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Inhibition of Thrombin and Factor Xa by *Fucus Evanescens* Fucoidan and Its Modified Analogs

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Specimens of fucoidan extracted from *Fucus evanescens* were purified from protein and polyphenols, deacetylated and depolymerized by fucoidanase for evaluation of their biological activity. Deacetylation did not modify the capacity of fucoidan to inhibit thrombin and factor Xa, while purification from protein and polyphenols reduced this capacity. Depolymerization of fucoidan increased its capacity to inhibit thrombin mainly through heparin cofactor II. All the studied specimens formed complexes with protamine sulfate.

Key Words: *fucoidans; anticoagulant activity; thrombin; factor Xa*

Anticoagulant (AC) activity of heparins is explained by their capacity to stimulate inhibition of serine proteases of the blood clotting system by activating plasma inhibitors, which leads to conformational changes in the structure of antithrombin and heparin cofactor II [9]. However, heparins are immunodepressants and possess high hemorrhagic activity [13], which necessitates the search for new sources of substances with AC and antithrombotic activities. Sea algae and invertebrates contain fucoidans, galactans, and other bioactive substances [5]. Fucoidans (brown algae sulfated polysaccharides) consist mainly from α-L-fucopyranose residues. In addition to α-L-fucose, they can contain galactose, mannose, xylose, glucuronic acid; their molecules can also contain acetates in addition to sulfates, depending on the natural source of fucoidans. Since fucoidans are substances of plant origin, the probability of infectious agents (such as prions) is low for them. We previously showed that fucoidans isolated from *Fucus evanescence* and *Laminaria cichorioides* (brown algae of the Okhotsk Sea) inhibit thrombin and blood clotting factor Xa [2,3].

Here we compared the capacities of initial fucoidan specimen isolated from *Fucus evanescens* and its analogs obtained by chemical or enzymatic modification of the initial preparation to inhibit thrombin and blood clotting factor Xa *in vitro* and evaluated the possibility of complex formation between these preparations and protamine sulfate, a well-known antidote of heparin AC activity.

MATERIALS AND METHODS

Fucoidan (FeF and FeF1) from *Fucus evanescens* brown algae was isolated [14]; enzymatic hydro-

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lysis of fucoidan was carried out as described previously [10]; the preparation was purified from polyphenols [4], from proteins by Sevag's method [1]; deacetylation was carried out by a previously described method [8]. Structural characteristics of fucoidan and its modified analogs are presented in Table 1.

Inhibition of the formation of plasma fibrin clot was evaluated by activated partial thromboplastin time (APTT) using ReaClot-heparin kit (Renam) [6,11]. Rabbit blood was obtained from the marginal ear vein and collected into plastic tubes with 0.11 M $C_6H_5O_7Na$ solution (9:1 blood-anticoagulant ratio). For obtaining platelet-poor plasma, the tubes were centrifuged at 1400*g* for 20 min. Commercial lyophilized human plasma (Renam) was used. Specific antithrombin (antifactor IIa; aIIa) and antifactor Xa (aXa) activities were calculated from calibration curves of International Reference Specimens of nonfractionated/low molecular weight heparins 5 and 1 [2].

Inhibition of amidolytic activities of thrombin and factor Xa with calculation of fucoidan concentrations, at which amidolytic activity decreased 2-fold (IC_{50}) in comparison with the control, was evaluated as described previously [11,12]. In order to measure aIIa and aXa activities in comparison with heparin reference samples, serial dilutions of fucoidan and its analogs in 0.05 M Tris-HCl buffer with 0.175 M NaCl (pH 7.4) in concentrations from 0.05 to 80.00 μg/ml were prepared. Specific activities (U/mg) were calculated by comparing changes in optical densities of solutions of the studied samples and reference samples during hydrolysis of chromogenic substrates S2238 or S-2222 at λ=405 nm. The following reagents were used: human antithrombin, human factor Xa, bovine thrombin, chromogenic substrates for thrombin (S-2238) and for factor Xa (S-2222) (Biopool, Renam, and Technology Standard).

Rocket biospecific electrophoresis of the test solutions was carried out at voltage gradient of 10 V/cm on glass plates in 1% agarose gel containing protamine sulfate [2]. Specimens of the studied preparations were put into 3-4 mm wells (0.31-5.00 μg). After 30 min, the plates with the gels were stained with 0.1% alcyan blue. Plates with dry gel were scanned and the precipitation peaks were measured in the jpg format. The height and areas of the precipitation peaks were evaluated using PhotoM-1.31 software.

