Sulfated Cellulose Derivatives Inhibit Activities of Thrombin and Activated Factor X

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Sulfated derivatives based on powdered cellulose were obtained, including those containing additional (carboxymethyl, ethyl amide or phosphate) groups, and their activity against blood clotting factors (thrombin and Xa) was studied. Maximum antithrombin activity of the test compounds, measured using a coagulation test, was 144±11 U/mg.

Key Words: *cellulose derivatives; anticoagulant activity; thrombin*

Anticoagulants based on sulfated plant polysaccharides can have the following advantages over widely used in clinical practice unfractionated and low-molecular weight heparins: nearly unlimited source of raw materials; raw material does not contain prion proteins that may be present in mammalian tissues; anticoagulant mechanism of action differs from that of heparin [5,7,11]. Anticoagulant activity (AA) of sulfated polysaccharides depends on chemical and supramolecular structure of the biopolymer [12]. An alternative way to obtain these polysaccharides is based on methods of chemical modification of common polysaccharides chitosan, mannans, cellulose, *e.g.* introduction of sulfate, carboxyl, and other functional groups and their combinations in macromolecular structure [5,9].

Our aim was to study AA of highly substituted cellulose derivatives containing sulfate groups and combined sulfated derivatives of cellulose containing sulfate groups and additionally carboxymethyl groups (carboxymethyl cellulose sulfates), amidoethyl (amidoethyl cellulose sulfates), aldehyde (dialdehyde cellulose sulfates), and phosphate groups (phosphate cellulose sulfates).

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MATERIALS AND METHODS

Microcrystalline cellulose (Company Polieks, TU 64-11-124-90; obtained from cotton cellulose, mean polymerization degree 220) was the starting material for subsequent chemical transformations. Cellulose sulfate was obtained by the reaction of cellulose with $ClSO_3H$ in dry pyridine [2]. Carboxymethylcellulose sulfate was obtained as a result of sequential processing of carboxymethylcellulose with *p*-toluenesulfonic acid monohydrate and $ClSO_3H$ in *N*,*N*-dimethylformamide at room temperature [1]. Amidoethyl cellulose sulfate was obtained by processing of cyanoethyl cellulose with $ClSO_3H$ in *N*,*N*- dimethylformamide at room temperature. Phosphocellulose sulfate was obtained by one-step esterification of cellulose with the mixture of phosphorus oxide (V) and $ClSO_3H$ in *N*,*N*- dimethylformamide. Dialdehyde cellulose sulfate was synthesized by oxidation of cellulose sulfate with sodium periodate. Average molecular weight was determined using gel permeation chromatography.

Specific AA of chemical combinations was determined *in vitro* by inhibition of amidolytic and fibrinogen clotting activities of thrombin (antithrombin, anti-IIa or aIIa activity) and Xa factor (anti-Xa or aXa activity) using International NIBSC standard V of nonfractionated heparin, based on the recommendations of national and European pharmacopoeia.

The effect of cellulose derivatives on clotting of human plasma was determined by thrombin time [9]

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and prothrombin time [6]. Antithrombin (aIIa) activity was determined in tests of activated partial thromboplastin time (APTT) by method [3]. aXa activity was evaluated by inhibition of fibrinogen clotting activity of factor Xa as described previously [14]. Antithrombin (aIIa) activity was evaluated as described elsewhere [8]. Antithrombin (1 U/mg; Sigma Aldrich) in 250 μl 0.05 M Tris-HCl buffer with 0.0075 M Na_2EDTA, 0.175 M NaCl, pH 7.4, was incubated with cellulose samples (final concentration of 0.1-1000 μg/ml) for 3 min at 37°C. Then 100 ml (2 NIH U/mg) bovine thrombin (Sigma Aldrich) in 2 mM Tris-EDTA buffer was added. After 30 sec, 200 μl thrombin-specific chromogenic substrate S-2238 (Sigma Aldrich) in Tris-EDTA 2 mM buffer was added. Activity of the compounds was assessed by changes in optical density of the solution at 405 nm for 1 min. Antifactor-Xa activity (aXa) was measured as described previously [10].

For evaluation of inhibition of factor Xa amidolytic activity, 250 μl mixture containing 8 U/ml factor Xa (Trinity Biotech) and 0.02 mg/ml dextran sulfate (Trinity Biotech) in Tris-EDTA buffer was added to 250 μl 0.05 M Tris-HCl buffer with 0.0075 M Na_2EDTA, 0.175 M NaCl, pH~8.4 containing 1 U/ml antithrombin and cellulose sulfates in a final concentration of 0.1-1000 μg/ml and incubated for 3 min at 37°C. Then, residual activity of factor Xa was determined by adding 50 μl chromogenic substrate S-2222 (Trinity Biotech). Activity was determined by changes in optical density of the solution at 405 nm.

