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Carbohydrate Polymers 66 (2006) 184–191
Carbohydrate Polymers
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An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*

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Received 13 December 2005; received in revised form 25 February 2006; accepted 1 March 2006
Available online 18 April 2006

Abstract

In this study, seven brown algal species (*Ecklonia cava*, *Ishige okamurae*, *Sargassum fulvellum*, *Sargassum horneri*, *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. According to the polyacrylamide gel electrophoresis, the purified compound showed a high molecular weight. The highly sulfated (0.92 sulfate/total sugar) active sample was mainly composed of fucose and small amount of galactose. 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.

Keywords: Brown algae; *Ecklonia cava*; Anticoagulant activity; Enzymatic hydrolysis; Sulfated polysaccharide

1. 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 & Pereira, 1999). Heparin is a 1,4 linked linear copolymer of glucosamine (2-amino-2-deoxyglucopyranose) 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 (Desai, 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 (Desai, 2004). The potential anticoagulant action of heparin is achieved mainly by potentiation of antithrombin and heparin cofactor II (Pereira, Mulloys, & Mourao, 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; Desai, 2004). Heparin also has a propensity to bind non-specifically to other plasma proteins. Because plasma levels of these heparin-binding proteins

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doi:10.1016/j.carbpol.2006.03.002

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, Water, Lam, Juneau, & Mccans, 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 anticoagulant agents, which can exhibit rapid and predictable onset and offset kinetics are in high demand.

In 1913, scientists investigated blood anticoagulant properties from marine brown algae (Killing, 1913). There is a grater incidence of anticoagulant activity in the extracts of marine brown algae than red and green algae (Chevolvet et al., 1999; Patanker, Oehninger, Barnett, Williams, & Clerk, 1993). As it has been previously reported, it is clear that polysaccharides from seaweeds are good alternative sources for anticoagulative drug production (Church, Meade, Treanor, & Whinna, 1989; Matsubara, 2004; Nishino, Yamauchi, Horie, Nagumo, & Suzuki, 2000). 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, Lee, Song, & Jeon, 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 *Ecklonia 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 *E. cava* and to characterize a purified compound with respect to its anticoagulant activity.

2. Materials and methods

2.1. 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 2004. Salt, sand and epiphytes were removed using tap water. Finally, seaweed samples were rinsed carefully with fresh water and freeze-dried at −20 °C for further experiments. APTT (ellagic + bovine phospholipid) and $CaCl_2$ solution were obtained from International Reagents Corporation (Japan), PT (rabbit thromboplastin) 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, β-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-α-D-glucosidase), Termamyl 120 L (a heat stable α-amylases), Ultraflo L (a heat stable multiactive β-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.

2.2. 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 °C to inactive the enzyme. Each sample was clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. All samples were kept in −20 °C for further experiments.

2.3. 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.

2.4. 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 °C (Kuda, Taniguchi, Nishizawa, & Araki, 2002; Matsubara, Matsuura, Hori, & Miyazawa, 2000).

2.5. 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 × 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 & 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.

2.6. Gel filtration chromatography

Purified sample (10 mg/ml in water) was applied to a Sepharose 4B column (72 × 2 cm) equilibrated and eluted with water at 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.

2.7. 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 1,3-diaminopropane/acetate buffer (pH 9.0) for 1 h at 110 V. The polysaccharide in the gel was fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in water. Polysaccharides were stained after 12 h using 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5 v/v).

2.8. 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.

2.9. 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 × 250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartridge (4.5 × 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.

2.10. Sulfate content analysis

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

2.11. 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.

2.12. 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, Je, Kim, and Kim (2002), Rajapaksha, Jung, Mendis, Moon, and Kim (2005).

3. Results and discussion

In this study, seven brown algal species (*E. cava*, *Ishige okamurae*, *Sargassum fulvellum*, *Sargassum horneri*,

Sargassum coreanum, *Sargassum thunbergii* and *Scytosiphon lomentaria*) were digested with carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo).

All representative results are shown in Table 1. 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 s of APTT activity at 80 μg/ml. Viscozyme, Celluclast and Ultraflo extract of *S. horneri* 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 2, *E. cava* sample that was digested by AMG extract showed the highest APTT activity compared to its other counterparts. In PT assay, almost all tested extracts did not show good activities, however AMG hydrolysate showed 15 s PT value with that of 11 s in control. However, in TT assay AMG hydrolysate showed 124 s 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 carbohydrases 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 carbohydrases (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 hydrolysis 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 3). 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. 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. 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 (Figs. 1A 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. 1B). 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 & Rys, 1999).

