

## A Novel Anticoagulant Protein from *Scapharca broughtonii*

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An anticoagulant protein was purified from the edible portion of a blood ark shell, *Scapharca broughtonii*, by ammonium sulfate precipitation and column chromatography on DEAE-Sephadex A-50, Sephadex G-75, DEAE-Sephacel, and Biogel P-100. *In vitro* assays with human plasma, the anticoagulant from *S. broughtonii*, prolonged the activated partial thromboplastin time (APTT) and inhibited the factor IX in the intrinsic pathway of the blood coagulation cascade. But, the fibrin plate assay did not show that the anticoagulant is a fibrinolytic protease. The molecular mass of the purified *S. broughtonii* anticoagulant was measured to be about 26.0 kDa by gel filtration on a Sephadex G-75 column and SDS-PAGE under denaturing conditions. The optimum activity in the APTT assay was exhibited at pH 7.0-7.5 and 40-45°C in the presence of Ca<sup>2+</sup>.

**Keywords:** Purification, Anticoagulant protein, *Scapharca broughtonii*, Factor IX

### Introduction

Blood coagulation is processed by coagulation factors in order to stop the flow of blood through the injured vessel wall whenever an abnormal vascular condition and exposure to non-endothelial surfaces at sites of vascular injury occur (Guyton, 1982; Grey and Meyer, 1988). As endogenous or exogenous anticoagulants interfered with the coagulation factors, the blood coagulation can be prolonged or stopped (MacFarlane, 1964; Davie and Ratnoff, 1964; Esmon, 2000). These anticoagulants have been used as convenient tools for the exploration of the complex mechanisms of coagulation cascade. Coincidentally, the importance of research for anticoagulants also arose with therapeutic purposes, for example, a cure for hemophilia. While it was studied for various exogenous anticoagulants (Woo *et al.*, 1996; Waidhet-Kauadio *et al.*, 1998; Kim *et al.*, 1999), anticoagulants from

marine organisms have rarely been isolated, except for several anticoagulant proteoglycans and polysaccharides from marine algae (Chargaff *et al.*, 1936 Kindness *et al.*, 1980; Maimone and Tollefsen, 1990; McLellan & Jurd, 1991; Jurd *et al.*, 1995) and ascidian tunic (Lee *et al.*, 1998).

During screening of the anticoagulant activity in marine animals, we recently detected anticoagulant activity from soluble extracts of the blood ark shell, *Scapharca broughtonii*. In the present paper, we report the purification and properties of the first anticoagulant protein from marine bivalves.

### Materials and Methods

**Materials** Fresh blood ark shells, *Scapharca broughtonii*, were obtained from a blood ark shell aquafarm (Tongyoung, Korea), and kept under -20°C until use. HEMOLAB Thrombomatt, HEMOLAB Silimat, and HEMOLAB Cofac reagent for inhibitory assay were obtained from the BioMerieux Co. (Marcy-l'Etoile, France). BioMerieux Coagulometer Option 8 was a product of Behnk Electronic Co. (Norderstedt, Germany). Fibrinogen (from bovine) and urokinase (from human urine) were purchased from the Sigma Chemical Co. (St. Louis, USA). Molecular weight markers for gel filtration and SDS-polyacryl amide gel electrophoresis were products of the Sigma Chemical Co. (Hercules, USA). DEAE-Sephadex A-50, Sephadex G-75, DEAE-Sephacel, and Biogel P-100 were purchased from the Sigma Chemical Co. (St. Louis, USA). ACE homogenizer AM-6 was a product of Nihonseiki Kaisa Ltd. (Tokyo, Japan). The ultramembrane filter, SM165 (Molecular weight, 10 kDa cut-off), for the concentration of protein solutions was obtained from the Sartorius Co. (Göttingen, Germany). All of the other chemicals that were used in this study were of analytical grade and the highest purity.

