Arch Pharm Res Vol 28, No 6, 667-674, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

The Anticoagulant Fraction from the Leaves of *Diospyros Kaki L.* Has an Antithrombotic Activity

You Seon Sa, Soo-Jin Kim*, and Hye-Seon Choi

Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea

(Received January 19, 2005)

The leaves of Persimmon (*Diospyros kaki L.*) has long been used for tea in Korea since it was thought to be effective against hypertension. An anticoagulant fraction was purified through gel filtration G-100, hydrophobic, gel filtration G-150, and FPLC, Phenyl superpose column chromatographies. The purified fraction was homogenous and its Mr was estimated 10,000 Da by gel filtration and SDS-PAGE. The purified fraction was sensitive to treatment of subtilisin B, but not to heat and its activity was not changed after periodate oxidation, indicating that the activity was not due to carbohydrates. It delayed thrombin time (TT), activated partial thromboplastin time (APTT), and prothrombin time (PT) using human plasma. TT was more sensitive than APTT and PT, suggesting that the anticoagulant activity may be caused by a degradation or a defect of fibrin or thrombin. It did not cause the hydrolysis of fibrin after incubation. However, it inhibited thrombin-catalyzed fibrin formation with a competitive inhibition pattern These results indicate that it may be an antithrombotic agent and that it is bound to fibrinogen binding sites of thrombin.

Key words: Anticoagulant, Antithrombotic, Leaves of persimmon (*Diospyros kaki L.*), Thrombin time

INTRODUCTION

Sweet persimmon is one of the fruits that are used in most Oriental countries such as Korea, Japan, and China. In Korea, it has also been considered to have therapeutic value against various diseases. Dried persimmons, for example, were applied to wounds for their anti-inflammatory and analgesic activities. It was also thought, from folk remedies, that persimmon reduced the symptoms of hypertension and strokes (Moon, 1991). Okonogi *et al.* (1979) found that the tannin from persimmon had the effect of detoxification against snake venoms and bacterial toxins.

Blood clots formed from fibrinogen by thrombin are effectively removed by plasmin. Under normal conditions, coagulatory and fibrinolytic processes are tightly regulated but the normal hemostatic process can be disturbed under pathological conditions, resulting in irreversible vessel occlusion and excessive morbidity. Thrombin plays a pivotal role in this process as a potent inducer of platelet activation, a fibrin generator, and an activator of coagulation factors (McGowan and Detwiler, 1986; Tsiang *et al.*, 1990; Stubbs *et al.*, 1992).

Using antithrombotic agents are promising for chemotherapy, for serious cardiovascular disease and for cerebral infarction (Tsiang *et al.*, 1990; Friedrich *et al.*, 1993; Holsat *et al.*, 1994; Rezaie *et al.*, 1995; Herbert *et al.*, 1998; Cho *et al.*, 2000; Yuk *et al.*, 2000; McClanahan *et al.*, 2001; Wall *et al.*, 2001; Wong *et al.*, 2002; Kang *et al.*, 2003). Several thrombin inhibitors have been introduced such as α2-macroglobulin, serpins antithrombin III (Olson *et al.*, 1992), heparin cofactor II, heparin, protease nexin I, hirudin, a protein obtained from the medicinal leech (Jakubowski and Maraganore, 1990; Naski *et al.*, 1990; Markwardt, 2002), triabin from a bug, *Triatoma pallidipennis* (Noeske-Jungblut *et al.*, 1995), haemadin from Indian leech (Strube *et al.*, 1993), and antithrombotic protein from *Spirodela polyrhiza* (Choi and Sa, 2001; Cho and Choi, 2003).

In the present study, we have purified and characterized an antithrombotic protein from leaves of sweet persimmon to provide logical explanation in biochemical basis for therapeutic value of persimmon.

*S. J Kim was supported by BK21 of University of Ulsan (2003-2005).

Correspondence to: Hye-Seon Choi, Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea
Tel: 82-52-259-2357, Fax. 82-52-259-1694
E-mail. hschoi@mail.ulsan.ac.kr

MATERIALS AND METHODS

Materials

Bovine fibrinogen, human fibrinogen, thrombin, heparin, protamine sulfate, snake venom, APTT reagents, trypsin, *N*-Tosyl-GPR pNA, *N*-Tosyl-GPK pNA, phenyl Sepharose, Sephdex G-100, Sephdex G-150, ammonium sulfate, trizma base were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). The leaves of persimmon (*Diospyros kaki L.*) was obtained from the University of Ulsan in May, washed several times, and frozen for storage.

