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Isolation and Purification of Anticoagulant Polysaccharide Compound from Fermented Edible Brown Seaweed, *Laminaria ochotensis*

Chamilani Nikapitiya, Mahanama De Zoysa, Prashani Mudika Ekanayake, Hojin Park and Jehee Lee*
Department of Marine Biotechnology, Cheju National University, Jeju 690-756, Korea

Anticoagulant activities of a fermented edible brown alga, *Laminaria ochotensis* was investigated. *L. ochotensis* was fermented with 15% sugar (w/v) at 25°C for 10 weeks. Anticoagulant activity was measured from the supernatant of algal mixture at biweekly intervals up to 10th week by activated partial thromboplastin (APTT), prothrombin time (PT) and thrombin time (TT) assay using citrated human plasma. Sample having high APTT activity (6th week) was filtered, ethanol precipitated and freeze-dried. The polysaccharide compound having anticoagulant activity was purified by DEAE ion exchange chromatography followed by Sepharose-4B gel filtration chromatography. Anticoagulant activity, polysaccharide concentration, and heparin like activity were determined for the collected fractions by APTT, phenol-H_2SO_4, and glycosaminoglycan assay, respectively. The anticoagulant activity assay showed that the activity was increased up to 6th week, and decreased thereafter. The concentration of our purified compound was 31.0 μg/ml and showed higher APTT activity than commercial heparin. At the same concentration of 31.0 μg/ml, the heparin showed 186.5 sec activity while our purified compound showed an activity of 386 sec. Single spot on agarose gel electrophoresis showed that the compound was purified and polyacrylamide gel electrophoresis (PAGE) results revealed that the molecular mass of the purified polysaccharide compound was between 60 and 500 kDa. Therapeutic interest of the algal polysaccharide as an anticoagulant has recently been in highlighted. This purified anticoagulant compound from fermented *L. ochotensis* can be used as a model for anticoagulant agent or could be developed as an anticoagulant agent. This study can be extended to identify the structure and chemical composition of the purified polysaccharide, and to establish a relationship between structure and the function of the identified anticoagulant compounds.

Keywords: *Laminaria ochotensis*, Anticoagulant activity, Fermentation

Introduction

Heparin, a highly sulfated polysaccharide present in mammalian tissues is used as a blood anticoagulant in laboratories as well as in therapeutics. The disadvantages occurred with heparin give rise to a new area of research for discovering novel substances with blood anticoagulant activity. Polysaccharides of plant origin have emerged as an important class of natural bioactive products and their blood anticoagulant, antimutagenic, anti-viral, anti-complementary and anti-inflammatory properties (Srivastava and Kulshreshtha, 1989) have been reported in recent years. Blood anticoagulant, antithrombic, fibrinolytic and platelet aggregation activities of several red and brown algal sulfated polysaccharides (SPS) have been reported and responsible structural compounds have been identified as sulfated galactans and sulfated fucans respectively (Grauffel et al., 1989). The anticoagulant activity of marine algae was first reported in 1936. The existence of structural similarities between SPS from marine algae and heparin has also been reported (Chargaff et al., 1936). However, the mechanism by which the algal fucans exerts their anticoagulant action remains controversial. Mechanism related to both antithrombin and heparin cofactor II-mediated activity has been described by many scientists for algal-sulfated fucans from different species (Church et al., 1989; Colliec et al., 1991). The action of heparin as plasma anticoagulant is the potentiation of the rates of inhibition of the activated clotting factors such as thrombin, factors XIIa, XIa, Xa, and IXa by antithrombin III (Kurachi et al., 1976; Vehar and Davie, 1977).