**Purification of the anticoagulant from *S. broughtonii*** All of the operations were carried out at 4°C unless stated otherwise. All of the edible parts (100 g) of *S. broughtonii*, except the shell, were minced and homogenized at 10,000 rpm for 10 min in 100 ml of 50 mM Tris-HCl that contained 0.1 mM EDTA, pH 8.0 (Tris-HCl/EDTA) buffer after washing in the same buffer to remove the blood. The insoluble materials were removed by centrifugation at 6,000 × g for 10 min, and the supernatant was treated by 150 ml of 20% CCl<sub>4</sub> (after cooling to -70°C) to remove lipid. Crude extract in

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the water layer was dialyzed against 5 mM Tris-HCl buffer (pH 8.0) for 12 h, and tested for their anticoagulant activity using a prothrombin time (PT) and activated partial thromboplastin time (APTT) assay (see below). The extract was desalted out in the range of 20-80% with ammonium sulfate. After centrifugation ( $8,000 \times g$ , 10 min), the precipitate was collected. The precipitate was dissolved in a Tris-HCl/EDTA buffer, then the dissolved solution was dialyzed against a 5 mM Tris-HCl buffer (pH 8.0) for 12 h. The dialyzed solution was loaded onto a DEAE-Sephadex A-50 column ( $3 \times 40$  cm) that was previously equilibrated with a Tris-HCl/EDTA buffer. It was then eluted (0.5 ml/min) with a linear gradient from 0 to 0.5 M NaCl in the same buffer. The eluted fractions at each purification step were tested as to their anticoagulant activity using an APTT assay. The active fraction was pooled, dialyzed against a 5 mM Tris-HCl buffer, and subsequently loaded onto a Sephadex G-75 column ( $2 \times 80$  cm, 0.9 ml/min) that was previously equilibrated with a Tris-HCl/EDTA buffer. The eluted fraction was pooled, dialyzed, and rechromatographed on a DEAE-Sephacel column ( $3 \times 40$  cm) that was previously equilibrated with a Tris-HCl/EDTA buffer. It was then eluted (0.5 ml/min) with a linear gradient from 0 to 0.5 M NaCl in the same buffer. The fraction showing the highest activity was pooled, dialyzed, and rechromatographed on a Biogel P-100 column ( $2 \times 80$  cm) that was previously equilibrated with a Tris-HCl/EDTA buffer. It was and then eluted (0.9 ml/min) with the same buffer. The major peaks with anticoagulant activity were collected, dialyzed against distilled water, concentrated using the ultramembrane filter (molecular weight, 10 kDa cut-off membrane), and then lyophilized.

**Measurement of APTT and PT** An *in vitro* coagulation assay of APTT and PT was performed according to the manufacturers instructions. The citrated normal human plasma (80  $\mu$ l) and *S. broughtonii* samples (20  $\mu$ l) were incubated for 3 min at 37°C. The mixture was activated with 100  $\mu$ l of HEMOLAB Silimat that contained rabbit brain cephaline or 100  $\mu$ l of kaolin/saline (5 mg/ml) for 3 min at 37°C. In the PT assay, 200  $\mu$ l of HEMOLAB Thrombomatt contained rabbit brain thromboplastin (thromboplastin c). After 100  $\mu$ l of 20 mM,  $\text{CaCl}_2$  was added. The prolonged time (sec) was measured as an inhibitory activity using a Coagulometer Option 8.

**Inhibitory activity for the activated coagulation factors** In the inhibition assay for activated factor IX, 100  $\mu$ l of the HEMOLAB Cofac IX (containing factor IX-deficient human plasma and 100  $\mu$ l of HEMOLAB Silimat containing rabbit brain cephaline) were incubated for 3 min at 37°C. Then the mixture of the citrated normal human plasma (80  $\mu$ l) and *S. broughtonii* samples (20  $\mu$ l) (previously incubated for 3 min at 37°C) was added. Subsequently, 100  $\mu$ l of 20 mM  $\text{CaCl}_2$  was added and the clotting time was measured. An inhibition activity was calculated as a percent (%) unit by a Coagulometer Option 8. Assays using *in vitro* diagnostic kits for activated factor XII, XI in the intrinsic pathway and activated factor X in the common pathway, were also performed according to similar methods from the manufacturers instructions. Inhibitory activity against thrombin (factor IIa) was tested with the thrombin assay method of Seegers (1964).

**Fibrinolytic activity** Fibrinolytic activity was assayed using the fibrin plate method (Jespersen and Astrup, 1983). Six milliliters of bovine fibrinogen (3.5 mg/ml in 150 mM barbital buffer, pH 7.75, containing 1 mM  $\text{CaCl}_2$  and gelatin 0.1%) were placed in a petri dish and clotted by adding 3.6 NIH U of bovine thrombin. After *S. broughtonii* samples (15  $\mu$ l, 3-6 replicates/ plate) were applied, it was incubated for 18 h at 37°C. Then the lysed areas ( $\text{mm}^2$ ) were measured as the product of two perpendicular diameters. Urokinase (15  $\mu$ l, 25 units/ml) was used as a positive control.