Purification

Leaves of persimmon (300 g) was washed with distilled water and suspended with 250 mL of 10 mM Tris-HCl, pH 7.5 (Buffer A). The cells were disrupted for 5 min with pulse in an ice-chilled bead-beater (Biospec Products, Bartleville, OKLA., U.S.A.), and the homogenate was centrifuged at 10,000×g for 40 min in a refrigerated centrifuge, T-324 using A-8.24 rotor (Kontron Instruments, Zurich, Sweden). The supernatant was concentrated completely by lyophilization and dialyzed against buffer A. Dailysate was applied on a Sephadex G-100 column (2.8×10^5 cm) equilibrated with 20 mM Tris-HCl, pH 7.5 and proteins were eluted at 10 mL/h. Fractions containing anticoagulant activity were pooled, concentrated by lyophilization, applied on a phenyl Sepharose column (2.0×10 cm) equilibrated with Buffer A containing 1.7 M $(NH_4)_2SO_4$. Anticoagulant fraction was eluted with Buffer A and then concentrated. The concentrated preparation was applied to a Sephadex G-150 column (1.8×100 cm) equilibrated with 20 mM Tris-HCl, pH 7.5 and fractions were eluted at 10 mL/h. Fractions containing anticoagulant activity were pooled, concentrated by ultrafiltration. The anticoagulant fraction was loaded on Phenyl superose (Pharmacia; H/R, 5/5), a hydrophobic interaction column equilibrated with 1.7 M $(NH_4)_2SO_4$. After washing the column with the same buffer, the active fraction was eluted with decreasing concentrations of $(NH_4)_2SO_4$, and then concentrated by ultrafiltration. The activity was determined by a conversion of purified fibrinogen to fibrin by thrombin. A coagulation fold of control was used as a unit. The delay of the clotting time by the anticoagulant was divided by the control in the absence of anticoagulant.

Anticoagulant activity

Fresh normal human blood (54 mL) was collected in 6 mL of 3.8% sodium citrate. Plasma was prepared with an initial whole blood centrifugation at 400×g for 10 min, and a second centrifugation of the plasma at 800×g for 20 min to eliminate residual platelets. Activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) were measured by an increase of absorbance at 420 nm. A typical sample assay for APTT contained 0.3 mL platelet-poor plasma, 0.3 mL APTT reagent, and various concentrations of fractions in a final volume of 1 mL. The reaction mixture was incubated at 37°C for 2 min and the reaction was started by adding a 8 μL of 1 M $CaCl_2$. For TT excluded APTT reagent and $CaCl_2$ the reaction began by adding thrombin (0.8 ug/mL). For PT it was started by adding the throbomax reagent (Sigma Chemical Co.). The clotting time was the time when a drastic increase of absorbance occurred ($\Delta A_{420}>0.010$). The same method was used for snake venom, reptilase activity, but 4 μg/mL of reptilase was added instead of thrombin. The reaction mixture contained the indicated concentrations of fibrinogen in 0.2 M borate buffer, pH 7.8 containing 50 mM NaCl and the reaction started by adding 1 ug/mL of thrombin to determine fibrinogen clotting time. The amidolytic activity was determined with several synthetic substrates. Initial rates of hydrolysis were determined using a Kontron spectrophotometer (UVKON 860) equipped with a thermostated cell compartment. Hydrolysis of substrates conjugated to *p*-nitroaniline were measured by monitoring the release of *p*-nitroaniline ($\Delta\varepsilon$≈10,000 $M^{-1}cm^{-1}$) at 405 nm. The reaction was monitored for up to 10 min and the rate of reaction was calculated from the linear portion of the curve. Rates of the hydrolysis of substrates containing 7-amido-4-methylcoumarin were measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a spectrofluorimeter (Hidachi). For SDS-PAGE analysis, human fibrinogen was treated with protease in 50 mM Tris-HCl, pH 7.5 at 37°C. The reaction stopped by boiling for 3 min. The reaction mixture was combined with an SDS-sample buffer and boiled for 3 min. Electrophoresis was carried out on 10% gel. Gels were stained by Coomassie brilliant blue R-250 and destained by 10% acetic acid and 7% methanol.

Statistical analysis

All values are expressed as mean ± SEM. Student's *t*-test were used to evaluate the differences between the samples of interest and the respective controls. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Anticoagulant activity was detected in sweet persimmon (Sa *et al.*, 2003). Since the leaves of persimmon contain substantial amounts of anticoagulant activity, we purified an anticoagulant through gel filtration, hydrophobic, and another gel filtration, and Phenyl superpose column chromatographies (Fig. 1). The final fraction was homogenous in SDS-PAGE and was purified 6.5-fold with 1.5% recovery (Table I). The purified fraction was estimated to be a monomer of 10,000 Da of *Mr* by SDS-PAGE and gel

