The Inhibitory Effect of Sulfated Schizophyllans and Related Oligosaccharides on Coagulation and Fibrinolysis¹⁾

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Sulfated schizophyllans and sulfated oligosaccharides were prepared and their inhibitory effects on coagulation and fibrinolysis were examined. Some sulfated schizophyllans, which had a sulfur content of more than 10%, prolonged prothrombin time (PT) and thrombin time (TT). Interestingly, some sulfated schizophyllans and related oligosaccharides were able to inhibit plasmin toward fibrinolysis and amidolysis.

Keywords sulfated schizophyllan; sulfated oligosaccharide; inhibitory activity; plasmin; fibrinolysis; coagulation

Schizophyllan (SPG) is a polysaccharide consisting of linearly linked β -1,3-glucose residues with one β -1,6-glucose side chain for every three main chain glucose residues, and SPG exhibited a potent antitumor effect on sarcoma 180 *in vivo*.²⁾

It was also revealed that sulfated SPG³⁾ exhibited potent anti-human immunodeficiency virus (anti-HIV) effects on Molt-4 clone No. 8 or MT-4 cells infected with HIV, as well as on lymphocytes from AIDS patients. It was suggested that the sulfur content of sulfated SPGs was the most important factor for inhibiting the growth of HIV, rather than molecular weight or the type of sugar component.⁴⁾

Because sulfated SPGs appear to be applicable as anti-HIV drugs, it is very important to investigate whether or not sulfated SPGs and related oligosaccharides exhibit effects other than anti-HIV activity. This report deals with the examination of the inhibitory effects of sulfated SPGs and related oligosaccharides on coagulation and fibrinolysis.

MATERIALS AND METHODS

Degradation of SPG Native SPG obtained from *Schizophyllum commune* Fries (molecular weight (MW): $>8 \times 10^6$) was degraded by the method described previously.^{5,6)} Namely, native SPG was degraded by ultrasonication to give SPG (MW: 4.5×10^5). This SPG was further degraded by formic acid to give SPG (MW: 3.5×10^3). The molecular weights of SPGs were determined by the viscosity–molecular weight relation.⁷⁾

Oligosaccharides were obtained as follows: SPG (MW: 3.5×10^3) was degraded by endo- β -1,3-glucanase treatment to give a mixture of oligosaccharides. This mixture in water was applied to a column of active carbon (100 g, 3.6×36 cm), equilibrated with water and eluted with the following solvents: 10% EtOH (2 l), 28% EtOH (4 l), 30% EtOH (4 l), 35% EtOH (2 l), 38% EtOH and 40% EtOH. Trisaccharide (G₃), tetrasaccharide (G₄) and pentasaccharide (G₅) were obtained from the effluents of 28% EtOH, 30% EtOH and 38% EtOH, respectively. Each oligosaccharide was further purified with HPLC [column: Polyspher CH-NA (Merck), solvent: water]. The structure

of each oligosaccharide was confirmed by the Hakomori method⁸⁾ (analysis of methylated oligosaccharide) and elemental analysis. *Anal.* Calcd for G₃·2.5H₂O: C, 39.4; H, 6.79. Found: C, 39.6; H, 6.66. Calcd for G₄·3.5H₂O: C, 39.5; H, 6.77. Found: C, 39.5; H, 6.70. Calcd for G₅·3.5H₂O: C, 40.4; H, 6.67. Found: C, 40.7; H, 6.59.

SPGs with different molecular weights (MW: 4.5×10^5 or 3.5×10^3) were sulfated in pyridine by chlorosulfonic acid.⁹⁾ Oligosaccharides were sulfated by the sulfur trioxide–pyridine complex method in pyridine.¹⁰⁾ The S-content was determined by the oxygen flask combustion method.¹¹⁾ Thus, we obtained G₃, sulfated G₃ (sulfur content: 12.5%, 1.0 SO₃Na residue is attached to each glucose unit), G₄, sulfated G₄ (S content: 12.8%, 1.0 SO₃Na residue per glucose unit), pentasaccharide (G₅) and sulfated G₅ (S content: 14.3%, 1.3 SO₃Na residues per glucose unit) (see Fig. 1). Additionally, we obtained four kinds of sulfated SPGs (S-1, MW: 3.5×10^3 , S content: 1.1%, 0.056 SO₃Na residue per glucose unit; S-2, MW: 3.5×10^3 , S content: 12.9%, 0.65 SO₃Na residue per glucose unit; S-3, MW: 4.5×10^5 , S content: 2.1%, 0.106 SO₃Na residue per glucose unit and S-4, MW: 4.5×10^5 , S content: 17.6%, 0.89 SO₃Na residue per glucose unit). The sulfated position was determined by measuring ¹³C-NMR as follows: The hydroxy group at C₆ was sulfated first, followed by that at C₂ (data not shown).