Brown seaweeds (Phaeophycophyta) produce different kinds of polysaccharides namely alginates, fucoidans and laminarans. Fucans are major sulfated polysaccharides of the brown seaweed cell wall and mainly composed of α-(1-3) and α-(1-4) fucosyl units and mostly sulfated at positions 2 and 3 with branching sulfate or additional monosachcharides at free positions (Chevolot et al., 1999). Fucoidans usually contains large pro-

*Corresponding author: jehee@cheju.ac.kr

portions of L-fucose and sulfate, with minor quantities of xylose, galactose, mannose and guluronic acid. The anticoagulant activity of polysaccharides is improved chemically by increasing the degree of sulfation (Shanmugam and Mody, 2000). Moreover, the composition of algal fucans varies according to species (Dietrich et al., 1995), extraction methods (Grauffel, 1989), climatic conditions and season of harvest (Percival and Mcdwell, 1967). Different techniques for extracting of fucoidans such as cold and hot water, mild acids, $CaCl_2$ (Pericival, 1979) and enzymes (Albuquerque et al., 2004) are used to extract of anticoagulant SPS from brown algae.

Fermentation involves the breaking down of complex organic substances into simpler ones and finally forms many end products such as lactate, acetate, ethanol, carbon dioxide (CO_2), hydrogen molecules (H_2) etc. The microbial enzymes play an active role in fermentation processes, and one or more of these end products formed by the action of one or more microorganism, either working together or in a sequence (Atlas, 1995). Few studies on seaweed fermentation products such as methane, organic acids or volatile compounds during seaweed fermentation have been reported (Uchida and Muata, 2004). However, screening of bioactive compounds from seaweed fermentation has not yet been studied.

Purpose of the present study was to isolate and purify an anticoagulant compound from fermented edible marine brown seaweed, *Laminaria ochotensis*.

Materials and Methods

L. ochotensis a marine brown alga was a gift from Prof Y. J. Jeon, Marine Bio-resource technology lab, Cheju National University, South Korea. The seaweed samples were washed with tap water to free it from sand, epiphytes and other contaminating materials. It was freeze-dried, powdered and stored at -70°C for further analysis.

Prothrombin time (PT) reagent, activated partial thromboplastin time (APTT) reagent, thrombin time (TT) reagent and 0.025 M calcium chloride ($CaCl_2$) solution were purchased from Fisher Scientific Co (Middletown, USA). Commercial heparin (183 USP unit/mg), Sepharose-4B and diethyl amino ethyl (DEAE) were purchased from Sigma Co. (St. Louis, USA). All the other reagents used were analytical grade as commercially available.

Preparation of fermented alga sample

Freeze dried *L. ochotensis* (1.5 g) was mixed with 15% sugar (w/v) in 300 ml water and kept at 25°C for 10 weeks in glass jars in an incubator. For further experiments, after shaking, samples (10 ml) were drawn from the supernatant of algal mixture for 10 weeks and before to the commencement of the fermentation process and during the fermentation, biweekly. Ethanol content (%) was measured by hand held refractometer (ATAGO Co., LTD., Honcho, Itabashi-ku, Tokyo, Japan).

Yield test

Yield was determined by oven drying the crude sample (1 ml) with and without sugar at 105°C for 6 +/-2 h in a pre-weighed aluminium plate. After reducing the sugar content, the weight difference was expressed as yield (mg/ml).

Anticoagulation assays

All coagulation assays (APTT, PT, and TT) were performed with coagulation machine (Dual-channel 1 clot-2, Seac, Italy). Human plasma was collected from healthy individual donors into conical tubes with 2.5% sodium citrate solution (9 parts of blood : 1 part of sodium citrate). The plasma was separated from blood cells by centrifuging at 3,000×g at 4°C for 20 min and was stored at –70°C for future use. All coagulation assays were carried out according to manufacturer's specification (Pacific Heomeostat Co.). For APTT assay, 90 μl of citrated human plasma was mixed with 10 μl of fermented sample (yield is given in Table 1) and incubated at 37°C for 1 min. After that, 100 μl of APTT reagent was added into the mixture and incubated for 5 min at 37°C. Clotting time was recorded after adding 0.025 M of 100 μl $CaCl_2$ solution. For the PT assay, 90 μl of citrated human plasma was mixed with 10 μl of fermented sample and incubated at 37°C for 10 min. After adding 200 μl PT reagent, clotting time was recorded. To determine TT, 190 μl of human plasma was mixed with 10 μl of the fermented sample and incubated for 2 min at 37°C. The clotting time was determined after inducing the clotting by adding 100 μl TT reagent.

