Preparation of Aminated Fucoidan and Its Evaluation as an Antithrombotic and Antilipemic Agent¹⁾

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Fucoidan, a sulfated poly(L-fucopyranose), is an effective anticoagulant *in vitro* and *in vivo*. In the present study, an aminated derivative of fucoidan was prepared and examined for its fibrinolytic and anticoagulant activities. The aminated derivative was more potent than native fucoidan as a stimulator of tissue plasminogen activator-induced plasma clot lysis, and its effectiveness was comparable to that of oversulfated fucoidan reported previously. Furthermore, the ability of aminated fucoidan to accelerate heparin cofactor II-mediated thrombin inhibition was 2.3 times more potent than that of native fucoidan. Aminated fucoidan effectively prevented endotoxin-induced hepatic vein thrombosis in hyperlipemic rats and decreased the elevated levels of serum cholesterol and triglyceride. The present results that the anticoagulant and antilipemic potency of fucoidan can be improved by charge modification may provide useful clues for the development of an ideal anticoagulant and antilipemic drug.

Keywords fucoidan; amination; antithrombotic activity; antilipemic activity; hyperlipemic rat

During the last decade, many materials have been proposed as potent anticoagulants. However, the most available material is still heparin, which is used for the prevention of reocclusion after initially successful thrombolysis with recombinant tissue plasminogen activator (t-PA). Several reports have suggested that heparin cannot prevent coronary reocclusion in patients treated with t-PA.^{2,3)} A possible explanation for the limited efficacy of heparin is that it promotes the binding of thrombin to fibrin polymer by forming a ternary complex,⁴⁾ where the rate constant for the inactivation of thrombin by heparin-antithrombin III (AT-III) is decreased 300-fold or more.⁵⁾ The altered reactivity of thrombin toward its substrate and inhibitor is attributable to the growing of thrombus. Thus far, the search for an ideal anticoagulant that maximizes the efficacy of the thrombolytic agent and minimizes undesirable side effects still continues.

Fucoidan, which is enriched in brown marine alga, has been reported previously to have anticoagulant activity.⁶⁾ Recently, Church *et al.* have shown that fucoidan enhances the heparin cofactor II (HC-II)-mediated thrombin reaction more than 3500-fold.⁷⁾ Furthermore, we have indicated that the fibrinolytic and anticoagulant activities of fucoidan can be improved by its chemical sulfation, and that the oversulfated fucoidan exhibits a potent antithrombotic activity in hyperlipemic rats, suggesting the importance of charge density for these actions of the polysaccharide.⁸⁾ To investigate further the structure-activity relationships in fucoidan binding to HC-II and fibrinolytic components, we prepared an amino group-introduced fucoidan derivative. The present study was also designed to evaluate the antithrombotic activity of the aminated fucoidan *in vivo*.

MATERIALS AND METHODS

Materials The following materials were commercially obtained: *Fucus vesiculosus* fucoidan, porcine intestinal mucosa heparin, biotinyl-N-hydroxysuccinimide ester and

Salmonella typhosa lipopolysaccharide (endotoxin) from Sigma Chemical Co., St. Louis, MO, U.S.A.; Sephadex G-100 from Pharmacia Fine Chemical Co., Uppsala, Sweden; Chromozyme TH from Boehringer Mannheim Yamanouchi Co., Tokyo, Japan; S-2251 from KabiVitrum AB, Stockholm, Sweden; HC-II from Diagnostica Stago, Asnieres, France; α_2 -plasmin inhibitor (α_2 -PI) from Protogen AG, Laufelfingen, Switzerland; human Glu-plasminogen from American Diagnostica Inc., Greenwich, CT, U.S.A.; streptavidine-peroxidase conjugate from Bethesda Research Labs. Inc., Gaithersburg, MD, U.S.A.; and bovine thrombin from Mochida Pharmaceutical Co., Tokyo. Recombinant human t-PA (TD-2061, 550000 IU/mg protein) was supplied by Daiichi Pharmaceutical Co., Tokyo. A stabilizing agent added to the t-PA preparation was removed by affinity chromatography on heparin-Sepharose.⁹⁾ Plasminogen-rich human fibrinogen was obtained from KabiVitrum AB and rendered plasminogen-free by lysine-Sepharose chromatography.¹⁰⁾