Determination of the Inhibitory Effects of Sulfated SPGs and Oligosaccharides on Coagulation, Fibrinolysis and Amidolytic Activity of Various Proteinases Human plasmin (Kabi Co.), human leukocyte elastase,¹²⁾ cathepsin G,¹³⁾ α -chymotrypsin (Sigma Chemical Co.) and bovine thrombin (Mochida Seiyaku Co.) were used. Heparin was purchased from Novo, Denmark.

PT and TT were measured in the usual manner, described as follows, in the absence and presence of sulfated SPGs and related oligosaccharides. PT: Human plasmin (100 μ l) and test solution (75 μ l) were mixed and the mixture was incubated at 37°C for 2 min. Thromboplastin (175 μ l, thromboplastin-C, Kokusaiyaku) was added to the mixture and PT was measured by a coagulometer at 37°C. TT: Fibrinogen solution (480 mg/dl, 150 μ l) and test sample (75 μ l) were mixed and the mixture was stirred at 37°C for 2 min. Thrombin

Fig. 1. Structure of Oligosaccharides (G₃, G₄ and G₅)

(thrombin, Mochida, 5 unit/ml, 75 μ l) was added to the mixture and TT was measured by a coagulometer at 37 °C. Measurements of PT and TT were done three times, respectively. Three experiments exhibited similar results concerning PT or TT. Using the data from three experiments, typical experimental data are cited in Table I.

The fibrin plate was prepared using plasminogen-free fibrinogen and bovine thrombin, as described previously.¹⁴⁾ Human plasmin and the compound to be tested were mixed, and 0.03 ml of the mixture was dropped on a plasminogen-free fibrin plate. After incubation for 24 h at 37 °C, its fibrinolytic activity was estimated by the lysed area (mm²).

The amidolytic activity of leukocyte elastase and cathepsin G and of α -chymotrypsin was measured with Suc-Ala-Tyr-Leu-Val-pNA and Suc-Ile-Pro-Phe-pNA, respectively.¹⁵⁾ The amidolytic activity of plasmin and thrombin was measured with D-Val-Leu-Lys-pNA (s-2251) and D-Phe-Pip-Arg-pNA (s-2238), respectively.¹⁶⁾

RESULTS AND DISCUSSION

The inhibitory effects of sulfated SPGs and oligosaccharides on coagulation are shown in Table I. Similarly to heparin prolongation of PT and TT, which are the parameters of the anticoagulant,¹⁷⁾ S-2 and S-4 prolonged both PT and TT significantly. S-3 prolonged only TT. S-1 did not exhibit any prolongation effect on PT or TT. Although G₃, G₄, and G₅ did not exhibit any effects on either PT or TT, sulfated G₃, sulfated G₄ and sulfated G₅ prolonged both PT and TT slightly. From these results, it can be deduced that their anticoagulant activity depends partly on the degree of sulfur content. The above compounds did not inhibit the amidolytic activity of thrombin (data not shown). Therefore, it is deduced that they exhibited anticoagulant activities in the presence of anti-thrombin III, as in the case of heparin, although the precise mechanism of their anticoagulant activities remains to be

TABLE I. Inhibitory Effect of Sulfated SPG and Related Oligosaccharides on Coagulation

Compounds	Conc. (μ g/ml) ^{a)}	TT (s) ^{b)}	PT (s) ^{b)}
Control		13.2	13.6
G ₃	2000	12.1	13.8
G ₄	2000	11.7	13.7
G ₅	2000	12.0	13.0
Sulfated G ₃	2000	48.1	36.4
	500	25.3	16.5
Sulfated G ₄	2000	28.0	45.1
	500	14.3	13.3
Sulfated G ₅	2000	34.0	95.7
	500	18.9	14.3
S-1	2000	15.2	12.9
S-2	2000	> 120	> 120
	50	> 120	22.9
	3	23.7	13.4
S-3	2000	> 120	12.8
	30	28.6	13.0
S-4	2000	> 120	> 120
	10	> 120	18.2
	0.4	65.2	13.1
Heparin ^{c)}	0.2 u	> 120	64.5
	0.02 u	33.1	15.0

a) The final concentration of a compound in the reaction mixture. b) Values given are those obtained in a typical experiment. c) One unit (u) of heparin is approximately 6 μ g.

clarified.