The results were presented as arithmetic mean± error of the mean. The significance of differences were evaluated by Student's *t* test.

RESULTS

Rabbit plasma clotting time in APTT and ReaClot tests increased with increasing the concentrations of the initial fucoidan sample 1 and its derivative free from proteins and polyphenols (sample 2). The effects of the studied preparations on the formation of plasma clot was evaluated by the concentration at which plasma clotting time was 2-fold longer in comparison with the control. The initial sample better inhibited plasma clot formation than sample 2. The concentrations of samples at which plasma clotting time increased 2-fold (2APTT) in comparison with the control (18.3±0.6 sec) were 0.60±0.05 and 13.5±1.3 μg/ml for samples 1 and 2, respectively. Specific aIIa activities of the samples in APTT test calculated by the heparin reference sample, were 60±11 U/mg for sample 1 and 12±5 U/mg for sample 2. Sample 1 stronger inhibited the formation of plasma clot in the ReaClot test than sample 2. The concentrations of samples at which plasma clotting time increased 2-fold (2ReaClot) in comparison with the control (30.8±0.7 sec) were 1.8± 0.5 and 11.0±1.0 μg/ml for samples 1 and 2, respectively. Specific aXa activities in the ReaClot

TABLE 1. Structural Characteristics of Fucoidan and Its Modified Analogs

Specimen No.	Code of sample	Molecular weight, kDa	Fuc: SO_3Na	Monosaccharide composition, mole %				
				Fuc	Man	Gal	Xyl	Glc
1	FeF	20-40	1.0:0.8	90.0	2.0	3.0	0	5.0
2	F2	10-40	1.0:1.0	98.0	0	2.0	0	0
3	FeF1	20-40	1.0:0.9	75.2	2.1	7.7	5.5	9.5
4	FeF1LMF	8-18	1.0:0.9	71.0	2.8	16.0	8.3	1.8
5	FeFDA	—	1.0:0.8	75.1	4.3	10.1	6.0	4.5

Note. Here and in Tables 2, 3: *1*) FeF: *F. evanescens* fucoidan; *2*) F2: fucoidan from *F. evanescens*, free from polyphenols and proteins; *3*) FeF1: *F. evanescens* fucoidan; *4*) FeF1LMF: low molecular-weight fraction of fucoidan from *F. evanescens*, obtained by enzymatic hydrolysis of the native FeF1 specimen; *5*) FeFDA: deacetylated specimen of FeF initial fucoidan from *F. evanescens*.

test were 36.5±4.0 U/mg for sample 1 and 20.3±3.0 U/ml for sample 2. Purification of *F. evanescens* fucoidan from polyphenols and protein decreased specific anticoagulant activity. The inhibitory activity of sample 2 towards thrombin decreased 5-fold in comparison with sample 1. Activity towards factor Xa decreased 1.8 times.

The initial fucoidan sample, its deacetylated derivative, and low-molecular-weight fraction obtained by enzymatic hydrolysis of the initial sample, increased human plasma clotting time (Fig. 1, *a*, *b*). The test samples in concentrations of 5 to 80 μg/ml changed the time of plasma clotting in the APTT test (Fig. 1, *a*). Sample 4 exhibited maximum capacity to prolong human plasma clotting time, while samples 3 and 5 were least effective in these experiments. 2APTT fucoidan concentrations for samples 3, 4, and 5 were 120±7, 15±3, and 110±5 μg/ml, respectively (Table 2).

The effects of the studied fucoidans on changes in human plasma clotting was shown in the ReaClot test (Fig. 1, *b*). Sample 5 most markedly prolonged plasma clotting time (Fig. 1, *b*). 2ReaClot concentrations of samples 3, 4, and 5 were 64.7±10.0, 88±7, and 56.5±7.0 μg/ml, respectively (Table 2).

Differences in effective concentrations and activities of native fucoidan samples (samples 1 and 3) can be explained by differences in structural characteristics and species specificities of rabbit and human plasma [7].