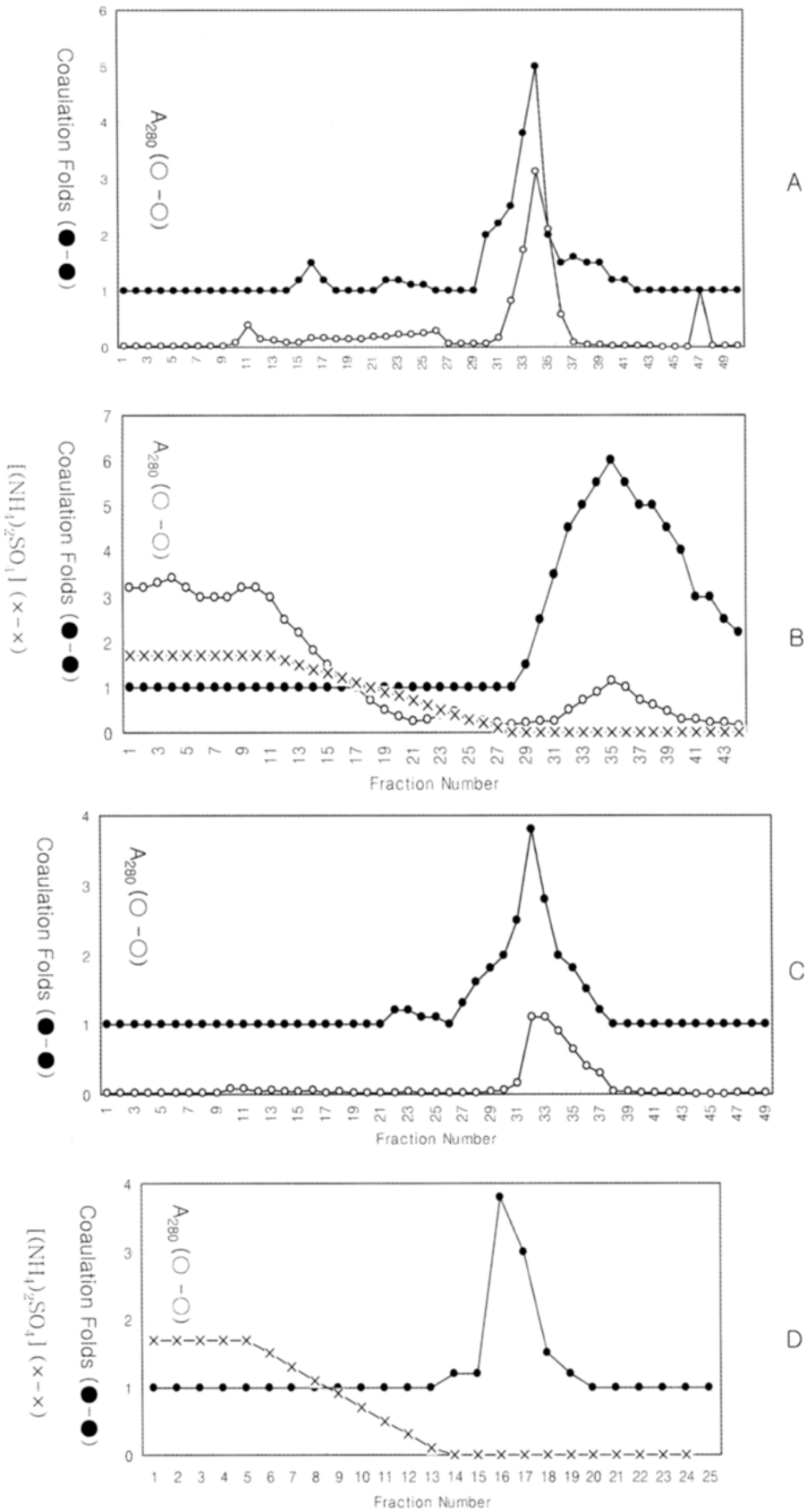

Fig. 1. Purification of the anticoagulant fractions from the leaves of persimmon (*Diospyros kaki L.*). The homogenates were purified through gel filtration G-100 (A), phenyl sepharose (B), G-150 (C), and FPLC, phenyl Sepharose (D) as described in Methods and Materials (coagulation times compared with control, ●-●, absorbance at 280 nm, ○-○).

Table I. Purification of the anticoagulant from the leaves of persimmon (*Diospyros kaki L.*)

Purification	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Homogenate	52	65000	1548	100	1
G-100	20	38000	1900	58	1.20
Phenyl sepharose	3.50	20000	5714	31	3.70
G-150	1.20	8000	6667	12	4.30
FPLC, Phenyl superose	0.10	1000	10000	1.50	6.50

filtration (Fig. 2). Thermal stability was determined after incubation of the fraction at different temperatures for 15 min followed by fibrinogen clotting assay. Remaining activities were 76 and 52% after incubation at 50 °C and 100°C, respectively, indicating that the purified fraction was relatively heat-stable (Fig. 3A). To investigate the characteristics of the purified fraction, it was treated with various enzymes that hydrolyze proteins or carbohydrates. As shown in Fig. 3B, substantial amounts of anticoagulating activity was diminished after exposure to fungal protease or subtilisin B, whereas not much change of activity was observed after a treatment of trypsin, glycosidase F, pectinase, hyaluronidase, and *N*-acetylhexoseaminidase. No significant difference was observed between control and heat-inactivated fungal protease or subtilisin when added alone (Fig. 3C). To exclude the possibility that the anticoagulant is a carbohydrate, periodate oxidation was examined. Less than 10% loss of activity was found after periodate oxidation, indicating that the active fraction is not a carbohydrate (Fig. 3D).

The anticoagulant activity of the purified fraction was measured with APTT, TT, and PT tests using human plasma (Fig. 4). TT was prolonged 2 and 3 times the control value at 5.3 and 13.1 μg/mL of the anticoagulant from persimmon leaf, respectively. Compared to its effects on TT, it delayed APTT and PT to a lesser degree. APTT and PT required 27.0 and 30.0 μg/mL of the anticoagulant to reach double the control value, respectively. Low molecular weight heparin prolonged clotting times twice at 4.5 and 10 μg/mL for APTT and TT, respectively, in our system. We tested other clotting-inducing agents to see whether or not the activity of the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) is specific to thrombin or not. The snake venom of *Bothrops atrox* (Hofmann and Bon, 1987), reptilase can catalyze plasma clotting. It did not, however, delay the clotting time induced by this enzyme (Fig. 4). The effect of another serine protease, trypsin, on the purified fraction was determined by an amidolytic assay. No inhibition was found on trypsin with a synthetic substrate, even though an excess amount of the purified fraction was added (data not shown). We have also determined whether or not the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) can cleave fibrinogen. It did not hydrolyze a purified fibrinogen under the test conditions (data not shown). These data suggest that the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) is specific to thrombin.

