed almost similar antihemostatic effect to that of heparin at same concentration level. In the saline-treated control group, the bleeding time averaged at 900 ± 20.1s. Bleeding time is an indicator of the platelets functioning; in this study at the injected ECA level a good tail transection bleeding time was recorded. Taken together, it is clear that ECA has good anticoagulant activity *in vitro* and *in vivo*, where it can be utilized in the field of pharmaceutical industry. We postulate that the number of sugar present in this compound and various kinds of sulfate groups present in the sample are important for their pharmacological activity. Although, the exact mechanism of action is to be investigated more, the data provided in this study clearly indicate its feasibility in future drug discovery.

Part II

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

ABSTRACT

An edible marine red alga, *Grateloupia filicina* collected from Jeju Island of Korea was hydrolyzed by cheep food grade carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) to investigate its anticoagulant activity. Among the tested enzymatic extracts of *G. filicina*, 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 polysaccharide (0.42 sulfate/total sugar) showed ~ 1357 kDa molecular weight and comprised mainly of galactose 98% and 1-2 % of glucose having 0.08% yield. The sample showed potential anticoagulant activity on activated partial thromboplastin time (APTT) and thrombin time (TT) assays. The purified *G. filifina* anticoagulant (GFA) inhibited the coagulation factor X (92%), factor II (82%) and factor VII (68%) of the coagulation cascade, the molecular interaction (protein-polysaccharide) was highly enhanced with the presence of ATIII. The dissociation constant of polysaccharide towards serine proteins decreased in the order of FXa (58.9 nM) > FIIa (74.6 nM)> and FVII (109.3 nM) respectively. The low/less cytotoxicity of the polysaccharide benefits its use in pharmaceutical industry, however further studies which would help us to elucidate the mechanism of its activity are needed to address.

INTRODUCTION

In the developing world, still cardiovascular diseases cause severe health problems. In 1995 heart diseases and stroke caused approximately 44% of the world's death toll (Mourano and Pereira. 1999). As a treatment, heparin has been utilized to treat thromboembolic disorders for more than 50 years. However, limitations associated with heparin therapy have created big demand for alternative drugs (Mourano and Pereira. 1999; Desai, 2004, Boneu, 2000). According to previous reports, algal polysaccharides are good natural compounds to replace this long felt need. Therefore, scientists have focused their especial attention on algal polysaccharide to utilize/modify them as a means of anticoagulative drugs (Chevolvet et al., 1999; Kuda et al., 2002).

Among other algal groups, Rhodophyta is well documented for its potential anticoagulant activity. Carrageenans and D-galactan have been identified as the key anticoagylative compounds of this seaweed group. Sulfated galactans/carrageenans are located in the cell wall of the seaweed (Shanmugam and Mody 2000). When algal samples are evaluated for their potential anticoagulant activity, the algal samples are normally extracted using water as a solvent, therefore water soluble active compounds may only come into water extract. Utilization of cell wall break-down 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 (Heo et al., 2003). But, so far few or less reports are available related to the enzymatic digestion of algal samples for the evaluation of their potential bioactive compounds. As we have observed before, the digestion of marine algal materials from the carbohydrases increase extraction yield, and thereby enhance bioactive availability. Furthermore, common problems associated with organic solvent

extraction can be over come by this technique (Athukorala, 2006). Therefore, investigation of bioactive compounds after an enzymatic digestion will be a fruitful way in pharmaceutical industry.

In this study we aim was to utilize several food grade carbohydrate enzymes (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) on *G. filicina* to extract plant material in order to its anticoagulant activity. The anticoagulant compound of the *G. filicina* enzymatic extract was purified, characterized and compared its activity with that of heparin. The compound exhibited good anticoagulant activity inhibiting coagulation factors of the coagulation cascade. Taken together, 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^{o}C for further experiments. APTT (ellagic + bovine pospolipid) and $CaCl_2$ solution were obtained from International Reagents Corporation (Japan) and PT (rabbit thromboplastion) and TT reagents were purchased from Fisher Scientific Company (USA). Carbohydrate-degrading enzymes (Table 1) such as Viscozyme L (a multi-enzyme complex containing a wide range of carbohydrate-degradating enzymes, including arabanase, heju ed , beta-glucanase, hemicellulase and xylanase), Celluclast 1.5L FG (catalyzing the breakdown of cellulose in to glucose, cellobiose and higher glucose polymers), AMG 300 L (an exo1, 4-alpha-d-glucosidase), Termamyl 120 L (a heat stable alpha–amylases), 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 grater purity.

Enzymatic extraction of *G. filicina*

The preparation of enzymatic extracts was followed as previously reported (Heo et al, 2003). The freeze-dried *G. filicina* (100 g) was homogenized with water (2 L), and then 1 g or 1 ml enzyme was mixed. The enzymatic hydrolytic reactions were performed for 12 h to achieve optimum degree of the hydrolysis. Before the digestion 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 (3000 rpm, for 20 min at 4°C) to remove the residue. The supernatant was kept in –20°C for further experiments.

Crude polysaccharide separation

Each algal enzymatic extract (240 ml) was mixed well with 480 ml of 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at a room temperature, and crude polysaccharides were collected by centrifugation at 10000 X g for 20 min at 4°C (Matsubara et al, 2000). The precipitated crude polysaccharide was freeze-dried and kept in –20°C for further experiments

Anion-exchange chromatography

The crude polysaccharide of Termamyl hydrolysate of *G. filicina* (500 mg) obtained using the procedures described above was applied to a DEAE-cellulose column (17 x 2.5 cm) equilibrated in 50 mM sodium acetate (pH 5.0) and washed with the same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate 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, carbazole reactions and by metachromatic property (Chaplin and Kennedy, 1994). Fractions showing strong anticoagulant activity were collected, dialyzed against distilled water, and concentrated to 5 ml by rotary evaporation under reduced pressure bellow 40°C. The partially purified concentrated polysaccharide fraction was re-chromatographed on new DEAE-cellulose column, under 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 X 2 cm) equilibrated and eluted with water at a room temperature at a flow rate 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 hr 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/v).

Molecular weight determination

In order to determine the molecular weight of the sample, the freeze dried sample was introduced into a PL-Aquaz OH 40 column and eluted with de-ionized water at 0.8 ml/min flow rate (23^{o}C). Dextran standards (48.6, 148, 273, 410, 830, and 2000 kDa) were also introduced into the column under same experimental condition for comparison purposes. The retention time was plotted against average molecular weight of the dextrans and thereby molecular weight of the sample was calculated.

Neutral sugar analysis

The purified GFA was hydrolyzed in a sealed glass tube with 2 M of trifluoroacetic acid for 4 h at 100^{o}C to analyze neutral sugars. In order to analyze the amino-sugars the

sample was digested using 6 N of HCl for 4 h. Then, 0.055 μg and 2.75 μg of sample were separately applied to CarboPacc PA1 (4.5 x 250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartridge (4.5 X 50 mm) column to analyze neutral and amino sugar respectively. The column was eluted using 16 mM of NaOH at 1.0 ml/min flow rate. Each sugar of the sample was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

Sulfate content analysis

After acid hydrolysis of the purified polysaccharide, the sulfate content was measured by the $BaCl_2$ / gelation method (Saito et al., 1968).

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 2400 x g, and the plasma was stored at -60^{o}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^{o}C, then APTT reagent (100 μl) was added to the mixture and incubated for 5 min at 37^{o}C. Thereafter clotting was induced by adding 0.025 mol/l $CaCl_2$ (100 μl) and 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^{o}C was added and 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 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) and was slightly changed according to the method of (Jung et al., 2002, Rajapaksha 2005).

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 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 the method of (Shobe et al., 1999) after pH scouting. Sensor chip surface was coated with ligand proteins in 10mM sodium acetate buffer at optimal pH, and un-reacted groups were blocked with 1 M ethanolamine, 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 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 (*ka*, *kd*, and *K*D), GSG solution at various concentrations (0–7,500 nM) with or without adding 1,000 nM of antithrombin III (ATIII) was injected to coagulation factors, and real-time sensorgrams were evaluated using BIAevaluation software (version 3.2). Association rate constant (*ka*) was calculated from multiple sensorgrams, representing at least five different concentrations of analyte for each experiment. Dissociation rate constant (*kd*) was calculated from the initial dissociation phase of the binding curves, and equilibrium dissociation constant (*K*D) equaled the ratio of *kd*/*ka*.

Cytotoxicity assay

The Chinese hamster fibroblast cell line/normal cell line (V79-4) was maintained in DMEM medium 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 GFP on V79-4 was measured by using MTT assay (Mossman 1983, 15, Carmichael 1987). The cells were seeded in a 96-well plate at the concentration of 2×10^4 cells/ml using DMEM medium. After 16 h (at 37°C, in a humidified atmosphere of 5% CO_2), GFP was treated to the wells at a concentration range 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., USA). For treated cells, viability was expressed as a percentage of control cells. All determinations were carried out in triplicate.

RESULTS AND DISCUSSION

A popular edible alga, *G. filicina* was hydrolyzed by several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) to examine its anticoagulative efficacy. All enzymatic extracts of *G. filicina* showed good anticoagulant potential compared to that of the control sample (Table 2-1). Of the tested extracts, Termamyl and AMG hydrolysates of *G. filicina* showed an outstanding anticoagulant activity on APTT assay. However, the former sample showed pronounced APTT activity selected for further purification purposes. All enzymatic extracts showed a slight activity on TT assay, however the *G. filicina* extracts were devoid of PT activity.

Due to high activity of the Termamyl extract, crude polysaccharide faction of the sample was separated by ethanol precipitation technique. The freeze-dried crude polysaccharide sample was introduced to DEAE-cellulose column with NaCl gradient to separate the anticoagulant fraction. According to the first anion-exchange chromatograph (Fig. 2-1), the main prominent peak eluted around 50-60 fractions showed high anticoagulant activity (APTT), however the rest of the peaks, eluted after the main peak were devoid of anticoagulant activity. Hence, we can assume that the anticoagulant compound present in the *G. filicina* as a main polysaccharide. The active fractions were collected, concentrated, dialyzed and freeze- dried. Then, the ~100 mg of the partially purified sample was re-chromatographed 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.2-2). After being evaporated under rotary evaporator, the dialyzed sample was further purified by gel-filtration chromatography on Sepharose 4B (Fig. 2-3).

Table 2-1. Anticoagulant activity of *G. filicina* enzymatic extracts

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 means of two determinations.

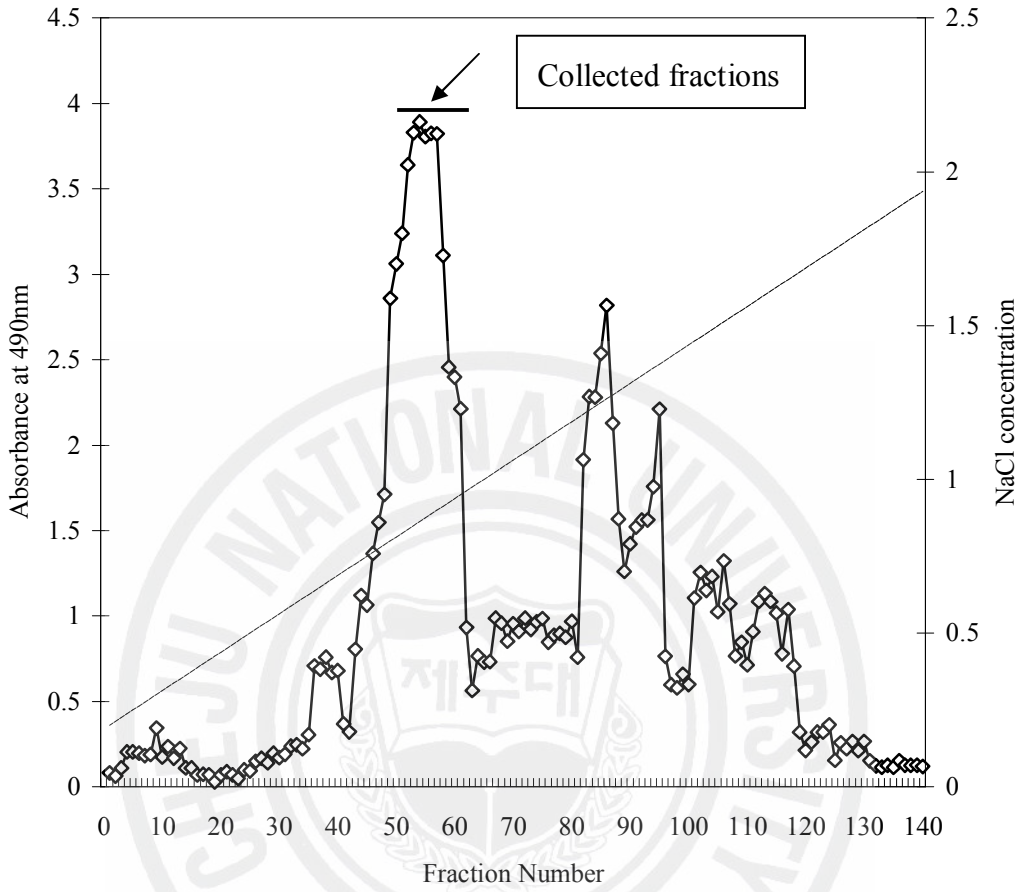

Fig. 2-1. Purification of the anticoagulant polysaccharide (GFA) from the red alga *G. filicina* by DEAE cellulose. A crude polysaccharide (500 mg) was applied to a DEAE-cellulose column (17 X 2.5 cm) equilibrated in 50 mM sodium acetate (pH 5.0) and washed with 500 ml of the same buffer containing 0.2 M NaCl. Thereafter, the column was eluted by a gradient prepared by mixing 50 mM sodium acetate containing from 0.2 M NaCl to 2 M NaCl in the same buffer. Fractions of 4 ml were collected and checked for carbohydrate content by phenol-H_2SO_4.