Polysaccharide extraction

Based on the results of anticoagulant assays, sample having high activity (6th week) was selected for purification. The crude sample was filtered through Watmann No. 1 filter paper and filtrate was subjected to precipitation with absolute ethanol (99.0%) and kept for 24 h at 4°C. The precipitate was collected by centrifugation at 10,000×g for 20 min at 4°C and freeze-dried. The supernatant was removed after testing for the anticoagulant activity.

Table 1. Anticoagulant activity and yield of *L. ochotensis* from 1st-10th week of fermentation period. Anticoagulant activity was measured at biweekly intervals up to 10th week by activated partial thromboplastin (APTT), prothrombin time (PT) and thrombin time (TT) assay using citrated human plasma. The activity was expressed by seconds (sec). Weights are in dry matter basis and yield was expressed as mg/ml. Data were average of two experiments

Sample	Fermentation period (weeks)	Yield mg/ml	Anticoagulant activity (sec)		
			APTT	PT	TT
L. ochotensis	0	2.41	89.0	18.1	100.0
	2	2.44	102.0	18.7	*>1000.0
	4	2.45	106.0	19.3	*>1000.0
	6	2.47	152.5	23.0	*>1000.0
	8	2.29	103.6	33.0	612.0
	10	1.92	67.6	30.0	201.0
Control (water)			41.7	11.4	12.3

*More than 1000 sec activity.

Purification of anticoagulant compound

The 80 mg of freeze dried precipitate was dissolved in 1.5 ml of 50 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl and applied to a DEAE column (1×18 cm) equilibrated with 500 ml of 50 mM sodium acetate (pH 5.0). The column was washed with 200 ml of same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate of 0.2 ml/min with a linear gradient of 0.2–2.0 M sodium chloride in the same buffer. Fractions of 4 ml were collected and measured anticoagulant activity by APTT, total polysaccharide by phenol-H_2SO_4 test (by measuring the absorbance at 490 nm) (Dubois et al., 1956; Chaplin, 1994) and heparin like activity by glycosaminoglycan test (by measuring the absorbance at 540 nm) (Farndale et al., 1986). Fractions showing high anticoagulant activity were combined, dialyzed against distilled water, and concentrated to 1 ml by rotar evaporation at 40°C. The concentrated sample was subjected to gel filtration chromatography on a sepherose column (2×45 cm) equilibrated with distilled water using fast performance liquid chromatography (ProTeam LC Gradient System, Isco Inc., USA). The elution was performed at a flow rate of 0.3 ml/min with distilled water. Fractions (3 ml) were collected and performed anticoagulant activity, glycosaminoglycan and phenol-H_2SO_4 tests. Fractions showing high anticoagulant activity were pooled, concentrated and freeze dried.

Agarose gel electrophoresis

Purified polysaccharide (10 μg) was applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3–diaminopropane/acetate buffer (pH 9.0), using dextran sulfate sodium salt from *Leuconostoc* spp. (8 kDa) as the standard to find out the degree of purity of the compound (Pereira et al., 1999). The polysaccharide in the gel was fixed with 0.1% N-acetyl-N,N,N-trimethylammonium bromide solution and after 4 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v).

Determination of molecular weight of the purified compound

The molecular mass of the purified polysaccharide was estimated by polyacrylamide gel electrophoresis (PAGE). The sample (10 μg) was applied to a 1 mm thick 6% polyacrylamide gel slab in 0.02 M sodium barbital (pH 8.6). After electrophoresis (100 V for 30 min), the gel was stained with 0.1% toluidine blue in 1% acetic acid and then for about 4 h washed in 1% acetic acid. The molecular weight of the markers used were 20 kDa (chondroitin sulfate B. sodium salt), 60 kDa (chondroitin 6 sulfate sodium salt from shark cartilage) and 500 kDa (dextran sulfate sodium salt).

Results and Discussion

Anticoagulant activity

Anticoagulant activities were measured biweekly from 0 to 10th week by APTT, PT, and TT assays during fermentation of *L. ochotensis* (Table 1). Fermented samples showed higher APTT, PT and TT values compared to control (water). Values obtained for APTT, PT and TT for the control were 41.7, 11.4 and 12.3 sec, respectively. It has been reported that the sensitivities of these three techniques of anticoagulant tests are different from each other (Siddhanta et al., 1999).