**Measurement of molecular weight** The molecular weight of *S. broughtonii* anticoagulant was measured by gel filtration on a Sephadex G-75 column ( $2.0 \times 80$  cm) that was previously equilibrated with a Tris-HCl/EDTA buffer. The protein was eluted (0.7 ml/min) with the same buffer. The molecular weight standards were bovine serum albumin (66.0 kDa), carbonic anhydrase (29.0 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). A SDS-poly acryl amide gel electrophoresis was performed on 7.5% slab gel by the method of Laemmli (1970). The protein bands were stained with Coomassie Brilliant Blue R-250. The molecular mass standards were fumarase (58.6 kDa), triosephosphate isomerase (32.5 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa).

**Amino acid composition** The anticoagulant was hydrolyzed with 6 N HCl at 110°C for 24 h in vacuum-sealed ampoules. The amino acids were analyzed with an amino acid analyzer (Biochrom 20, Biochrom Ltd., Cambridge, UK). Cysteine residues were estimated by the method of Spencer and Wold (1969).

**Effects of pH, temperature, and cations on anticoagulant activity** The effects of pH, temperature, and cations of the *S. broughtonii* extract (20  $\mu$ l, 25  $\mu$ g/ml) were measured on the anticoagulant activity.

The pH effect of the anticoagulant protein was measured after the protein was incubated in various buffers for 48 h at 4°C and neutralized. The following buffers were used: 10 mM glycine-HCl, pH 2.5-3.5; 10 mM acetate, pH 4.0-5.5; 10 mM phosphate, pH 6.0-7.5; 10 mM Tris-HCl, pH 8.0-9.0; 10 mM glycine-NaOH, pH 9.5-11.0. The residual activity was measured by an APTT assay.

The effect on temperature of the anticoagulant was examined after the protein was incubated in a Tris-HCl/EDTA buffer for 15 min at various temperatures. After cooling to room temperature, the residual anticoagulant activity was examined by an APTT assay.

To test the dependency of the anticoagulant activity on cations, the sample solution was dialyzed against 25 mM EDTA in a Tris-HCl/EDTA buffer at 4°C overnight. Then the solution was dialyzed against deionized water for 24 h in order to remove EDTA. The effect of the cations on APTT was assayed in the presence of various metal cations.

**Other methods** The protein concentration was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The protein content in all of the fractions that were obtained during the chromatographic process was estimated by the absorbance at 280 nm.

The sugar content of the purified anticoagulant was determined by the phenol sulfuric acid method of Dubois *et al.* (1956) using a

**Table 1.** Purification steps of anticoagulant protein from *S. broughtonii*

Step	Total protein (mg) <sup>a</sup>	Total anticoagulation (sec) <sup>b</sup>	Specific anticoagulation Activity (sec/mg)	Purification fold	Yield (%)
Crude extraction	496.4	324414.4	653.6	1.0	100.0
Ammonium persulfate precipitation	196.2	149605.8	762.4	1.2	46.1
Ion exchange chromatography on DEAE-Sephadex A-50	21.5	28476.8	1324.5	2.0	8.8
Gel chromatography on Sephadex G-75	2.2	5705.6	2641.5	4.0	1.8
Ion exchange rechromatography on DEAE-Sephacel	0.3	1233.0	3977.5	6.1	0.4
Gel rechromatography on Biogel P-100	0.09	538.8	5986.5	9.2	0.2

<sup>a</sup>Protein was measured by the method of Lowry *et al.* (1951).

<sup>b</sup>The clotting time was measured using the mixture of citrated normal human blood plasma (80  $\mu$ l) and *S. broughtonii* anticoagulant (20  $\mu$ l) by activated partial thromboplastin time assay.

mixture of D-glucosamine and D-galactosamine (1:1) as a standard.