The application of the sample on sepharose 4B yielded a single polysaccharide peak with high metalachcomatic property. Metalachromatic property is an indicator of the polyanioninc power of the active compound. Samples with high metalachromatic power lead strong binding property with other compounds. However, the samples were devoid with hexauronic acid like compounds (data not shown). As a method to confirm the homogeneity of the purified compound, we introduced the purified sample on 0.5% agarose gel electrophoresis. After stained with toluidine blue, the sample appeared as a clear single spot on agarose gel (Fig. 2-4). The sugar composition of the active compound was investigated by HPLC and compared the absorption spectra with standard sugars (Fig. 2-5A). The purified compound composed with high amount of galactose (~98%) and less amount of fucose (1-2%) respectively (Fig. 2-5 and Table 2-2). The average molecular weight of the polysaccharide sample was calculated as 1357 kDa according to the calibration curve with standard dextrans (Fig. 2-6). Then the polysaccharide from the red alga was analyzed by FTIR spectrometer to elucidate its some structural features (Fig. 2-7). The main absorption band was observed at 3489 cm^{-1} is responsible for O-H stretching. Hemiacetal group of the sample was distinguished by the 1030 cm^{-1} absorption band while the presence of S=O was identified by the band at 1246 cm^{-1}. A shoulder at 835 cm^{-1} is believed to be due to sulfate at the axial C-4 positions. The data almost in the line with previous publication as it has been published by Patanker et al 1993.

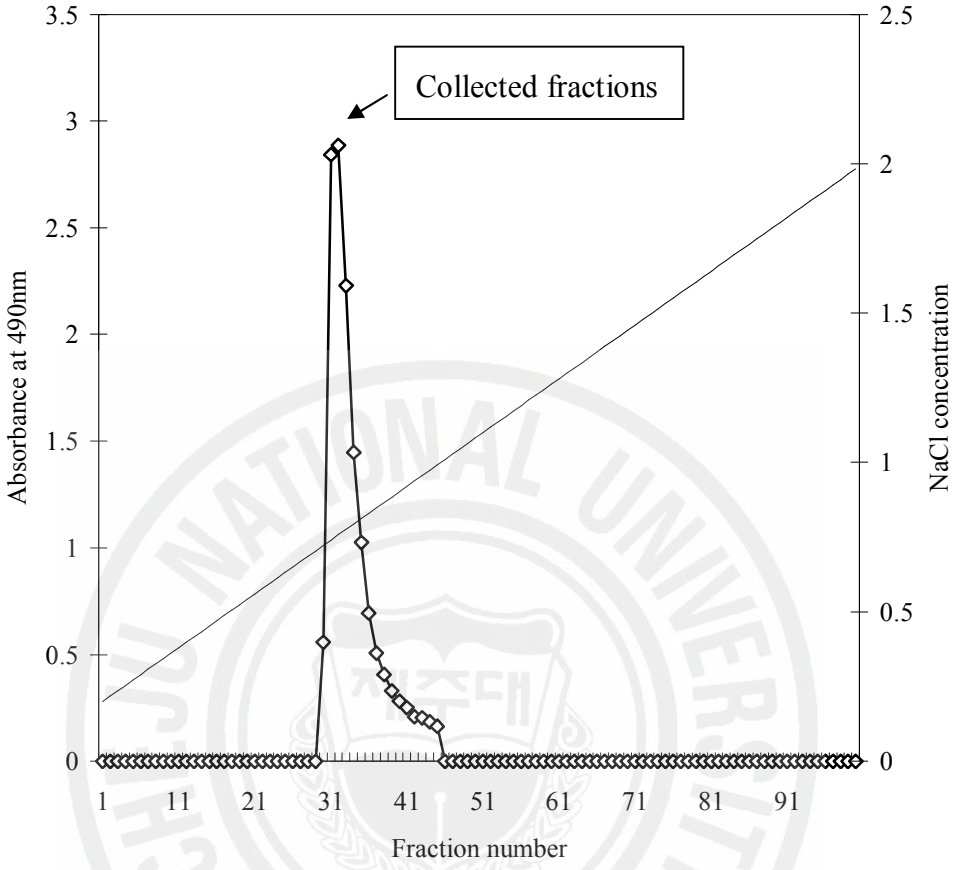

Fig. 2-2. Partially purified anticoagulant polysaccharide of *G. filicina* (100 mg) were re-chromatagraphed on a new DEAE cellulose column (10 x 1.7 cm). Partially purified polysaccharide was applied to a new DEAE-cellulose column equilibrated in 50 mM sodium acetate (pH 5.0) and washed with 500 ml of the same buffer containing 0.2 M NaCl. Thereafter, the column was eluted by a gradient prepared by mixing 50 mM sodium acetate containing from 0.2 M NaCl to 2 M NaCl in the same buffer. Fractions of 4 ml were collected and checked for carbohydrate content by phenol-H_2SO_4.

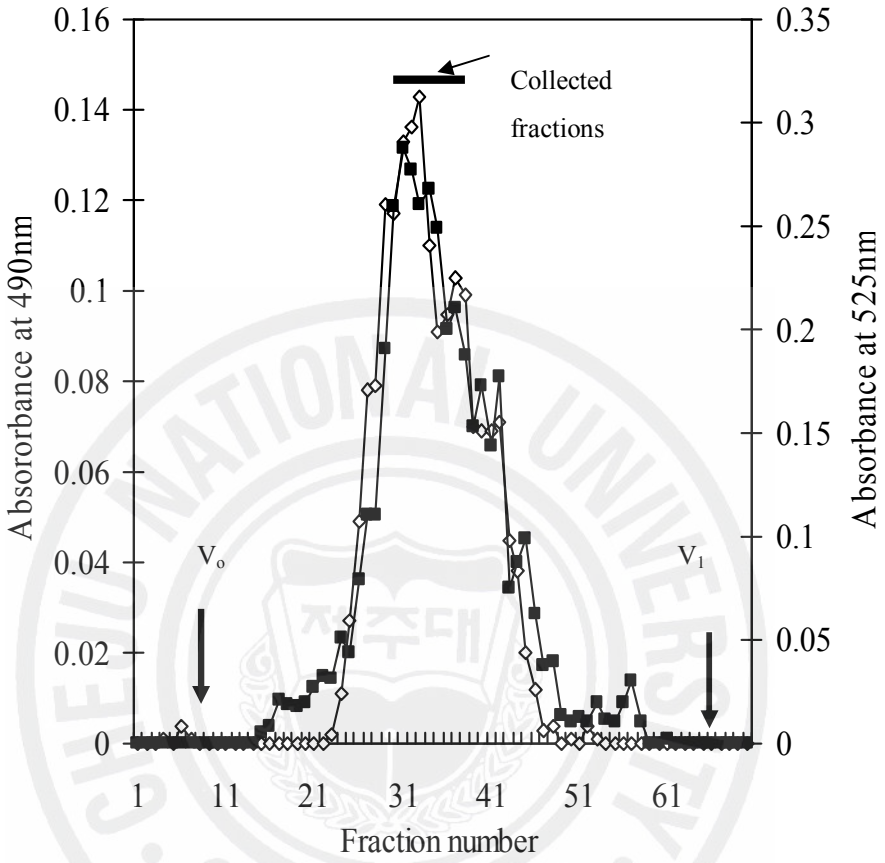

Fig. 2-3. Purification of the anticoagulant polysaccharide from the red alga *G. filicina* by Sepharose 4B. Solution fraction (1 mg/ml in water) was applied to a Sepharose 4B column (72 x 2 cm) equilibrated and eluted with water at a room temperature at a flow rate 1 ml/min. Fractions (2 ml) were collected and assayed for metachromatic property at 525 nm and for total carbohydrate contents. Carbohydrate content (◊); Metachromatic property (■); Blue dextran (V_o); Cresol red (V_1).

Furthermore, according to the $BaCl_2$ technique, the purified polysaccharide constituted considerable amount of sulfate (0.42 sulfate/total sugar). 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 2-3). Furthermore, the purified compound showed considerable activity on TT assay, but had very 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. 2-8). Hence, presumably the sample selectively inhibits coagulation cascade enzymes and thereby potentates its anticoagulant activity. However sample had less/slight inhibition potential on other tested coagulation factors (factor V, IX and XI). In order to dissect the mechanism of the GFP for its anticoagulant activity, a SPR study was carried out and the binding affinities on the human blood coagulant factors were analyzed by real-time sensorgraphic information (BIAcore 2000 system). The blood coagulations factors, ATIII were immobilized into the CM5 sensor chips using amine coupling after determining the optimal pH. Fig 2-9, illustrates the difference of the binding affinity of the blood coagulant factors with the GFA alone and with the presence of ATIII. The coagulant factors were interacted with GFA in a varying degree considerably, however with the presence of ATIII the biomolecular interaction enhanced rapidly. Especially, the addition of ATIII accelerated GFA-factor II interaction by two fold. Presumably, the active compound has strong interaction with ATIII mediated anticoagulant pathway. The kinetic constants (*ka, kd* 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 of SPR program.

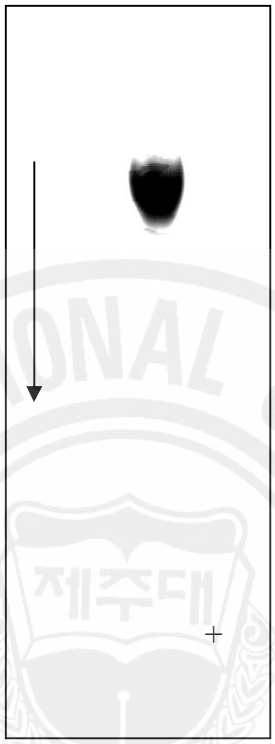

Fig. 2-4. Agarose gel electrophoresis of the anticoagulant polysaccharide of *G. filicina*. The purified polysaccharide (~10 μg) was applied to a 0.5% agarose gel and run for 1h at 110 V in 0.05 M 1, 3-diaminopropane/acetate (pH 9.0). The sulfated polysaccharides were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution.

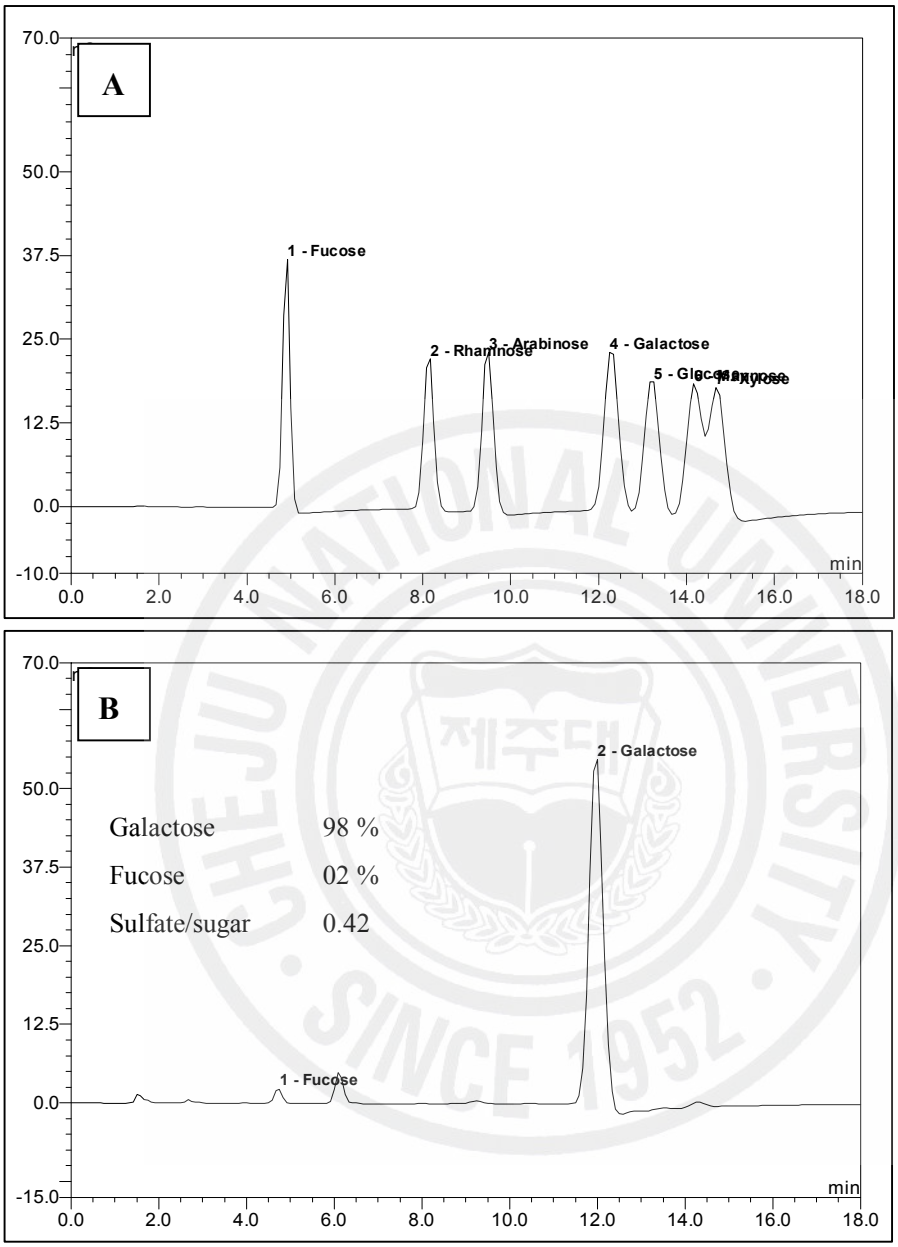

Fig. 2-5. The HPLC chromatograms for the monosugar composition of the purified polysaccharide of *G. filicina*. Chromatogram for the sugar standards (A); Chromatogram for the *G. filicina* polysaccharide sample (B).