RESULTS

Addition of cellulose sulfate and combined sulfated derivatives of cellulose human plasma (Table 1, Fig. 1) increased clotting time in APTT test, prothrombin, and thrombin time (Table 2), which attested to the influence of these compounds on blood clotting.

Antithrombin activity of cellulose sulfate with a molecular weight of 10-150 kDa and degree of sulfation (DS) 0.6-2.5 ranged from 23.3±5.9 to 144±11 U/mg (according to APTT test) and from 0.05±0.01 to 130±12 U/mg (according to thrombin time measure-

TABLE 1. Sulfated Derivatives of Cellulose

Code of the sample	Structure; substitute R_1, R_2, R_3	Degree of substitution by sulfate groups	Average molecular weight, kDa	Additional group and the degree of substitution by this group
CS 256/3	SO_3Na, H	0.6	150	No
CS 339	SO_3Na, H	1.0	30-50	No
CS 79	SO_3Na, H	1.3	100	No
CS 328	SO_3Na, H	1.6	30-50	No
CS 261	SO_3Na, H	1.7	100	No
CS 469	SO_3Na, H	1.8	10-30 No	
CS 348	SO_3Na, H	2.5	10-30	No
CMCS 76	SO_3Na, H	0.3	50	CH_2COONa; 0.1
CMCS 267	SO_3Na, CH_2COONa, H	1.1	100	CH_2COONa; 1.0
CMCS 82	SO_3Na, CH_2COONa, H	2.5	30-50	CH_2COONa; 0.1
PCS 289	SO_3Na, $PO(ONa)_2$, H	$PO(ONa)_2$; 1.2	40	0.8
PCS 292	SO_3Na, $PO(ONa)_2$, H	$PO(ONa)_2$; 1.1	40	1.7
PCS 298	SO_3Na, $PO(ONa)_2$, H	$PO(ONa)_2$; 1.2	75	1.0
PCS 312	SO_3Na, $PO(ONa)_2$, H	$PO(ONa)_2$; 1.3	75	1.2
AECS 233	SO_3Na, $CH_2CH_2CONH_2$, H	$CH_2CH_2CONH_2$; 1.4	120	0.3
AECS 264	SO_3Na, $CH_2CH_2CONH_2$, H	$CH_2CH_2CONH_2$; 0.8	150	0.3
DACS 81a	SO_3Na, CHO, H	CHO; 0.3	100	0.8
DACS 266	SO_3Na, CHO, H	CHO; 0.6	150	0.5

Note: CS, cellulose sulfate; CMCS, carboxymethyl cellulose sulfate; PCS, phosphocellulose sulfate, AECS, amidoethyl cellulose sulfate, DACS, dialdehyde cellulose sulfate.

ment, Table 2). The relationship between aIIa activity measured in units of standard unfractionated heparin and concentrations of the test compounds 2-fold lengthening the plasma clotting time in comparison with the control (30.1±4.2 sec for APTT and 9.3±1.8 sec for thrombine time) is moderately negative (r= -0.70 and -0.63; p<0.05, relatively). Cellulose sulfates inhibit fibrinogen clotting activity of factor Xa with activity from 0.10±0.07 to 45.0±3.3 U/mg (Table 2; $r_{2ReaClot}$=-0.69 [10]; p<0.05). These correlation coefficients indicate that AA should be determined in two ways (by comparison with the standard and by effective concentrations). Xa activity increases with increasing DS and more sharply in DS range of 0.5-1.6. For instance, when DS=0.5, aXa activity is 0.1 U/mg; at DS=1.3 it increases to 7.5 U/mg; при DS=1.6, to 12.1 U/mg. Further increase in DS leads to a less significant increase in aXa activity, which reached the maximum at about 2.0 DS. We can conclude that, first, aXa activity of the polymer grows with increasing the negative charge of the macrochain and, second, the optimal value of DS, apparently, lies in the range of 1.5-2.0. The effect of DS on aXa-related activity of cellulose sulfate and other cellulose derivatives is probably due to electrostatic interactions between the polysaccharide macromolecule and specific sites of antithrombin when inhibiting factor Xa activity. The increase in antithrombin activity within the group of cellulose sulfate with increasing DS is consistent with the data [12]. Increase in aXa activity with increasing the number of sulfate groups per statistical polysaccharide moiety was noted for several sulfated animal and plant polysaccharides [5,7,11,15]. The concentrations of cellulose sulfates 2-fold lengthening the plasma clotting time in the prothrombin time test were 3.5-5.3 mg/ml for the most active compounds (vs. 15.3±3.7 in the control; Table 2).