It showed that the APTT activity increases with the fermentation time from 0 to 6th week and decreases there after (Table 1). The APTT values of fermented seaweed sample on 0 and 6th week were 89 sec and 152.5 sec, respectively and total polysaccharide content were 232.14 μg/ml and 227.3 μg/ml, respectively. Prolongation of APTT suggests that the

active anticoagulant compound of fermented *L. ochotensis* was able to inhibit the intrinsic and/or common pathways of the coagulation cascade of human body, excluding factor VII. On 0 week the anticoagulant activity was higher than the control and this may be due to the water soluble compounds responsible for the anticoagulant activity. Increased values of the anticoagulant activity may be due to enzymatic breakdown of the seaweed by microorganisms, which involved during fermentation processes. They may break down the complex polysaccharide compounds to smaller units with special structures (Yang et al., 2002; Yoon et al., 2002). The decreasing values with increasing time may be due to further breaking down of those to smaller units of polysaccharides. Moreover, similar pattern was obtained in yield estimation (Table 1) during fermentation process. The ethanol content of the fermented seaweed sample was increased from 0 to 6th week (0% to 9.0%) and decreased thereafter (no data indicated). Production of ethanol is an indicator of fermentation process (Atlas, 1995) and it indicates that fermentation has been occurred and optimized during the period of 6th week.

In the present study, TT values from 0 to 10th week varied from 100 to >1000 sec (Table 1) and prolongation of TT indicates the inhibition of thrombin activity or fibrin polymerization (Matsubara et al., 2000) by active compounds from the seaweed. TT is a simple screening technique for the fibrin polymerization process and it is a function of fibrinogen concentration and sensitive to heparin and direct thrombin inhibitors. It has been reported that sulfate rich and uronic acid poor fractions in fucose containing SPS showed relatively high anticoagulant activities compared to fractions with high uronic acid and poor sulfate contents when tested with TT and APTT assays (Shanmugam and Mody, 2000).

PT is used as a screening tool and quantitative test for coagulation factors in the extrinsic and common pathways. PT measures the presence and activity of five different blood clotting factors (factor I, II, V, VII, and X). In the present study, PT assay showed higher values compared to control and values varied from 18.1 to 30 sec during the fermentation period from 0 to 10th week (Table 1). Therefore, it can be predicted that fermented *L. ochotensis* has potential activity for prolonging the prothrombin time in normal human citrated plasma. Moreover, prolongation of PT may indicate that active compound of *L. ochotensis* inhibit one or more activity of blood clotting factor. Even though anticoagulant isolated from a marine green alga, *Codium cylindrical* significantly prolonged the TT and APTT using normal human plasma, no activity was observed in PT assay at the same concentration of 15 μg/ml (Matsubara et al., 2001).

Therefore, we can predict that the active polysaccharide fraction of this fermented alga inhibit intrinsic and/or common pathways as well as extrinsic pathway of the blood coagulation cascade and the thrombin activity or conversion of fibrinogen to fibrin. APTT is useful, simple and versatile test as a screening tool and a quantitative test for the intrinsic coagulation factors, we selected APTT assay to measure anticoagulant activity on proceeding experiments.

Isolation and purification of the anticoagulant

Purification of the anticoagulant was achieved by two steps. The first step was done using DEAE ion exchange chromatography (Fig. 1A). DEAE chromatography separated the eluted polysaccharides into a single major peak from the column with linear gradient of 0.2–2.0 M sodium chloride. The anticoagulant activity of each fraction was determined by measuring the clotting time of plasma as calculated by APTT. Fractions F81, F82 and F83 showed higher APTT values compared to other eluted fractions and values were 91.6, 309.0 and 139.0 sec, respectively (Fig. 1B). Those fractions had high polysaccharide content, when measured by phenol-H_2SO_4 method and the fractions showed strong metachromasia produced with 1,9-dimethyl methylene blue, when measured at 540 nm (Fig. 1A). Moreover, those active fractions were pooled (12.0 ml) and concentrated to 1 ml (209.5 μg/ml). The concentrated sample showed APTT value >1000 sec and was subjected to further purification by sepherose-4B gel filtration chromatography.