Table 2-2. Sugar constituents and the degree of sulfation of the potential anticoagulant polysaccharide isolated from *G. filicina.*

Sugar	**%**
Galactose	98.0
Fucose	2.0
Sulfate/total sugar [a]	0.42

[a] The mean degree of substitution of sulfate ester per anhydro sugar residue.

Table 2-3. Comparison of anticoagulant activity of *G. filicina* with that of Heparin

	Clotting time (sec)						
		G. filicina anticoagulant (μg/ml)			Heparin (μg/ml)		
	Control	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 means of three determinations.

The optimum pH values and RU units of the coagulant factors for the coupling reaction on sensor chip are illustrated in Table 1-9. 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 (58.9 nM)> FIIa (74.6 nM)> and FVII (109.3 nM) respectively Table 2-4. In pharmaceutical research, the compounds with low equilibrium dissociation constant are in high demand, in our previous research a sulfated polysaccharide purified from *E. cava* showed 15.1 nM of K_D value towards FVIIa inhibition. Taken together, it's clear that GFA exert high anticoagulant activity by inhibiting the both extrinsic and common pathway 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 (Travis and Salvesen, 1983). Therefore, the ability of a compound to accelerate ATIII mediated pathway is much important for further studies. As summary of this research, the GFA triggered anticoagulation effect can be roughly illustrated as shown in Fig 2-10. The compound exerts high activity by governing several coagulant factors in a human coagulation pathway; however, there are some weak negative effects as indicated by dotted lines of the figure. Natural compounds are diverse with multiple bioactivities, sometimes the *in-vivo* application of those compounds are limited due to their side effects. Especially, natural compounds should not induct cytotoxic effects on normal human cell line while binding to their target compounds. To elucidate the potential cytotoxic activity of the GFA, in this study we introduced samples at different concentration on normal cell lines and estimated its effects on cell growth by MTT assay (Fig. 2-11). Obviously, the sample had no any side effects on normal cell growth indicating its applicability in *in vivo* assays.

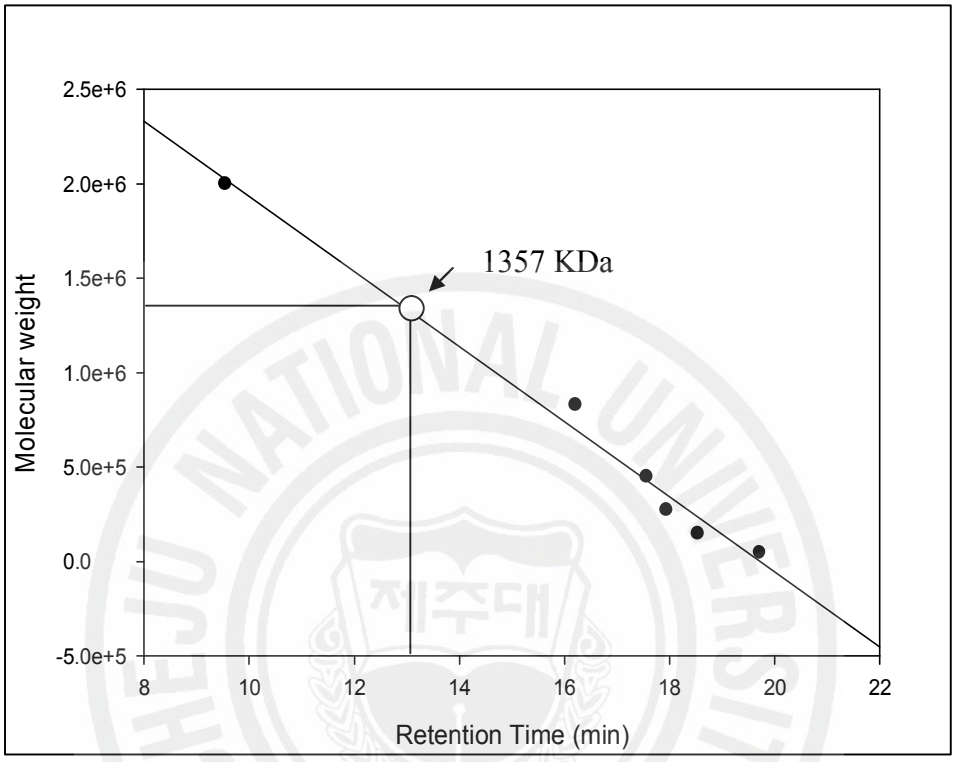

Fig. 2-6. Calibration curve of dextran standards for the determination of the average molecular weight of the *G. filicina* sample. The retention time is plotted against the molecular weight of the dextrans.

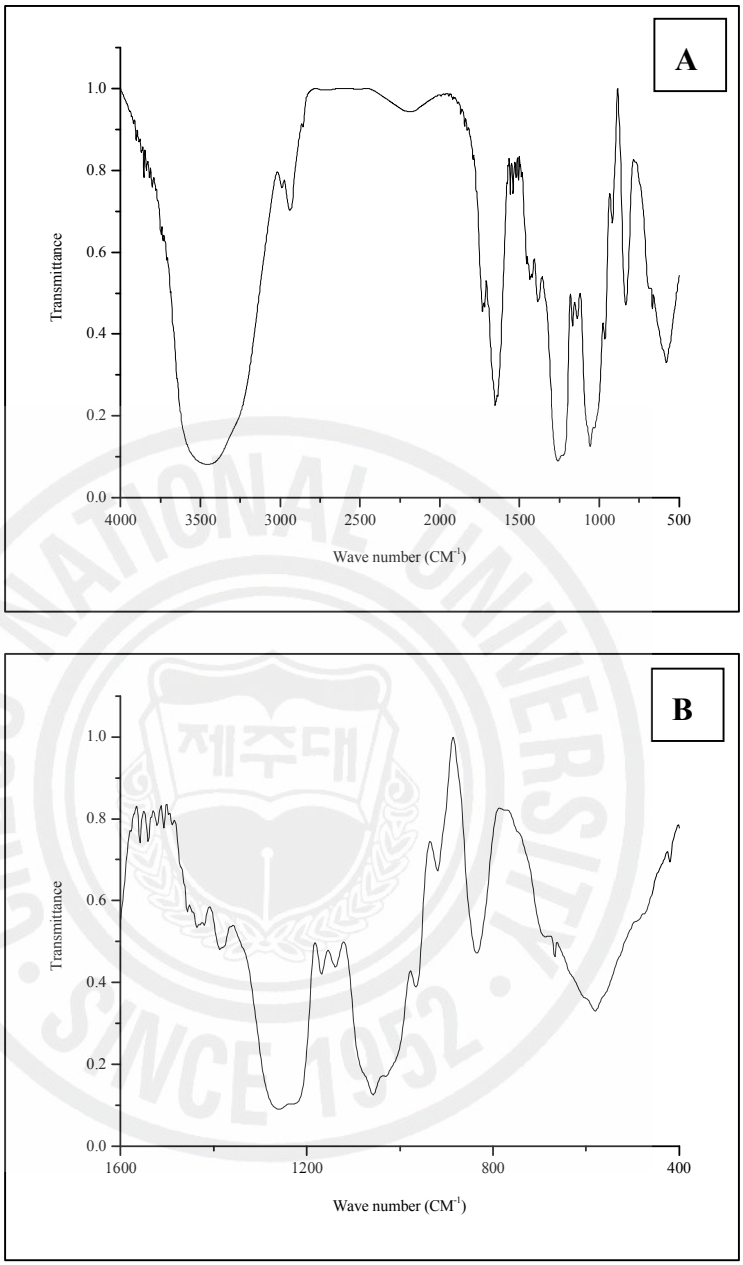

Fig. 2-7. Infrared analysis of the purified sulfated polysaccharides isolated from *G. filicina*. The sample was scanned between 4000 and 400 cm^{-1}(A); The sample scanned between 1600 and 400 cm^{-1}(B).

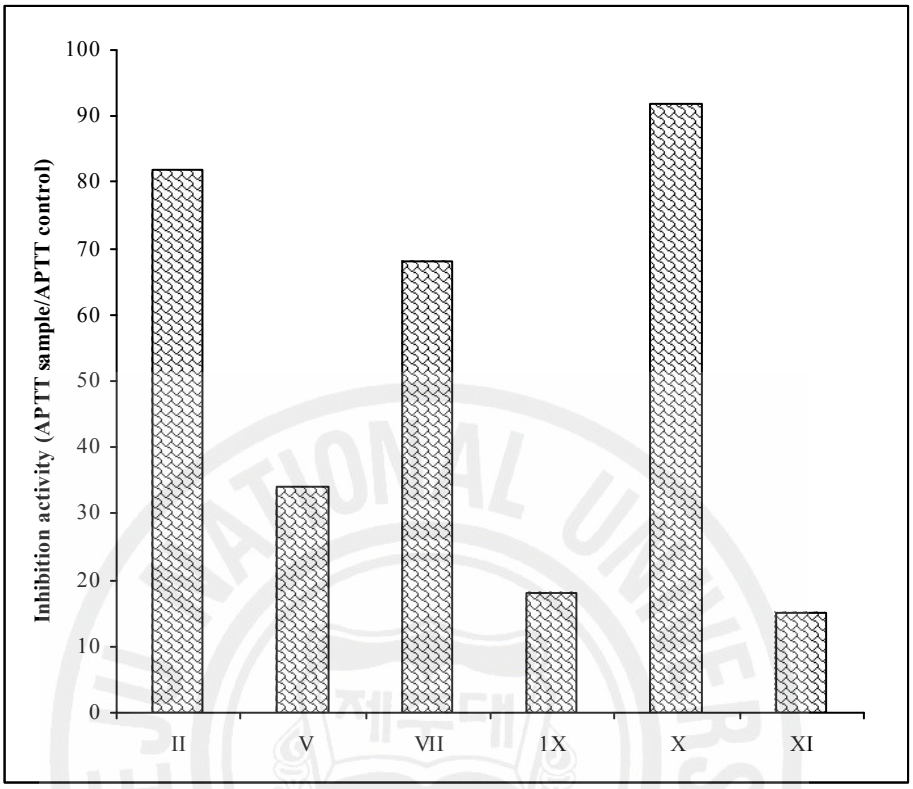

Fig. 2-8. Specific factor inhibitory pattern of the purified anticoagulant on blood coagulant factors.

Algal polysaccharides have been widely tested for their biological activities *in vitro* and *in vivo*. Due to their high biological activities, sulfated polysaccharides contained in algae are an alternative natural source for synthetic compounds in pharmaceutical industry. Therefore, studies related to marine compounds are continuously increasing in recent history (Uehara et al., 1992, Siddhanta et al., 1999). However, studies related to algal enzymatic extraction preparation for their biological activity are quite limited. Interestingly enzymatic digestion gains 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 also this method do not adapt any toxic chemicals.

Therefore, enzymatic digestion can be utilized well for the biological activity evaluation of algae (Athukorala et al., 2007). In this study, the anticoagulant activity of *G. filicina* collected from Jeju Island was investigated by utilizing enzymatic hydrolysis technique. Several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) as cheap food grade enzymes were used in this study to investigate 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 tailor-made compounds to breakdown special linkages of the polysaccharides, and this may explain their different affinities to extend coagulation time. Taken together, in this study, tested all enzymatic extracts showed good anticoagulant activity, especially the Termamyl extract showed the highest anticoagulant activity. Termamyl, a popular food grade enzyme is widely used in food industry, may break down plant cell wall materials such as polysaccharides or glycoproteins by hydrolyzing 1, 4-alpha glycosidic linkages of the polysaccharides.