A relationship between inhibition of thrombin and factor Xa amidolytic activities and the con-

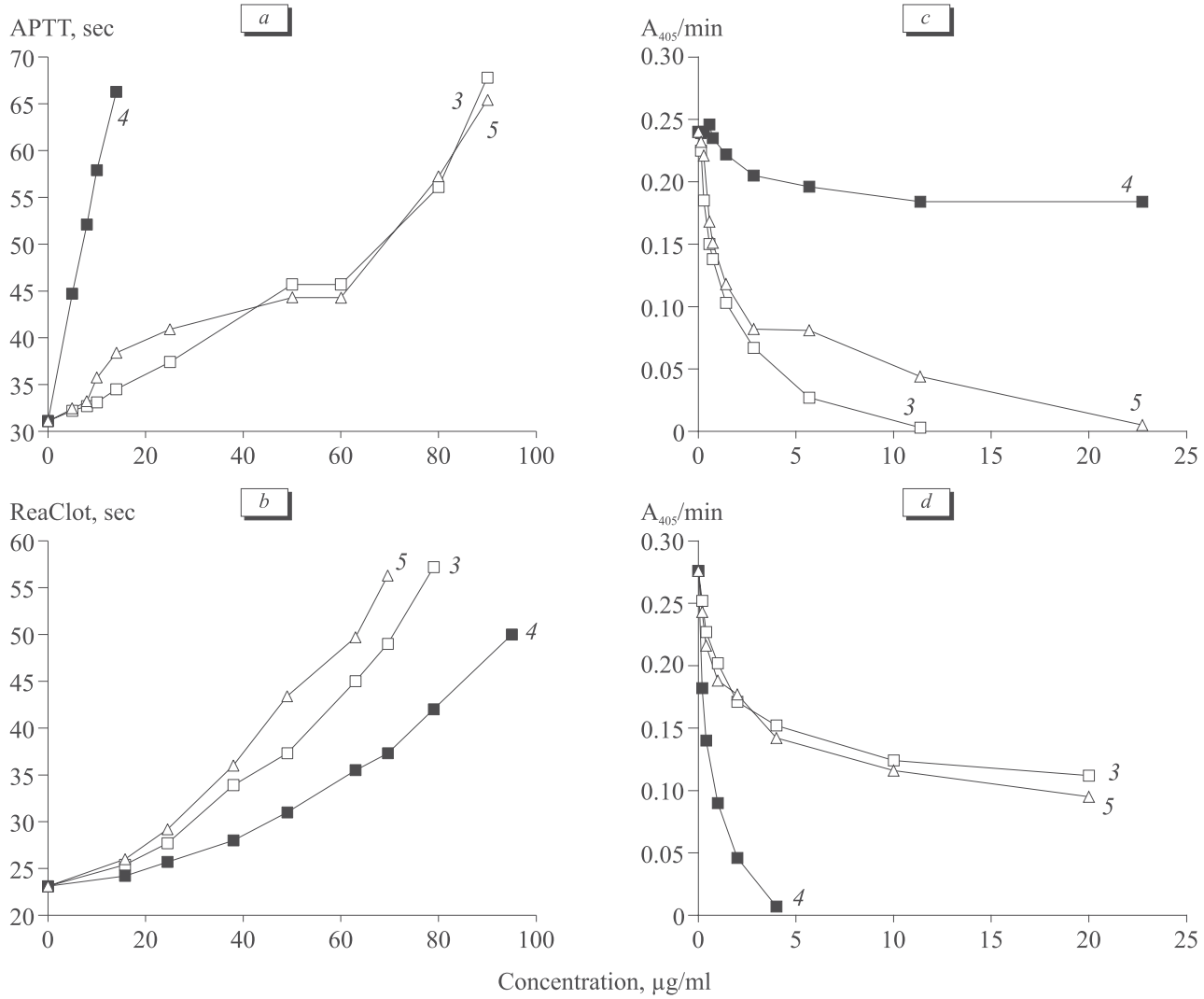

Fig. 1. Anticlotting activities of *F. evanescens* fucoidan and its modified products *in vitro*: effects of the studied samples on the time of fibrin clot formation in human plasma in clotting tests and on amidolytic activities of factors IIa and Xa. *a*) APTT test; *b*) inhibition of factor Xa activity using ReaClot-heparin kit; *c*) amidolytic activity of factor IIa; *d*) amidolytic activity of factor Xa. *3*) *F. evanescens* fucoidan; *4*) low-molecular-weight fucoidan fraction obtained by enzymatic hydrolysis of native specimen (FeF1); *5*) deacetylated specimen derived from *F. evanescens* native fucoidan (FeF).