Preparation of Fucoidan Derivatives Fucoidan (200 mg) was purified on a Sephadex G-100 column (2.5 × 36 cm), equilibrated with 0.5 M NaCl. Fractions corresponding to an 80–100 kDa molecular mass region were collected and dialyzed against water at 25 °C overnight. The dialysate (purified fucoidan) was lyophilized and stored at –20 °C until use. Oversulfated fucoidan with a molecular mass of 100–130 kDa was prepared as described previously.⁸⁾ Amination of the purified fucoidan was carried out as follows. To introduce a spacer (–O–CH₂CH(O)CH₂–), the fucoidan (500 mg) was dissolved in 750 μ l of water and mixed with 250 μ l of 2.6 N NaOH and 150 μ l of epichlorohydrin. The mixture was stirred at 40 °C for 2 h and dialyzed against water at 25 °C for 2 d. After lyophilizing the dialysate, the residue was dissolved in 3 ml of 30% ammonia water and aminated at 40 °C for 90 min. The reaction mixture was dialyzed against water and lyophilized. The aminated product (–O–CH₂CH(OH)CH₂NH₂) was dissolved in 0.5 M NaCl and purified on the Sephadex G-100 column. Fractions corresponding to a 100–130 kDa molecular mass region

were collected.

Determination of Fucoidan Fucoidan concentration was determined by the anthrone- H_2SO_4 method,¹¹⁾ using D-fucose as standard.

Determination of Amino and Sulfate Groups The introduced amino group onto fucoidan was estimated by elemental analysis with a CHNO-rapid Analyzer (Heraeus Co., Germany). The sulfation degree of fucoidan was estimated turbidimetrically as barium sulfate after HCl hydrolysis. Gelatin was used as a cloud-stabilizer and absorbance was measured at 360 nm.¹²⁾

Amidolytic Assays All enzyme assays were carried out in 96-well plates, and their activities were measured at 25°C with a microplate reader (EIA Reader model MPR-A4, Toyo Soda, Tokyo, Japan). The inhibitory effect of α_2 -PI on the plasmin generation by t-PA was determined as described previously.⁸⁾ The assay mixture contained 0.75 mM S-2251, 150 nM Glu-plasminogen, 0 to 6 $\mu\text{g}/\text{ml}$ α_2 -PI, 250 IU/ml t-PA, 50 mM phosphate buffer (pH 7.5)/0.15 M NaCl/0.05% (v/v) Tween 80 and, if indicated, 50 $\mu\text{g}/\text{ml}$ fucoidan or its derivative in a total volume of 200 μl . The assay was started by addition of t-PA and the absorbance at 405 nm was measured after 30 min. The fucoidan-catalyzed HC-II-thrombin reaction was determined as follows. Thrombin (0.05 NIH units) and HC-II (0.008 units) were incubated for 10 min with various amounts of fucoidan or its derivative in a total volume of 150 μl of 50 mM Tris-HCl buffer (pH 7.5)/0.15 M NaCl/0.1% (w/v) poly(ethylene glycol) 4000. To the reaction mixture was added 50 μl of 0.64 mM Chromozyme TH, and the change in absorbance at 405 nm was monitored for 10 min.

Determination of Clot Lysis Time Human plasma (250 μl) was mixed at 4°C with 50 μl of a t-PA solution (100 IU/ml in 20 mM sodium phosphate buffer (pH 7.2)/0.15 M NaCl/0.05% (v/v) Tween 20 (TPBS), and the mixture was clotted by adding 50 μl of thrombin (50 NIH units/ml). Various amounts of fucoidan or its derivative dissolved in 150 μl of 20 mM TPBS were overlaid on the clots, and then the clot lysis time was determined at 37°C.

Protein Determination Protein concentration was determined by the method of Lowry *et al.*,¹³⁾ using bovine serum albumin (BSA) as standard.

Preparation of Biotin-Labeled Aminated Fucoidan Aminated fucoidan (20 mg) was dissolved in 5 ml of 0.1 M NaHCO_3 (pH 8.4) and mixed with 200 μl of a biotinyl-N-hydroxysuccinimide ester solution (1 mg/ml in dimethylsulfoxide). The mixture was stirred at 25°C for 2 h and dialyzed against water at 4°C overnight. The dialysate (biotin-labeled aminated fucoidan) was stored at -20°C until use.