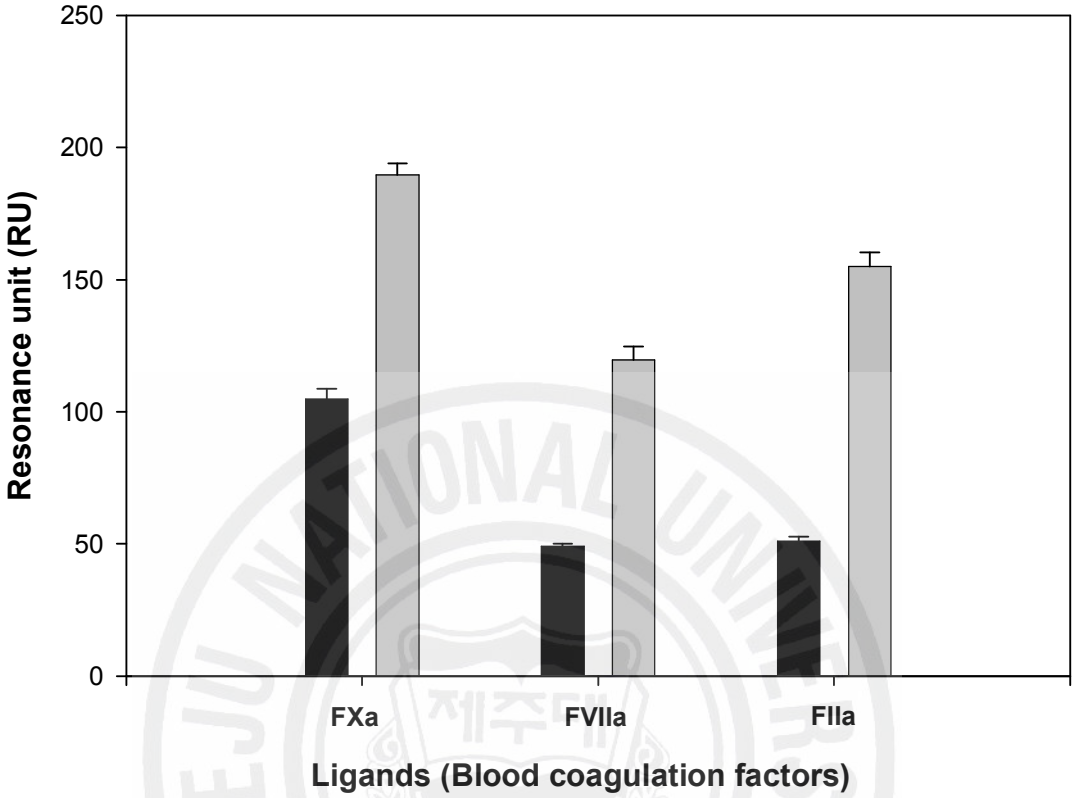

Fig. 2-9. Binding affinity assay of *G. filicina* anticoagulant (GFA) to blood coagulation factors using surface plasmon resonance (SPR) spectrometer. Black columns express resonance unit (RU) values of real-time sensorgram in the absent of tissue factor (ATIII). Gray columns express resonance unit (RU) values in the present of tissue factor (ATIII).

Table 2-4. Kinetic parameters for the blood coagulation factors with *G. filicina* anticoagulant (GFA) using SPR sensorgraphy.

Analyte	**Ligand**	**Association rate constant (*ka*)**	**Dissociation rate constant (*kd*)**	**Equilibrium dissociation constant (K_D = *kd*/*ka*)**
		$M^{-1}s^{-1}$	s^{-1}	***nM***
***G. filicina* anticoagulant**	**FVIIa**	5.33×10^{3}	5.83×10^{-4}	109.3
	Fxa	1.97×10^{3}	1.16×10^{-4}	58.9
	FIIa	2.18×10^{3}	1.63×10^{-4}	74.6

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

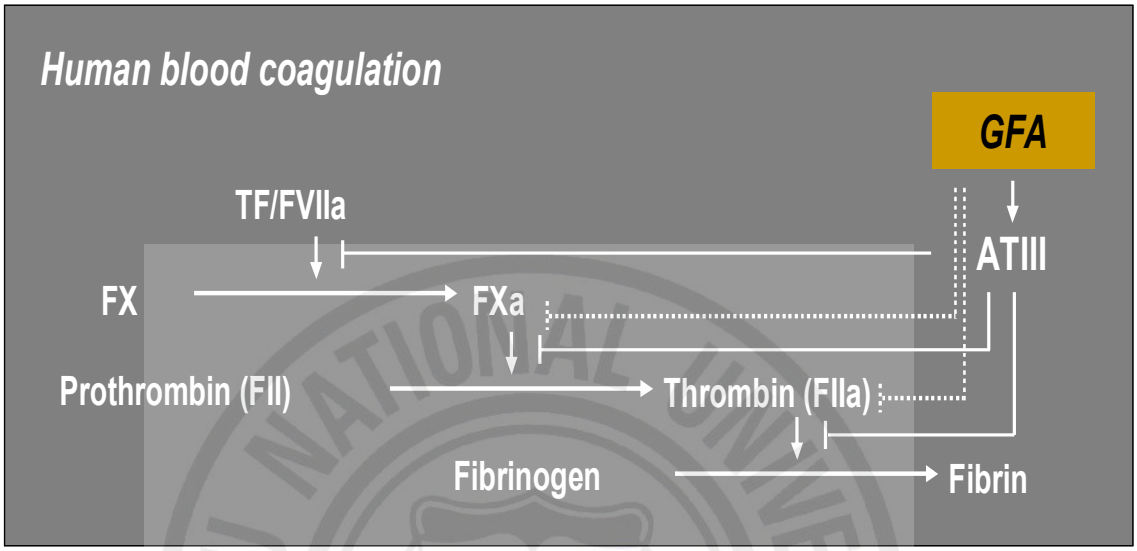

Fig. 2-10. Anticoagulation mechanism of *G. filicina* anticoagulant (GFA) in human blood coagulation pathway. The dotted line means weak-negative effects of GFA on target factor (Fxa).

This leads a variety of biochemical compounds and some of them may be strong anticoagulants. Sulfated galactans, available in marine red algae and in marine invertebrates have been studied for their biological activities. The structure and the nature of galactans (carrageenans and agarans) present in the alga sample can be changed according to the species, collected area and the harvesting season. However the structure of most galactans is composed with alternating 3-linked β-galactopyranose and 4-linked α-galactopyranose (Pereira et al., 2005). Sulfated galactans are known for its high anticoagulant activity. In this study, galactose rich- sulfated polysaccharide showed considerable anticoagulant activity compared to 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 (Pereira et al., 2005). Also, Gelidium crinale a popular red alga composed with sulfated galactan (-4-α-Galp-(1(3)-β-Galp1) exhibited potent anticoagulant activity (Farias et al., 2000). 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 x 10^4) showed higher anticoagulant potential than those with low sulfur content with low molecular weight (1.0 x 10^4) (Shanmugam and Mody. 2000).

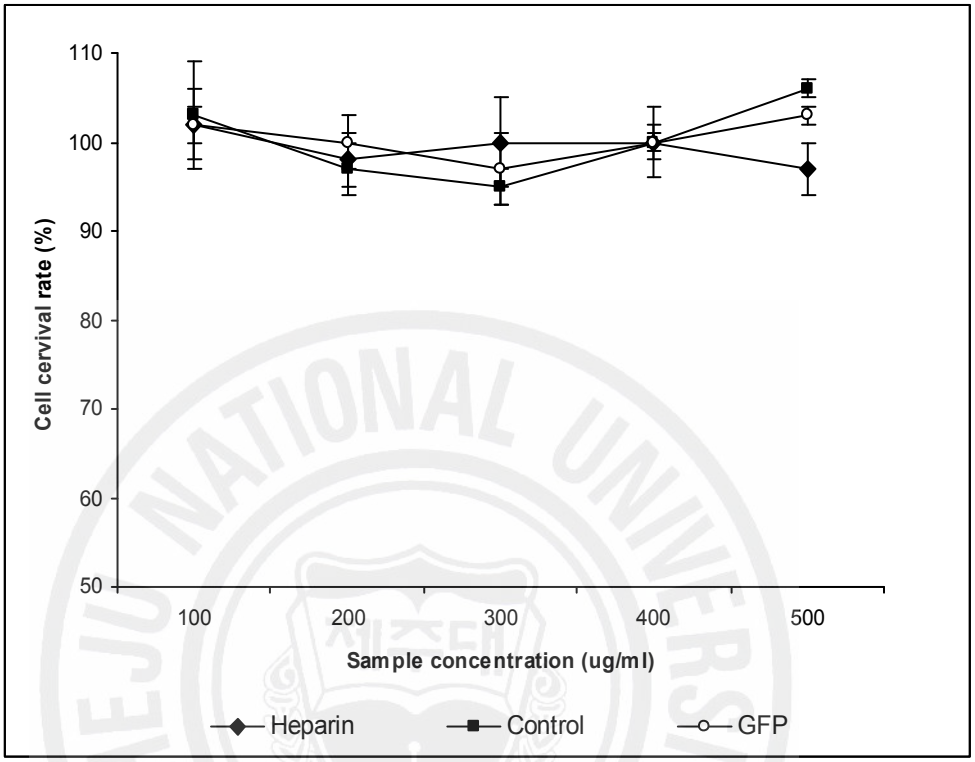

Fig. 2-11. Cytotoxicity of GFA on monkey kidney cell line (V79-4) in dose-dependant manner. The control expresses cell viability (%) without any dose.

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. Blood coagulation pathway of human has several pathways; the potentiality of the anticoagulant on intrinsic/common pathway can be estimated by APTT assay, while the ant-thrombin 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 extrinsic pathway (Dave et al., 1979). 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 with PT activity. Therefore the active compound selectively controls the blood serine proteases associated intrinsic/common pathway of the blood coagulation cascade. However, to study 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 an excellent activity on factor X (staurt factor), factor II (thrombin) and VII (proconvertin) respectively. Among the tested factors, the purified compound showed the most potent activity (92%) on factor X, that catalyze the hydrolysis 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 (Travis and Salvesen 1983). As it is shown in specific factor inhibition assay results, X (stuart factor), factor II (prothrombin), and VII (proconvertin) were strongly inhibited by the tested purified polysaccharide, respectively, and showed low activity on factors IX, V and XI. Therefore, several coagulation factors are

associated with the observed activity of GFP. Hence, the interaction of GFP with serine proteases was investigated with the presence of ATIII. The presence of ATIII rapidly accelerated molecular interaction of GFP with serine proteases both in extrinsic and common coagulation pathway. GFP showed no side effect on normal cell growth, therefore the compound is a promising agent for future drug discovery as a model compound in pharmaceutical industry, and however sample should be evaluated in other possible coagulation mechanism pathway to investigate sample's detailed interactions on the coagulation pathway.

Part III

Anticancer activity of sulfated polysaccharide isolated from *Ecklonia cava*

ABSTRACT

The sulfated polysaccharide purified from a brown alga, *E. cava* having high anticoagulant activity was investigated for its antiproliferative effect on murine colon carcinoma (CT-26), human leukemic monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60) and mouse melanoma (B-16) cell lines. The sulfated polysaccharide of *E. cava* (ECSP) had good selective tumor cell growth inhibition effect, especially its effect on human leukemic monocyte lymphoma cell line (U-937) and human promyelocytic leukemia cell (HL-60) was promising. The IC_{50} values for the commercial fucoidan and ECSP on U-937 were 43.9 and 36.2 μg/ml respectively. The presence of the sample in the cell culture media stimulated the induction of apoptosis as it was revealed by nuclear staining with Hoechst 33342. The apoptosis induction was confirmed by the cell cycle analysis, clearly a pronounced sub G1 phase arrest was observed when the cells were treated at 15 μg/ml and 30 μg/ml both in U-937 and HL-60 cell lines. The sample didn't show any cytotoxic effect even at a high sample concentration on venous endothelial cell (ECV-304). After 24 hr incubation period, ECSP dose dependently enhanced the DNA fragmentation on U-937 cell line as observed in the agarose gel electrophoresis assay. To rule out the mechanism of action of the polysaccharides for its anticancer activity, a western blot analysis was conducted with antibodies (caspasase-7, caspasase-8, Bax, and Bcl-xL and PARP), and the sample had clear effect on the caspase -7 and 8 which cleave protein substrates, including PARP; an inducer of apoptosis responsible for DNA cleavage. Moreover, ECSP controlled the cellular transmembrane molecules like Bax, and Bcl-xL. Taken together of the above results the apoptosis for antiproliferative effect of sulfated polysaccharide was clearly induced on U-937 cells.

INTRODUCTION

Marine algae have been reported to contain diverse classes of biologically active compounds which are useful in pharmaceutical industry. Antitumor activity is one of the important biological activities in marine algae, some of the marine algae and their metabolites showed promising activities and hence it's a great source to investigate new antitumor drugs.

Most of the natural anticancer compounds are able to manipulate the growth of cancer cells with reduced side effects. Therefore, novel natural anticancer drugs have been commercially developed successfully to treat the cancer patients (Mans at al., 2000). As it has been reported, around 60% of the current commercial anticancer drugs have been originated from the natural sources. It is noteworthy to highlight that, during last decade, about 2500 new marine derived metabolites with anti-proliferative activity have been discovered, however the mode of action for their activity are remained to be elucidated. Moreover, recently marine-derived three compounds (Ecteinascidin-743, isostatine (aplidin) and kahalalide F) have proven to be feasible in cancer patients (Jimeno et al., 2003).

Sulfated polysaccharide isolated of marine brown algae are also promising compounds with multiple biological activities. Some Japanes scientists investigated extracts of edible alge for their protective effect against L-1210 leukemia cell line *in vivo*. Of the tested species, the crude fucoidan separated from *Eisenia bicyclis* sample showed considerable activity on that study (Yamamoto et al., 1984). The polysaccharide fraction of *Capsosiphon fulvescens* (Chlorophyta) consisted of xylose (19.1%), fucose (15.3%), mannose (4.2%) and galactose (8%) inhibited sarcoma-180 growth successfully and exhibited immunostimulation activity *in vitro* (Park et al., 2006). Due to numerous health benefits, the compound is currently investigated both *in vivo* and *in*

vitro to utilize as a therapeutic drug. At the moment, there are several nutrient supplements containing fucoidan (a sulfated polysaccharide) are available in the market.

Ecklonia cava, a kind of brown seaweed is plentifully produced in Jeju Island in Korea (30,000 tons per year), is not available in Europe; however, it is popular in Korea and Japan where this valuable brown algae is utilized in the field of food ingredients, animal feed, fertilizers and medicine. In addition, *E. cava* has xanthopophyll, pigment, fucoxathin, fucoidan, phycocolloid and especially is a good source of alginates, which can use in viscosifiers of thickeners in a wide variety of products (Ahn et al., 2006). *E. cava* has been reported for scavenging activity (Kang et al., 2005; Kang et al., 2005; Kang et al., 2003), anti-plasmin inhibiting activity (Fukuyama et al., 1990; Fukuyama et al., 1989) antimutagenic activity (Lee et al., 1998; Han et al., 2000), bactericidal activity (Nagayama et al., 2002), HIV-1 reverse transcriptase and protease inhibiting activity (Ahn et al., 2004) and tyrosinase inhibitory activity (Kang et al., 2004).