With decreasing the molecular weight of carboxymethyl cellulose sulfate samples from 100 to 30-50 kDa and increasing DS by 10 times, aIIa $_{APTT}$ activity increased by 2-20 times and reached 84.0±3.5 U/mg. However, $aIIa_{TT}$ activity >70 U/mg was detected in samples with a molecular weight below 50 kDa (Table 2). aIIa activity in these samples was similar by concentrations of doubled APTT and thrombin time. It could mean that the mechanisms of action of unfractionated heparin and these compounds in APTT test are somewhat different. Maximal aXa activity of carboxymethyl cellulose sulfates with a molecular weight <50 kDa was 27.2±0.4 U/mg.

Antithrombin activity in the samples of phosphate cellulose sulfate compared to the standard of unfractionated heparin in the APTT test was 2-3 times higher than $aIIa_{TB}$-activity (Table 2). Antithrombin activity assessed by effective concentrations in two coagulation tests is comparable, which also may indicate different effects of phosphate cellulose sulfate and unfractionated heparin on the internal pathway of blood clotting. Among the studied sulfated cellulose derivatives, the samples of phosphate cellulose sulfates were characterized by maximum aXa/aIIa activity ratio (0.1-0.7).

Since anticoagulant properties of cellulose sulfate are determined by the formation of electrostatic bonds and, consequently, violation of the conformation of some blood clotting proteins [12,13,15]. We can assume that those anticoagulant properties of sulfated cellulose derivatives are primarily determined by the presence of sulfate groups in the macromolecule. These groups are more electronegative than phosphate and carboxyl ones, and therefore they form stronger electrostatic bonds with antithrombin [13]. Phosphate groups are also involved in polysaccharide—protein interactions. Although they are to a lesser extent responsible for AA manifestation [4], phosphate cellulose sulfate preparations exhibit significant aIIa activity even at relatively low DS.

AA in the samples of amidoethyl cellulose and dialdehyde cellulose sulfates is negligible (Table 2).

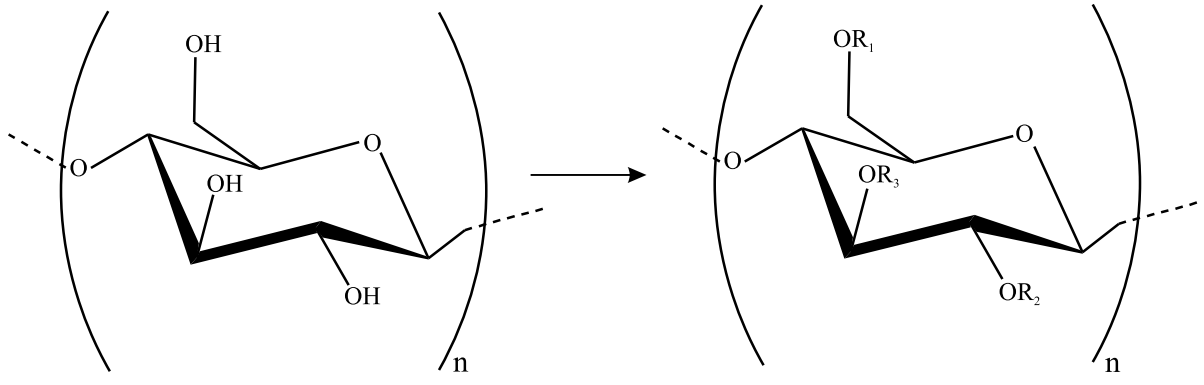

Fig. 1. The scheme of cellulose modification

Group 1: R=H, SO_3Na (CS). Group 2: R=H, SO_3Na CH_2COONa (CMCS). Group 3: R=H, SO_3Na, $PO(ONa)_2$ (PCS). Group 4: R=H, SO_3Na, $CH_2CH_2COHH_2$ (AECS).