Sepherose-4B gel filtration chromatography showed a single peak of polysaccharide (Fig. 2A) and elution at initial stage of the chromatography showed that the purified polysaccharide may be a compound with a high molecular weight. The fractions from F8 to F12 showed higher polysaccharide content (Fig. 2A) than the other fractions and fractions from F8 to F11 showed APTT activity ranging from 60.0 to 92.0 sec (Fig. 2B). Further, these fractions (F8 to F11) showed strong metachromasia produced with 1,9-dimethyl methylene blue (Fig. 2A) and those fractions were pooled (12 ml). Pooled fractions were concentrated to 2.7 ml (31.0 μg/ml) and it showed 386 sec of APTT activity. The resulted strong metachromasia of the fractions may indicate the presence of heparin like activity in the purified polysaccharide compounds as 1,9-dimethyl methylene blue assay is used to determine the heparin like activity. Several brown seaweeds such as *Hizikia*

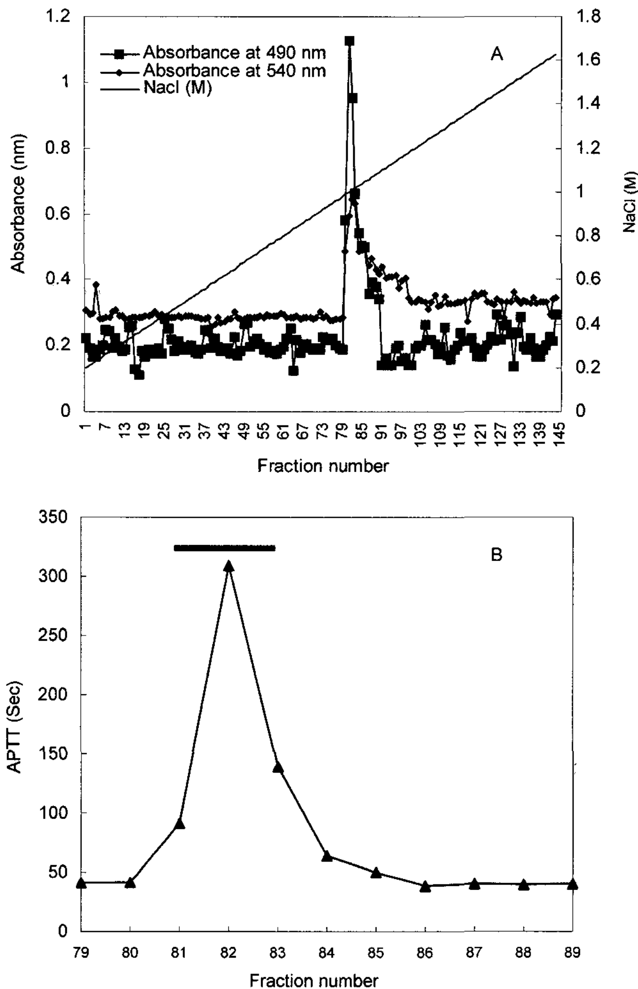

Fig. 1. Purification of anticoagulant polysaccharide from *L. ochotensis* by anion exchange chromatography on DEAE with a linear gradient of 0.2–2.0 M sodium chloride (–) containing 50 mM sodium acetate (pH 5.0) (A) and anticoagulant activity of the fractions giving high absorbance for both phenol-H_2SO_4 assay and glycosaminoglycan assay after anion exchange chromatography (B). **A**, Fractions were collected and assayed total polysaccharide content by phenol-H_2SO_4 method (by measuring the absorbance at 490 nm) (■), heparin like activity using glycosaminoglycan test (by measuring the absorbance at 540 nm) (◆) and **B**, anticoagulant activity was measured by APTT assay (▲) and activity was reported by seconds (sec). The fractions showing high anticoagulant activity (F81-F83) indicated by bold bar were pooled, dialyzed against distilled water and concentrated.