TABLE 2. Anticlotting Activities of Fucoidans in Tests with Human Plasma

Sample No.	Sample code	Clotting activity with rabbit or human plasma				Amidolytic activity with antithrombin	
		2APTT, μg/ml	2ReaClot, μg/ml	alla, U/mg	aXa, U/mg	factor IIa inhibition (IC_{50}), μg/ml	factor Xa inhibition (IC_{50}), μg/ml
3	FeF1	120.0±7.0	64.7±10.0	48.0±4.0	11.0±0.7	1.500±0.100	0.800±0.050
4	FeF1LMF	15.0±3.0*	88.0±7.0*	47.0±3.0	10.0±0.5	>1000.000*	0.100±0.020*
5	FeFDA	110.0±5.0	56.5±7.0	50.1±6.0	12.5±0.6	2.000±0.1000	0.700±0.040
NFH reference sample		0.6±0.1	—	203.1	—	0.029±0.009	
LMH reference sample		—	0.43±0.10	—	168.0	—	0.019±0.007

Note. NFH: nonfractionated heparin; LMH: low molecular weight heparin. *p<0.05 *vs.* initial value for FeF1 fucoidan (n=3-5).

centrations of the samples was found (Fig. 1, *c*, *d*). The rate of hydrolysis of chromogenic substrate by thrombin decreased with increasing the concentrations (Fig. 1, *c*). The concentrations of substances, at which the rate of chromogenic substrate hydrolysis by thrombin decreased 2-fold (IC_{50}), were 1.5, more than 1000, and 2.0 μg/ml for samples 3, 4, and 5, respectively. Increasing sample concentrations led to greater inhibition of amidolytic activity of factor Xa (Fig. 1, *d*). Hydrolyzed sample 4 exhibited maximum activity (Table 2; IC_{50}=0.10±0.02 μg/ml).

Low-molecular-weight fraction FeF1 (sample 4) obtained by enzymatic hydrolysis of native fucoidan exhibited maximum activity with human plasma containing antithrombin and heparin cofactor II. The same sample exhibited minimum activity in a "pure system" in the presence of antithrombin alone. Deacetylated fucoidan FeF (sample 5) showed no appreciable changes in comparison with the initial fucoidan.

The anticlotting effect has to be neutralized in profuse hemorrhages, after aortocoronary bypass surgery, and in hyperheparinemia [13]. Protamine sulfate is a classical antidote for heparin. Scanned electrophoregrams of fucoidan samples in agarose gel with protamine sulfate are presented on Fig. 2. Zones of precipitation of stained polysaccharide-protein complexes are shaped as rockets. Precipitation peaks indicate the formation of complexes of nonfractionated heparin and the studied fucoidans with protamine sulfate during electrophoresis. All the studied samples added to the gel (0.31, 0.63, 1.25, 2.50, and 5.00 μ) formed complexes with protamine sulfate. The higher was the concentration, the larger precipitation peaks formed (Table 3). Precipitation zones of hydrolyzed low-molecular-weight fraction FeF1 (Fig. 2: sample 5; Table 1: sample 4) had blurred contour characteristic of low-molecular-weight sulfated polysaccharides.

Hence, purification of *F. evanescens* fucoidan from polyphenols and protein leads to reduction of its capacity to inhibit thrombin and factor Xa. Depolymerization of fucoidan by the specific enzyme increases its capacity to inhibit thrombin by predominantly heparin cofactor II. The capacity to inhibit thrombin amidolytic activity by antithrombin virtually disappears with decreasing polymerization degree. Presumably, the realization of antithrombin activity at the expense of serpins (anti-

TABLE 3. Electrophoresis of Fucoidans in Agarose Gel Containing Protamine Sulfate

Fucoidans, μg	Sample 3 (FeF1)		Sample 4 (FeF1LMF)		Sample 5 (FeFDA)	
	h	S	h	S	h	S
0.31	22±5	424±35	21±3	557±32	26±5	646±34
0.63	59±10	1199±92	49±6	1237±27	65±8	1445±66
1.25	113±12	2678±95	110±12	3089±81	132±10	2841±29
2.50	179±23	3391±22	179±19	4015±52	222±23	5798±95
5.00	315±38	6919±131	307±11	8779±116	305±20	8038±101

Note. Height (h) and area (S) of precipitation peaks in pixels (unit of measurement, determining the volume of data in a scanned image).

