Pharmacodynamic Parameters of Anticoagulants Based on Sulfated Polysaccharides from Marine Algae

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Fucoidans isolated from *Fucus evanescens* and *Laminaria cichorioides* kelp can inhibit thrombin and factor Xa of the blood coagulation system. In rats, intravenous injection of fucoidans dose-dependently increased anticoagulant activity of the plasma. Fucoidans can form complexes with protamine sulfate. The observed quantitative differences in the action of fucoidans can result from different sulfation degree and the presence of various types of glycoside bonds in polysaccharide molecules.

Key Words: *fucoidans; thrombin inhibition; Xa factor inhibition; pharmacodynamics; protamine sulfate*

Sulfated polysaccharides (fucoidans) extracted from algae demonstrate anticoagulant and antithrombotic activities [5-8]. We previously showed that sulfated polysaccharide from brown algae *F. evanescen* (Sea of Okhotsk) exhibits heparin-like anticoagulant activity *in vivo* and *in vitro* mediated by thrombin inhibition [2].

Our aim was to study the mechanisms of anticoagulant action of the fucoidans isolated from *F. evanescens* (Sea of Okhotsk) and *L. cichorioides* (Japanese Sea), in particular, specificity of anticoagulant activity, pharmacodynamics after intravenous injection, and ability to form complexes with known heparin antidote protamine sulfate.

MATERIALS AND METHODS

The fucoidans were extracted from *F. evanescens* and *L. cichorioides* kelp as described previously [13].

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Specific anticoagulant activity of fucoidan samples was determined *in vitro* by inhibition of Xa factor (anti-Xa- or aHa-activity) [12] and thrombin (anti-IIa or aIIa-activity) [9]. For measuring aIIa- and aXa-activity, we prepared a series of fucoidan solutions (0–10.0 mg/ml) and solutions of standards (International NIBSC standard I of low-molecular-weight heparin and International NIBSC standard V of non-fractionated heparin, respectively). Specific activity was calculated in U/mg by comparison of optical density of the test and standard solutions during hydrolysis of chromogenic substrates (S-2238 and S-2222, λ=405 nm). Human antithrombin, human Xa factor, bovine thrombin, chromogenic substrates S-2238 and S-2222 for thrombin and Xa-factor, respectively, were from Dade Behring and Sigma.

Plasma anticoagulant activity was measured after administration of fucoidans (5 or 10 mg/kg) into the jugular vein of male Wistar rats weighing 350-400 g. The blood was collected from the contralateral jugular vein into a plastic tube containing 0.11 M $C_6H_5O_7Na$ (9:1). Platelet-poor plasma was obtained by centrifugation at 1400*g* for 20 min. Anti-IIa- and aXa-activities of rat plasma were accessed 5-180 min postinjection. Elimination half-

life was calculated by linearization of the pharmacokinetic curves of plasma activity [4].

Rocket electrophoresis [1] of fucoidan solutions was carried out on glass plates in 1% agarose containing protamine sulfate. The height and area of the precipitation peaks were evaluated using PhotoM 1.31 software.

The data were processed statistically using Student's *t* test.

RESULTS

Fucoidans isolated from *F. evanescens* and *L. cichorioides* differed by the structure of the main chain, molecular weight, and sulfation degree. Fucoidan isolated from *F. evanescens* kelp is a polymer with main chain consisting of alternating (1→3)- and (1→4)-bound fucose residues sulfated mainly by C-2. In addition to fucose (Fuc, 90%), this fucoidan contains insignificant amounts of xylose (Xyl, 3%), mannose (Man, 2%), and galactose (Gal, 5%), the ratio of SO_4^{2-}:Fuc being 0.8:1.0. Fucoidan isolated from *L. cichorioides* kelp is (1→3)-α-L-fucan (Fuc, 100%) sulfated by C-2 and by C-4, the ratio of SO_4^{2-}:Fuc being 0.8:1.0. The molecular weight distribution peaks of polysaccharides derived from *F. evanescens* and *L. cichorioides* correspond to 30 and 50 kDa, respectively [3,13].

Inhibitory activities against thrombin (aIIa-activity) of *F. evanescens* and *L. cichorioides* fucoidans were 22±7 and 43±8 U/mg, respectively, while activities against Xa-factor were 28±7 and 31±8 U/mg, respectively (Table 1). The potency of direct anticoagulants to inhibit activity of IIa- and Xa-factors of the blood coagulation system is their significant feature, which is especially important in drugs used to prevent thrombosis. The aXa/aIIa ratio for *F. evanescens* fucoidan was 1.3, which attests to its practical prospects. The corresponding values for widely used non-fractionated and low-molecular weight heparins are 1.0 and 1.5-6.0 [12].