TABLE 2. Effect of Cellulose Sulfates on Clotting Time of Human Plasma

Code of the sample	Inhibition of fibrinogen clotting activity of factor Xa		Effects on human plasma clotting in tests:				
			thrombin time		prothrombin time	APTT	
	aXa, U/mg	2ReaClot, μg/ml	aIIa, U/mg	2TT, μg/ml	2PT, μg/ml	aIIa, U/mg	2 APTT, μg/ml
CS 256/3	0.10±0.07	>1000	0.05±0.01	>1000	>1000	23.3±5.9	7.90±1.00
CS 79	7.5±3.0	27.40±0.41	43±6	2.24±0.08	556±38	27.8±6.2	1.60±0.34
CS 261	12.1±2.4	2.5±0.8	205±11	0.89±0.25	4.7±0.6	122±17	0.56±0.09
CS 328	**32.9±3.7**	0.063±0.003	115±8	1.19±0.20	6.1±0.3	86.5±5.2	1.13±0.08
CS 339	**20.3±1.9**	1.260±0.090	65.1±4.4	2.11±0.09	8.8±1.0	77.9±5.5	1.30±0.05
CS 348	**23.3±2.1**	0.790±0.040	99.0±6.2	1.52±0.08	5.3±0.9	118.5±6.9	0.77±0.06
CS 469	45.0±3.3	0.033±0.010	130±12	0.99±0.10	3.5±0.4	144±11	0.47±0.09
CMCS 76	27.2±0.4	2.28±0.09	135±19	1.05±0.08	148±7	33.7±5.4	1.16±0.03
CMCS 267	0.43±0.09	794±93	0.17±0.04	794±33	>1000	4.1±0.7	89.1±15.3
CMCS 82	21.9±3.4	0.50±0.04	99.0±8.3	1.75±0.50	6.6±1.9	84.0±3.5	1.23±0.09
AECS 233	3.4±0.5	10±3	18.6±2.3	6.92±0.44	>1000	22.2±4.1	2.60±0.54
AECS 264	0.14±0.07	126.0±15.5	1.34±0.30	39.8±2.8	>1000	6.0±1.8	50.0±7.7
DACS 81a	21.2±2.2	3.04±0.30	42.2±2.8	2.69±0.30	341±28	31.0±4.1	1.4±0.1
DACS 266	0.03±0.01	>1000	0.11±0.06	363±17	>1000	22.6±5.3	199.5±21.3
PCS 289	19.6±1.6	4.47±1.90	27.0±3.9	2.04±0.90	5.6±1.5	56.20±9.64	1.89±0.01
PCS 292	9.6±0.9	12.60±3.40	20.0±1.5	4.18±1.70	13.7±2.0	37.90±8.41	3.50±0.12
PCS 298	11.9±1.1	5.62±2.10	25.0±1.8	2.82±1.20	5±1	80.30±12.46	1.29±0.19
PCS 312	31±17	2.82±1.80	32.0±3.3	2.24±1.00	7.2±1.4	77.10±13.91	1.21±0.01
UFH	220.0	0.08±0.03	203	0.36±0.11	1.3±0.8	203.00	0.39±0.07

Note: UFH: unfractionated heparin. ReaClot: clotting method using factor Xa; TT: thrombin time; PT: prothrombin time. 2ReaClot, 2TT, 2PT, 2APTT, sulfate cellulose concentrations 2-fold lengthening plasma clotting time compared with the values in samples without cellulose sulfate.

This can be explained by low compared to other studied derivatives DS (0.3-1.4), high molecular weight (100-150 kDa), weak charge of introduced functional groups, and macrochain structure that prevents interaction with proteins. For example, introduction of aldehyde groups leads to breakage of the elementary cellulose moiety between its 2nd and 3rd carbons.

Relatively low values of aIIa activity and inhibition of factor Xa in carboxymethyl and amidoethyl cellulose sulfates can be explained by lower electronegativity of carboxymethyl and amidoethyl groups compared to sulfate and phosphate and, consequently, less intense electrostatic interactions with blood clotting factors. At the same time, carboxymethyl and amidoethyl groups shield the charged polymer chain preventing the formation of electrostatic bonds with the clotting factors.

Considering the amidolytic activity of thrombin (IIa) and factor Xa by several groups of cellulose sulfates, it was found that in most of compounds aIIa activity assessed by unfractionated heparin as compared with the methods of coagulation, is reduced 3-20 times, hence another serpin, other than antithrombin is needed. In one case, activity increased 2-fold; hence, something in the plasma prevents polysaccharides to display AA. In 2 cases AA does not change (antithrombin is just sufficient). Probably, there are different mechanisms of IIa activity inhibition with antithrombin for the samples in the sulfate cellulose group. Only for three cellulose samples, the capacity to inhibit factor Xa amidolytic activity by studied compounds increased significantly (Table 2). This suggests that certain plasma factors can inhibit AA of cellulose compounds. In the analysis of amidolytic

activity of IIa and factor Xa by the group of phosphate cellulose sulfates, a decrease in aIIa activity by was 5-50 times and aXa activity by 3-40 times were revealed. It can be noted that for the implementation of AA in the plasma of this compound group, other serpins are necessary apart from antithrombin. No direct inhibition of thrombin activity without antithrombin was observed in any of the cellulose samples in this concentration range.

Thus, aIIa- and aXa activity assessed with coagulometric tests is determined by several parameters: i) amount of sulfate groups (the higher DS, the more AA); ii) additional constitutients (amidoethyl and carboxymethyl groups reduce these values under comparable DS, phosphate group appears to be involved in complexation); iii) molecular weight (decrease of molecular weight leads to an increase in AA).

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