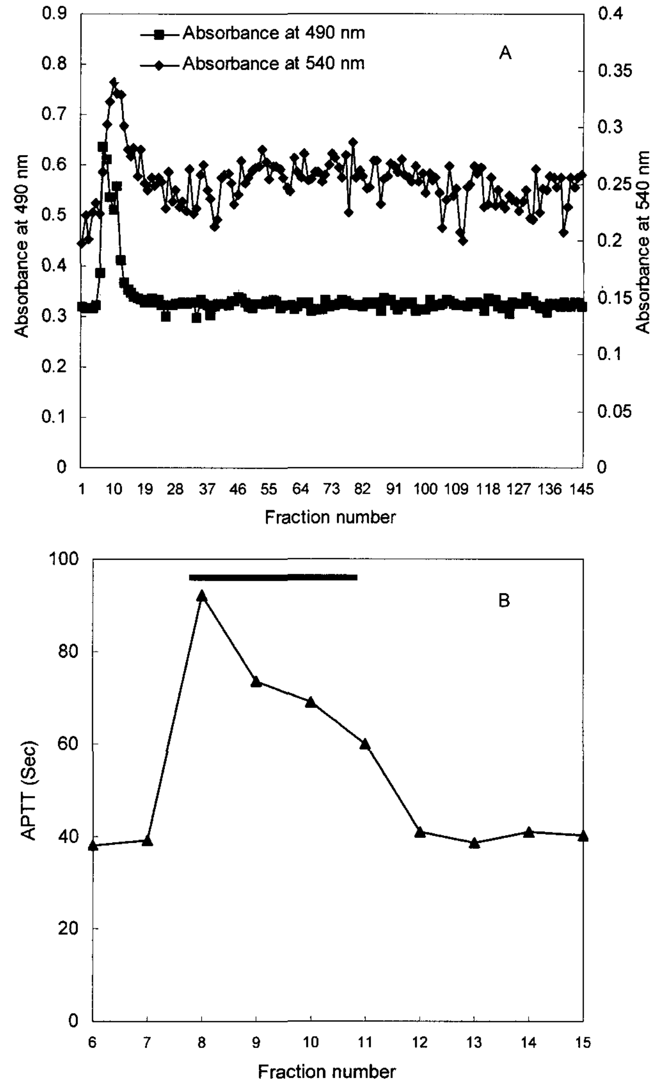

Fig. 2. Gel filtration chromatography of the anticoagulant polysaccharide from *L. ochotensis* (chromatographed fractions of F81-F83 from Fig. 1) on sephrose -4B column (A) and anticoagulant activity of the fractions giving high absorbance for both phenol-H_2SO_4 assay and glycosaminoglycan assay after gel filtration chromatography (B). **A**, Fractions were collected and assayed total polysaccharide content by phenol-H_2SO_4 method (by measuring the absorbance at 490 nm) (■), heparin like activity using glycosaminoglycan test (by measuring the absorbance at 540 nm) (◆) and **B**, anticoagulant activity was measured by APTT assay (▲) and activity is reported by seconds (sec). The fractions showing high anticoagulant activity (F8-F11) indicated by bold bar were collected, pooled, concentrated and used as purified anticoagulant.

fusiforme, Laminaria angutata, L. religios and *Eisenia bicyclis* containing fucoidans (pure fucans) showed considerably high anticoagulant activity (Shanmugam and Mody, 2000). Finally, 83.7 μg of polysaccharide was purified from 80 mg of ethanol precipitated freeze-dried fermented seaweed sample (purified yield was 0.1%). The concentration of our purified compound was 31.0 μg/ml and it showed higher APTT activity than commercial heparin (Table 2). At the same concentration of 31.0 μg/ml, the heparin showed 186.5 sec activity while our purified compound showed an activity of 386 sec. This shows that purified anticoagulant compound contain stronger activity than heparin. Moreover, similar activity was shown in sargassan, which is a sulfated polysaccharide composed of galactose, mannose, xylose, fucose, glucouronic acid and sulfate. Due to the presence of high uronic acid content, the polysaccharide was shown to have higher anticoag-

Table 2. APTT activity values of commercial heparin and purified anticoagulant compound after sepherose–4B chromatography at different concentrations

Concentration (μg/ml)	Anticoagulant activity (sec)	
	Commercial heparin	Purified compound
15.0	72.2	133.4
31.0	186.5	386.0

ulant activity than heparin (Abdel-Fattah et al., 1974). Similar high anticoagulant activity has been reported for highly purified fucan sulfates from *Padina tetrastromatica, P. pavonia, Eisenia bicyclies and Dictyota dichotoma* (Shanmugam and Mody, 2000). Therefore, algae are good alternative source for the production of anticoagulative drugs. In contrast to our results, an anticoagulant proteoglycan purified from the *C. pugniformis* showed weaker anticoagulant activity than that of heparin (Matsubara et al., 2000).