Plasma anticoagulant activity in experimental rats increased with increasing the intravenous dose of fucoidans from *F. evanescens* and *L. cichorioides*. Injection of *F. evanescens* fucoidan resulted in therapeutically sufficient aIIa plasma activity (up to 2.5 U/ml) and a low aXa-activity (up to 0.3 U/ml), while *L. cichorioides* fucoidan increased aIIa- and aXa-activities to 3.1 and 0.95 U/ml, respectively (Table 2). This anticoagulant effect (assessed by aIIa-activity) persisted for 3 h and longer for both fucoidans. The elimination half-life of fucoidans was dose-dependent, being 15 and 60 min in the first case and 60 and 90 min in the second case. Despite higher ratio of aXa/aIIa activities for *F. evanescens* fucoidan, the intensity and duration of the anticoagulant effect of *L. cichorioides* fucoidan was more pronounced and persisted for a longer time. This paradox can be explained by differences in specific antithrombin activity of the agents (2-fold higher aIIa activity of *L. cichorioides* fucoidan) and similar inhibitory activities against Xa-factor.

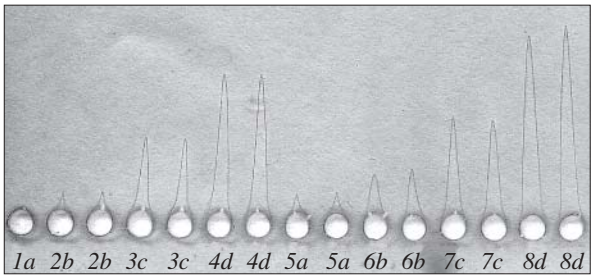

Fig. 1. Electrophoregram of fucoidans isolated from *F. evanescens* (1-4) and *L. cichorioides* (5-8) obtained in agarose gel with protamine sulfate. *a*) 0.625 μg; *b*) 1.25 μg; *c*) 2.5 μg; *d*) 5 μg.

Neutralization of the anticoagulant effect is required in some patient at risk of bleeding resulting after long-term administration of anticoagulant preparations, during the extracorporeal circulation used in open-heart surgery or in programmed hemodialysis (applied in the course of substitution renal therapy) where non-fractionated or low-molecular weight heparins are administered [11]. Elimination of heparin-induced anticoagulant effect results from electrostatic interaction between negatively charged polysaccharide and positively charged protamine sulfate (heparin antidote) followed by complex formation [10]. The result of rocket electrophoresis of both examined fucoidans in agarose gel with protamine sulfate is shown in Figure 1, where stained areas of precipitation complexes

TABLE 1. Anticoagulant Activity of Fucoidans and Mobility of Fucoidan—Protamine Sulfate Complexes in Electric Field ($\bar{X}$±*SD*, *n*=7)

Index		*F. evanescens*	*L. cichorioides*
Activity, U/mg			
aHa		28±7	31±8
aIIa		22±7	43±8
aHa/aIIa		1.3	0.7
Fucoidan concentration, μg/ml			
500	h, px	49±6	46±2
	S, px	361±56	373±23
1000	h, px	94±7	104±3
	S, px	1229±80	1230±75

Note. *h* and *S* are the height and area of precipitation peaks.

TABLE 2. Anticoagulant Activity of Rat Plasma after Intravenous Injection of Fucoidans Isolated from Brown Seaweed *F. Evanescens* and *L. Cichorioides* Help (U/ml, $X \pm SD$)

Fucoidan dose, mg/kg	Activity, U/mg	Postinjection time, min					
		5	15	30	60	120	180
F. evanescens							
5	aHa	0.20±0.02	0.11±0.03				
	aIIa	2.30±0.23***	1.10±0.15***	1.30±0.34***	0.70±0.22**	0.60±0.02**	0.5±0.09*
10	aHa	0.30±0.02	0.15±0.02				
	aIIa	2.50±0.36***	2.00±0.27***	1.70±0.15***	1.20±0.14***	0.70±0.09**	0.20±0.01
L. cichorioides							
5	aHa	0.81±0.06**	0.31±0.05*	0.12±0.03			
	aIIa	2.80±0.25***	2.40±0.21***	2.10±0.22***	1.4±0.1***	1.10±0.09***	0.60±0.05**
10	aHa	0.95±0.06***	0.77±0.05***	0.61±0.13**	0.43±0.09**	0.29±0.10*	0.11±0.06
	aIIa	3.10±0.24***	2.70±0.21***	2.4±0.2***	1.60±0.11***	1.30±0.13***	0.90±0.08**

Note. Prior to injection, aIIa- and aXa-plasma activity were 0.10±0.04 and 0.12±0.03 U/ml, respectively. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to preinjection values.

look like the rockets. The examined fucoidans applied in concentrations of 125, 250, 500, and 1000 μg/ml formed complexes with protamine sulfate. The height of peaks increased with increasing fucoidan concentration (Table 1). The formation of fucoidan-protamine sulfate complexes makes it possible to test the possibility of neutralization of anticoagulant activity of the examined fucoidans *in vitro* (using chromogenic substrates, fibrinogen, or human plasma) and *in vivo* (neutralization during intravenous injection to animals).

Thus, fucoidans isolated from *F. evanescens* and *L. cichorioides* kelp can inhibit factor IIa (thrombin) and factor Xa. Intravenous injection of fucoidans to rats increased plasma anticoagulant activity. The examined fucoidans can form complexes with protamine sulfate. The revealed individual peculiarities in the action of examined fucoidans could be related to various degree of sulfation and various types of glycoside bonds in them.

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