Contrary to the above inhibitory effect by thrombin, the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) did not significantly inhibit thrombin activity in an

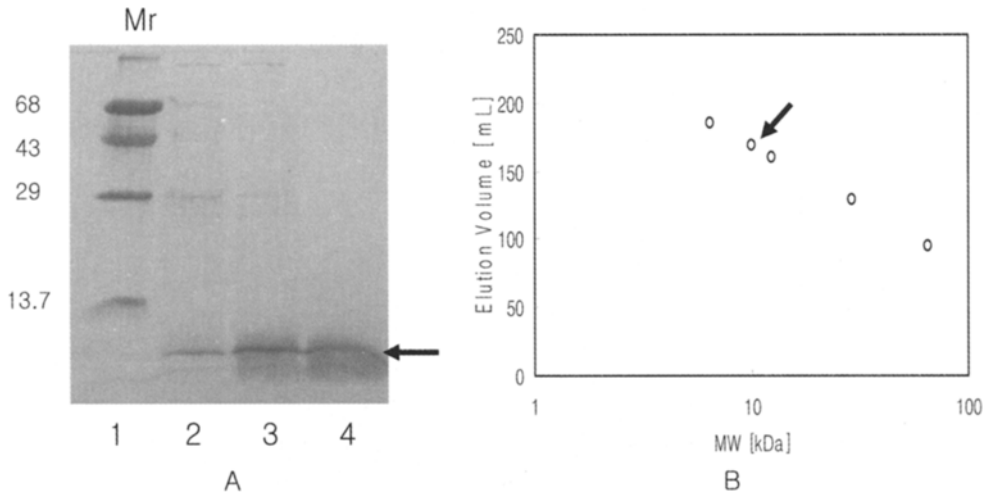

Fig. 2. Determination of *Mr* of the anticoagulant fractions from the leaves of persimmon (*Diospyros kaki L.*). The active fraction after FPLC phenyl Superose column chromatography (lane 4) was applied on a 18% SDS-PAGE (A) and G-150 gel filtration column (B). The *Mr* standards consisted of bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), and ribonuclease (13,700) for SDS-PAGE. The *Mr* standards consisted of bovine serum albumin (66,000), carbonic anhydrase (29,000), horse heart cytochrome C (12,400), and bovine lung aprotinin (6,500) for gel filtration. The *Mr* of the purified fraction (lane 4) was estimated to be 10,000 (lane 2, homogenates; lane 3, the active fraction after phenyl Superose).