## Results and Discussion

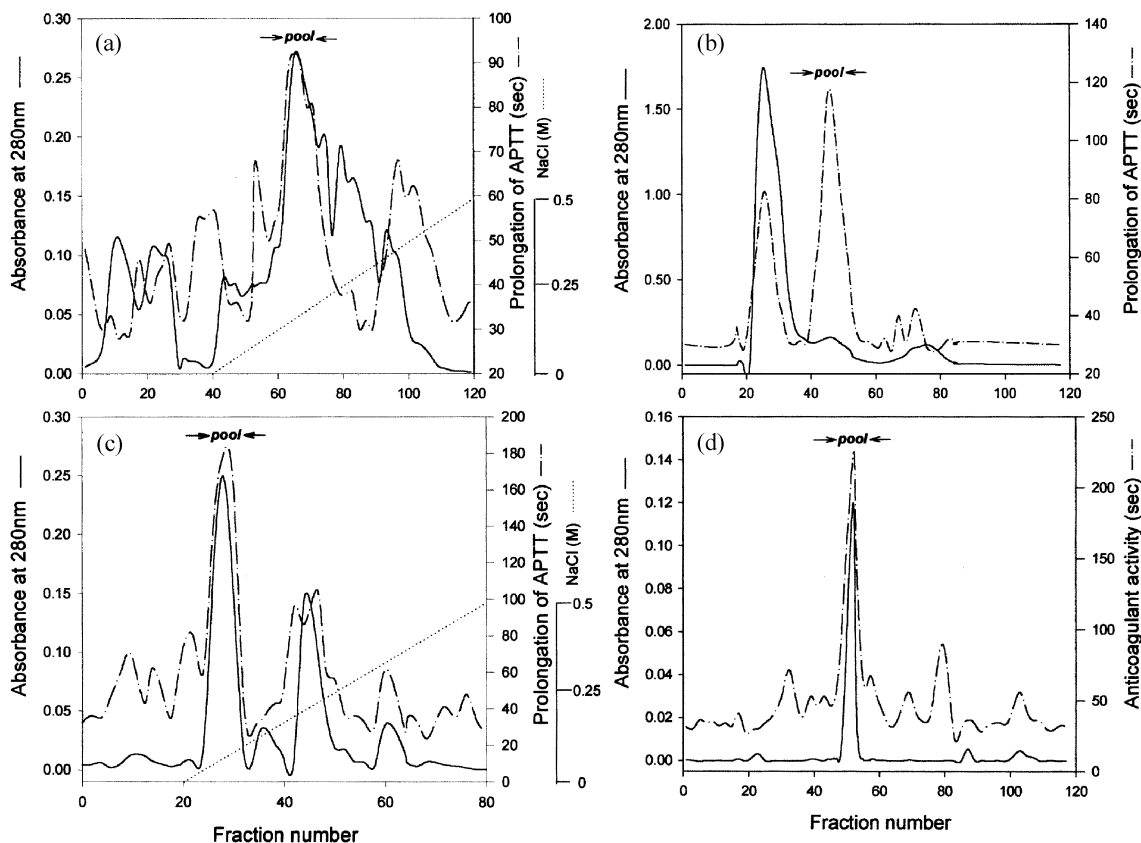
**Purification of anticoagulant from *S. broughtonii*** As summarized in Table 1, purification of the anticoagulant protein from *S. broughtonii* was accomplished by the following five steps: desalting out with ammonium sulfate, anion exchange chromatography on a DEAE-Sephadex A-50, gel filtration on a Sephadex G-75, and rechromatography on DEAE-Sephacel and Biogel P-100.

A hundred grams of the samples (all edible parts of *S. broughtonii* except the shell) were homogenized at 10,000 rpm for 10 min in a 50 mM Tris-HCl buffer that contained 0.1 mM EDTA, pH 8.0 (100 ml, Tris-HCl/EDTA buffer), after washing in the same buffer to remove the blood. Insoluble materials were removed by centrifugation and the supernatants were treated by 150 ml of 20% CCl<sub>4</sub>, stored at -70°C to remove lipid in order to prevent deleterious effect, and keep at an optimum anticoagulant activity. The crude extracts, after dialysis, prolonged the clotting time on the activated partial thromboplastin time (APTT) assay, but not on the prothrombin time (PT) assay. Thus, the anticoagulant activities at every step were also shown as a prolonged APTT (sec). A 35-s control (100  $\mu$ l of normal plasma) clotting time was prolonged to 65 s by the addition of 20  $\mu$ l of the crude extract (15.7 mg/ml). This implies that the soluble extracts from *S. broughtonii* contain an anticoagulant, which inhibits a specific factor in the intrinsic pathway of the coagulation cascade. The extracts were desalted out in the range of 20-80% saturation with an ammonium sulfate. After dialyzing the precipitate, the sample was applied onto a DEAE-Sephadex A-50 column for the anion exchange chromatography. The results of anion exchange