Binding of Aminated Fucoidan to Fibrinolytic Components Fibrinolytic components were immobilized on the wells of a polystyrene microtiter plate. Each protein was dissolved in 20 mM PBS at a concentration of 0.8 nmol/ml, and 200 μl of the solution was incubated at 4°C overnight in the wells. The wells were washed 5 times with 200 μl of 20 mM TPBS and incubated at 4°C overnight with 200 μl of 20 mM PBS containing 1% (w/v) BSA. After washing exhaustively with 20 mM TPBS, various amounts of biotin-labeled aminated fucoidan (200 μl) were added to

the wells. The wells were incubated at 25°C for 2 h and washed 5 times with 200 μl of 20 mM TPBS. To detect the bound fucoidan, 200 μl of a streptavidin-peroxidase conjugate solution (0.7 $\mu\text{g}/\text{ml}$) was added to each well and incubation followed at 25°C for 1 h. The activity of bound peroxidase was measured with *o*-phenylenediamine as described previously.¹⁴⁾

Animal Experiments Wistar male rats (5 weeks old, 110–120 g) were obtained from the Animal Center of Fukuoka University. The animals were divided into three groups (4–6 rats each) and maintained for 50 d on water and a butter-rich diet as described previously.⁸⁾ Rats received a weekly intravenous injection of aminated fucoidan or heparin at the dosage of 1 mg/kg of body weight. Control rats were injected with vehicle (saline) alone. Thrombosis in the hepatic veins was induced in each animal by intravenous injection of 0.3 mg/kg of body weight of endotoxin 2 h after the last polysaccharide injection. The animals were deprived of the diet, but not water, for 17 h before the endotoxin injection and killed with chloroform 24 h after the injection. The liver was weighed before and after removal of the red infarcted parts. Severity of thrombus was quantitatively expressed in terms of percentage in grams of infarcted liver. Blood samples were taken from the tail veins at definite days after an overnight fasting. Serum cholesterol and triglyceride concentrations were determined using the reagent kits, Iatrolipo TC and Cleantech TG-S (Iatron Labo. Inc.), respectively. Other experimental details were as described previously.⁸⁾

Statistical Analysis of Data All values are expressed either as mean or mean $\pm$ S.E. (as indicated), and the significance level between groups was calculated by Student's *t* test.

RESULTS

Preparation of Aminated Fucoidan Derivative Native fucoidan (100 kDa) is a polymer of L-fucose linked primarily α 1 $\rightarrow$ 2 with either a C₃- or a C₄-sulfate.⁶⁾ Therefore, the non-sulfated (hydroxylated) C₃- or C₄-position is predicted to accept easily epoxyactivation with epichlorohydrin. Aminated fucoidan was prepared by introducing amino groups onto the epoxyactivated fucoidan (see Materials and Methods). Elemental analysis data were as follows. *Anal.* Calcd for C₁₂H₂₀O₁₄S₂ (fucose-fucose unit of fucoidan): C, 31.86; H, 4.42. Found: C, 29.73; H, 4.83. *Anal.* Calcd for C₁₈H₃₄N₂O₁₆S₂ (aminated fucose-fucose unit): C, 36.12; H, 5.69; N, 4.68. Found: C, 28.70; H, 5.32; N, 1.75. Also, the sulfate contents of native and aminated fucoidans were estimated to be 31.0% and 29.4%, respectively, suggesting no loss of sulfate groups during the amination reaction. Because fucoidan itself is originally a heterogenous polymer, we judged that the introduction of amino groups is not perfect but adequate to investigate the characteristics as an aminated derivative of fucoidan.

In Vitro Fibrinolytic and Anticoagulant Activities of Aminated Fucoidan Figure 1 shows the effects of fucoidan and its derivatives on human plasma clot lysis by t-PA. The lysis time of plasma clot was shortened from 133 to

Fig. 1. Effects of Native (○), Aminated (△) and Oversulfated (●) Fucoidans on Human Blood Plasma Clot Lysis Caused by t-PA

Experimental details are described in Materials and Methods. Each point represents the mean $\pm$ S.E. of four experiments.

Fig. 2. Effects of Fucoidan and Its Derivatives on α_2 -PI Inhibition of Plasmin Activity

Each assay was performed in the presence or absence of 50 μ g/ml fucoidan or its derivative. Other experimental details are described in Materials and Methods. Activities obtained by native fucoidan (○), aminated fucoidan (△), oversulfated fucoidan (●) and without fucoidan (▲) are presented. Each point is the mean of duplicate experiments.

84 min by addition of 5 μ g/ml of oversulfated or aminated fucoidan. The maximal fibrinolytic activity of t-PA was 2.5 times that of the control in the presence of each fucoidan derivative, as judged from the calibration curve of t-PA concentration-dependent lysis time (data not shown). However, with less than 5 μ g/ml, none of the fucoidan derivatives affected the clot lysis. When more than 10 μ g/ml of each fucoidan derivative was added, the lysis time began to be prolonged. Native fucoidan was less potent as a stimulator than the two derivatives. The effects of fucoidan and its derivatives on α_2 -PI inhibition of t-PA-catalyzed plasminogen activation were next examined using S-2251 as substrate. As shown in Fig. 2, in the absence of α_2 -PI, oversulfated fucoidan increased plasmin generation 2.8-fold more effectively than the aminated derivative. Oversulfated fucoidan exerted its stimulatory effect on the plasminogen activation even in

Fig. 3. Dose-Dependent Binding of Aminated Fucoidan to Various Fibrinolytic Components

t-PA (△), Glu-plasminogen (▲), α_2 -PI (□), fibrinogen (○) and BSA (●) were immobilized on the wells of a polystyrene microtiter plate. Other experimental details are described in Materials and Methods. Each point is the mean of duplicate experiments.