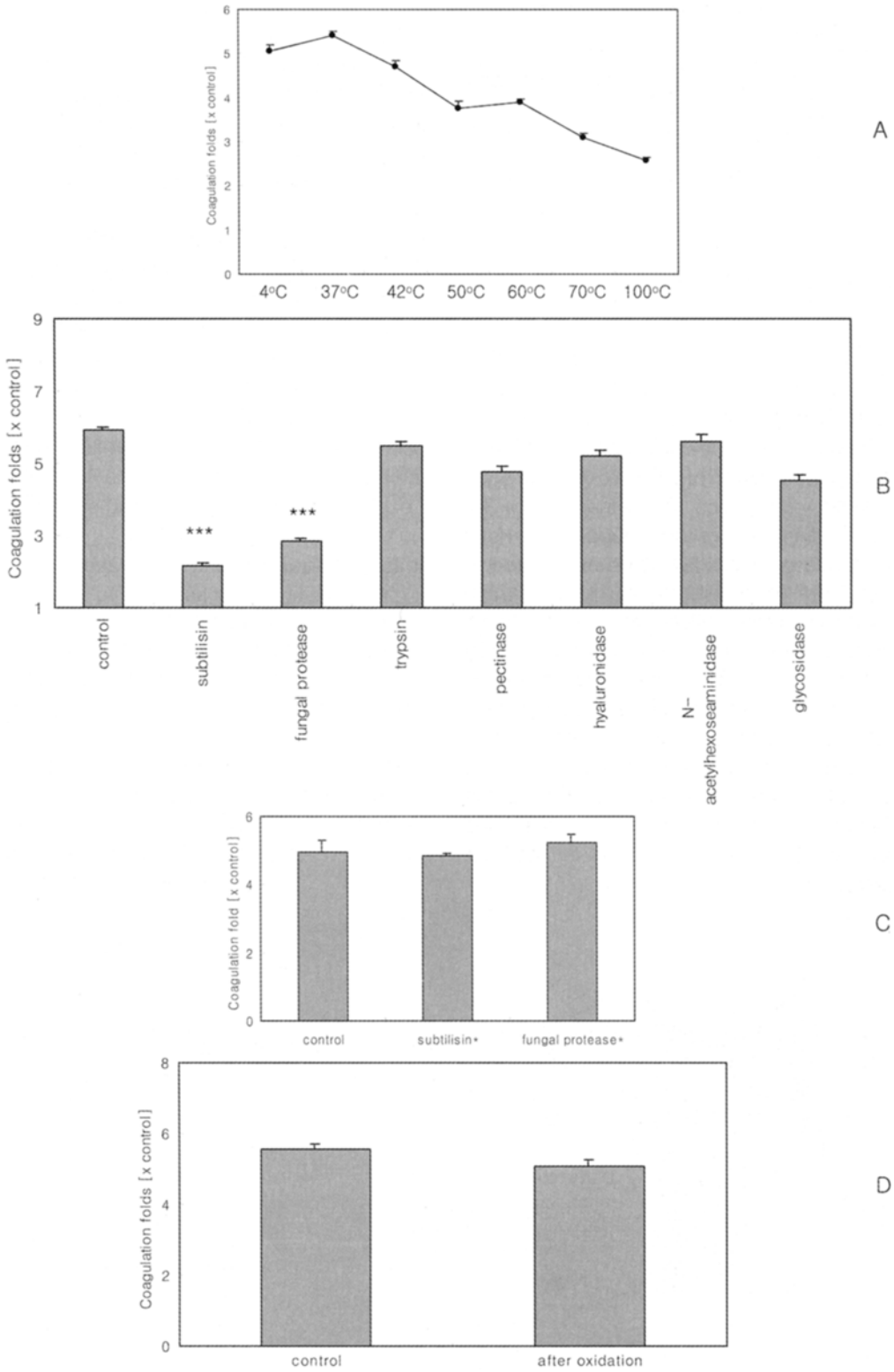

Fig. 3. Effects of heat, enzymes, and periodate oxidation of the purified fractions from the leaves of persimmon (*Diospyros kaki L.*). The purified fraction was incubated at each indicated temperature for 15 min (A), or incubated at one unit of each enzyme for 18 h at the appropriate buffer conditions (B, C). Sutilisin* or fungal protease* indicates that heat-inactivated enzyme, alone, was added to reaction mixture. Periodate oxidation was performed as described previously (Sa *et al.*, 2003) (D). Fibrinogen clotting time was measured by adding 23 5 uM of fibrinogen in 0.2 M borate, pH 7.8 containing 50 mM NaCl, 2 min before initiating activation and coagulation at 37°C using the increase the absorbance at 420 nm. Control time was TT, 20.0±2.0 seconds. Results are means of SEM. The differences between subtilisin-treated or fungal protease-treated one and control were statistically significant (***, $p<0.001$, n=3).

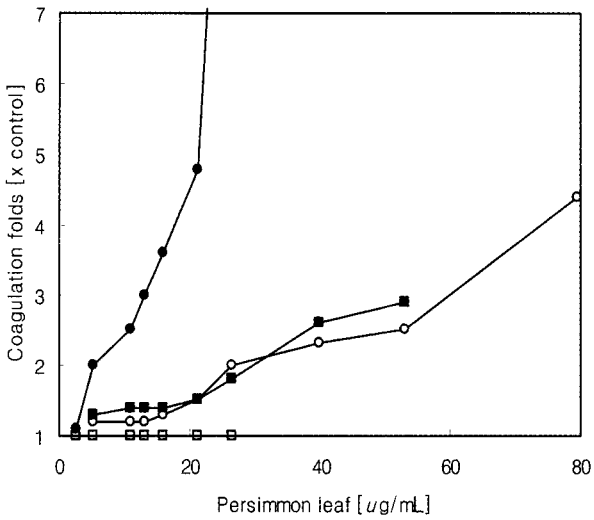

Fig. 4. Anticoagulant activity of the purified fractions from the leaves of persimmon (*Diospyros kaki L.*). The effects of the anticoagulation activity from leaf of Persimmon on thrombin time (TT, ●-●), APTT (○-○), PT (■-■), and clotting time induced by snake venome (°‡-°‡) was measured by adding the preparation to 0.3 mL of citrated plasma 2 minutes before initiating activation and coagulation at 37°C, using the increase the absorbance at 420 nm. Data was expressed as the mean ±SD. Control time were TT, 25.0±0 sec; APTT, 35.0±0.0 sec; and PT, 14.0±2.0 sec; and clotting time induced by snake venome (0.5 ug), 20.0±2.0 sec.. Each set of assays was performed in single subject.

amidolytic assay using *N*-Tosyl-Gly-Pro-Arg AMC as a synthetic substrate. Even at an excess molar concentration of the purified fraction, only 5% inhibition was observed, indicating that it is not an active site-directed inhibitor (data not shown). The purified fraction from the leaves of persimmon (*Diospyros kaki L.*) could bind to electropositive regions of thrombin. Since TT was more pronounced, the effect of protamine sulfate, which is a positively charged protein, was examined using TT. As shown in Fig. 5, protamine sulfate shifted the TT curve to the right. The concentration which reached double the control value was moved 5.3 to 15.0 and 20.0 μg/mL with 2 and 5 μg/mL of protamine sulfate, respectively.