chromatography are shown in Fig. 1a. The anticoagulant activities of the fractions gave several strong peaks. The pooled fraction for gel filtration was eluted from 0.13 to 0.23 M NaCl in a Tris-HCl/EDTA buffer. Although the other fractions showed stronger activities, these activities were deleted in the following process. The gel chromatography, or the quantity, was too small to completely purify to the final process. After dialysis, the active fraction was loaded onto a Sephadex G-75 column and eluted with a Tris-HCl/EDTA buffer. The result of the gel filtration is shown in Fig. 1b. The major anticoagulant active fractions (42-54<sup>th</sup>) were collected and dialyzed. The dialyzed fraction was rechromatographed on a DEAE-Sephacel, and a major anticoagulant fraction was eluted at 0.1 M NaCl in a Tris-HCl/EDTA buffer (Fig. 1c). Finally, the solution was further fractionated on a Biogel P-100 column after pooling and dialyzing (Fig. 1d). Subsequently, a strong active fraction was eluted with the same buffer and showed a potent anticoagulant activity on an APTT assay.

### Properties of anticoagulant protein from *S. broughtonii*

The result of SDS-PAGE was also shown as a homogeneous protein (Fig. 2, insert). The results of the migration of the incubated anticoagulant protein with 2-mercaptoethanol for 10 min at 95°C revealed only one band with a molecular mass that corresponded to about 26.0 kDa on gel (Lane 1). In addition, the molecular weight of the intact anticoagulant was also estimated to be 26.0 kDa by gel filtration (Fig. 2). The presence of a carbohydrate in the purified anticoagulant was not detected by the phenol-sulfuric acid method. In conclusion, the purified anticoagulant from *S. broughtonii* is considered to be a pure monomeric protein, while most of natural anticoagulant proteins had a high molecular weight. This is shown as a heterogeneous band pattern in SDS-PAGE, and had been found in most glycoproteins (Garfin, 1990).



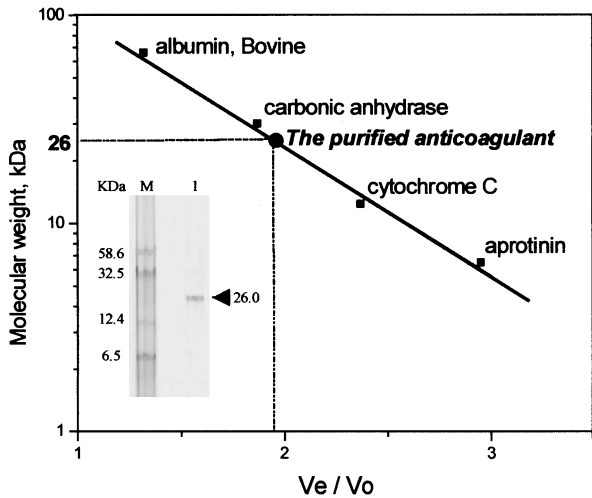
**Fig. 1.** Purification profiles of *S. broughtonii* anticoagulant. (a) Anion exchange chromatography on a DEAE-Sephadex A-50 column ( $3 \times 40$  cm). After being previously equilibrated with 50 mM Tris-HCl that contained 0.1 mM EDTA, pH 8.0 buffer, the fractions were eluted (0.5 ml/min) with a linear gradient from 0 to 0.5 M NaCl in the same buffer. The eluted fractions were collected and tested for their anticoagulant activity using an APTT assay. (b) Gel filtration on a Sephadex G-75 column ( $2 \times 80$  cm). The pooled DEAE-Sephadex A-50 fraction was applied to the Sephadex G-75 and eluted with 50 mM Tris-HCl that contained 0.1 mM EDTA, pH 8.0 buffer at a flow rate of 0.9 ml/min. The active fraction was pooled for next step of purification. (c) Rechromatography on a DEAE-Sephacel column ( $3 \times 40$  cm). The sample from the G-75 was loaded onto a DEAE-Sephacel column and eluted (0.5 ml/min) with linear gradient from 0 to 0.5 M NaCl in 50 mM Tris-HCl that contained 0.1 mM EDTA, pH 8.0 buffer. The fraction showing the highest activity was pooled and processed for the next step. (d) Biogel P-100 column rechromatography of *S. broughtonii* anticoagulant. The pooled fraction from the DEAE-Sephacel column was applied to a Biogel P-100 column ( $2 \times 80$  cm) and eluted (0.9 ml/min) with 50 mM Tris-HCl that contained 0.1 mM EDTA, pH 8.0 buffer. The active fraction was pooled and contained a homogeneous protein.