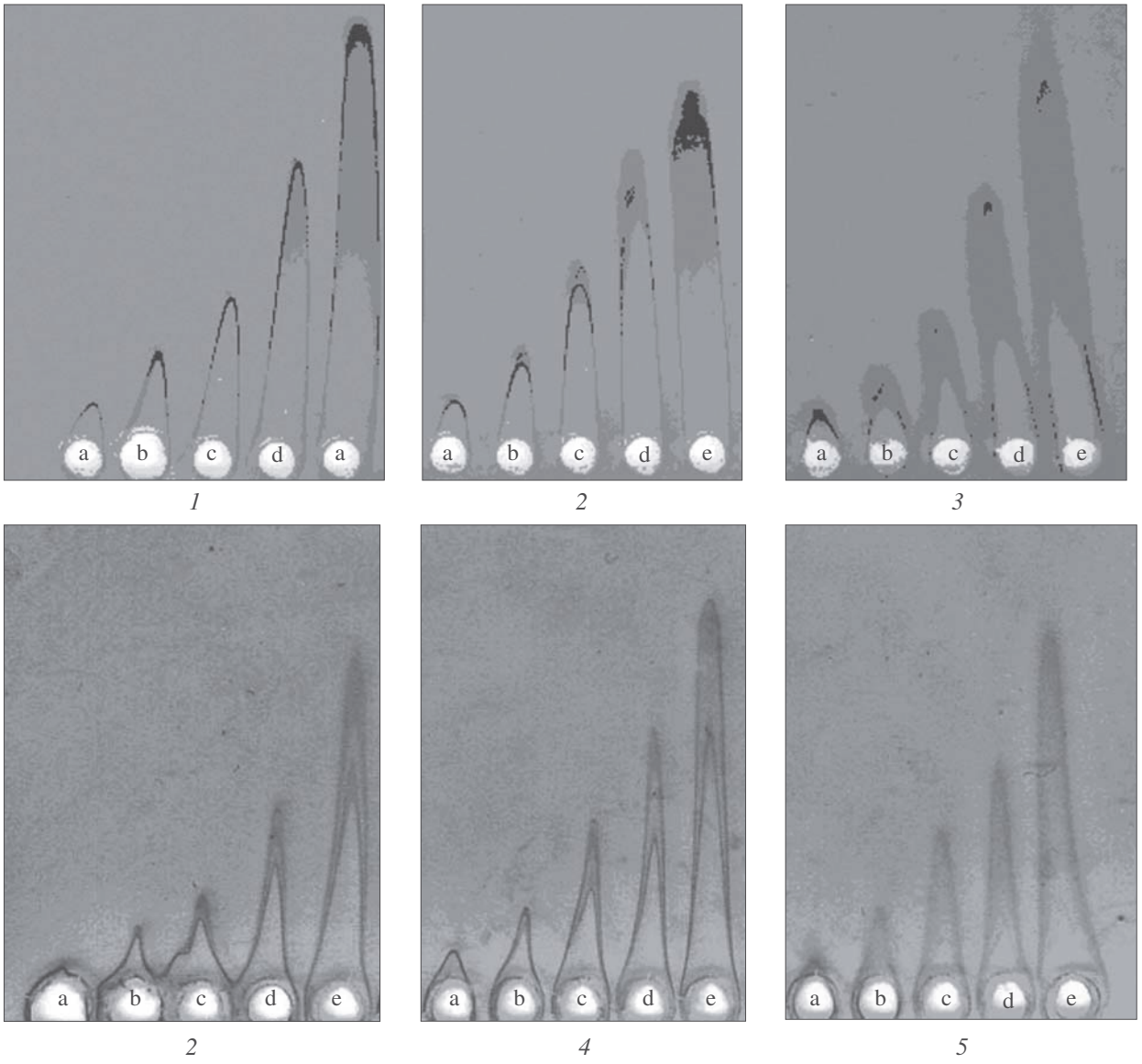

Fig. 2. Biospecific electrophoresis of nonfractionated heparin, *F. evanescens* fucoidan, and its chemically modified products in agarose gel with protamine sulfate. *1*) nonfractionated heparin; *2*) *F. evanescens* fucoidan; *3*) *F. evanescens* fucoidan free from polyphenols and proteins; *4*) deacetylated fucoidan from *F. evanescens*; *5*) hydrolysis product from *F. evanescens* fucoidan. *a*) 0.31 μ; *b*) 0.63 μg; *c*) 1.25 μg; *d*) 2.5 μg; *e*) 5 μg.

thrombin and heparin cofactor II) involves various sites of fucoidan molecule, and reduction of thrombin amidolytic activity by antithrombin is caused not by reduction of the molecular weight, but largely by destruction of the polysaccharide molecule site responsible for serpin binding under the effect of the enzyme. Deacetylation of the initial fucoidan sample does not lead to modification of its capacity to inhibit thrombin or factor Xa; it seems that acetates are inessential for manifestation of biological effect of fucoidans. All the studied samples formed complexes with protamine sulfate.

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