The effect on the conversion of purified fibrinogen to fibrin by thrombin was also tested. The purified fraction from the leaves of persimmon (*Diospyros kaki L.*) also prolonged the clotting time of purified fibrinogen. Fig. 6 demonstrates that a delay of clotting time was more pronounced at lower concentrations of fibrinogen. Coagulation folds of control were used as 1/velocity to determine K_i value. Analysis of the effects of the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) as an inhibitor of thrombin-catalyzed fibrin-formation gave an apparent K_i value of 0.5 μg/mL with a competitive mode. The effect of the purified fraction on coagulation times for the thrombin-catalyzed fibrin formation indicated that the binding of the purified fraction and fibrinogen to thrombin is exclusive.

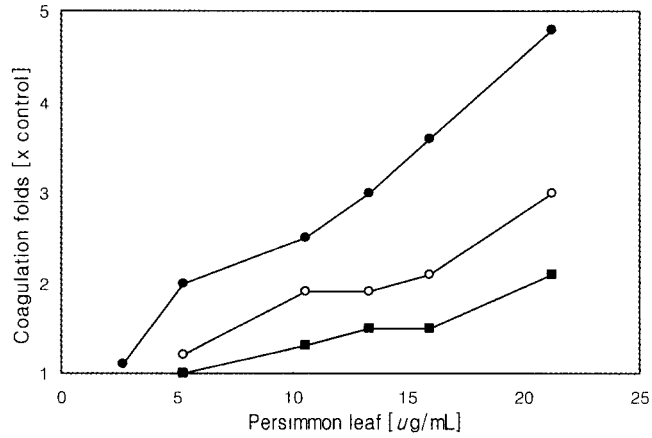

Fig. 5. Effects of protamine on anticoagulant activity of the purified fractions from the leaves of persimmon (*Diospyros kaki* L.). The anticoagulation activity from leaf of Persimmon on thrombin time (TT, ●-●) was measured by adding the preparation to 0.3 mL of citrated plasma 2 minutes before initiating activation and coagulation at 37°C, using the increase the absorbance at 420 nm. The effects of protamine (2 ug/mL, ○-○; 5 ug/mL, ■-■), were measured. Data was expressed as the mean (SEM<5%). Control time were TT, 25.0±0 sec. Each set of assays was performed in single subject.

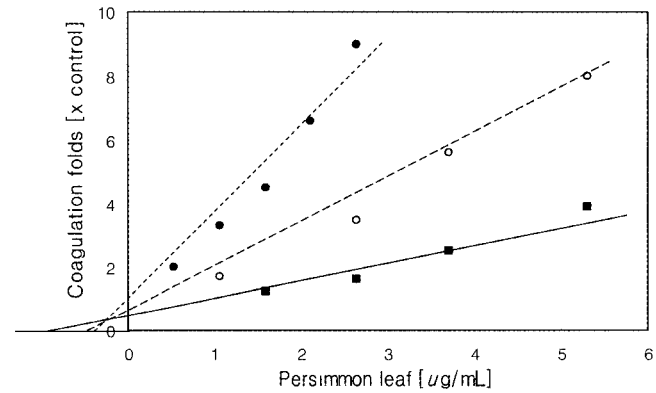

Fig. 6. Inhibition of Coagulation by the the purified fractions from the Leaves of Persimmon (*Diospyros kaki L.*) at Different Fibrinogen Concentrations. The effects of the anticoagulant from Persimmon leaf on clotting time (at 1.9 μM of fibrinogen, ●-●; at 3.8 μM of fibrinogen, ○-○; at 7.6 μM of fibrinogen, ■-■) was measured by adding different concentrations of fibrinogen in 0.2 M borate, pH 7.8 containing 50 mM NaCl, 2 minutes before initiating activation and coagulation at 37°C using the increase the absorbance at 420 nm. The value of *Ki* of the anticoagulant from Persimmon leaf was calculated to be 0.5 μg/mL. Control time was TT, 20.0±2.0 seconds.

DISCUSSION

We have isolated an antithrombotic protein from the leaves of persimmon (*Diospyros kaki L.*) with *Mr* of 10,000. It was relatively heat-stable, but it was sensitive to subtilisn and fungal protease. Since the anticoagulant

activity remained after periodate oxidation and it was not sensitive to several carbohydrate metabolizing enzymes, it could not be a carbohydrate. In human plasma, TT was more sensitive than APTT and PT, suggesting that anticoagulating activities may be caused by a degradation or defect of fibrin, fibrinogen, or thrombin rather than those of other coagulation factors. No effect on clotting time induced by snake venom of *Bothrops atrox* suggested that the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) is specific to thrombin. Insensitivity of the purified fraction on other serine protease such as trypsin also supports its specificity to thrombin.