In our previous study (Athukorala et al, 2006), we isolated a highly sulfated polysaccharide from an enzymatic hydrolysate of marine brown alga (*E. cava*). The highly sulfated (0.92 sulfate/total sugar) active sample was mainly composed of fucose and a small amount of galactose. The polysaccharide showed high anticoagulant activity and strongly interfered with coagulation cascade by inhibiting biological activity of the activated blood coagulation FII, FX and FVII as serine proteases.

Having an idea of investigating its biological activity, in this study, we investigated the purified sample on several cancer cell lines to investigate its antipoliferative activity on on murine colon carcinoma (CT-26), human leukemic monocyte lymphoma (U-937), human promyelocytic leukemia (HL-60) and mouse melanoma (B-16) cell lines. More over, its effect on protein expression was investigated by western blot analysis.

MATERIALS AND METHODS

Purification of the sulfated polysaccharide from *E. cava*

The purification of the sulfated polysaccharide was followed as it has been previously published (Athukolara et al., 2006). Briefly, freeze dried AMG hydrolysate of *E. cava* sample was introduced to DEAE-cellulose ion exchange chromatography and separated high anticoagulant fractions according to activated partial thromboplastin time (APTT) assay, and then the sample was further purified on a new DEAE-cellulose column to improve the purity of the sample. Thereafter, the sample was applied into a gel-permeation chromatography on Sepharose-4B to purify sample according to its molecular weight. The purity of the sample was confirmed by agarose gel electrophoresis, and the molecular weight of the sample was determined by GFC system. The purified sulfated polysaccharide (0.92 sulfate/total sugar) showed 1381 kDa molecular weight and comprised mainly of fucose and small amount of galactose.

Assessment of cell viability

Cell viability was then estimated via an MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The cells were seeded in 96-well plates at a concentration of 1.0 x 10^5 cells/ml, and treated with the sulfated polysaccharides at different concentrations. After 72 hr, MTT stock solution (50 ml; 2 mg/ml) was applied to each of the wells, to a total reaction volume of 200 ml. After 4 h of incubation, the plates were centrifuged for 5 min at 800 x g, and the supernatants were aspirated. The

formazan crystals in each well were dissolved in 150 ml of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100 % viability. The data are expressed as mean percentages of the viable cells versus the respective control.

Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995, Lizard et al., 1995). The U-937 cells were placed in 24-well plates at a concentration of 1.0 x 10^5 cells/ml and samples were treated with sulfated polysaccharides. After 24 h, 1.5 ml of Hoechst 33342 (stock 10 mg/ml), a DNA-specific fluorescent dye, were added to each well (1.5 ml), followed by 10 min of incubation at 37 ^oC. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.

Flow cytometry analysis

Flow cytometry analyses were conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells (Nicoletti 1991). The U-937 cells were placed in 6-well plates at a concentration of 1.0 x 10^5 cells/ml, and samples were treated with sulfated polysaccharides. After 24 h, the cells were harvested at the indicated time, and fixed for 30 min in 1 ml of 70% ethanol at 4^oC. The cells were then washed twice with

PBS, and incubated for 30 min in darkness in 1 ml of PBS containing 100 mg PI and 100 mg Rnase A, at 37°C. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). Effects on the cell cycle were determined by measuring changes in the percentage of cell distribution at each phase of the cell cycle, and were assessed by histograms generated by the Cell Quest and Mod-Fit computer programs (Wang et al., 1999).

DNA gel electrophoresis (DNA laddering)

The U-937 cells (5 x 10^5 cells/ 60 mm culture dish) was incubated with the presence of sulfated polysaccharide sample at different concentrations (30, 60, 120 μg/ml) and investigated its effect on inter-nucleosomal DNA cleavage of U-937 cells by agarose gel electrophoresis. The cells DNA was separated and purified according to the manufacturer's guidelines. 4 μl of the DNA was applied into a 1.5 % agarose gel containing ethidium bromide, the DNA damage was observed followed by observation under ultraviolet illumination.

Western blot analysis

U-937 cells (2×10^5 cell/ml) were treated with sulfated polysaccharide and incubated for 24 hr and harvested. The cell lysates were prepared with lysis buffer (50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/l EDTA). Cell lysate were washed in PBS, centrifuged and the protein content of the supernatant was determined by BCA™ protein assay kit. The lysate containing 40 μg of protein were subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibody against

Bax, Bcl-xL, cleaved caspase-3, -7 and β-actin (Cell Signaling Technology, Inc, Massachusetts, USA) in TTBS (25 mmol/l Tris-HCl, 137 mmol/l NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% nonfat dry milk at 1 h. Membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL western blotting detection kit and exposed to X-ray films.

RESULTS AND DISCUSSION

Algal polysaccharides are renowned for their unique mode of action which attracted them to be used in food and pharmaceutical industry for a long time. Most popular algal polysaccharides include agar, carrageenans, alginates, laminaran, rhamnan sulfate and fucoidan are commercially produce and use actively in food, agriculture and other related industries. Recently, as an abundant, renewable natural source with multiple biological activities, the latter compound has been playing an important key role in the life science research. Owing to its special structural features, fucoidan isolated from marine algae are known to exert anticoagulant, anticancer, antimitogentc, anticompliment, antiviral, anti-adhesive, anti-inflammatory and antioxidant activities. The composition of the fucoidan or other polysaccharides can vary according to its algal species, extraction procedure, harvesting season and the local climate conditions. In our previous study, a fucoidan and a sulfated galactan with promising anticoagulant activity was isolated from enzymatic hydrolysates of *E. cava* and *G. filicina*. The present work was devoted to investigate the anticancer activities of the isolated polysaccharides *in vitro* with several important cancer cell lines including CT-26, B-16, HL-60 and U-937.

In this study, sulfated galactan purified from the *G. filicina* was devoid of anticancer activity in all tested cancer cell lines. Interestingly, the sulfated polysaccharide purified from the *E. cava*, selectively and dose-dependently suppressed the proliferation of all cancer cell lines *in vitro*. The sample showed a strong anticancer activity on murine colon carcinoma cell (CT-26) line (Fig. 3-1). The growth inhibition rate of CT-26 cells increased consistently with the heju ed sample concentration, in which the highest activity (40%) was recorded at 100 structural functional studies sample concentrations. In the previous study, *Codium contractum* and *Codium fragile* collected from Jeju

Island exhibited strong cytotoxic activities against HL60 or CT26 cells (Kim et al., 2006 a). Moreover, in the same study, *Sagassum coreanum* and *Sagassum siliquastrum* aqueous extracts obtained at a low temperature exhibited strong cytotoxic activities against U937, HL60, HeLa cells. In this study, the presence of the sulfated polysaccharide dose-dependently delayed the growth on U-937 cell line, the IC_{50} of ECSP on human leukemic monocyte lymphoma cell line was 43.9 μg/ml(Fig. 3-2). A sulfated polysaccharide (B-1) isolated and purified from the culture filtrate of marine *Pseudomonas* sp. Induced apoptotic changes in the morphology of U937 cells at concentrations greater than 0.1 μg/ml (Matsuda et al., 2003).

When the human promyelocytic leukemia cell (HL-60) line was treated with different concentration of polysaccharide samples, a promising antiproliferative effect was observed (Fig. 3-3). Around 50% cell growth inhibition was recorded when the sample was treated at 100 μg/ml concentration, as depicted in the figure the inhibition rate gradually decreased with decreasing sample concentrations. The sample ability to control the growth of mouse melanoma cell line (B-16) was less, but all most similar pattern to that of HL-60 cell line (Fig. 3-4). As it has been reported previously, *Polysiphonia japonica* showed interesting cytotoxic activity against U-937, HL-60 and B-16 cells. In that study, more over, the extracts of *Scinaia okamurae* and *Chondrus crispus* showed cell growth inhibition activity more than 50% against HL-60 and B-16 cells. Especially, the former sample showed over 80% growth inhibition activity on against B-16 cells (Kim et al., 2006 b)

Taken together, the fucoidan purified from the *E. cava* showed a promising antiproliferative effect on tested all cancer cell lines. It is noteworthy to mention that sample had no cytotoxic effect on the normal cell growth (Fig. 3-11).

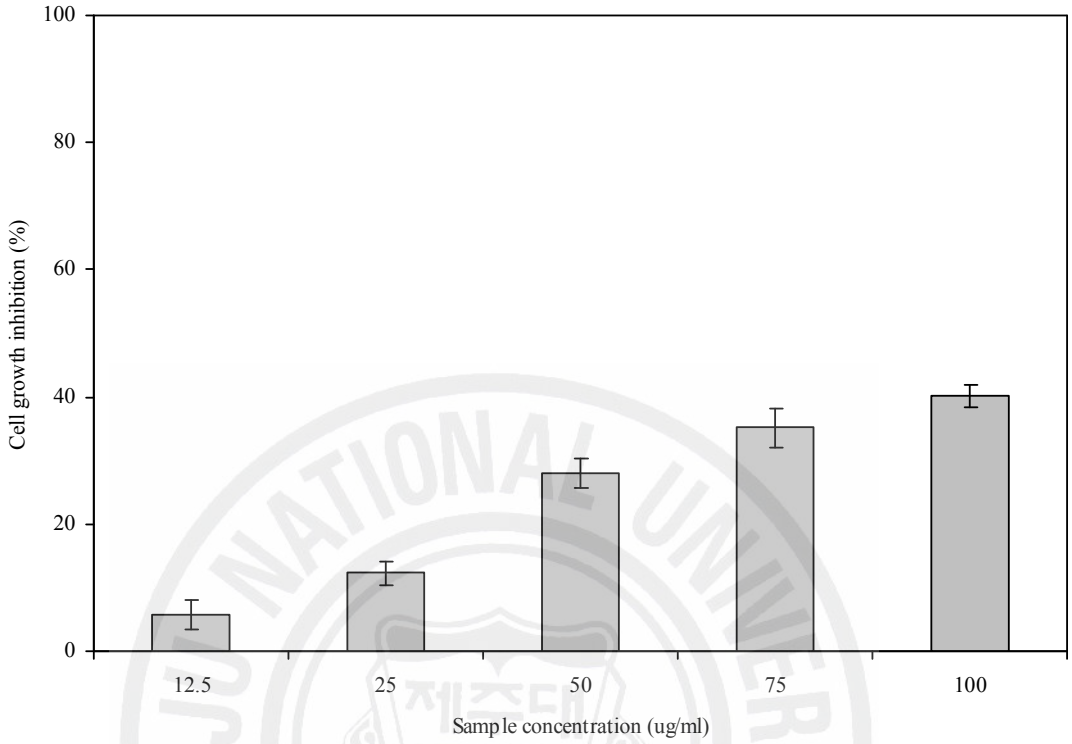

Fig. 3-1. The effect of the *E. cava* sulfated polysaccharide on the growth of the murine colon carcinoma (CT-26) cancer cell line. Cells were seeded onto 96-well plate at 1×10^3 cells/well and were treated with the sulfated polysaccharides at different concentrations, and percentage of cell viability was determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

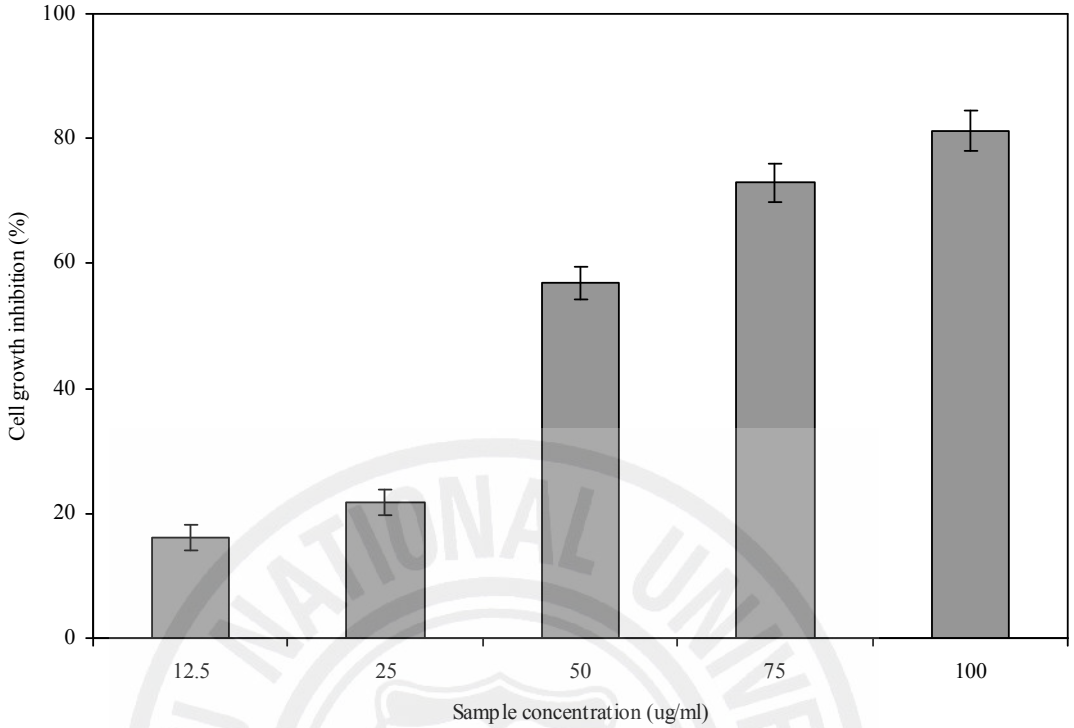

Fig. 3-2. The effect of the *E. cava* sulfated polysaccharides on the growth of the human leukemic monocyte lymphoma (U-937) cancer cell line. Cells were seeded onto 96-well plate at 1×10^3 cells /well and were treated with sulfated polysaccharides at different concentrations and percentage of cell viability was determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