Table 1
Anticoagulant activities of brown algae hydrolyzed with carbohydrases

Species	Activated partial thromboplastin time (s), APTT				
	1[a]	2	3	4	5
Ecklonia cava	>300	>300	>300	300	>300
Ishige okamurae	35	32	38	31	35
Sargassum fulvellum	32	35	32	32	33
Sargassum horneri	>300	>300	120	165	>300
Sargassum coreanum	125	152	114	131	115
Sargassum thunbergii	30	35	32	31	36
Scytosipon 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; 5, Ultraflo.

Table 2
Anticoagulant activities of enzymatic hydrolysates of *E. cava*, measured by APTT, PT and TT assays (s)

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 3
Anticoagulant activities of molecular weight fractions of AMG hydrolyzed *E. cava*, measured by APTT, PT and TT assays (s)

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.

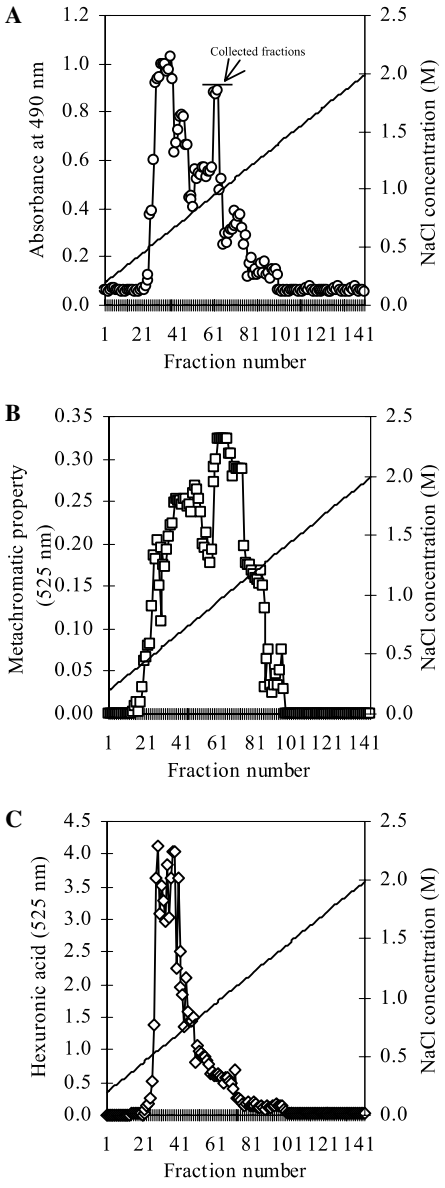

Fig. 1. Purification of the sulfated polysaccharides from the brown alga, *E. cava* by DEAE-cellulose. Fractions of 4 ml were collected and checked by phenol–H_2SO_4 (A) metachromacia using 1,9-dimethylmethylene blue (B) and carbasole reaction (C).

Normally, anticoagulant compounds isolated from brown algae have relatively high metachromatic 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. 2). In re-chromatography experiment the active compound appeared as a very clear and distinct one peak with strong metachromatic property (Figs. 2A and B). Moreover, the final active compound composed with low hexuronic acid content as it was revealed by the carbazole reaction results. After being under vacuum, the dialyzed sample was applied on Sepharose 4B column to purify active compound in terms of molecular mass (Fig. 3). 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. 4 shows the electrophoretic mobility of the purified polysaccharide on agarose gel. The clear one spot on