**Anticoagulant activities and factor specific inhibitory** As shown in Fig. 3, the purified anticoagulant from *S. broughtonii* prolonged the activated partial thromboplastin time (APTT), cephaline-induced (or kaolin-induced) clotting times. The addition of 20  $\mu$ l of the purified anticoagulant (0–100  $\mu$ g/ml) prolonged a 32 s control-clotting time from 49 s to 325 s (Fig. 3). These results suggest that the soluble extract from *S. broughtonii* contains an anticoagulant, which inhibits a specific factor in the intrinsic pathway of the coagulation cascade.

As shown in Fig. 4, specific inhibition against factors in the intrinsic pathways' assay showed that the anticoagulant is a potent inhibitor for the activated factor IX. The addition of 20  $\mu$ l of the purified anticoagulant (0–100  $\mu$ g/ml) may interfere with the occurrence of coagulation in normal plasma from 20 to 99% in the specific factor IXa inhibition assay. However,

the activated factor XI, XII, and others in the intrinsic pathway were not significantly inhibited.

In addition, inhibitory assays against factor Xa and thrombin (factor IIa) in common pathways suggest that the anticoagulant could not suppress the activation of factor X and the ability of thrombin to convert fibrinogen to fibrin in the common pathways. Apitz-Castro *et al.* (1995) reported that draculin, an anticoagulant glycoprotein of the activated factor IX, was isolated from the saliva of a vampire bat, *Desmodus rotundus*. The factor IX-binding protein, a potent anticoagulant against factor IX, was also isolated from the venom of *Trimeresurus flavoviridis* (Atoda & Morita, 1989) and *Deinagkistrodon acutus* (Atoda *et al.*, 1998). But, the anticoagulant against factor IX from marine organisms has not been reported to our knowledge. The fibrinolytic activity of *S. broughtonii* extract was undetected in the fibrin plate assay,

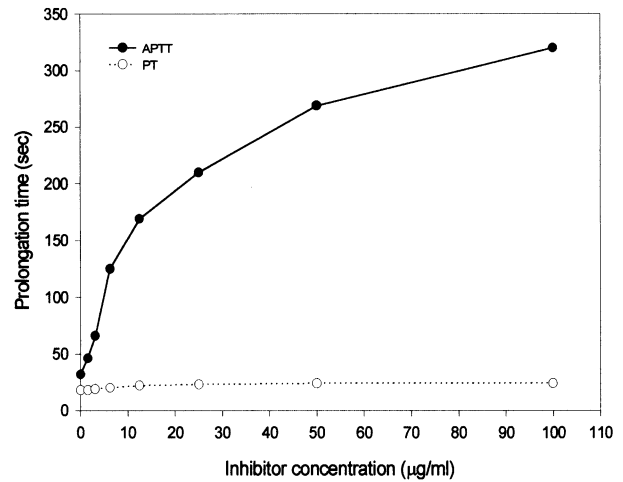


**Fig. 2.** Gel filtration of intact *S. broughtonii* anticoagulant on a Sephadex G-75 column (2.0×80 cm). After being previously equilibrated with 50 mM Tris-HCl that contained 0.1 mM EDTA pH 8.0 buffer, the anticoagulant protein was eluted (0.7 ml/min) with the same buffer.  $V_e/V_o$  ( $V_e$ , volume of eluted protein;  $V_o$  volume of eluted Blue dextran). Molecular weight standards bovine serum albumin (66.0 kDa), carbonic anhydrase (29.0 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). The inserted figure shows the results of the SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed on 7.5% slab gel and the protein bands were stained with Coomassie Brilliant Blue R-250. Lane M (molecular markers): fumarase (58.6 kDa) triosephosphate isomerase (32.5 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa). Lane I: *S. broughtonii* anticoagulant (26.0 kDa).

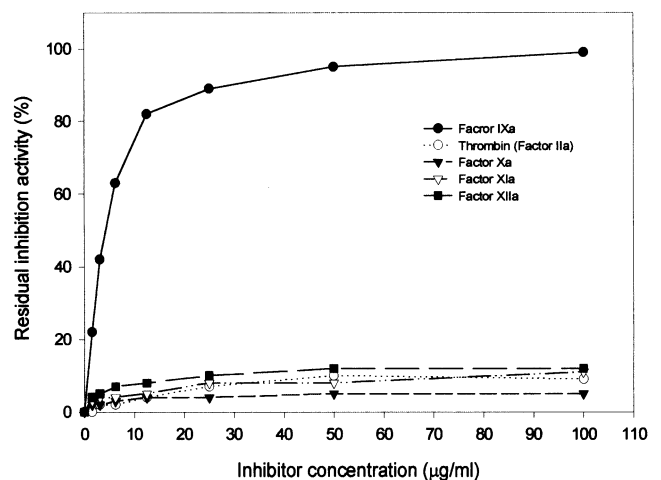
while urokinase lysed the circular area (0.9 mm radius) of the fibrin plate. Some anticoagulant protein, such as the isolated anticoagulant from *Lumbricus rubellus* (Jeon *et al.*, 1995), did cleave to both the coagulation factor and fibrin.