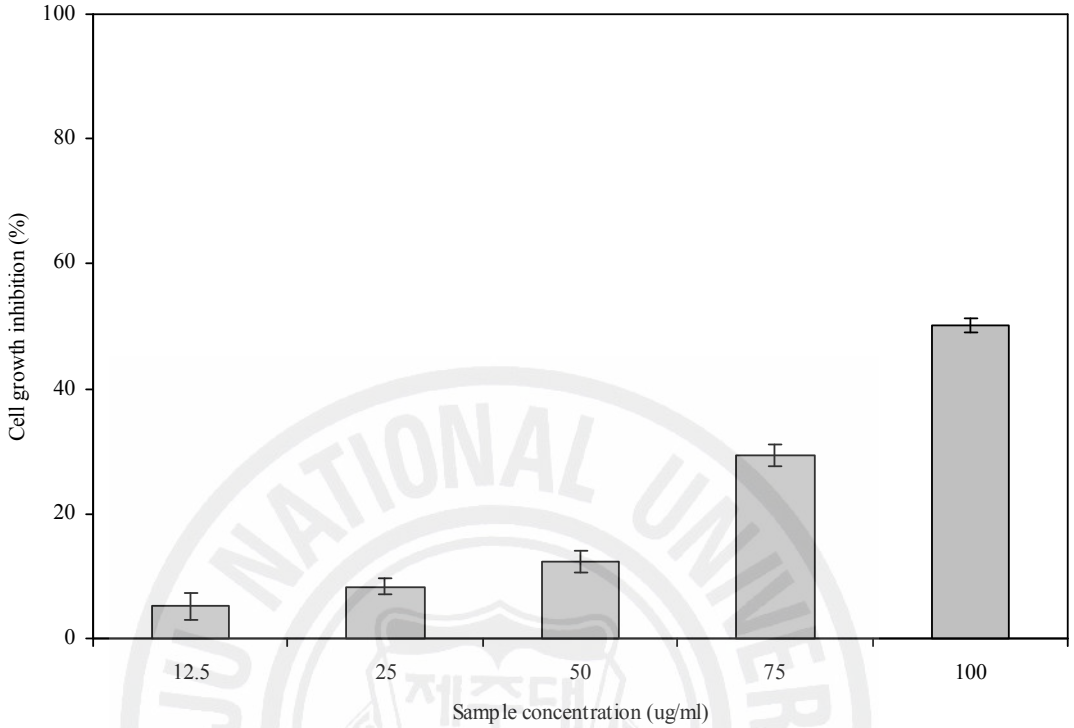

Fig. 3-3. The effect of the *E. cava* sulfated polysaccharides on the growth of the human promyelocytic leukemia (HL-60) cancer cell line. Cells were seeded onto 96-well plate at 1×10^3 cells /well and were treated with the sulfated polysaccharides at different concentrations, and percentage of cell viability was determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

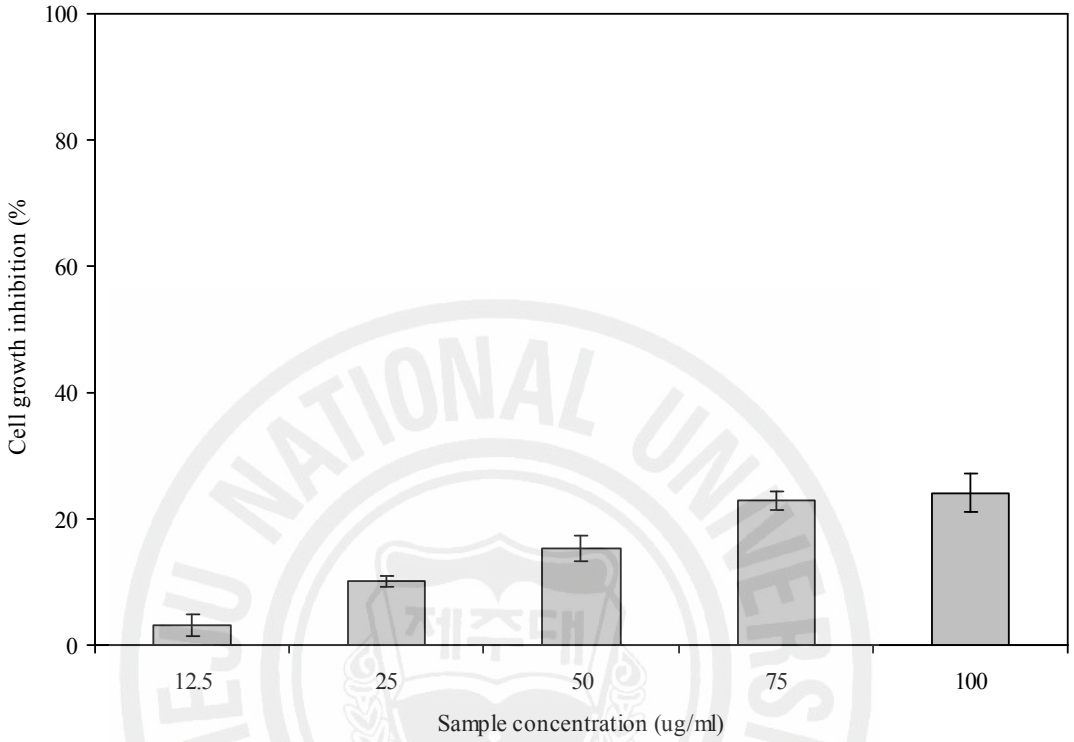

Fig. 3-4. The effect of the *E. cava* sulfated polysaccharide on the growth of the mouse melanoma (B-16) cancer cell line. Cells were seeded onto 96-well plate at 1×10^3 cells /well and were treated with the sulfated polysaccharides at different concentrations, and percentage of cell viability was determined by MTT assay after 72 hr of the treatment. The cell growth inhibition was calculated as function of the control. The standard error was calculated using Microsoft Excel software with data obtained from triplicate experiments.

In a cell when the mitrochrondrilal reductase enzymes are active, the presence of MTT (yellow colour formazan) converts into purple colour, hence the technique could be applied in cancer research to investigate the anticancer activity of unknown samples. The depicted results of this study, clearly demonstrated the ability of the sulfated polysaccharides to suppress the growth of cancer cells *in vitro*. ECSP showed selective inhibition against four cancer cell lines, and showed the its growth inhibitiory efficacy in a decreasing order of U-937>HL-60>CT-26 and B-16. Hence, the further experiments were carried out on U-937 cell line to investigate activity mechanism of action for the antiproliferative activity of ECSP.

Since there is a big demand for cancer chemotheraphy, anticancer action mode of ECSP is worthy to study. Hence, in order to dissect the mechanism of action for its high anticancer activity, following the sample treatment, cells were stained with Hoechest 33342. The latter dye is able to migrate into the cells and stain the DNA in a quantitative manner. Through a florescent microscope, the apoptotic cells can be distinguished easily. Normally, live cells appear with normal nuclei, blue/green pale chromatin with organized structure. Apoptotic cells (early apoptotic cells) can be identified by the presence of chromatin condensation within the nucleus and intact nuclear boundaries, bright blue chromatin that is highly condensed, marinated (late apoptotic cells) exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasmic membrane (Cavas et al., 2006). The morphological evaluations of chromatin condensation and/or fragmentation induced with the sulfated polysaccharides on U-937 cells are shown in Fig 3-5. Both ECSP and the commercial polysaccharide sample induced apoptotic bodies in the U-937 cells successfully than that of non-treated control group.

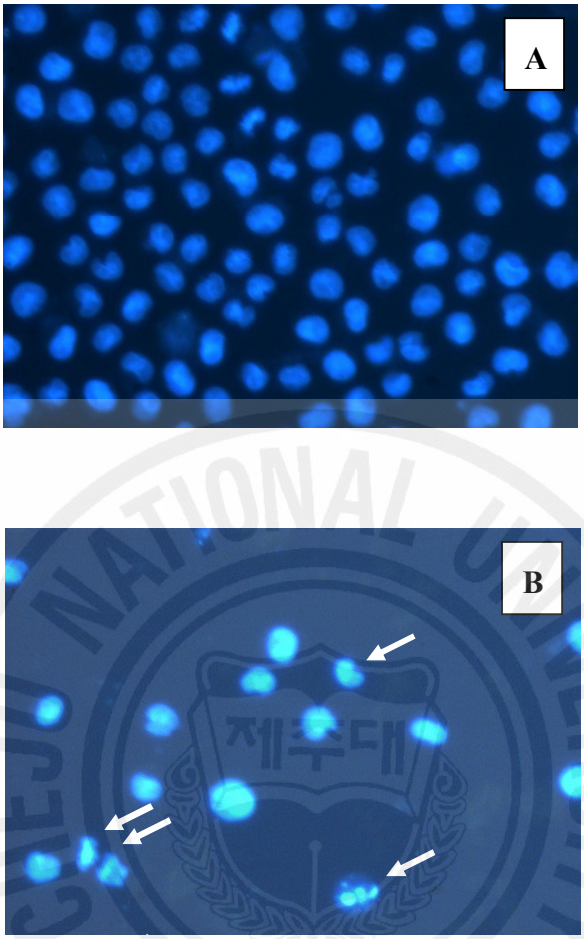

Fig. 3-5. Effect of *E. cava* sulfated polysaccharide on morphological changes in U-937 cell line. Cells were treated in the absence of (**A**) or in the presence of 75 μg/ml of sulfated polysaccharide for 24 hrs, stained with Hoechst 33342, and observed by fluorescence microscopy. Arrows in photo **B** indicate a typical apoptotic cell with apoptotic body. Apoptotic nuclei appear brighter and more fragmented than healthy nuclei, which are less bright and intact.

Therefore, apoptosis induction of the sulfated polysacchatides probably may be the reason for its anticancer activity. The polysaccharide isolated from *Misgurnus anguillicaudatus* sowed a high antiproliferative effect associated with apoptosis on HL-60 cell line, apart from that the polysaccharide enhanced the content of nitric oxide (NO) and activity of lactate dehydrogenase (LDH). Therefore sometimes, multiple mechanism are collaborated with the anticancer activity of sulfated polysaccharides (Zhang et al, 2005). The ability of a compound to induced apoptosis in which cells are programmed to die is an interesting and therapeutically useful way of an anticancer agent to govern the growth of a cancer cell cycle. The unique morphological, biochemical and cellular changes associated with the apoptotic cells are helpful to identify them from the normal cells. Flow cytometry technique is a quick and rapid technique to analyse the cell cycle. After anticancer treatment, as a result of the apoptosis, DNA fragmentation can be taken place. The small fragments of the DNA are able to elute by washing with PBS. The remaining DNA quantitatively binds with the binding dye (Propidium Iodide). Cells that have lost DNA take up less stain and will appear to the left of the G1 peak. Hence, the size of G1 peak of the histogram is directly proportional with the inducted apoptosis of a given treatment. In this study, after the sample treatment, cells were harvested, fixed and processed for DNA fragmentation by flow cytometry to elucidate its efficacy for apoptosis induction. On HL-60 cell line, when the sample treated at 15 and 30 μg/ml, there were ~ 6 and ~ 23 % sub G1- peak arrest respectively (Fig. 3-6). The effect of the sulfated polysaccgarides on the cell cycle of U-937 was investigated. The treatment of *E. cava* showed around ~ 13% cell cycle arrest on U-937 cells at the two sample concentrations tested (Fig. 3-8). The sample effect on the other growth phases of the cell cycle was very low or less, (Fig. 3-7, 3-9) this phenomenon clearly showed that the ECSP introduced apoptosis formation.