The data shown in Fig. 5 demonstrate that protamine sulfate substantially reduces the effectiveness of the purified fraction from the leaves of persimmon (*Diospyros kaki L.*), indicating that protamine sulfate could compensate for the negative charge and interrupt the binding to thrombin. It also suggests that the purified fraction could bind electropositive regions of thrombin rather than an active site. The purified fraction from the leaves of persimmon (*Diospyros kaki L.*) did not affect the interaction of a small peptide substrate with thrombin, indicating that it is not an active site-directed inhibitor of thrombin. The delay of fibrinogen clotting time by the purified fraction was more pronounced at lower concentrations of fibrinogen, indicating that the bindings of the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) and fibrinogen to thrombin are mutually exclusive. Glycoprotein IIb/IIIa blockers are also powerful antithrombotic agents and high fibrinogen concentration reduced platelet inhibition by them (Liu *et al.*, 2000; Renda *et al.*, 2003). A plot of the purified fraction from the leaves of persimmon (*Diospyros kaki L.*) as an inhibitor of thrombin-catalyzed fibrin-formation gave an apparent K_i value of 0.5 μg/mL. These results are consistent with the fact that it binds to thrombin at fibrinogen binding sites which are distant from the active site.

Thrombin inhibitors that reversibly block the fibrinogen-binding exosite of thrombin could have better therapeutic value than reversible active site-directed inhibitors (Muramatsu *et al.*, 1997; Lettino and Toschi, 2004). Haemadin from *Haemadipsa sylvestris* is also an exosite biding inhibitor, but it interacts with the heparin binding exosite, not with the fibrinogen-recognition exosite (Richardson *et al.*, 2000). Reversible active site-directed inhibitors are expected to simultaneously inhibit the rate of conversion of fibrinogen to fibrin (Carter *et al.*, 2004) and the rate of the irreversible inactivation of thrombin by antithrombin III, because both were processed through an active site. This was supported by the findings of Naski *et al.* (1990) that the amount of fibrin clot formed by thrombin was not affected by a reversible active site directed inhibitor when the reaction of thrombin with thrombomodulin was insignificant. However, a reversible exosite-directed inhibitor of thrombin could decrease the amount of fibrin clot, if it had a better inhibitory effect on fibrinogen processing than on the reaction of thrombin with antithrombin. Our results showed that purified fraction from the leaves of persimmon (*Diospyros kaki L.*) has an anticoagulant activity by inhibiting thrombin *via* binding to fibrinogen binding sites which are also anion-binding exosites. Since this step represents a choice target for pharmacological intervention, it could be useful in clinical applications of antithrombosis.

ACKNOWLEDGEMENT

This work was supported by 2005 grants from the University of Ulsan in Korea.

REFERENCES

Carter, W. J., Myles, T., Gibbs, C. S., Leung, L. L., and Huntington, J. A., Crystal structure of anticoagulant thrombin variant E217K provides insights into thrombin allostery. *J. Biol. Chem.*, 279, 26387-26394 (2004).

Cho, J., Seo, H., Yun, C., Koo, B., Yoshida, S., Koga, T., Dan, T., and Kim, H., *In vitro* and *in vivo* stuies of AT-1362, a newly synthesized and orally active inhibitor of thrombin. *Thromb. Res.*, 100, 97-107 (2000).

Cho, H. R. and Choi, H. S. Effects of anticoagulant from *Spirodela polyrhiza* in rats. *Biosci. Biotechnol. Biochem.*, 67, 881-883 (2003).

Choi, H. S. and Sa, Y. S., Fibrinolytic and antithrombotic protease from *Spirodela polyrhiza*. *Biosci. Biotechnol. Biochem.*, 65, 781-786 (2001).

Friedrich, T., Kroger, B., Bialojan, S., Lemaire, H. G, Hoffken, H. W., Reuschenbach, P., Otte, M., and Dodt, J., A kazal-type inhibitor with thrombin specificity from *Rhodnius prolixus*. *J. Biol. Chem.*, 268, 16216-16222 (1993).

Herbert, J. M., Herault, J. P, Bernat, A., van Amsterdam, R. G. M., Iormeau, J. C , Petitou, M., van Boeckel, C., Hoffmann, P., and Meuleman, D. G, Biochemical and pharmacological properties of Sanorg 34006, a potent and long-acting synthetic pentasaccharide. *Blood*, 91, 4197-4205 (1998).

Hofmann, W. and Bon, C., blood coagulation induced by the venom of Bothrops atrox. 1. Identification, purification, and properties of a prothrombin activator *Biochemistry*, 26, 772-776 (1987).

Holsat, J., Lindblad, B., and Bergqvist, D., Antithrombotic effect of recombinant truncated tissue factor pathway inhibitor (TFPI1-161) in experimental venous thrombosis-a comparison with low molecular weight heparin. *Thromb. Haemost.*, 71, 214-219 (1994).

Jakubowski, J. A. and Maraganore, J. M., Inhibition of coagulation and thrombin-induced platelet activities by a

synthetic dodecapeptide modeled on the carboxy-terminus of hirudin. *Blood,* 75, 399-406 (1990).

Kang, K. T., Jung, B. I., Bae, O. N., Lee, M. Y., Chung, S. M., Lee, J. Y., Lee, S. K., Kim, I C , and Chung, J. H., Anti-thrombotic activity of LB30057, a newly synthesized direct thrombin inhibitor. *Thromb. Haemost.,* 89,104-111 (2003).

Lettino, M. and Toschi, V., Direct antithrombins: new perspectives in cardiovascular medicine. *Curr. Med. Chem. Cardiovasc. Hematol. Agents,* 2, 267-275 (2004)

Liu, F., Craft, R. M., Morris, S. A., and Carroll, R. C., Lotrafiban: an oral platelet glycoprotein IIb/IIIa blocker. *Expert. Opin. Investig. Drugs*, 9, 2673-2687 (2000).