**Amino acid composition analysis** The amino acid composition of the anticoagulant protein is summarized in Table 2. It revealed a high content of Asx, Glx, Leu, and a low content of Cys, His. As reported by Esmon (2000), the hydrophobic interaction, or hydrogen bond between the functional groups (like the carboxyl and hydroxyl groups in the amino acid of the anticoagulant and coagulation factors) might lead to inhibit the coagulation pathway in the present of calcium ion, vitamin K, or phospholipid.

**Effects on pH, temperature and metal cations** The inhibitory activity of the anticoagulant protein from *S. broughtonii* was stable in the range of pH values between 7.0 and 8.0, but the activity at below pH 4.0 and above pH 10.5 was completely destroyed (Fig. 5). The anticoagulant retained its full inhibition activity when maintained at temperatures up to 45°C, but a gradual decrease of activity occurred between 45 and 55°C. The activity was completely inhibited at 55°C (Fig 6). As summarized in Fig 7, the anticoagulant activity



**Fig. 3.** Prolongation of APTT and PT by the purified anticoagulant from *S. broughtonii*. Citrated normal human plasma (80 µl) and *S. broughtonii* samples (20 µl) were incubated with 100 µl of HEMOLAB Silimat that contained rabbit brain cephaline for APTT, and 200 µl of HEMOLAB Thrombomatt that contained rabbit brain thromboplastin (thromboplastin c) for PT. After 100 µl of 20 mM  $\text{CaCl}_2$  was added, the prolongation time (sec) was measured using a Coagulometer Option 8, and exhibited as an inhibitory activity.

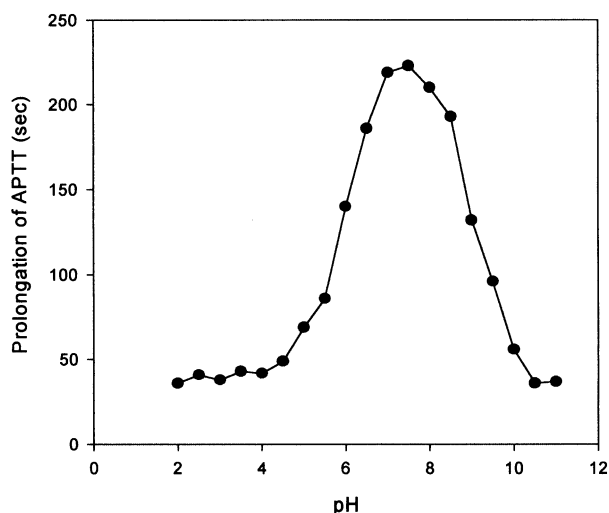


**Fig. 4.** Inhibition of blood coagulant factors by the purified anticoagulant from *S. broughtonii*. Inhibition activities for coagulant factors were measured according to the manufacturers instructions. In the inhibition assay for factor IX, 100 µl of HEMOLAB Cofac IX that contained factor IX-deficient human plasma and 100 µl of HEMOLAB Silimat that contained rabbit brain cephaline were incubated with a mixture of citrated normal human plasma (80 µl) and *S. broughtonii* samples (20 µl). After 100 µl of 20 mM  $\text{CaCl}_2$  was added and the clotting time was measured. The inhibition activity was calculated as a percent (%) unit by a Coagulometer Option 8.

was completely deleted, and coagulation did not occur after dialysis with EDTA. Then, the addition of 20 mM of metal divalent cations fairly recovered  $\text{Cu}^{2+}$  after their removal by