Fig. 4. Effects of Fucoidan and Its Derivatives on HC-II-Mediated Thrombin Inhibition

Experimental details are described in Materials and Methods. Symbol key: (○), native fucoidan; (△), aminated fucoidan; (●), oversulfated fucoidan; and (□), heparin. Each point is the mean of duplicate experiments.

the presence of high concentrations of α_2 -PI. In the presence of 50 μ g/ml of aminated fucoidan, the generated plasmin was somewhat insensitive to α_2 -PI. Native fucoidan had a weak effect on the t-PA potentiation and the protection of plasmin activity from α_2 -PI. Figure 3 shows the binding ability of aminated fucoidan to various fibrinolytic components. Biotin-labeled aminated fucoidan (0.1–20 μ g/well) bound to the immobilized t-PA, plasminogen, fibrinogen and α_2 -PI in a dose-dependent manner, while it did not bind to the immobilized BSA used as reference. This result suggests the specific binding of aminated fucoidan to these fibrinolytic components.

Figure 4 shows the effects of fucoidan and its derivatives on HC-II-mediated thrombin inhibition. Assessment of the anticoagulant activities of these fucoidans with the concentration required for 50% inhibition revealed it to be in the order of oversulfated fucoidan (0.19 μ g/ml) > aminated fucoidan (0.29 μ g/ml) > native fucoidan (0.66

$\mu\text{g/ml}$). Heparin exerted 50% inhibition at a concentration of $0.19 \mu\text{g/ml}$. Also, aminated fucoidan inhibited the thrombin activity in the presence of AT-III by 50% at a concentration of $3.2 \mu\text{g/ml}$, and its potency was almost the same as that of native fucoidan (data not shown).

In Vivo Antithrombotic and Antilipemic Activities of Aminated Fucoidan To examine the effect of aminated fucoidan on the prevention of coagulation in an animal model, we created hyperlipemic and hepatic vein thrombosis model rats as described previously.⁸⁾ The animals were injected weekly with aminated fucoidan or heparin at a dose of 1 mg/kg of body weight, which is within the clinical dosage of heparin. As shown in Fig. 5A, the serum levels of triglyceride in the control animals fed a butter-rich diet reached maximum (177 mg/dl) at 20 d and then returned to the basal value. A similar pattern

Fig. 5. Changes in Serum Triglyceride (Panel A) and Cholesterol (Panel B) Levels in Hyperlipemic Rats

The animals were treated with aminated fucoidan (Δ), heparin ($\square$) or vehicle alone ($\bullet$). Other experimental details are described in Materials and Methods. Each point represents the mean $\pm$ S.E. (4–6 rats per group). Significantly different from the corresponding control value: a) $p < 0.05$; b) $p < 0.01$.

was seen in the serum triglyceride levels in heparin-treated rats, except that the maximal value (127 mg/dl) at 20 d was different from that in control rats. The serum triglyceride levels in aminated fucoidan-treated rats, however, were generally lower than those in the control animals, and maximal value was 108 mg/dl at 10 d. The data indicate that aminated fucoidan is effective in reducing the serum triglyceride. The serum cholesterol levels in control rats increased up to 518 – 531 mg/dl after 10–20 d and then decreased gradually to 180 mg/dl at 50 d (Fig. 5B). Aminated fucoidan effectively lowered the elevated cholesterol levels, while heparin seemed to have a less potent effect. These results indicate that the aminated derivative has an ability to lower the elevated levels of serum triglyceride and cholesterol in hyperlipemic rats. Table I shows the effects of aminated fucoidan and heparin on endotoxin-induced hepatic vein thrombosis in hyperlipemic rats. All of the control animals (group A) developed thrombosis with a severity of $16.5 \pm 1.5\%$. Sixty-seven percent of group B rats given 1 mg/kg of heparin weekly had thrombosis with $1.1 \pm 0.5\%$ severity. Aminated fucoidan effectively prevented the thrombus formation (group C, thrombosis in 40% of the animals with $2.2 \pm 1.1\%$ severity).