Agarose gel electrophoresis and polyacrylamide gel electrophoresis (PAGE)

Agarose gel electrophoresis is used to determine the degree of purity in purified compound and single spot on gel electrophoresis confirmed that the compound was purified (Fig. 3A). PAGE was carried out to determine the molecular weight of the purified anticoagulant compound and it revealed that the molecular mass of the purified polysaccharide compound is between 60 and 500 kDa (Fig. 3B). The molecular weight of hot water extracted anti-viral sulfated polysaccharide component from brown alga *Sargassum patens* and an acidic polysaccharide of brown algae *Spathglossum schreoderi* were 424 kDa (Wen et al., 2003) and 21 kDa respectively (Leite et al., 1998). Moreover, molecular size of potent anticoagulant fucan sulfate components is approximately 50-100,000 Da, and fractions with a high molecular size (>850,000 Da) showed lower activity (Grauffel et al., 1989).

The present work was carried out as a preliminary study to identify and purify the anticoagulant compound from *L. ochotensis* with non-specific bacteria, which was naturally living in the fermentation medium (natural fermentation). This study could be extended to identification of chemical composition and structure of the purified polysaccharide, and to establish the relationship between structure and the function of the identified anticoagulant compound. Further, bacteria responsible for the fermentation of *L. ochotensis* also could be isolated and identified. The technique of fermentation is an inexpensive process and the therapeutic interest of the algal

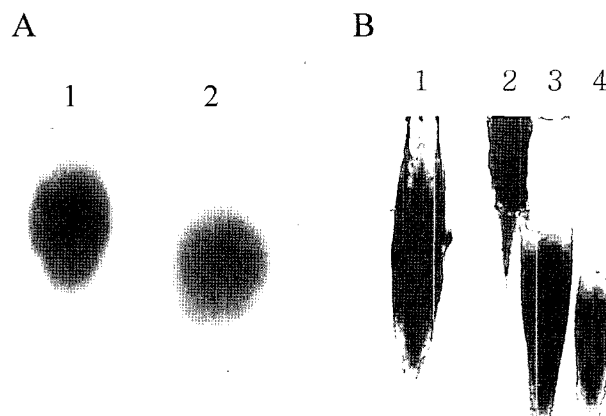

Fig. 3. Agarose and polyacrylamide gel electrophoresis of the purified polysaccharide obtained from *L. ochotensis*. A. Purified polysaccharide (10 μg) was applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0). Lane 1-purified polysaccharide, Lane 2-dextran sulfate sodium salt from Leuconostoc spp. (8 kDa) was used as the standard. The polysaccharide in the gel was fixed with 0.1% N-acetyl-N,N,N-trimethylammonium bromide solution and after 4 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v). **B.** The sample (10 μg) was applied to a 1 mm thick 6% polyacrylamide gel slab in 0.02 M sodium barbital (pH 8.6). The gel was stained with 0.1% toluidine blue in 1% acetic acid. Lane 1-purified polysaccharide, 60-500 kDa. Lane 2-4, markers (2-chondroitin sulfate B sodium salt, 500 kDa; 3-chondroitin 6 sulfate sodium salt from shark cartilage, 60 kDa; 4-chondroitin sulfate B sodium salt, 20 kDa were used as standards for molecular mass determination.

polysaccharide as an anticoagulant has recently been in highlighted. This purified anticoagulant compound from fermented *L. ochotensis* can be used as a model for anticoagulant agent. Moreover, it could be developed as an anticoagulant agent and could yield a greater potential in the field of medical and in pharmaceutical industry.

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