The sulfated SPGs and oligosaccharides examined did not show any inhibitory effects on leukocyte elastase, cathepsin G or α -chymotrypsin toward fibrinolysis and amidolysis, while some of them exhibited inhibitory activity against plasmin toward fibrinolysis and amidolysis as summarized in Table II. However, heparin did not exhibit such inhibitory effects on plasmin.

Sulfated G₄ and sulfated G₅ inhibited the amidolytic activity of plasmin in an uncompetitive manner with K_i values of 8.4×10^{-5} and 3.5×10^{-4} M, respectively, while sulfated G₃ did not show any inhibitory effect on ami-

TABLE II. Inhibitory Effect of Sulfated SPGs and Related Oligosaccharides on Fibrinolytic and Amidolytic Activity of Plasmin

Compounds	Fibrinolysis ^{a)} (Lysed area, mm ²)	Amidolysis ^{b)} K_i (M)
Control	205 ± 10	$>2.0 \times 10^{-3}$
G ₃	204 ± 3	$>2.0 \times 10^{-3}$
G ₃	245 ± 7	$>2.0 \times 10^{-3}$
G ₅	238 ± 14	$>2.0 \times 10^{-3}$
Sulfated G ₃	77 ± 5	$>2.0 \times 10^{-3}$
Sulfated G ₄	42 ± 6	8.4×10^{-5}
Sulfated G ₅	100 ± 2	3.5×10^{-4}
S-1	63 ± 9	3.0×10^{-5}
S-2	52 ± 2	2.9×10^{-5}
S-3	93 ± 2	1.4×10^{-7}
S-4	88 ± 2	1.5×10^{-7}
Heparin ^{c)}	210 ± 15	$>2.0 \times 10^{-3}$

Figures in fibrinolysis show mean ± S.D. for three experiments. a) The final concentration of compounds dropped with plasmin (5 cu/ml) was 5 mg/ml. b) The final concentration of plasmin was 0.05 cu/ml. c) The final concentration of heparin dropped with plasmin was 100 u/ml.

dolysis by plasmin. Since the C₆ position of the glucose units of G₃, G₄ and G₅ are sulfated as determined by ¹³C-NMR measurement, it was deduced that molecular size might affect their inhibitory activity against plasmin toward amygdolysis.

It is of interest that although S-1 and S-3 inhibited plasmin uncompetitively with K_i values of 3.0×10^{-5} and 1.4×10^{-7} M, respectively, S-2 and S-4 inhibited plasmin competitively with K_i values of 2.9×10^{-5} and 1.5×10^{-7} M, respectively. Since heparin did not inhibit plasmin, it can be deduced that the structure of the sulfated SPGs and related oligosaccharides (the types of sugar components) has some effect on the interaction between SPGs or oligosaccharides and plasmin, and the degree of the sulfation on SPGs has an effect on their inhibitory mechanism against plasmin. Although S-3 did not inhibit plasmin toward fibrinogenolysis, S-4 could inhibit it (data not shown), demonstrating that S-4 is an active center-directed plasmin inhibitor.¹⁸⁾

In conclusion, sulfated SPGs prolonged PT and TT,

although they did not inhibit thrombin activity itself. Unexpectedly, sulfated SPGs and related oligosaccharides exhibited inhibitory activity against plasmin. The relationship between their anti-HIV activity and antiplasmin activity remains to be studied.

REFERENCES AND NOTES

- 1) The customary L indication for amino acid is omitted; only D isomers are indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967); **11**, 1726 (1972)]. Other abbreviations used are: Suc, succinyl; pNA, *p*-nitroanilide; Pip, pipercolic acid; PT, prothrombin time; TT, thrombin time; cu, casein unit.
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