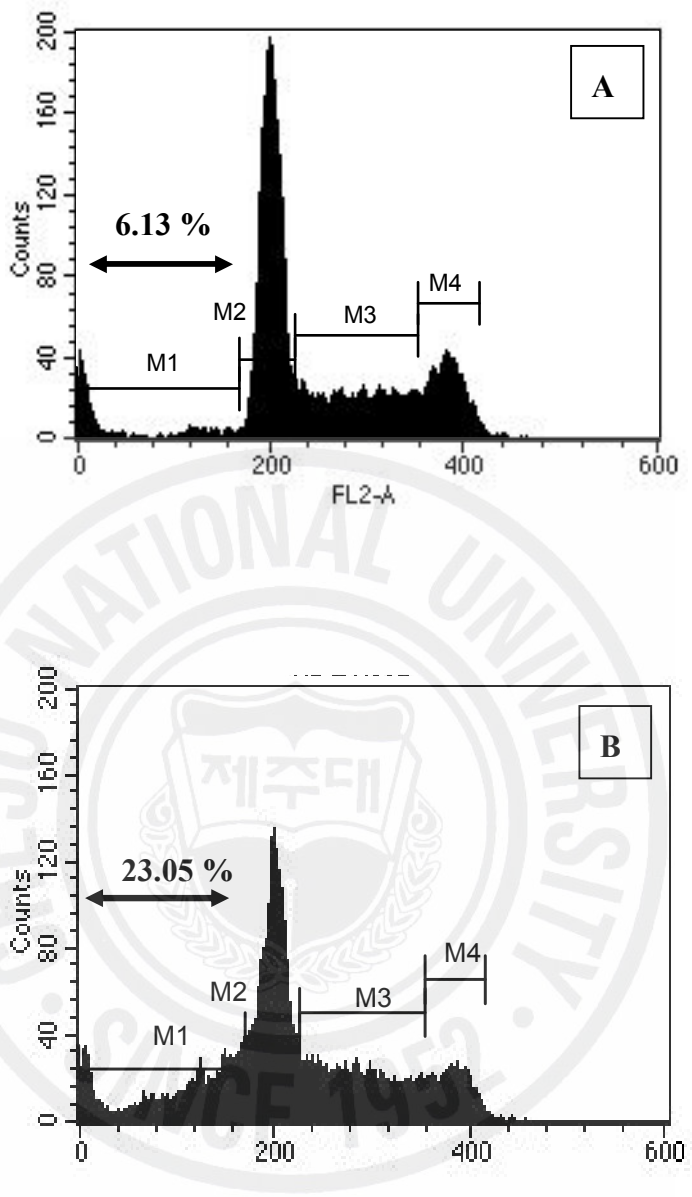

Fig. 3-6. The effect of *E. cava* sulfated polysaccharide on the cell cycle distribution of HL-60 cancer cell line. The analysis of cell cycle distribution was performed on an equal number of cells (1×10^5) by flow cytometry after the staining of DNA by propidium iodide. (A) The effect on the cell cycle after 15 μg/ml of sample, (B) The effect on the cell cycle after 30 μg/ml of sample.

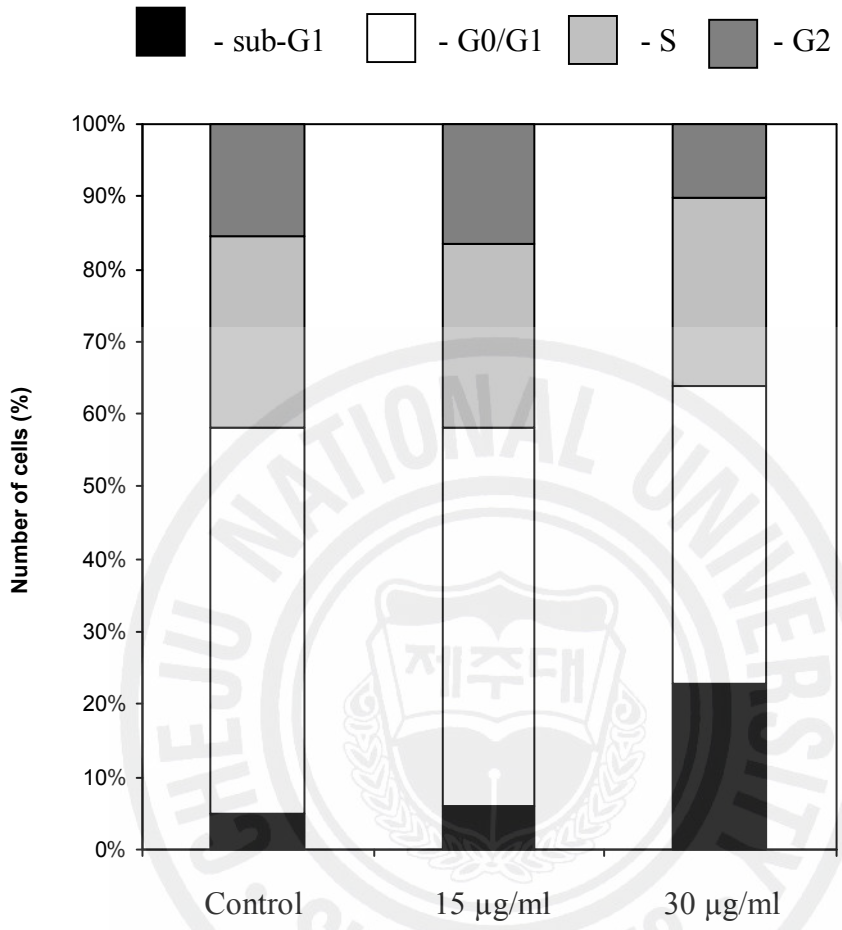

Fig. 3-7. The cell cycle pattern of HL-60 after the treatment of sulfated polysaccharides purified from *E. cava*. Percentages of cells in the sub-G1, G0/G1, S, and G2/M phases were determined using established CellFIT DNA analysis software.

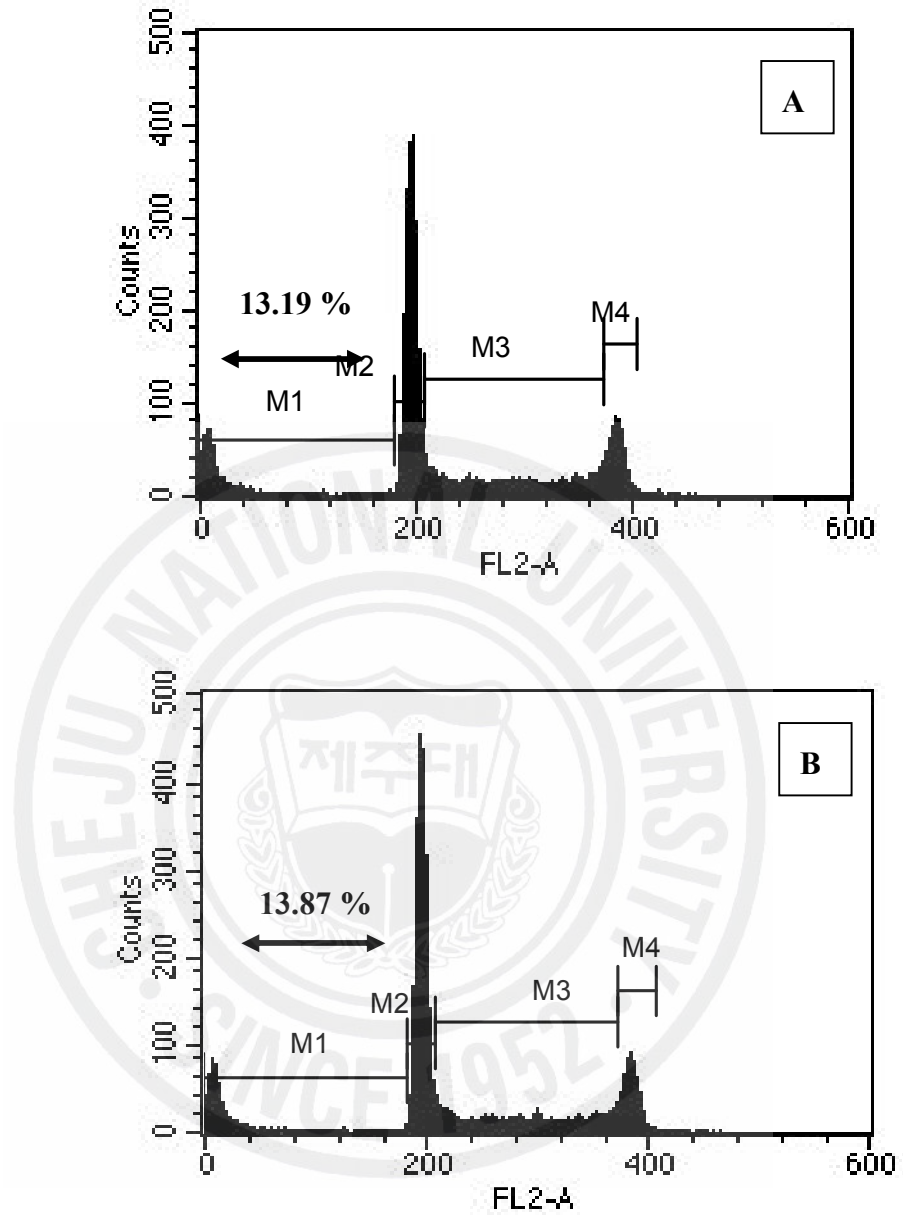

Fig. 3-8. The effect of *E. cava* sulfated polysaccharide on the cell cycle distribution of human leukemic U-937. The analysis of cell cycle distribution was performed on an equal number of cells (1×10^5) by flow cytometry after the staining of DNA by propidium iodide. (A) The effect on the cell cycle after 15 μg/ml of sample, (B) The effect on the cell cycle after 30 μg/ml of sample.

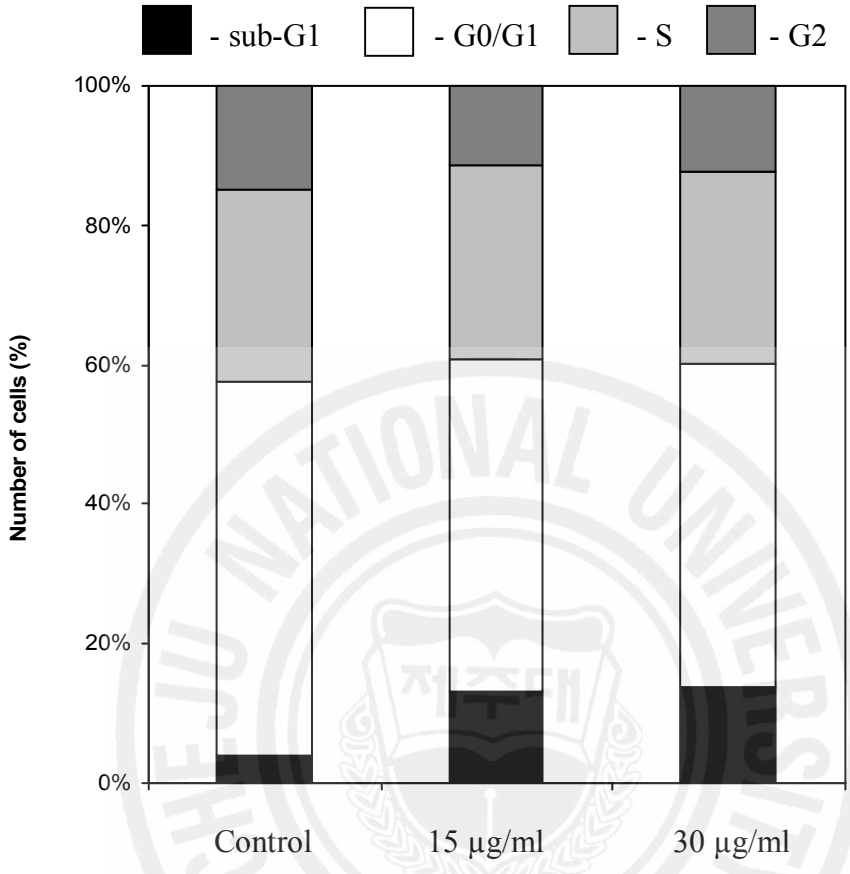

Fig. 3-9. The cell cycle pattern of U-937 after treatment of sulfated polysaccharides purified from *E. cava*. Percentages of cells in the sub-G1, G0/G1, S, and G2/M phases were determined using established CellFIT DNA analysis software.

The normal growth phase of the average cell cycle is depicted in Fig. 3-10. Of the five phases of a cell cycle (mitosis, M; gap1, G1; resting, G0; synthesis, S and gap2, G2) the G1 phase is important and most anticancer drugs target this phase to control the growth of the cancer cells. A commercial fucoidan sample purified from *Fucus vesiculosus* enhanced sub-G1 phase arrest in a time-dependent manner (0 hr, 1.1%; 24 hr, 4.0%; 36 hr, 28.7%; 48 hr, 89.0%) after stimulation with 100 mg/mL fucoidan. Moreover, the results indicate that the fucoidan induced apoptosis through caspase and mitochondrial pathways (Asia et al., 2005).

Taken together, the results of this study clearly demonstrate the ability of the purified sulfated polysaccharide sample of *E. cava* to control the cell proliferation effect of tumor cells. The apoptosis induction of the sulfated polysaccharide is assumed to be the reason for its anticancer effect. In order to clarify, the apoptosis formation of the cultured cells by sulfated polysaccharide, the DNA fragmentation effect of the cells was investigated by agarose gel electrophoresis. DNA fragmentation is a hallmark of the programmed cell death (apoptosis), in this study sample treated cells were harvested after 24hr incubation time period and the cell DNA was extracted. On the agarose gel, a ladder of small fragments of double-stranded DNA was dose-dependently observed and the damage was enhanced with the sample concentration (Fig. 3-12). The control sample where there is no sulfated polysaccharide clearly indicated very low DNA damage, in contrast the cells treated with polysaccharide showed pronounced double-stranded DNA damage dose dependently. As it has been reported, fucoidan extract derived from *Cladosiphon novae-caledoniae kylin* effectively reduced both intracellular and released H_2O_2 of HT1080 cells, which might lead to the suppression of MMP-2/9 expression and subsequent inhibition of the invasive ability.