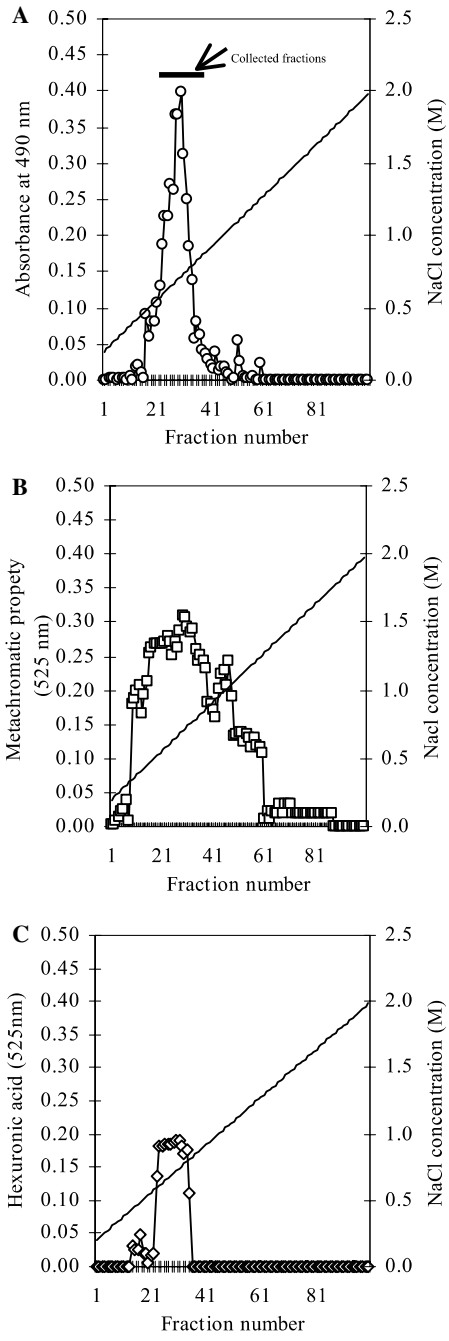

Fig. 2. Re-chromatography of the sulfated polysaccharides from the brown alga, *E. cava* on new DEAE-cellulose. Fractions of 4 ml were collected and checked by phenol–H_2SO_4 (A) metachromacia using 1,9-dimethylmethylene blue (B) and carbasole reaction (C).

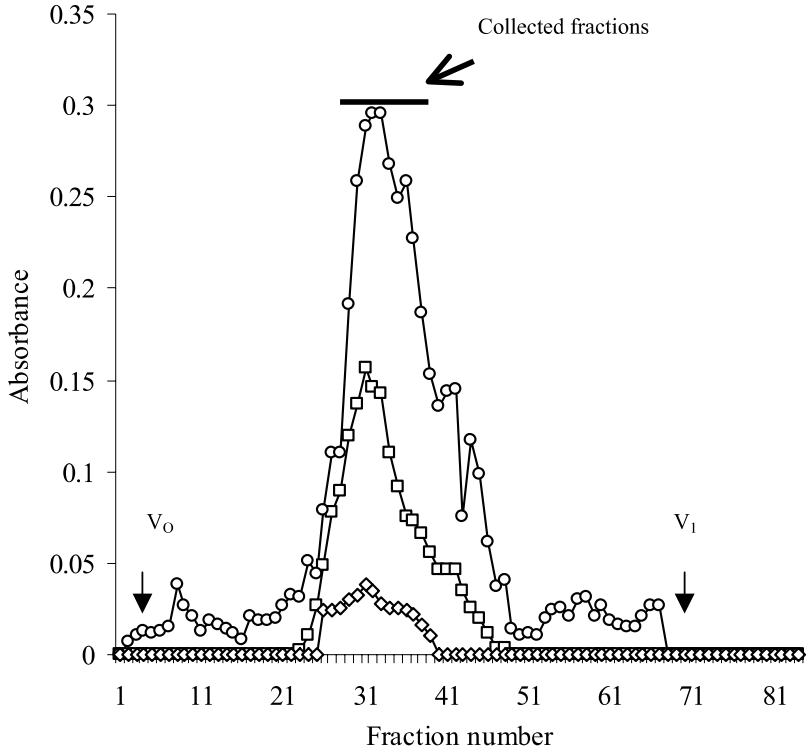

Fig. 3. Purification of the sulfated polysaccharides from the brown alga *E. cava* on Sepharose 4B. Fractions (2 ml) were collected and assayed for metachromatic property at 525 nm, for total polysaccharide contents and for hexuronic acid contents. Carbohydrate content (○), metachromatic property (□), hexuronic acid content (◇), Blue dextran (V_o); Cresol red (V_1).

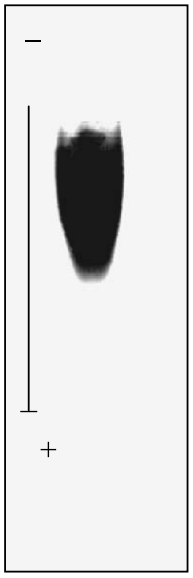

Fig. 4. Agarose gel electrophoresis of the sulfated polysaccharides from *E. cava*.

agarose gel after stained with toluidine 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. 5). 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. 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 & Mody, 2000). However, fucoidan isolated from *Fucus vesiculosus*, with molecular weight of 7.4×10^4 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 fucose, glucosamine, galactose and mannose in approximate molar ratio of 0.81, 0.01, 0.16 and 0.01, respectively (Table 4). The total sulfate content of the tested sample was 0.95 (sulfate/total sugar). Therefore, this compound belongs to the group of fucogalactan sulfate. Moreover this compound contains a slight amount of hexouronic acid. 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 & Mody, 2000).