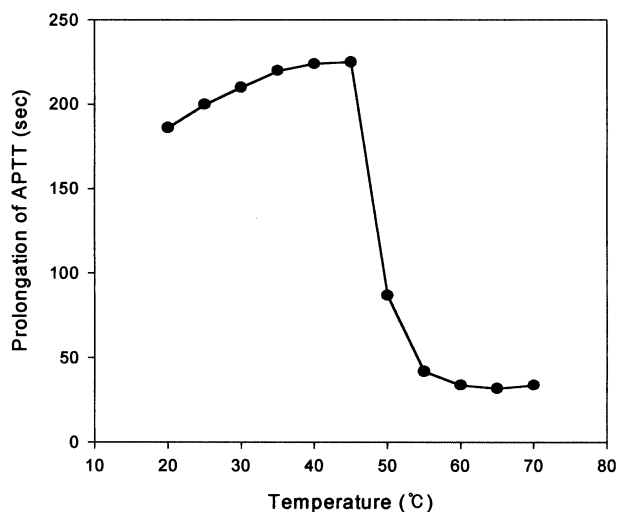
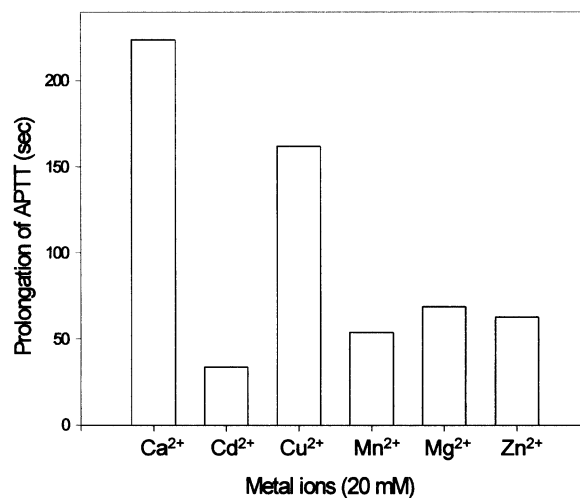
**Table 2.** Amino acid composition of the anticoagulant protein from *S. broughtonii*

Amino acids	mol%
Asx	10.379
Thr	7.063
Ser	6.734
Glx	7.136
Pro	5.956
Gly	9.649
Ala	5.556
Cys	5.828
Val	4.523
Met	7.238
Ile	4.908
Leu	4.090
Phe	8.585
His	2.150
Lys	6.584
Arg	3.621

**Fig. 5.** Effects of pH on anticoagulant activity. Residual activity on the APTT assay was measured after the anticoagulant (20  $\mu$ l, 25  $\mu$ g/ml) was incubated in the various buffers for 48 h at 4 and neutralized. The following buffers were used: 10 mM glycine-HCl, pH 2.5-3.5; 10 mM acetate, pH 4.0-5.5; 10 mM phosphate, pH 6.0-7.5; 10 mM Tris-HCl, pH 8.0-9.0; 10 mM glycine-NaOH, pH 9.5-11.0.

EDTA. But, the activity in the presence of  $\text{Ca}^{2+}$  was more excellent than that of  $\text{Cu}^{2+}$ . This result suggests that copper could play a role as a substitute of the cofactor,  $\text{Ca}^{2+}$ , on the blood coagulation system. As reported by Broze *et al.* (1990) and Davie *et al.* (1991), the factor IXa activated the factor X in the present of  $\text{Ca}^{2+}$  in the intrinsic pathway of the coagulation cascade.

In this study, we discovered that the inhibition mechanism of the anticoagulant from *S. broughtonii* interrupted the factor

**Fig. 6.** Thermostability of the anticoagulant from *S. broughtonii*. was examined after the anticoagulant (20  $\mu$ l, 25  $\mu$ g/ml) was incubated in 50 mM Tris-HCl that contained 0.1 mM EDTA, pH 8.0 buffer for 15 min at various temperatures. After cooling at 4 $^{\circ}$ C, the residual anticoagulant activity was examined by APTT assay.**Fig. 7.** Dependency of anticoagulant activity on cations. The anticoagulant solution (20  $\mu$ l, 25  $\mu$ g/ml) was dialyzed against 25 mM EDTA in 50 mM Tris-HCl, pH 8.0 buffer at 4 $^{\circ}$ C overnight. The solution was then dialyzed against deionized water for 24 h in order to remove EDTA. Prolongation of APTT was measured in the presence of each cation.

IXa activity in the intrinsic pathway of the human coagulation cascade in a  $\text{Ca}^{2+}$ -dependent manner. Its partial properties were also demonstrated. For a complete structure determination and the molecular mechanisms of the factor IX inhibition, further study will progress in our laboratory.

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