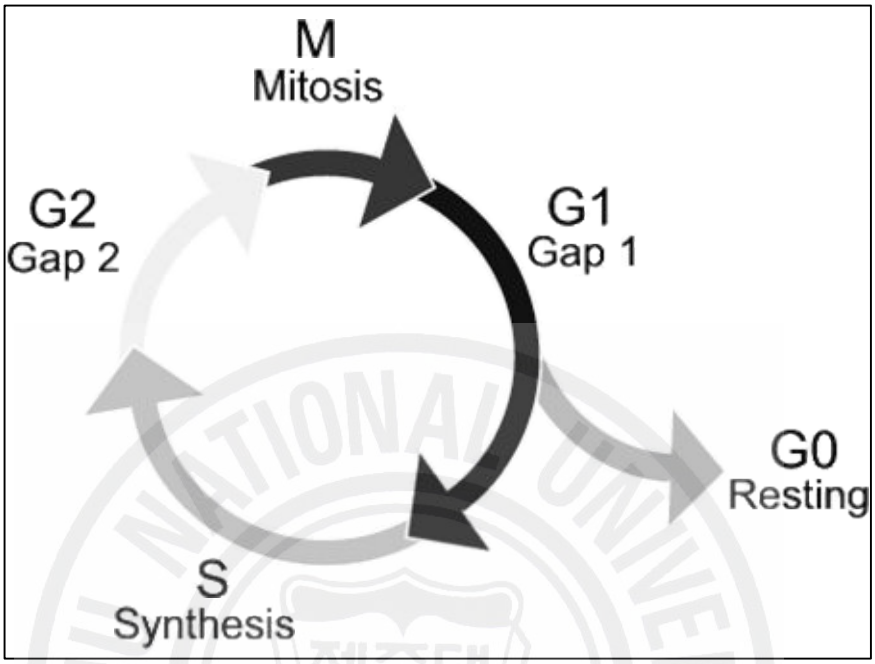

Fig. 3-10. Schematic illustration of the average cell cycle (the various phases of the cell cycle)

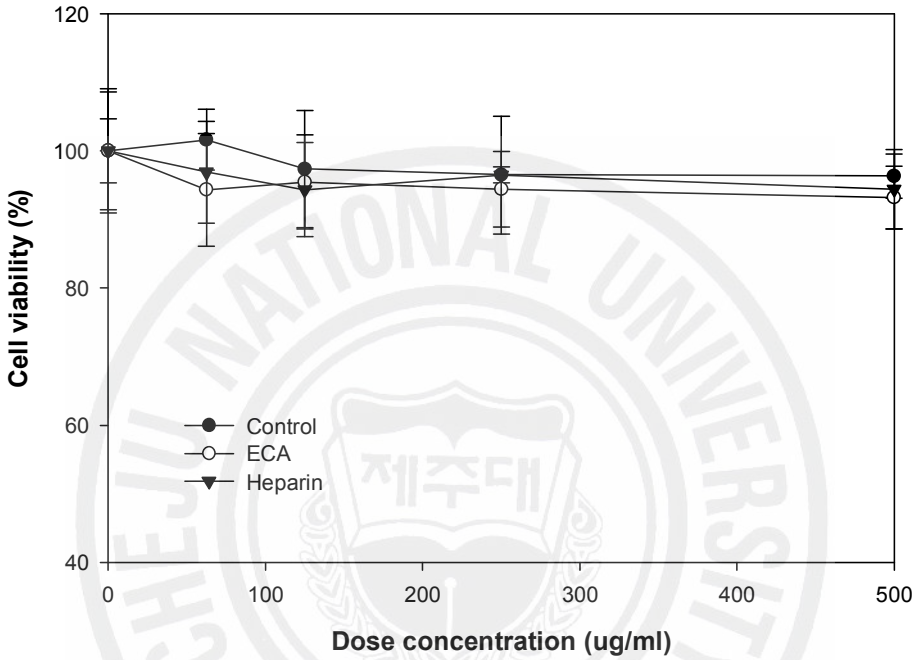

Fig. 3-11. Cytotoxicity of *E. cava* sulfated polysaccharide on venous endothelial cell (ECV-304) in dose-dependant manner. The control means non-treated cells with samples.

Control 30μg/ml 60μg/ml 120 μg/ml

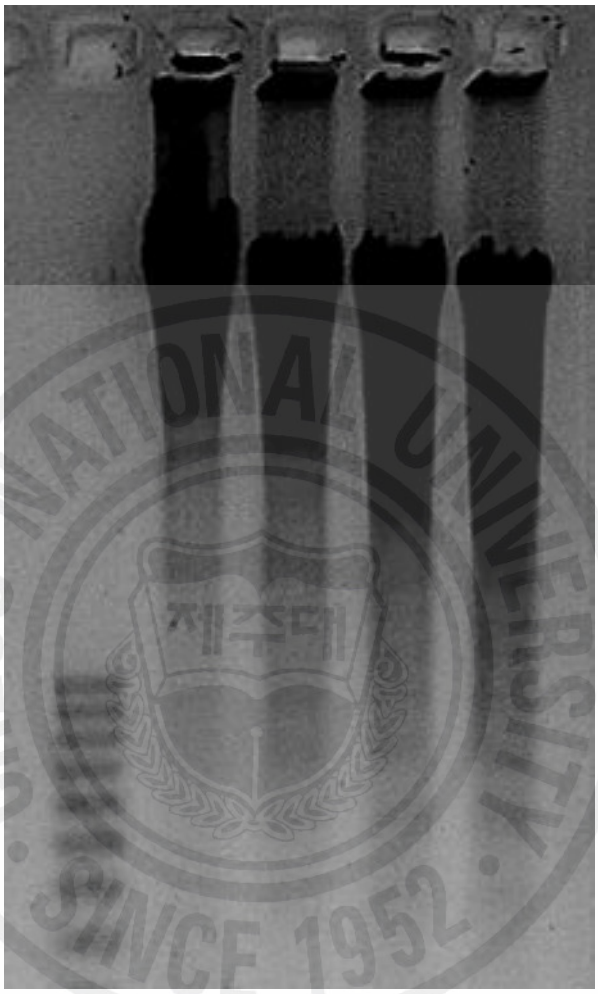

Fig. 3-12. The dose-dependant (30, 60 and 120 μg/ml) effete of the sulfated polysaccharide isolated from *E. cava* on DNA fragmentation of U-973 cells after 24 hr incubation period.

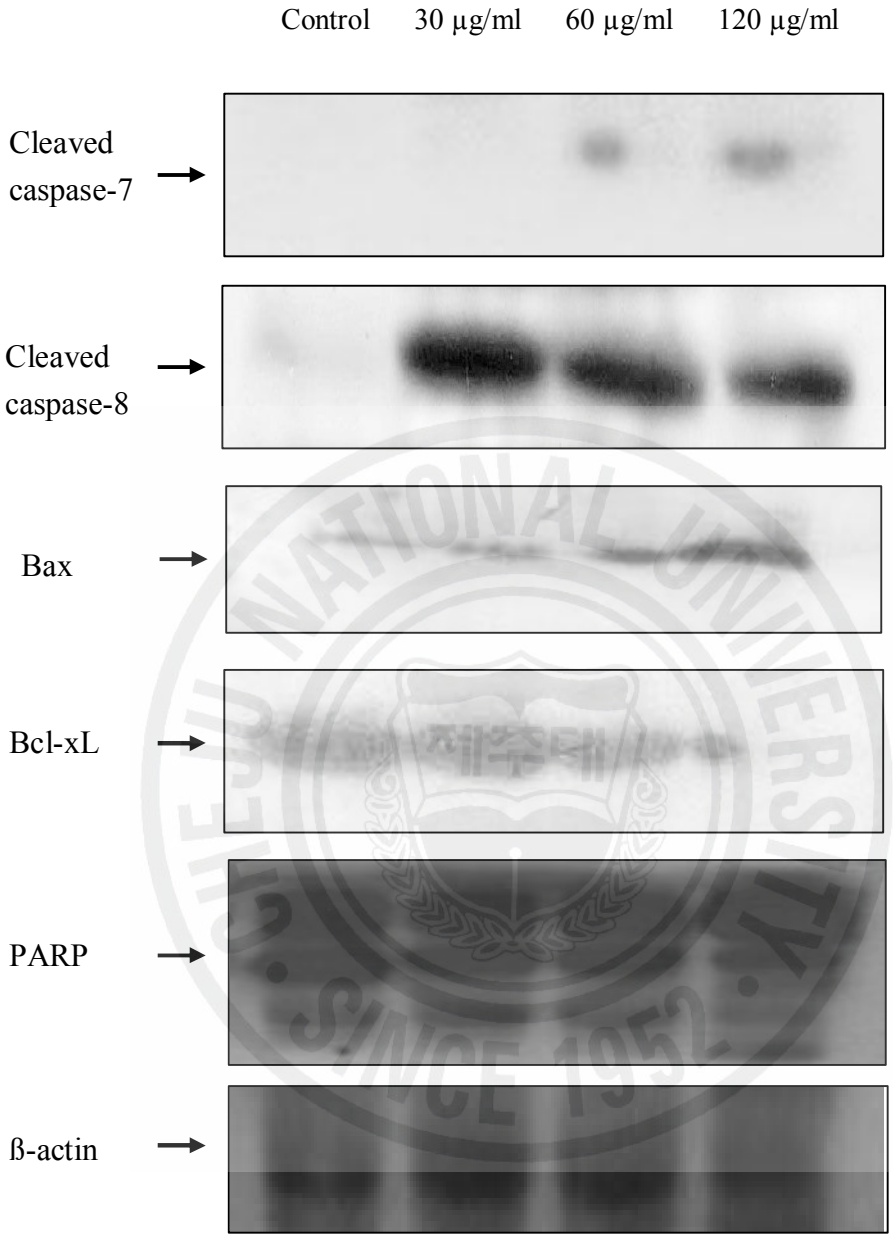

Fig. 3-13. The effect of the sulfated polysaccharide isolated from *E. cava* on the expressions of caspase-7, caspase-8, Bax, Bcl-xL, PARP and ß-actin in U-937 cells treated with various sample concentrations (30 μg/ml, 60 μg/ml, and 120 μg/ml) after 24 hr of incubation.

Moreover the scientists regarding seaweed polysaccharides predicted the ability of fucoidan extract for stimulating antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Ye et al., 2005). Therefore, probably several factors may be associated with the anticancer activity of the sulfated polysaccharide.

Apoptosis induction is followed by the activation of several proteins. In order to evaluate roles of the sulfated polysaccharide from *E. cava* on the expression of cytosolic proteins of U-937 cells some western blot studies were conducted. The results shown in Fig 3-13, the presence of the sulfated polysaccharide had clear effect on the expression of apoptosis related proteins, and this may explain the molecular mechanism of sample for its anticancer effect. PARP (ADP-ribose) is an important compound to maintain the health cells. Upon stress or anticancer activity the latter compound can breakdown into small molecular weight compounds and thereby directly stimulate apoptosis. In this study, when the U-937 cells were treated with the sulfated polysaccharide, a clear PARP breakdown (89 KD) was observed dose-dependently. As it is believed, the cleaved PARP can be taken place due to the activation of cleaved caspase-7 (20 KD), according to western blot results of this study, after 24 hr incubation period and the cleaved caspase-7 was clearly observed in 60 and 120 μg/ml sample treated groups indicating caspase pathway involved for the PARP cleavage. Also, in this study caspase-8 of the U-937 was cleaved by the sulfated polysaccharide treated, the cleaved caspase-8 is able to activate downstream caspase like caspase-7. As it has been observed previously, caspase pathway associated with PARP was regulated by the highly sulfated polysaccharide of *Cladosiphon okamuranus* (Teruya et al., 2007). Moreover, in this study, Bax, a key component of apoptosis, was highly expressed after treatment of the sample, and Bcl-xL which prevented apoptosis was clearly down regulated. Taken together, the western blot result of this study clearly shows signaling

pathway of apoptosis stimulated by ECSP on U-937 cells. However, additional signaling pathways which are related to apoptosis need to be mention to have a clear picture of the sulfated polysaccharide isolated from *E. cava* for its anticancer activity.

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(APPENDIX)

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EDUCATIONAL BACKGROUND

2004.09 - 2007.08	**Ph.D.** Cheju National University, Faculty of Applied Marine Science.
2002.09 - 2004.08	**M.Sc.** Cheju National University, Faculty of Applied Marine Science.
1995.06 - 1999.11	**B.Sc.** (Special Hons): University of Ruhuna, Faculty of Agriculture, Sri Lanka: Second Class Honors.
1990.09 - 1993.05	**Advanced level:** K/Taxila Central Collage, Horana.
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WORKING EXPERIENCES

2001.11- 2002.07	Research Assistant (on contract basis), National Institute of Technical Education (NITE), Sri Lanka.
2001.01- 2001.05	Research Assistant (on contract basis), Industrial Technology Institute (ITI), Sri Lanka.
1999.10 - 2000.06	Research Assistant (on contract basis), University of Ruhuna, Sri Lanka.

AWARDS AND SCHOLARSHIPS

1. Winner of the 2007 foreign student award of Cheju National Univerity.
2. The poster tilted "Identification of molecular interactions between blood coagulation factors and sulfated polysaccharide from *Ecklonia cava* using surface plasmon resonance spectroscopy" was awarded as one of the outstanding posters of the session at Korea-China-Japan international symposium (Asian summit for world foods), June 14-16, 2006, Jeju ICC.
3. NURI fellowship sponsored by regional industry development research program funded by ministry of commerce, Korea (Ph.D).
4. Second place, poster presentation competition at the annual BK21 workshop which was held at Ocean Science Collage, Cheju National University, 2005/ 05/ 31.
5. Brain Korea 21 Fellowship sponsored by the Korean Research Foundation (M.Sc).

CURRENT LIST OF PUBLICATIONS

1. Kim SH, Choi DS, **Athukorala Y**, Jeon YJ, Senevirathne M, Rha K. 2007. Antioxidant activity of sulfated polysaccharides isolated from *Sargassum fulvellum*. Journal of Food Science and Nutrition 12: (In press)
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11. Jeon YJ**, Athukorala Y,** Lee J. 2005. Development of novel technique for high quality agarose production from agar. Algae. 20:61-67
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