DISCUSSION

We have previously shown that fucoidan and its oversulfated derivatives stimulate t-PA-catalyzed plasminogen activation and plasma clot lysis, and decrease the polymerization rate of fibrin, in proportion to the increase in sulfation degree.⁸⁾ The present study aimed to investigate further the structural feature of fucoidan which acts as a potent anticoagulant *in vitro* and *in vivo*.^{6–8)} As the experimental results indicate, aminated fucoidan, as well as oversulfated fucoidan, was a potent stimulator of t-PA-induced plasma clot lysis (Fig. 1). The effectiveness of fucoidans was limited only at a low concentration range (less than $10 \mu\text{g/ml}$). At higher concentrations, fucoidans had a tendency to prolong the clot lysis time. This phenomenon may be caused by the tight binding of fucoidans to fibrin(ogen).⁸⁾ However, a totally desulfated fucoidan⁸⁾ and its aminated derivative had no potency to stimulate the clot lysis (data not shown). These findings suggest that sulfate groups are still essential for the binding of aminated fucoidan to fibrinolytic components such as t-PA and plasminogen (Fig. 3). The question arises why amination, as well as oversulfation, endows fucoidan with improved fibrinolytic activity. Although the precise mechanism by which these fucoidans stimulate t-PA-catalyzed plasminogen activation (Fig. 2) and plasma clot

TABLE I. Effects of Aminated Fucoidan and Heparin on Endotoxin-Induced Hepatic Vein Thrombosis in Hyperlipemic Rat

Group	Polysaccharide treatment (1 mg/kg/week)	No. of rats	Severity of thrombosis (%)		$p^a)$
			Individual value	Mean $\pm$ S.E.	
A	None	4	16.7, 12.4, 16.9, 19.9	16.5 ± 1.5	
B	Heparin	6	0.6, 0, 1.8, 1.4, 0, 2.9	1.1 ± 0.5	< 0.01
C	Aminated fucoidan	5	4.7, 0, 0, 0, 6.6	2.2 ± 1.1	< 0.001

a) Statistical significance of differences compared to A group.

lysis (Fig. 1) is not known, they may serve as a "template" on which both molecules align in a conformation suitable for the plasminogen activation by t-PA. As assumed from the data of Fig. 2, aminated fucoidan may bind to α_2 -PI more tightly and protect plasmin activity from the inhibitor more effectively than native or oversulfated fucoidan, while its stimulatory effect on the plasminogen activation was weaker than that of oversulfated fucoidan. Thus, the amino groups may also play a role in such protection of plasmin activity from α_2 -PI.

The ability of fucoidan to accelerate HC-II-mediated thrombin inhibition was also improved by its amination (Fig. 4), although its ability to accelerate AT-III-thrombin reaction was not (data not shown). Fucoidan- and heparin-binding sites of HC-II or AT-III may be located in the same region of each protein.⁷⁾ The introduction of positively charged groups onto fucoidan may improve its binding affinity for the heparin-binding sites of HC-II which are proposed to be Lys-173 to Leu-190 and Phe-183 to Arg-200.⁷⁾

We showed that weekly administration of aminated fucoidan to hyperlipemic rats resulted in a significant diminution of the venous thrombi (Table I). The fucoidan derivative also had an antilipemic effect (Fig. 5A). One mechanism may be the displacement of lipolytic enzymes by the competition of aminated fucoidan with heparan sulfate, which is believed to be the natural ligand present on the endothelial cell surface.¹⁵⁾ The release of the enzymes into the circulation minimizes the vascular damage caused by cholesterol-rich lipoproteins.¹⁶⁾ Aminated fucoidan lowered the elevated serum cholesterol levels, and its ability seemed to be more potent than that of heparin (Fig. 5B). Therefore, such actions of aminated fucoidan may have contributed to prevention of the development of atherosclerosis and thrombus formation in the hyperlipemic rats, together with its fibrinolytic and anticoagulant effects. Finally, we conclude that positive charges added onto native fucoidan improve both fibrinolytic and anticoagulant actions of this polysaccharide, and that the aminated derivative is newly endowed with antilipemic potency. We are continuing

efforts to synthesize and investigate a variety of well-defined fucoidan derivative(s) for an improved anticoagulant therapy.

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REFERENCES AND NOTES

- 1) Abbreviations used: t-PA, tissue plasminogen activator; Glu-plasminogen, native human plasminogen with NH₂-terminal glutamic acid; HC-II, heparin cofactor II; AT-III, antithrombin III; α_2 -PI, α_2 -plasmin inhibitor; S-2251, H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide; Chromozyme TH, tosylglycyl-L-prolyl-L-arginine *p*-nitroanilide; BSA, bovine serum albumin.
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