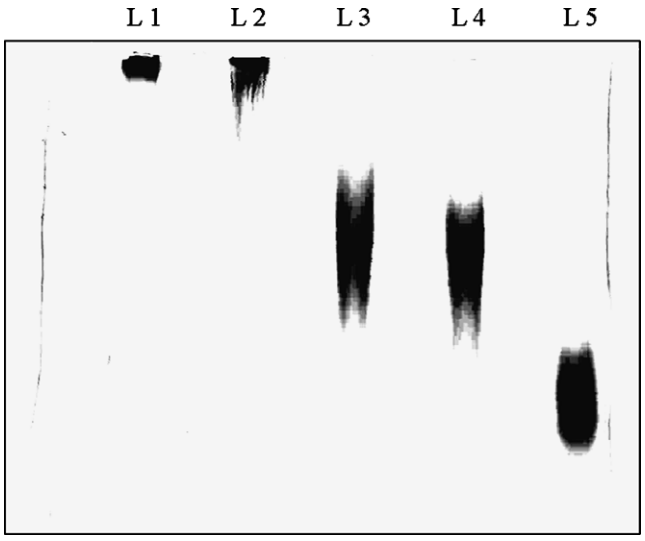

Fig. 5. Polyacrylamide gel electrophoresis of the sulfated polysaccharides from *E. cava*. Purified sample (L1); dextran sulfate, 500 kDa (L2); chondroitin 6-sulfate, 60 kDa (L3); chondroitin sulfate, 50 kDa (L4) and dextran sulfate, 8 kDa (L5).

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 5). 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

Table 4
Sugar constituents and degree of sulfation of sulfated polysaccharide isolated from *E. cava*

Sugar	(%)
Fucose	82.1
Galactosamine	ND
Glucosamine	0.52
Galactose	16.7
Glucose	0.52
Mannose	0.07
Sulfate/total sugar[a]	0.95

ND, not determined.
[a] The mean degree of substitution of sulfate ester per anhydro sugar residue.

Table 5
Comparison of anticoagulant activity of *E. cava* with that of heparin

	Control	Clotting time (s)					
		E. cava anticoagulant (μg/ml)			Heparin (μg/ml)		
		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.

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 & 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.

Blood coagulation pathway involves a series of zymogen (precursor protein) activation reactions. 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, Fujikawa, Kurachi, & Kisiel, 1979). Some natural polysaccharides can interfere with blood coagulation because they inhibit the biological activity of some plasmatic serine proteases either by direct or plasmatic factor interaction. Therefore, to understand the inhibitory mechanism, polysaccharide treated plasma was examined on specific clotting factors. The results of this study are shown in Fig. 6. 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, Magnani, Lamponi, & Albanese, 1996). Therefore, more

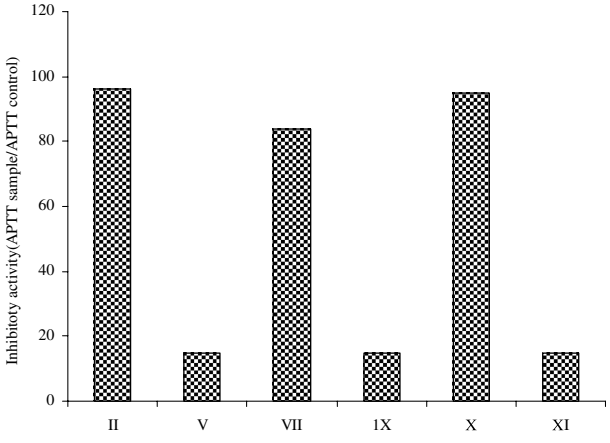

Fig. 6. Specific factor inhibitory pattern of the purified anticoagulant on blood coagulant factors.

intensive studies are needed to understand the anticoagulant behavior of the purified compound of *E. cava*. Hence, surface plasmon resonance (SPR) studies are being investigated to examine the active compound for its high anticoagulant activity.

Acknowledgements

This work was supported by the a grant of Regional Industry Development Research Program funded by Ministry of Commerce, Industry and Taerim Trade Co., Ltd. Also, this work was supported by the New Frontier Education Center for Eco-Friendly Marine Industry in 2005.

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