Markwardt, F., Hirudin as alternative anticoagulant-a historical review. *Semin. Thromb. Hemost.*, 28, 405-414 (2002).

McClanahan, T. B., Hicks, G. W., Morrison, A. L., Peng, Y. W., Janiczek-Dolphin, N., Mertz, T. E., Sullivan, M. E., Morser, J , Juneau, P. L., and Leadley, R., The antithrombotic effects of CI-1031 (ZK-807834) and enoxaparin in a canine electrolytic injury model of arterial and venous thrombosis. *Eur. J. Pharmacol.,* 432,187-194 (2001)

McGowan, E. B. and Detwiler, T. C., Modified platelet responses to thrombin. Evidence for two types of receptors or coupling mechanisms. *J. Biol. Chem.*, 261, 739-746 (1986).

Moon, K. S., In Usage of Herbal Medicine (ed. Choi, O. J.), pp 466-468, Ilwolseogak (1991).

Muramatsu, R., Sasaki, M., Watanabe, N., Goto, Y., Okayama, T., Nukui, E., Morikawa, T., and Hayashi, H., Antithrombotic effects of NE-6505, a novel anion-binding exosite inhibitor. *Thromb. Res.*, 86, 453-460 (1997).

Naski, M. C., Fenton II, J. W., Maraganore, J. M., Olson, S. T., and Shafer, J. A., The COOH-terminal domain of hirudin. *J. Biol. Chem.,* 265, 13484-13489 (1990).

Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L., and Schleu ning, W-D , Triabin, a highly potent exosite inhibitor of thrombin. *J. Biol. Chem.,* 270, 28629-28634 (1995).

Okonogi, T., Hattori, Z , Ogiso, A., and Mitsui, S., Detoxification by persimmon tannin of snake venoms and bacterial toxins *Toxicon,* 17, 524-527 (1979)

Olson, S. T., Bjo, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J., Role of the antithrombin-binding pentasaccharides in heparin acceleration of antithrombin-proteinase reactions. *J. Biol. Chem.,* 267, 12528-12538 (1992).

Rezaie, A. R., Cooper, S. T., Church, F C., and Esmon, C. T., Protein C inhibitor is a potent inhibitor of the thrombin-thrombomodulin complex. *J. Biol. Chem.,* 270, 25336-25339 (1995).

Renda, G., Rocca, B., Crocchiolo, R., Cristofaro, R.D., and Landolfi, R., Effect of fibrinogen concentration and platelet count on the inhibitory effect of abciximab and tirofiban. *Thromb. Haemost* , 89, 348-354 (2003)

Richardson, J. L., Kroger, B., Hoeffken, W., Sadler, J. E., Pereira, P., Huber, R., Bode, W., and Fuentes-Prior, P , Crystal structure of the human alpha-thrombin-haemadin complex: an exosite II-binding inhibitor. *EMBO J.*, 19, 5650-5660 (2000).

Sa, Y. S., Kim, K. A., and Choi, H. S., Purification and characterization of anti-coagulant activity from persimmon stem. *J. Kor. Soc. Food Sci. Nutr.*, 32, 1323-1327 (2003).

Strube, K.-H., Kroger, B , Bialojan, S., Otte, M., and Dodt, J., Isolation, sequence analysis, and cloning of haemadin. *J. Biol. Chem.,* 268, 8590-8595 (1993).

Stubbs, M. T., Oschkinat, H., Mayr, I., Huber, R., Angliker, H., Stone, S. R., and Bode, W., The interaction of thrombin with fibrinogen. A structural basis for its specificity. *Eur. J. Biochem.,* 206, 187-195 (1992).

Tsiang, M , Lentz, S. R., Dittman, W. A., Wen, D., Scarpati, E. M., and Sadler, J. E., Equilibrium binding of thrombin to recombinant human thrombomodulin: effect of hirudin, fibrinogen, factor Va, and peptide analogues. *Biochemistry,* 29, 10602-10612 (1990)

Wall, D., Douglas, S., Ferro, V., Cowden, W., and Parish, C., Characterisation of the anticoagulant properties of a range of structurally diverse sulfated oligosaccharides *Thromb. Res.*, 103, 325-335 (2001)

Wong, P. C., Crain, E. J., Watson, C. A., Zaspel, A. M., Wright, M. R., Lam, P. Y., Pinto, D. J., Wexler, R R., and Knabb, R M., Nonpeptide factor Xa inhibitors III· effects of DPC423, an orally-active pyrazole antithrombotic agent, on arterial thrombosis in rabbits *J. Pharmacol. Exp. Ther.*, 303, 993-1000 (2002).

Yuk, D. Y., Ryu, C. K., Hong, J T., Chung, K. H., Kang, W. S., Kim, Y., Yoo, H S., Lee, M. K., Lee, C. K , and Yun, Y. P., Antithrombotic and antiplatelet activities of 2-chloro-3-[4-(ethylcarboxy)-phenyl]-amino-1,4-naphthoquinone (NQ12), a newly synthesized 1,4-naphthoquinone derivative. *Biochem. Pharmacol.*, 60, 1001-1008 (2000).